

# Dynamic T cell migration program provides resident memory within intestinal epithelium

David Masopust,<sup>1,2</sup> Daniel Choo,<sup>2</sup> Vaiva Vezys,<sup>1,2</sup> E. John Wherry,<sup>2</sup> Jaikumar Duraiswamy,<sup>2</sup> Rama Akondy,<sup>2</sup> Jun Wang,<sup>3</sup> Kerry A. Casey,<sup>1</sup> Daniel L. Barber,<sup>2</sup> Kim S. Kawamura,<sup>2</sup> Kathryn A. Fraser,<sup>1</sup> Richard J. Webby,<sup>5</sup> Volker Brinkmann,<sup>4</sup> Eugene C. Butcher,<sup>6</sup> Kenneth A. Newell,<sup>3</sup> and Rafi Ahmed<sup>2</sup>

<sup>1</sup>Department of Microbiology, Center for Immunology, University of Minnesota, Minneapolis, MN 55455

<sup>2</sup>Emory Vaccine Center and Department of Microbiology and Immunology and <sup>3</sup>Department of Pathology, Emory University School of Medicine, Atlanta GA 30322

<sup>4</sup>Autoimmunity, Transplantation, and Inflammation, Novartis Institutes for Biomedical Research, CH-4002 Basel, Switzerland

<sup>5</sup>Department of Infectious Diseases, St. Jude Children's Research Hospital, Memphis, TN 38105

<sup>6</sup>Laboratory of Immunology and Vascular Biology, Department of Pathology, Stanford University School of Medicine, Stanford, CA 94305

**Migration to intestinal mucosa putatively depends on local activation because gastrointestinal lymphoid tissue induces expression of intestinal homing molecules, whereas skin-draining lymph nodes do not. This paradigm is difficult to reconcile with reports of intestinal T cell responses after alternative routes of immunization. We reconcile this discrepancy by demonstrating that activation within spleen results in intermediate induction of homing potential to the intestinal mucosa. We further demonstrate that memory T cells within small intestine epithelium do not routinely recirculate with memory T cells in other tissues, and we provide evidence that homing is similarly dynamic in humans after subcutaneous live yellow fever vaccine immunization. These data explain why systemic immunization routes induce local cell-mediated immunity within the intestine and indicate that this tissue must be seeded with memory T cell precursors shortly after activation.**

## CORRESPONDENCE

David Masopust:  
masopust@umn.edu  
OR  
Rafi Ahmed:  
rahmed@emory.edu

Abbreviations used: CLA, cutaneous lymphocyte-associated antigen; GALT, gastrointestinal-associated lymphoid tissue; IEL, intraepithelial lymphocyte; iLN, inguinal LN; LCMV, lymphocytic choriomeningitis virus; mLN, mesenteric LN; YFV, yellow fever virus.

Naive T cell recirculation is primarily restricted to secondary lymphoid tissues, blood, and lymph (Butcher and Picker, 1996; von Andrian and Mackay, 2000). However, most infections are initiated at body surfaces, including the gut (Neutra and Kozlowski, 2006). Before CD8 T cells can participate in the eradication of local infections, microbial antigen must get from the site of infection to secondary lymphoid tissue, cognate antigen-specific T cells must undergo activation and expansion, and then CD8 T cells must migrate to the site of infection to effect pathogen clearance. Because these events consume valuable time, it is perhaps not surprising that a portion of memory T cells remain distributed at body surfaces after primary infections, where they may be better positioned to respond immediately in the event of reinfection (Hogan et al., 2001; Masopust et al., 2001b; Reinhardt et al., 2001). In support of this hypothesis, memory CD8 T cells within the gut epithelium (intraepithelial lymphocytes [IELs]) are function-

ally distinct from those in lymphoid tissue and exhibit immediate cytotoxicity upon reinfection (Kim et al., 1999; Masopust et al., 2001a). The vulnerability of the intestinal mucosa to infection because of its proximity with the microbial world, large surface area, and identity as a major reservoir of HIV infection, and the putative role that in situ memory T cells play in protection, have provided substantial motivation to understand how memory is established and maintained at such body surfaces (Hayday et al., 2001; Nagler-Anderson, 2001; Belyakov and Berzofsky, 2004; Cheroutre, 2005; Haase, 2005; Liu et al., 2006; Gebhardt et al., 2009).

T cells use  $\alpha 4\beta 7$  integrin and CCR9 to migrate to small intestine epithelium, and they use different homing receptors, such as cutaneous

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lymphocyte-associated antigen (CLA), to migrate to skin (von Andrian and Mackay, 2000; Johansson-Lindbom and Agace, 2007). Gut and skin homing receptor expression is coupled to the location of T cell activation (Campbell and Butcher, 2002; Stagg et al., 2002; Mora et al., 2003, 2005). Priming within mesenteric LN (mLN) induces  $\alpha 4\beta 7$  expression. In contrast, priming within inguinal LN (iLN) induces expression of skin homing molecules. These observations support the hypothesis that local infection and subsequent priming within gastrointestinal-associated lymphoid tissue (GALT) is required for dissemination of CD8 T cells into the small intestine. Although numerous observations are consistent with this hypothesis (Gallichan and Rosenthal, 1996; Belyakov et al., 1998), there are several examples in which alternative routes of immunization result in effector T cell responses within the intestinal mucosa (Stevceva et al., 2002; Masopust et al., 2004; Liu et al., 2006). Reconciling these seemingly disparate observations is essential for understanding the relationship between immunization route and the establishment of local protective cellular immunity.

Another issue of relevance to protective immunity is whether memory CD8 T cells continuously recirculate between blood, lymphoid tissue, and mucosal epithelium or whether IELs are long-term sessile residents. Numerous studies support the general concept that effector memory CD8 T cells recirculate between nonlymphoid tissues and blood (Butcher and Picker, 1996). For instance, if the vasculature of two mice is conjoined via parabiosis, preexisting memory CD8 T cells originating from both mice can be isolated from a single lung, liver, or peritoneal cavity (Jungi and Jungi, 1981; Klonowski et al., 2004), demonstrating that memory cells enter certain tissues via the blood. The memory T cell population in lung airways is thought to be short lived but maintained by the continuous recruitment of the systemic population (Ely et al., 2006). Memory CD8 T cells also exit tissues using afferent lymphatic vessels, which drain interstitial fluid from peripheral tissues (Olszewski et al., 1995; Lehmann et al., 2001; Bromley et al., 2005; Debes et al., 2005). Indeed, Mackay et al. (1990) isolated antigen-experienced lymphocytes directly from the hind limb afferent lymphatics of sheep. Memory T cells putatively egress with the afferent lymph as it filters through proximal LNs, pools within the thoracic duct, and then rejoins the blood supply. Thus, memory CD8 T cells in most peripheral tissues may be transient visitors, constantly patrolling peripheral organs in the event of reinfection.

However, it is unclear whether the recirculation paradigm applies to virus-specific memory CD8 $\alpha\beta$  IEL. Unlike most tissues, including the subjacent lamina propria, this avascular compartment consists of T cells residing among a monolayer of columnar epithelial cells that lies above a basement membrane and lacks blood vessels and afferent lymphatics. Although antigen specificity was not determined, parabiosis studies showed limited mixing of IEL between conjoined mice (Klonowski et al., 2004). Lastly, memory IELs are phenotypically and functionally distinct from CD8 T cells in blood (Masopust et al., 2006). As these issues impact our understanding of how

epithelial memory CD8 T cell populations are established, instructed to express unique properties, and maintained, we investigated whether priming within GALT is required for CD8 T cell migration to small intestine and also whether IELs routinely recirculate or are long-term residents.

## RESULTS

### Dynamic T cell migration program in spleen

To examine the dynamics of CD8 T cell homing, we used the lymphocytic choriomeningitis virus (LCMV) and P14 transgenic CD8 T cell system, which provides access to large numbers of naive, effector, and memory CD8 T cells of known specificity. To generate effector and memory CD8 T cells, naive Thy1.1<sup>+</sup> P14 were transferred to naive mice, which were then challenged i.p. with the Armstrong strain of LCMV. In response to infection, P14 underwent clonal expansion, which peaked 7 d after challenge, followed by contraction of the population and formation of stable memory by  $\sim 1$  mo later (Fig. 1 A). We first examined the trafficking potential of cells isolated from spleen. We transferred  $2 \times 10^6$  naive P14 splenocytes or  $2 \times 10^6$  P14 splenocytes isolated 4.5, 7, or 60 d after LCMV Armstrong infection into naive Thy1.2<sup>+</sup> recipients. It should be noted that day-4.5 CD8 T cells represent early effectors that are still undergoing clonal expansion. The next day, lymphocytes were harvested from recipient tissues (Fig. 1 B).

As expected, naive CD8 T cells populated blood and secondary lymphoid tissues but were excluded from the IEL compartment of the small intestine (Fig. 1 C and Fig. S1). When early effector P14 splenocytes isolated from spleen (or blood; Fig. S2) were transferred, they were recovered from spleen and the epithelium of the intestinal mucosa. These data demonstrated that early effector CD8 T cells were capable of homing from spleen to gut. Pertussis toxin treatment of donor cells inhibited effector cell homing to intestinal epithelium, demonstrating that this migration was chemokine dependent (Fig. S1).

Remarkably, CD8 T cells lost the ability to migrate to the IEL compartment by 7 d after infection (Fig. 1 C). Memory CD8 T cells were entirely excluded from migrating to the intestinal epithelium. Thus, CD8 T cells were endowed with the ability to migrate to intestinal epithelium only early during the response.

We then tested whether effector cells were contaminated with a cofactor that permitted T cells, regardless of differentiation state, to migrate to the intestinal epithelium. When day-4.5 and memory P14 splenocytes were mixed and transferred to the same naive recipient, both populations of cells were equivalently recovered from spleen. However, memory CD8 T cells were  $\sim 20$ -fold more efficient at trafficking to LN and early effectors were  $>50$ -fold more efficient at migrating to the small intestinal epithelium (Fig. S3). This indicated that effector and memory CD8 T cells exhibit intrinsically different homing properties.

To test this conclusion further, we examined the dynamics of  $\alpha 4\beta 7$  expression, which is required for homing to the small intestine. Fig. 1 D shows that  $\alpha 4\beta 7$  expression is transiently up-regulated on all early effector LCMV-specific CD8

T splenocytes and is then rapidly down-regulated. Thus, the timing of  $\alpha 4\beta 7$  expression reflected the transient ability of T cells to traffic to the gut epithelium.

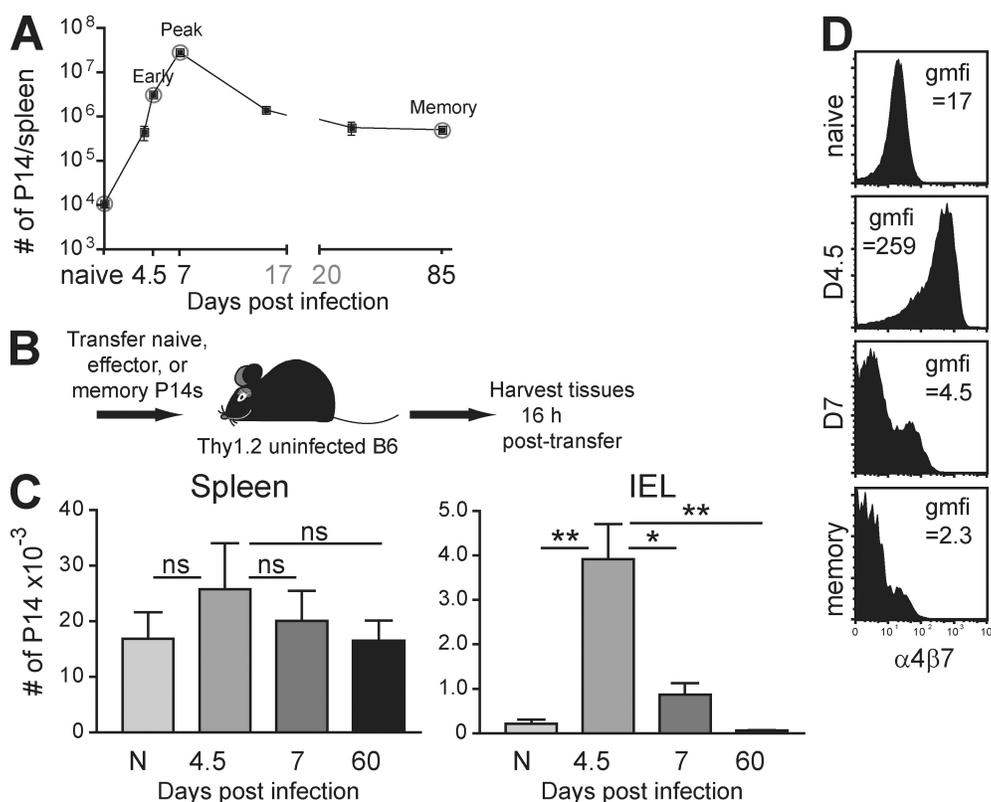
We then tested whether reactivation of memory CD8 T cells would result in transient reexpression of  $\alpha 4\beta 7$ . Splenocytes isolated from LCMV-immune Thy1.1<sup>+</sup> P14 chimeras (Fig. 1) were transferred to naive mice. Recipients were boosted the next day with pathogens that contained cognate gp33 antigen, including LCMV Armstrong strain, LCMV clone 13 strain, or recombinant *Listera monocytogenes* (LM-gp33). In each case, secondary donor CD8 T cell responses were coupled with transient reexpression of  $\alpha 4\beta 7$  and dissemination into the small intestinal epithelium (Fig. 2). Endogenous H-2Db/gp33-specific reactivated memory CD8 T cells also transiently up-regulated  $\alpha 4\beta 7$  upon boosting (Fig. 2), so this phenomenon was not unique to transgenic CD8 T cells. In contrast, challenge with LM that lacked recombinant cognate gp33 antigen (LM-WT) failed to induce  $\alpha 4\beta 7$  expression among memory P14, suggesting that the infectious milieu alone was insufficient and that cognate antigen was required.

It should be noted that LCMV infects the spleen, almost 100% of early effector splenocytes expressed  $\alpha 4\beta 7$ , and there are  $\sim 20$ -fold more early effectors in spleen than GALT

( $7.2 \times 10^6$  early effector P14 in spleen versus  $3.4 \times 10^5$  in mLNs). Thus, it is unlikely that activation in GALT was required for  $\alpha 4\beta 7$  expression. To examine this issue more stringently, mice were infected intranasally with influenza virus. 5 d later, almost no activated virus-specific CD8 T cells could be detected within GALT, yet flu-specific CD8 T cells in spleen- and lung-associated tissues expressed  $\alpha 4\beta 7$  and memory CD8 T cells were established within small intestinal epithelium (Fig. 3, A and B). Intramuscular injection of a DNA vaccine into the anterior tibialis (located within lower hind limb) also led to transient  $\alpha 4\beta 7$  expression and the establishment of CD8 T cells within intestinal epithelium (Fig. 3, C and D). Moreover, disruption of lymphocyte egress from GALT via treatment of mice with FTY720 did not affect  $\alpha 4\beta 7$  expression among effector CD8 T cells in spleen (Fig. 3 E). Collectively, these data demonstrate that activation in GALT is not required for  $\alpha 4\beta 7$  expression among CD8 T cells.

#### Homing potential among distinct lymphoid tissues

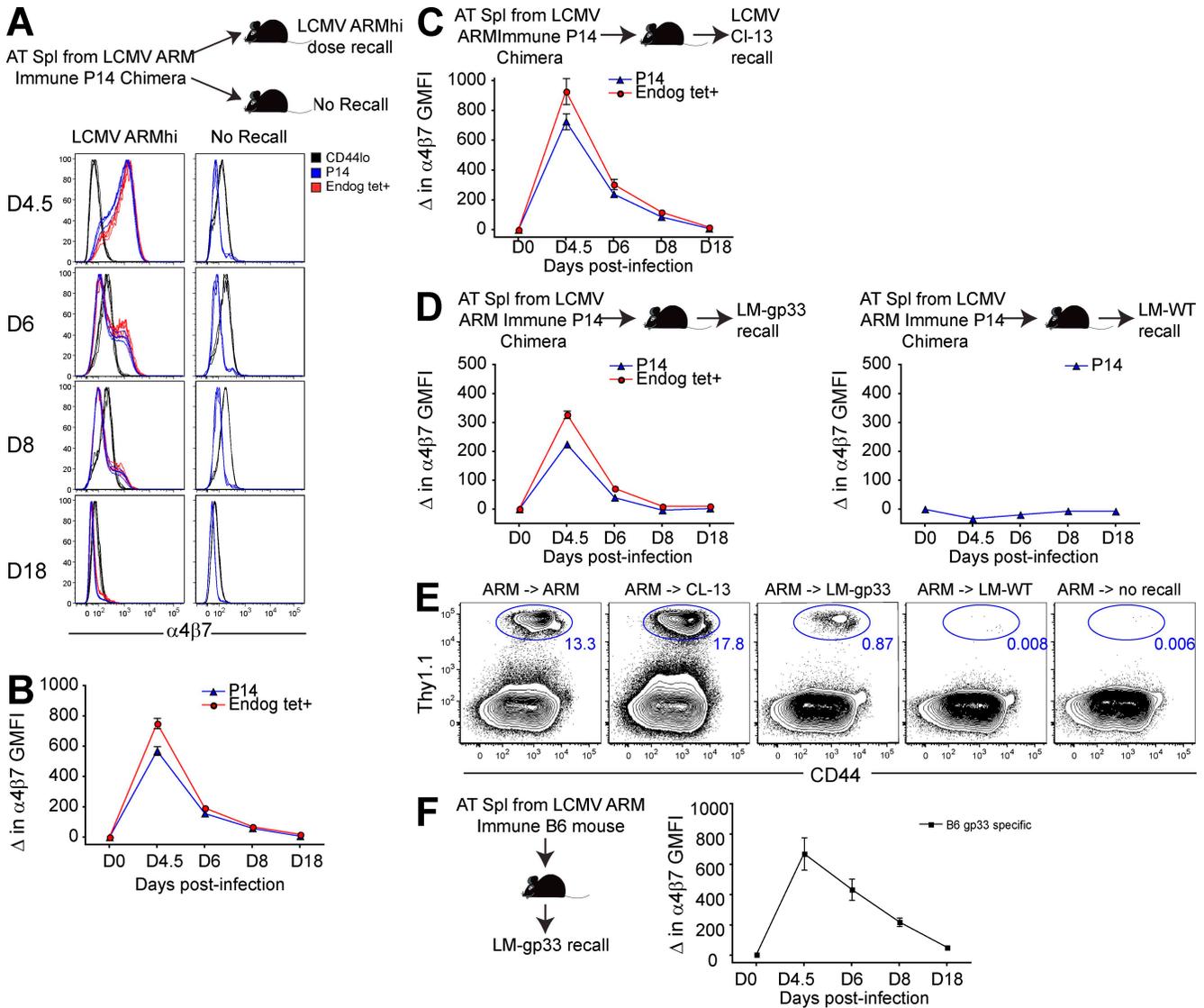
We next tested whether memory CD8 T cells in mucosal tissues also lacked  $\alpha 4\beta 7$ , and we determined the dynamics of CCR9 expression, which is also involved in migration to gut (Zabel et al., 1999; Kunkel et al., 2000; Johansson-Lindbom



**Figure 1. Only early effector CD8 T cells migrate to intestinal epithelium and express  $\alpha 4\beta 7$ .** (A) Dynamics of P14 response to LCMV. The day 37 time point represents 12 mice analyzed from days 31 to 50 after infection. (B) Experimental design consists of transferring P14 at different stages of differentiation and harvesting tissues the next day. (C) Numbers of naive (N), effector (isolated 4.5 or 7 d after infection), and memory (isolated 60 d after infection) cells isolated from recipient spleen and intestinal epithelium (IEL). ns, not significant, \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ , unpaired Student's *t* test. Error bars indicate SEM. (D) Virus-specific P14 CD8 T cells were analyzed for expression of  $\alpha 4\beta 7$ . gmfi, geometric mean fluorescence intensity of  $\alpha 4\beta 7$  staining. All plots are gated on Thy1.1<sup>+</sup> CD8<sup>+</sup> lymphocytes and are representative of at least three independent experiments totaling >10 mice/time point.

and Agace, 2007). Fig. 4 A demonstrates that neither  $\alpha 4\beta 7$  nor CCR9 expression is maintained on memory CD8 T cells isolated from any lymphoid tissue. Although IEL memory CD8 T cells expressed CCR9,  $\alpha 4\beta 7$  was down-regulated by day 7 after infection (Fig. 4 and Fig. S4). Thus, LCMV-specific memory CD8 T cells, regardless of location, did not coexpress the essential homing molecules required for entry into the intestinal mucosa.

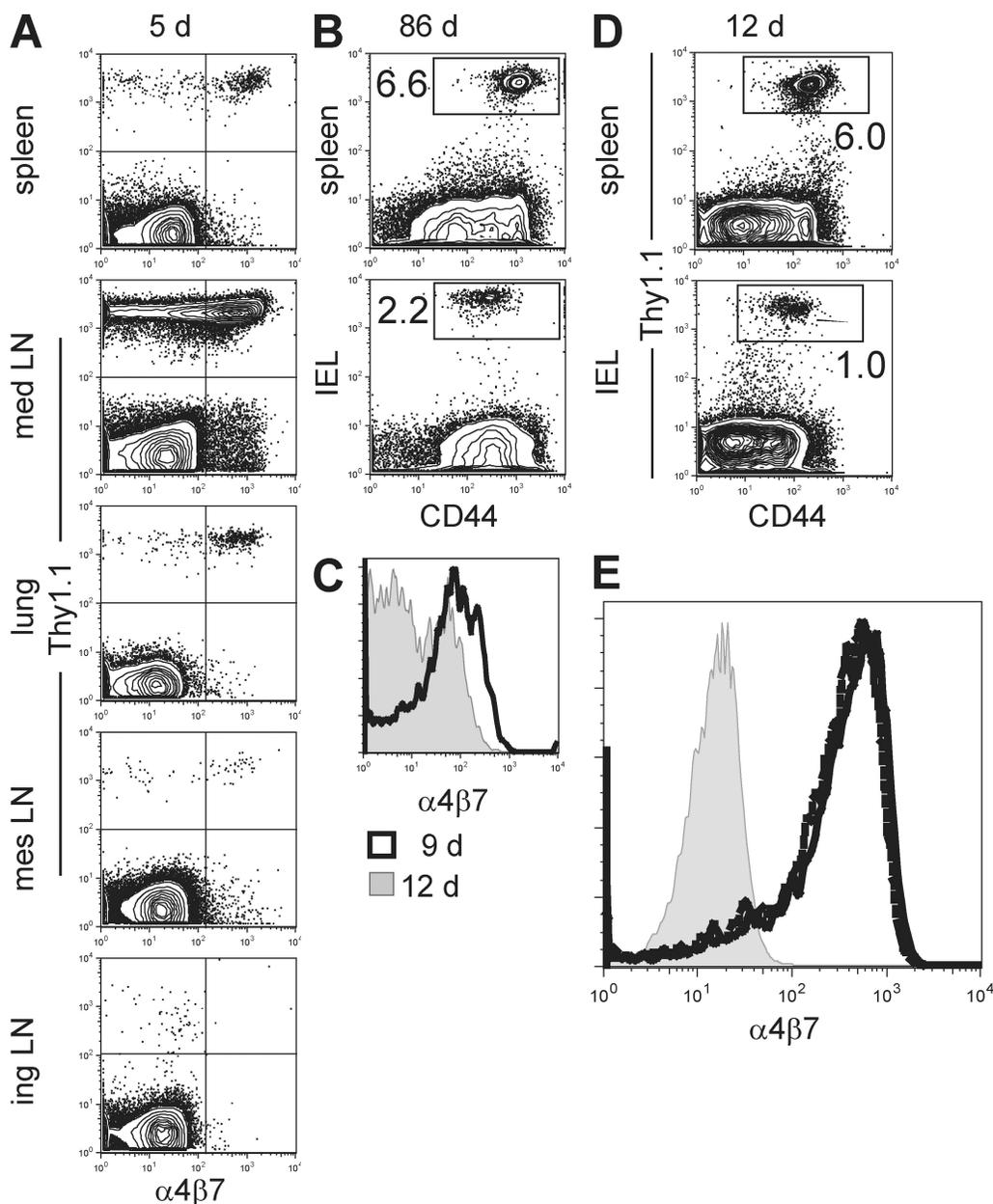
We then determined whether  $\alpha 4\beta 7$  expression was maintained on memory CD8 T cells after oral infection. OT-I transgenic CD8 T cells specific for ovalbumin were transferred to mice before oral infection with recombinant LM expressing ovalbumin (LM-ova; Masopust et al., 2001a; Pope et al., 2001). As shown in Fig. 4 B, memory CD8 T cells did not retain  $\alpha 4\beta 7$  after oral infection in any tissue analyzed, including the IEL compartment.



**Figure 2. Antigen-dependent reexpression of  $\alpha 4\beta 7$  by spleen-derived transgenic and endogenous memory CD8 T cells upon infection with virus or bacteria.** (A–D) Splenocytes isolated from P14 immune chimeras (>30 d after LCMV Arm infection) were transferred to naive recipients. (A and B) The next day, recipients were challenged with high-dose LCMV Arm or left unchallenged. (A)  $\alpha 4\beta 7$  expression was monitored in blood among donor P14 (Thy1.1<sup>+</sup>/gp33 tetramer<sup>+</sup>), nontransgenic gp33-specific cells (Thy1.1<sup>-</sup>/gp33 tetramer<sup>+</sup>), and CD44<sup>lo</sup> (naive) CD8 T cells. Representative flow cytometry data are shown. (B) Change in GMFI of  $\alpha 4\beta 7$  expression relative to  $\alpha 4\beta 7$  GMFI of memory P14 transferred to unchallenged mice that were analyzed on the same day. (C and D) As in A and B, except mice were challenged with LCMV CI-13 (C) or LM-gp33 (D), or the noncognate antigen bearing inflammation control LM-WT. (E) 19 d after infection, small intestine IEL of these mice were examined for the presence of donor P14. (F) Splenocytes from LCMV Arm-immune C57BL/6J mice (which did not contain P14) were transferred to naive CD45.1<sup>-</sup> gp33-tetramer<sup>+</sup> CD8 T cells was monitored in blood. At least three mice were analyzed at each time point in each experiment. Error bars indicate SEM. One of two experiments with similar results is shown.

Expression of gut homing molecules on early effector CD8 T cells varied by location (Fig. 4, A and B). Those isolated from skin-draining iLN up-regulated less  $\alpha 4\beta 7$  than cells in spleen and mLN. And early effectors in mLN expressed higher levels of

CCR9 than those isolated from spleen or iLN. We compared the homing potential of each population on a per cell basis by transferring  $10^6$  P14 isolated from each lymphoid tissue 4.5 d after LCMV infection to separate recipients (Fig. 4 C). With regard to



**Figure 3. Memory CD8 T cells do not retain  $\alpha 4\beta 7$  expression regardless of anatomical location or immunization route.** Naive Thy1.1<sup>+</sup> P14 were transferred to naive mice. (A and B) The next day, mice were infected intranasally with 500 pfu of recombinant influenza virus that expresses gp33. 5 (A) or 86 (B) d later, lymphocytes were isolated from the indicated tissues and stained with  $\alpha$ -Thy1.1, CD8, and  $\alpha 4\beta 7$  or CD44. Plots are gated on CD8<sup>+</sup> lymphocytes. (C and D) Alternatively, 200  $\mu$ g DNA that expresses the glycoprotein of LCMV under control of the CMV promoter was administered intramuscularly into both anterior tibialis muscles. (C) 9 and 12 d later, Thy1.1<sup>+</sup> cells were examined for expression of  $\alpha 4\beta 7$  (gated on CD8<sup>+</sup> Thy1.1<sup>+</sup> lymphocytes). (D) 12 d after immunization, lymphocytes were isolated from spleen and IEL and stained with  $\alpha$ -CD8 $\alpha$ , Thy1.1, and CD44 antibodies. Plots are gated on CD8<sup>+</sup> lymphocytes. (E) Naive Thy1.1<sup>+</sup> P14 were transferred to naive C57BL/6J mice. Control mice received normal drinking water, whereas treated mice were exposed to 2  $\mu$ g/ml FTY720 in the drinking water ad libitum for the duration of the experiment. The next day, both groups of mice were immunized with LCMV, and  $\alpha 4\beta 7$  expression among Thy1.1<sup>+</sup> P14 in spleen was compared among control mice (black line), FTY720-treated mice (dashed line), and CD44<sup>lo</sup> CD8 T cells isolated from spleens of naive mice (gray histogram). Plots are gated on Thy1.1<sup>+</sup> CD8<sup>+</sup> lymphocytes. All data are representative of two experiments with at least three mice per group in each experiment.

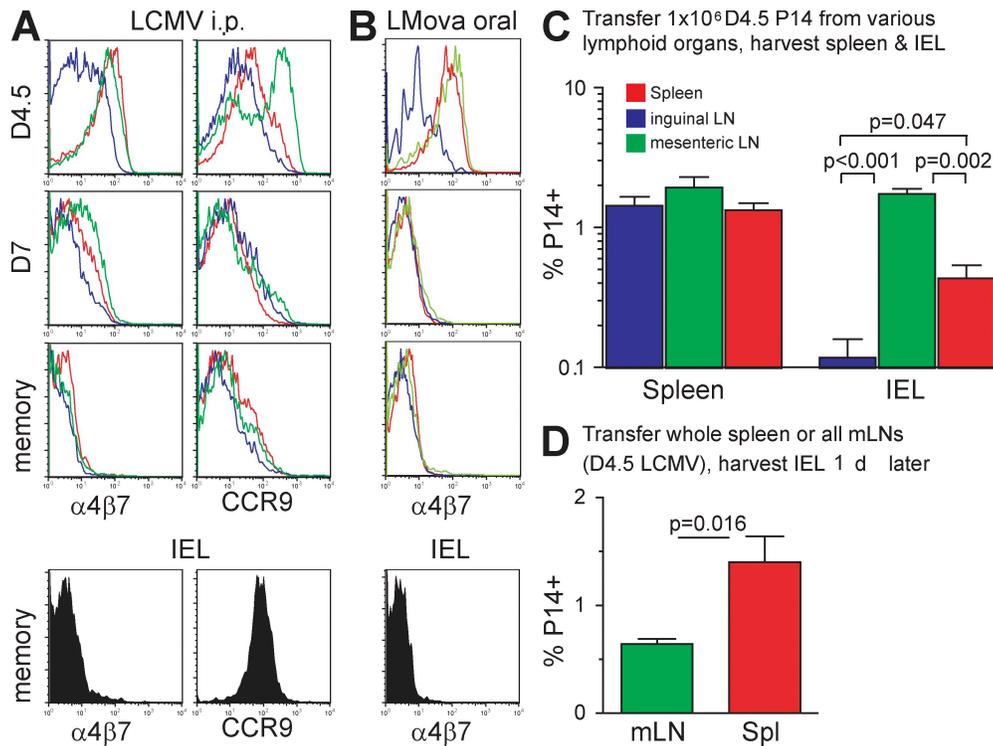
homing to intestinal epithelium, P14 cells derived from mLN were the most efficient, and iLN-derived cells migrated poorly. P14 derived from spleen exhibited an intermediate ability to traffic to mucosal epithelium. When one considers the fact that there are ~20-fold more early effector T cells in spleen than mLN, it is likely that more IEL effectors are derived from spleen than from mLN after an LCMV infection. To test this hypothesis, all lymphocytes derived from one spleen versus all mLNs derived from one mouse were transferred into separate recipients. Although recovery varied among different experiments, transfer of spleen consistently resulted in 2.5–4-fold more P14 within the gut epithelium than the transfer of mLN on a per-organ basis (Fig. 4 D,  $n = 5$  per group for the experiment shown).

**Memory IELs are resident**

FTY720 treatment causes an accumulation of recirculating lymphocytes within lymphoid tissue and a corresponding decrease within other tissues that contain recirculating lymphocytes (Schwab and Cyster, 2007). To further test whether IELs recirculate, we treated LCMV-immune mice with FTY720 and examined whether treatment induced a reduction in memory CD8 T cells within the intestinal epithelium.

LCMV-immune P14 chimeras (generated as described in Fig. 1 A) were treated with FTY720 for 2 or 30 d in drinking water. As shown in Fig. 5 A, treatment led to a rapid depletion of memory CD8 T cells in blood within 2 d, and this reduction was maintained for 30 d with continuous FTY720 treatment (Fig. 5 B). As expected, a reciprocal increase in P14 was observed in iLN, suggesting that lymphocyte recirculation through the iLN was interrupted. However, there was no reduction in the number of P14 IEL, even after 30 d of treatment (Fig. 5, A and B). These data support the hypothesis that IELs do not recirculate via afferent lymphatics.

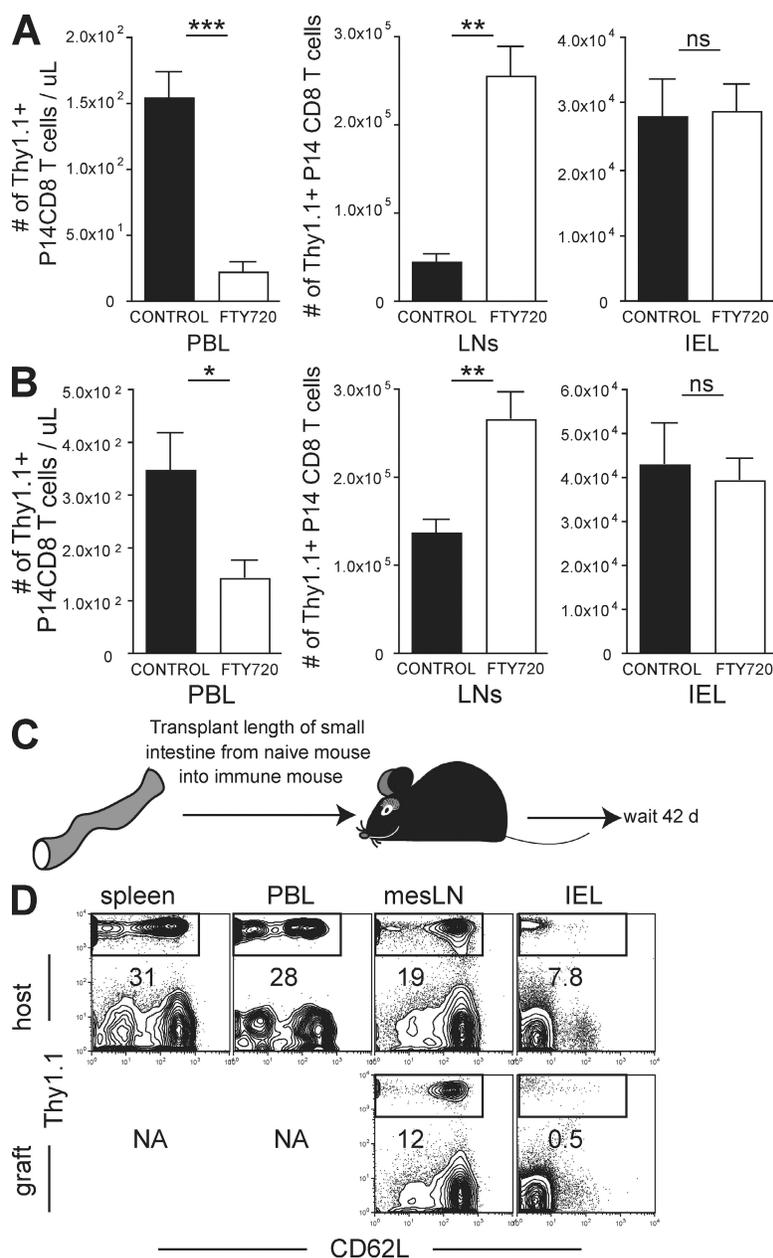
We wished to independently test whether memory CD8 T cells recirculated through gut epithelium over a period of several weeks. To this end, we removed 7 cm of small intestine from naive C57BL/6J mice and transplanted this tissue into C57BL/6J mice that had been immunized with LCMV 2 mo previously and contained large numbers of Thy1.1<sup>+</sup> memory P14 CD8 T cells (Fig. 5 C). Blood vessels were ligated upon transplantation and donor gut remained viable and appeared healthy by visual inspection upon cessation of the experiment. This method allowed us to test whether memory CD8 T cells, which are present in all tissues of the recipient



**Figure 4. Memory CD8 T cells do not retain  $\alpha 4\beta 7$  expression regardless of anatomical location or immunization route.** (A and B) Expression of  $\alpha 4\beta 7$  and/or CCR9 by P14 isolated from various tissues 4.5, 7, or 60 d after i.p. LCMV infection (A) or by OT-I after oral LMova infection (B). All plots are gated on Thy1.1<sup>+</sup> CD8<sup>+</sup> lymphocytes. (C) 10<sup>6</sup> Thy1.1<sup>+</sup> P14 isolated from spleen (red), iLN (blue), or mLN (green) 4.5 d after LCMV infection was transferred to naive mice. The next day, lymphocytes were harvested from recipient spleen and small intestinal epithelium, and the proportion of CD8<sup>+</sup> lymphocytes that were Thy1.1<sup>+</sup> was determined. Only p-values of <0.05 are shown. (D) Recipient mice received the entire single cell suspension derived from either one spleen or the complete cluster of mLN derived from one mouse isolated 4.5 d after LCMV infection. The next day, the proportion of CD8<sup>+</sup> lymphocytes that were Thy1.1<sup>+</sup> was determined. Error bars indicate SEM. All data are representative of at least two experiments with at least three mice per group in each experiment.

mouse, would equilibrate with donor gut epithelium. Association of donor small intestine with donor mLN remained intact and served as a positive control for recirculation between graft and host. 42 d after transplantation, host and donor tissues were removed and assessed for the presence of host mem-

ory P14. As shown in Fig. 5 D, donor mLNs have similar frequencies of memory P14 CD8 T cells compared with host mLNs, as expected. Within the donors' IEL compartment, there were far fewer host memory P14 CD8 T cells compared with host epithelium, indicating minimal recirculation.



**Figure 5. Memory CD8 T cells in intestinal epithelium do not recirculate.** Naive P14 cells were transferred to naive C57BL/6J mice, and recipients were infected with LCMV. 90 d later, 2  $\mu$ g/ml FTY720 was dissolved in drinking water (white bars) or mice were maintained on normal drinking water (black bars). (A and B) 2 (A) or 30 (B) d after FTY720 treatment, the number of LCMV-specific P14 memory CD8 T cells was determined in blood (PBL), iLNs, lung, or small intestinal epithelium (IEL). Data shown is one of five experiments with three mice per group with similar results. Immune mice were generated by transferring naive Thy1.1<sup>+</sup> P14 into naive C57BL/6J mice and infecting recipients with LCMV. Error bars indicate SEM. \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ ; \*\*\*,  $P < 0.001$ , unpaired Student's  $t$  test. (C) 2 mo later, 7 cm of small intestine, along with associated mesentery and mLN, were transplanted from naive mice into immune mice. (D) 42 d after transplantation, lymphocytes were isolated from host spleen, blood (PBL), mLN, and intestinal epithelium (IEL), as well as donor mLN and IEL. The presence of host memory P14 was determined in each tissue by Thy1.1 staining and flow cytometry. All plots are gated on CD8<sup>+</sup> lymphocytes and are representative of one of a total of three mice examined in two independent experiments. NA, not applicable.

### Dynamic $\alpha 4\beta 7$ and CLA expression after human s.c. yellow fever vaccination

Our observations in mice revealed that  $\alpha 4\beta 7$  was expressed by early effector CD8 T cells but not memory CD8 T cells. Expression of the putative skin homing molecule, functional P-selectin ligand, was similarly dynamic and was coexpressed with  $\alpha 4\beta 7$  on effector splenocytes shortly after LCMV infection (Fig. S5). We wished to examine whether expression of peripheral homing molecules was also dynamic after a primary immunization of humans. To follow a primary CD8 T cell response in humans, we examined the response to the live attenuated yellow fever virus (YFV)-17D vaccine (Miller et al., 2008). We identified an HLA-A2-restricted immunodominant epitope that we used to construct MHC class I tetramers. Volunteers were vaccinated s.c. and a tetramer<sup>+</sup> population in PBMC could be visualized by day 11. Because immunization was delivered s.c., we examined expression of CLA, which is involved in skin homing. CLA expression on YFV-specific T cells was remarkably transient, peaking on day 11, decreasing substantially by day 14, and was not detectable by day 30 after immunization (Fig. 6 A). When CD8 T cells were costained for both CLA and  $\alpha 4\beta 7$  molecules, early effectors expressed detectable levels of both homing molecules (Fig. 6 B). However, by day 30, YFV-specific effector CD8 T cells had predominantly down-regulated CLA and  $\alpha 4\beta 7$ , and this phenotype persisted among memory CD8 T cells (90 d after infection; Fig. 6 C). Thus, human virus-specific CD8 T cells also express a very dynamic pattern of homing molecule expression and support the hypothesis that CD8 T cell homing to body surfaces occurs predominantly upon recent activation.

### DISCUSSION

Our data support a model by which early effector CD8 T cells migrate into the intestinal mucosa and then down-regulate  $\alpha 4\beta 7$ , differentiate into long-lived memory CD8 T cells in situ, and remain resident without recirculating. Analysis of a primary human immune response to s.c. immunization suggests that these migration dynamics reflect what occurs at other body surfaces, such as skin, which may also harbor resident memory CD8 T cells (Clark et al., 2006). This observation has several implications. Obviously, analysis of blood-borne lymphocytes will not reflect the quantity and quality of nonrecirculating T cells in tissues. Defining correlates of cell-mediated protection after vaccination will require sampling T cells directly from portals of pathogen entry. The fact that memory CD8 T cells may remain sessile within intestinal epithelium supports the hypothesis that microenvironmental cues instruct their unique differentiation profile (Masopust et al., 2006). Most importantly, these data indicate that body surfaces must be seeded with memory T cell precursors shortly after priming or boosting, as memory T cells in other compartments will not equilibrate into this tissue. Consequently, in the absence of cognate antigen restimulation, it is unlikely that peripheral memory is maintained by a stem cell-like population of central memory CD8 T cells

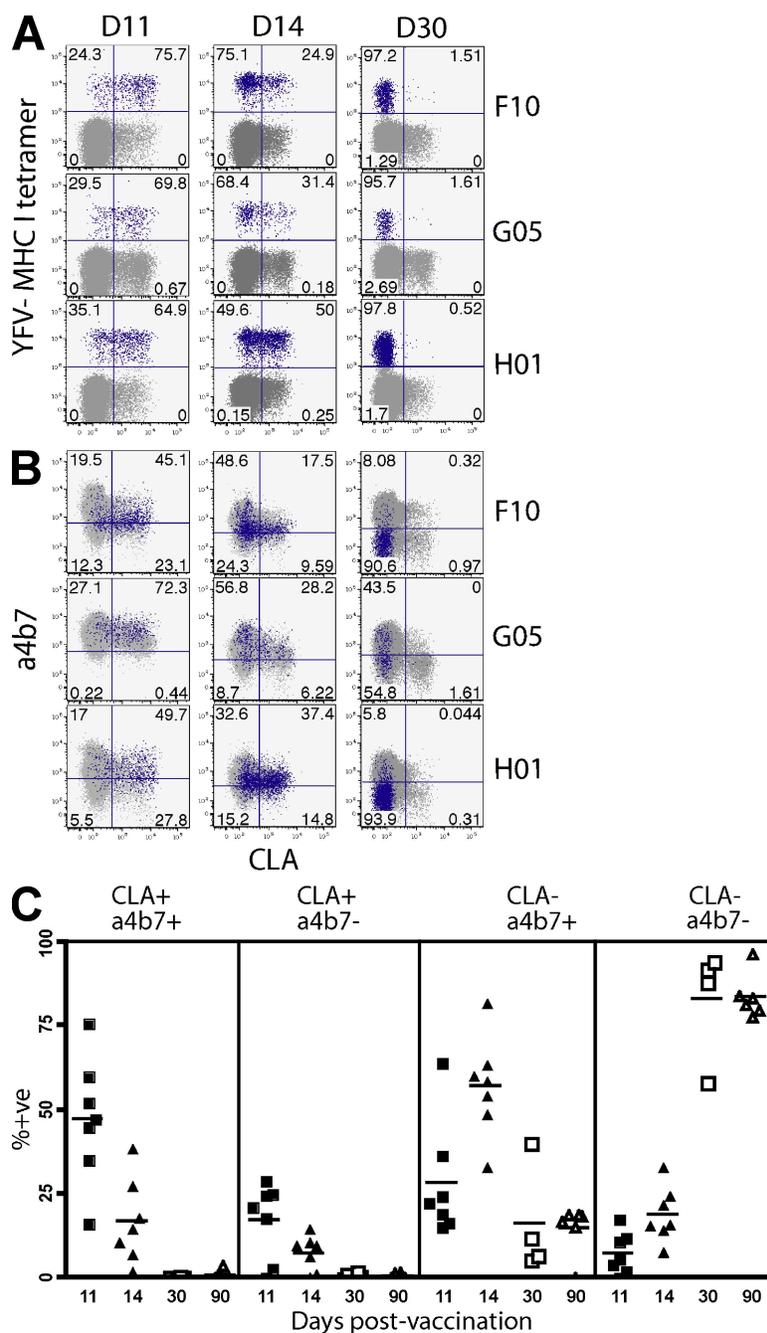
located in other tissues (Sallusto et al., 2004). Rather, the population is maintained in situ, which may have implications for the longevity of in situ protective immunity.

Local lymphoid tissue preferentially induces expression of homing molecules that help target activated T cells to associated nonlymphoid tissues (Butcher and Picker, 1996; von Andrian and Mackay, 2000). This has been best characterized in response to immunization with nonreplicating agents or in vitro. Our data extend these findings by demonstrating that T cells activated in vivo in response to a viral or bacterial infection also exhibit distinct homing properties that are dependent on the lymphoid environment within which they are primed (Fig. 4). However, our data demonstrates that priming within local lymphoid tissue may not be required for homing into the gut epithelium (Fig. 1–4). Rather, priming in spleen, a central lymphoid organ which contains the majority of antigen-experienced CD8 T cells even after local infections (Haanen et al., 1999; Marshall et al., 2001; Masopust et al., 2004), results in a promiscuous homing program that drives migration to the intestinal mucosa with intermediate efficiency (Fig. 4 and Fig. S5). This observation may help reconcile the many studies demonstrating the distinctive homing properties of T cells primed in intestinal versus skin draining lymphoid tissue (Campbell and Butcher, 2002; Mora et al., 2003, 2005) and those studies that have demonstrated promiscuous T cell distribution after local infections (Stevceva et al., 2002; Masopust et al., 2004; Liu et al., 2006). Our results do not exclude other possible mechanisms that could contribute to the dissemination of effector T cell responses, including recirculation of dendritic cells or early effector T cells through different LNs, where they may access a variety of homing signals (Belyakov et al., 2004; Liu et al., 2006). Defining what conditions regulate the establishment of intestinal CD8 T cell memory after nonoral routes of immunization will have obvious ramifications for vaccination.

In contrast to LNs, spleen does not drain tissues via the lymphatic system, nor is it directly associated with any particular body surface. Therefore, induction of a promiscuous homing program after activation within this tissue makes sense both teleologically and mechanistically and may enhance host immunity by ensuring that adaptive T cell responses examine all tissues for infection. Of course it remains quite possible that repeated infections, which may limit T cell reactivation to local tissues, could enhance the preferential accumulation of memory T cells specifically at the original site of infection. It is more difficult to speculate on the advantage of restricting recirculation through intestinal epithelium (Fig. 5), as this presumably limits the immediate availability of memory CD8 T cells in the event of local reinfection. It may be related to the fact that the function and differentiation state of small intestine IELs differs markedly from memory CD8 T cells in blood (Masopust et al., 2006). Regardless, the observation that the potential to home to the gut epithelium dissipates shortly after pathogen control highlights how quickly CD8 T cell homing properties change throughout the response (Figs. 1–4). These rapid changes in homing potential and  $\alpha 4\beta 7$  expression

were defined in mouse studies that used various immunogens (LCMV, *L. monocytogenes*, influenza virus, and DNA vaccination) and routes of immunization (i.p., oral, intranasal, and intramuscular). When this principle was tested in humans in the context of a live attenuated vaccine, we found that homing

molecule expression was similarly dynamic (Fig. 6). It remains important to determine whether long-term tissue residence will define the paradigm for memory T cells at other body surfaces, and how this relates to both control and pathogenesis of mucosal infections, such as HIV.



**Figure 6. Primary human CD8 T cell response to s.c. yellow fever vaccine results in only short-term expression of  $\alpha 4\beta 7$  and CLA.** Blood was isolated from HLA-A2–positive volunteers 11, 14, 30, and 90 d after s.c. vaccination with YFV-17D. (A) Expression of CLA versus staining with MHC class I tetramers that recognize YFV-specific CD8 T cells from three representative patients. (B) CLA versus  $\alpha 4\beta 7$  expression. HLA2-YFV tetramer<sup>+</sup> cells are blue and HLA2-YFV tetramer<sup>-</sup> cells are gray. Numbers indicate percentage of tetramer<sup>+</sup> cells in each quadrant. All plots are gated on CD3<sup>+</sup> CD8<sup>+</sup> lymphocytes. (C) Summary of CLA and  $\alpha 4\beta 7$  expression among YFV tetramer<sup>+</sup> cells. Longitudinal analysis is shown, although some patients were not examined on days 30 and 90. Horizontal bars show the mean. Day 11,  $n = 7$ ; day 14,  $n = 7$ ; day 30,  $n = 4$ ; day 90,  $n = 6$ .

## MATERIALS AND METHODS

**Generation of virus-specific naive, effector, and memory donor CD8<sup>+</sup> T cells and infections.** Animal protocols were approved by the Institutional Animal Care and Use Committees at the University of Minnesota and/or Emory University. Naive donor antigen-specific CD8 T cells were isolated directly from spleens of naive Thy1.1<sup>+</sup> transgenic P14 mice. For effector and memory cell generation,  $2 \times 10^5$  naive Thy1.1<sup>+</sup> transgenic P14 CD8 T cells isolated from spleen were transferred i.v. into C57BL/6 recipients. The next day, recipients were infected with  $2 \times 10^5$  pfu LCMV (Armstrong strain). Single cell suspensions of lymphocytes isolated from challenged mice were prepared 4.5, 7, and 30–60 d after infection. For cotransfer experiments (Fig. S3), Ly5.1<sup>+</sup> P14 were used to generate memory CD8 T cells. For analyzing influenza specific responses,  $2 \times 10^5$  naive Thy1.1<sup>+</sup> transgenic P14 CD8 T cells isolated from spleen were transferred i.v. into C57BL/6 recipients. The next day, mice were infected intranasally with 500 pfu of recombinant influenza virus that expresses gp33 in a total volume of 30  $\mu$ l. Alternatively, 200  $\mu$ g DNA was administered intramuscularly into both anterior tibialis muscles in a total volume of 100  $\mu$ l of sterile phosphate-buffered saline. The DNA plasmid used in these experiments expresses the glycoprotein of LCMV under control of the CMV promoter (pCMV-GPm; gift from J.L. Whitton [Scripps research Institute, La Jolla, CA] and H. Ertl [Wistar Institute, Philadelphia, Pennsylvania]). DNA stocks were prepared using the endotoxin-free gigaprep kit (QIAGEN).

For secondary infections, splenocytes from LCMV-immune C57BL/6J mice were transferred to CD45.1 mice (Fig. 2 F) or splenocytes from LCMV P14 immune chimeras (generated as described in the previous paragraph) were transferred to naive C57BL/6J mice (Fig. 2, A–E). Recipients were challenged the next day with  $2 \times 10^6$  pfu LCMV Arm i.v.,  $2 \times 10^6$  pfu LCMV CI-13 i.v.,  $2 \times 10^3$  cfu LM-gp33 i.v., or  $2 \times 10^3$  LM-WT i.v. LM-gp33 was provided by H. Shen (University of Pennsylvania School of Medicine, Philadelphia, PA).

**In vivo migration assays.** Before transfer, donor cells were positively selected for CD8 expression by magnetic separation (>95% purity), and total cells transferred (into C57BL/6J mice by tail vein injection) were normalized between groups via addition of splenocytes isolated from naive nontransgenic mice (Figs. 1 and 4). The next day (12–18 h later, depending on the experiment) recipient mice were perfused and lymphocytes were harvested from a variety of tissues (Masopust et al., 2004). In Fig. 4, donor lymphocytes were derived from spleen, iLN, or mLN as indicated. IEL were isolated as follows: small intestine was removed, Peyer's patches were dissected, and the intestines were cut longitudinally and then into 1-cm pieces. Gut pieces were incubated with 15.4 mg/ml DTE in 10% HBSS/Hepes Bicarbonate (30 min at 37°C, shaking at 200 rpm). Lymphocytes were purified on a 44/67% percoll gradient (800 g at 20°C for 20 min). For assessing the role of chemokine signaling on migration, after CD8 enrichment, lymphocytes were treated with 25 ng/ml pertussis toxin (R&D Systems) at a concentration of  $15 \times 10^6$  cells/ml for 1 h at 37°C. Control cells were treated equivalently except for the addition of pertussis toxin.

**Identification and phenotyping of mouse donor CD8 T cells.** Recovered mouse cells were stained with fluorochrome-conjugated anti-CD8, -Thy1.1, -CD62L, and - $\alpha$ 4 $\beta$ 7 (BD) or with anti-CCR9 (Pabst et al., 2004), followed by secondary staining with mouse anti-rat IgG (Jackson Immuno-Research Laboratories, Inc.). Anti-CCR9 antibody was provided by R. Forster (Institute for Immunology, Hannover Medical School, Hannover, Germany). Functional P-selectin binding was determined by staining with purified P-selectin-IgG fusion protein (BD) followed by secondary staining with allophycocyanin-conjugated AffiniPure goat anti-human IgG (Fc $\gamma$  fragment specific; Jackson ImmunoResearch Laboratories, Inc.). Cells were analyzed on a FACSCalibur or LSR II (BD).

**FTY720 treatment.** Mice were exposed ad libitum to drinking water containing dissolved FTY720 at a concentration of 2  $\mu$ g/ml.

**Intestinal transplantation.** Heterotopic small intestinal transplantation was performed as previously described (Newell et al., 1997; Wang et al., 2006).

In brief, intestinal grafts were transplanted by anastomosing the graft portal vein and superior mesenteric artery to the recipient inferior vena cava and infrarenal aorta, respectively. A stoma was formed from the proximal graft and the distal graft was anastomosed to the recipient jejunum.

**Study subjects and blood samples.** Blood samples were obtained from healthy volunteers (18–40 yr old) recruited after informed consent in a double-blind controlled trial. The live-attenuated YFV-17D vaccine (YF-Vax) was administered alone or in combination with pooled immune serum globulin. Approval for all procedures was obtained from the Emory University Institutional Review Board. Exclusion criteria for subjects were a previous history of vaccination for or exposure to flaviviruses as indicated by serology, travel to endemic areas, and those recommendations established by the Advisory Committee on Immunization Practices and Vaccination. Samples were analyzed before and at various times after vaccination as indicated in the text. PBMCs were purified from CPT (BD) containers.

**Phenotyping of human YFV-specific CD8 T cells.** HLA2-YFV-specific tetramers conjugated to streptavidin APC were prepared in house and used to stain 200–300  $\mu$ l of whole blood. A 10-min incubation at room temperature was followed by addition of appropriate antibodies and a further 30-min incubation. The antibodies used were anti-CLA, -CD3, -CD8 (BD), or - $\alpha$ 4 $\beta$ 7 (clone ACT-1). Red blood cells were removed by lysing with FACS lysis buffer (BD) before washing cells with PBS. Samples were acquired on an LSR II flow cytometer (BD) and analyzed with FlowJo software (Tree Star, Inc.).

**Online supplemental material.** Fig. S1 shows that migration of early effector CD8 T cells to small intestinal epithelium is chemokine dependent. Fig. S2 shows that early effector CD8 T cells migrate from blood to small intestinal epithelium. Fig. S3 shows that effectors do not elicit bystander migration of memory cells to small intestinal epithelium. Fig. S4 shows that CD8 T cells gradually lose  $\alpha$ 4 $\beta$ 7 expression after entry into small intestinal epithelium. Fig. S5 shows transient coexpression of functional P-selectin binding ligand and  $\alpha$ 4 $\beta$ 7 among effector splenocytes. Online supplemental material is available at <http://www.jem.org/cgi/content/full/jem.20090858/DC1>.

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