The Journal of Immunology

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J Immunol 2006; 176:2079-2083; ;
doi: 10.4049/jimmunol.176.4.2079
http://www.jimmunol.org/content/176/4/2079

The Journal of Immunology is published twice each month by
The American Association of Immunologists, Inc.,
1451 Rockville Pike, Suite 650, Rockville, MD 20852

Print ISSN: 0022-1767 Online ISSN: 1550-6606.

THE JOURNAL OF IMMUNOLOGY

CUTTING EDGE

Cutting Edge: Gut Microenvironment Promotes Differentiation of a Unique Memory CD8 T Cell Population

David Masopust,[1] Vaiva Vezys,[†] E. John Wherry,[‡] Daniel L. Barber,[*] and Rafi Ahmed[1]**

Whether tissue microenvironment influences memory CD8 T cell differentiation is unclear. We demonstrate that virus-specific intraepithelial lymphocytes in gut resemble neither central nor effector memory CD8 T cells isolated from spleen or blood. This unique phenotype arises in situ within the gut, suggesting that anatomic location plays an inductive role in the memory differentiation program. In support of this hypothesis, memory CD8 T cells changed phenotype upon change in location. After transfer and in vivo restimulation, gut or spleen memory cells proliferated, disseminated into spleen and gut, and adopted the memory T cell phenotype characteristic of their new environment. Our data suggests that anatomic location directly impacts the memory T cell differentiation program. *The Journal of Immunology,* **2006, 176: 2079–2083.**

The gut must solve the problem of maintaining a large surface area for nutrient absorption in the face of chronic exposure to microbes (1). Recent studies have demonstrated that pathogen-specific CD8 T cells persist within the intraepithelial compartment of the intestinal mucosa (intraepithelial lymphocytes; IEL)[2] following the clearance of infection (2–5). Because most pathogens infect mammalian hosts through mucosal surfaces, maintenance of immunological memory within the gut might provide a first line of defense against reinfection (6, 7). Indeed, the development of cellular mucosal memory may be requisite for successful vaccination against certain pathogens, such as HIV (8–12).

Current understanding of memory CD8 T cell differentiation is derived mostly from analyses of spleen and blood. These studies have identified two subsets of memory CD8 T cells, most often delineated in mice by expression of the lymph node homing receptor CD62L (13, 14). Central memory CD8 T cells (T_{CM}) express CD62L, traffic through spleen, blood, and lymph nodes, and comprise a long-lived population of memory cells with high proliferative capacity upon reinfection (15). Effector memory CD8 T cells (T_{EM}) lack CD62L, are present in spleen and blood, and putatively recirculate through the parenchyma of nonlymphoid tissues (16). The distribution of cellular memory into T_{CM} and T_{EM} has important consequences for immunity, and the factors that govern this fate decision are of major interest (14–20).

It is unclear whether memory IEL fit neatly into the T_{CM}/T_{EM} dichotomy that has been proposed for recirculating memory CD8 T cells. Recent data suggest that memory CD8 T cells do not continuously recirculate between the gut and other anatomical compartments (21). The intestinal mucosa differs from other tissues in cytokine milieu, the quantity and quality of leukocyte populations, and microbial exposure; properties that may impact CD8 T cell differentiation (6, 7). Historical data as well as serial analysis of gene expression among bulk CD8$\alpha\beta$ TCR$\alpha\beta$ IELs has suggested that many retain an activated phenotype, although it is unclear whether recent or persistent stimulation through the TCR maintains this phenotype (6, 7, 22, 23). Only recently have Ag-specific IEL been analyzed after clearance of Ag (2–5). Surprisingly, virus-specific IEL retain cytolytic activity and CD69 expression (markers of recent activation) long after Ag clearance (2, 4, 21). Otherwise, relatively little is known about how virus-specific memory IEL compare with the recirculating T_{CM} and T_{EM} subsets described previously. Therefore, we compared function, expression of differentiation markers, homeostatic division, proliferative potential, and phenotypic plasticity among virus-specific CD8 T cells derived from spleen and the small intestinal mucosa.

Materials and Methods

Analysis of lymphocytic choriomeningitis virus (LCMV)-specific CD8 T cells

Thy1.1$^+$ (B6.PL-Thy1^a/CyJ) mice were bred to P14 transgenic mice (24) and maintained in our colony. Splenocytes (5×10^5) from naive Thy1.1$^+$ transgenic P14 mice were transferred i.v. into C57BL/6J recipients (The Jackson Laboratory). The following day, recipients were infected i.p. with 2×10^5 PFU LCMV (Armstrong strain). At various times postinfection, cells were recovered from spleen and the intestinal mucosa. Single-cell suspensions were stained with anti-CD8α, anti-CD8β, Thy1.1, CD69, Ly6C, CD62L, 1B11, CD27, IL-15Rβ, IL-7Rα, $\alpha_4\beta_7$, CD103, and β_7 Abs (directly conjugated to FITC,

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Received for publication October 11, 2005. Accepted for publication December 30, 2005.

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[2] Abbreviations used in this paper: IEL, intraepithelial lymphocyte; LCMV, lymphocytic choriomeningitis virus; GMFI, geometric mean fluorescence intensity.

0022-1767/06/$02.00

PE, PerCP, or allophycocyanin). Intracellular staining for granzyme B and Bcl2 (directly ex vivo), or IFN-γ, TNF-α, and IL-2 (after 5-h stimulation with 0.1 μg/ml gp33 peptide) was performed using the Cytofix/cytoperm kit in accordance with the manufacturer's directions (BD Pharmingen). All staining reagents were purchased from BD Pharmingen, with the exception of anti-human granzyme B (Caltag Laboratories). Samples were analyzed on a FACSCalibur flow cytometer (BD Pharmingen).

Isolation of lymphocytes

Lymphocytes were isolated as described previously (4). In brief, mice were perfused with PBS, and livers were homogenized through a 100-μm filter (Falcon) in 5% RPMI 1640. Lungs were treated with 1.3 mM EDTA in HBSS (30 min/37°C, shaking at 200 rpm) followed by treatment with 100 U/ml collagenase (Invitrogen Life Technologies) in 5% RPMI 1640/2 mM $MgCl_2$/2 mM $CaCl_2$ (45 min/37°C, shaking at 200 rpm). IEL were isolated as follows; small intestine was removed, Peyer's patches dissected, and the intestines were cut longitudinally, and then into 1-cm pieces. Gut pieces were incubated with 15.4 mg/ml dithioerythritol in 10% 1× HBSS/HEPES bicarbonate (30 min/37°C, shaking at 200 rpm) to remove IEL. To isolate lamina propria (LP), gut pieces were then treated with EDTA followed by collagenase treatment (as in isolation of lymphocytes from lung). Lymphocytes from liver, lung, and gut were purified on a 44/67% Percoll gradient (800 × *g* at 20°C for 20 min).

In vivo proliferation assays

For analysis of homeostatic proliferation, mice were fed BrdU (0.8 mg/ml in drinking water) for 8 days, beginning 85 days postinfection. Lymphocytes were isolated from spleen and IEL and stained using the BrdU staining kit (BD Pharmingen) according to the manufacturer's instructions. For analysis of in vivo proliferation potential following restimulation, lymphocytes were isolated from spleen and IEL 160 days postinfection. $CD8^+$ lymphocytes were purified via anti-CD8α magnetic beads according to the manufacturer's instructions (Miltenyi Biotec). LCMV (2×10^4)-specific P14 isolated from spleen or IEL were transferred into naive mice. Recipients were challenged with 2×10^5 PFU LCMV Armstrong i.p. the following day.

Results

Virus-specific memory CD8 T cells in spleen and intestinal mucosa differ in phenotype, function, and homeostatic proliferation

We transferred naive P14 transgenic CD8 T splenocytes, specific for an immunodominant epitope of LCMV, to recipient mice, and then infected the mice with LCMV. Use of transgenic CD8 T cells allowed us to track a monoclonal homogenous starting population at all stages of the response, including early during clonal expansion.

Infection induced a robust CD8 T cell response that resulted in a long-term stable memory population within spleen and small intestinal mucosa (Fig. 1*A*). Consistent with previous reports (25), LCMV was undetectable in spleen and gut by 8 days postinfection (data not shown). P14 IEL remained $CD8\beta^+$ at all time points (Fig. 1*B*). We compared the phenotypic and functional properties of memory cells within each compartment 2–8 mo postinfection. IEL memory CD8 T cells differed from those in spleen by almost all properties examined, including effector function, differentiation state, homing receptors, and cell cycle (Fig. 1, *C*–*G*). For example, memory IEL constitutively expressed granzyme B, an important component of the cytolytic machinery, as well as 1B11 and CD69, markers that are typically associated with an effector rather than memory differentiation state (26). In contrast to splenocytes, IEL remained $CD27^{low}$ and never regained CD62L expression (as late as 250 days postinfection; data not shown). IEL also differed from

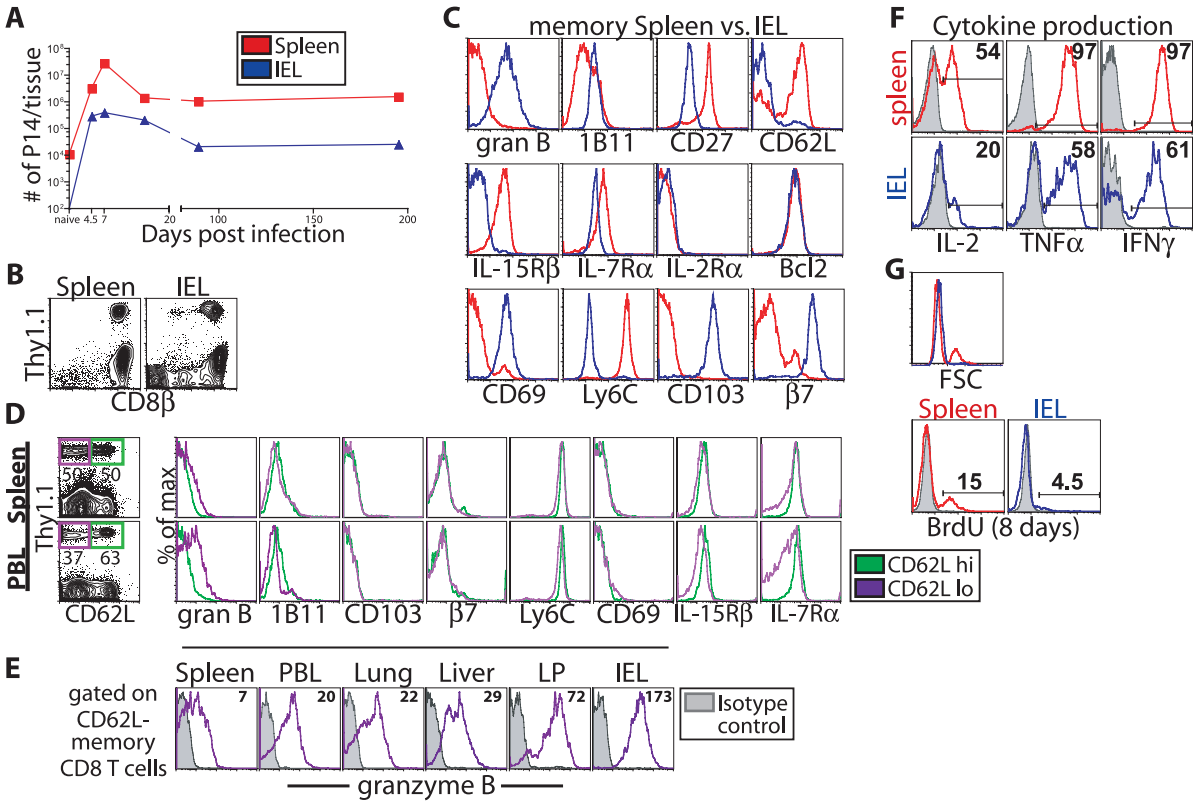

FIGURE 1. Virus-specific memory CD8 T cells in spleen and intestinal mucosa differ in phenotype, function, and homeostatic proliferation. *A*, Kinetics of H-$2D^b$-gp33-specific CD8 T cell (P14) response within spleen and IEL following LCMV infection. *B*, CD8β expression 85 days postinfection (gated on $CD8\alpha^+$ lymphocytes). *C*, Phenotype in spleen (red) and IEL (blue) 85 days postinfection (gated on P14 lymphocytes). *D*, Phenotype of $CD62L^+$ (green) and $CD62L^-$ (purple) P14 isolated from spleen and blood 60 days postinfection. *E*, Granzyme B expression among $CD62L^-$ P14 isolated from the indicated tissues 81 days postinfection. Geometric mean of fluorescence intensity is indicated. Gray histograms indicate isotype control Ab staining. *F*, Intracellular cytokine staining 85 days postinfection (gated on P14). Gray histograms indicate unstimulated controls. *G*, Cell cycle was measured by cell size (forward scatter; FSC) and BrdU incorporation 85 days postinfection (gated on P14). Gray histograms indicate anti-BrdU staining on spleen and IEL P14 from mice that were not fed BrdU.

canonical memory cells with respect to expression of Ly6C, and integrins CD103, and β_7. Similar data were observed following analyses of endogenous vesicular stomatitis virus-specific memory CD8 T cells (data not shown), suggesting that these phenotypes were a general feature of gut memory, and not unique to transgenic CD8 T cells nor LCMV infection.

In mice, T_{CM} and T_{EM} subsets are most routinely delineated by CD62L expression (15, 19, 20, 27). We examined the phenotype of both the $CD62L^-$ and $CD62L^+$ memory subsets isolated from spleen and blood (Fig. 1*D*), and neither subset was comparable to mucosal memory CD8 T cells (Fig. 1*C*). We also compared granzyme B expression among $CD62L^-$ memory P14 isolated from spleen, blood, lung, liver, IEL, and the lamina propria of the small intestinal mucosa (Fig. 1*E*). $CD62L^-$ memory CD8 T cells varied considerably within different anatomical compartments, with IEL's expressing the most granzyme B (geometric mean fluorescence intensities (GMFI) = 173 ± 25) and spleen T_{EM} expressing the least (GMFI = 7.3 ± 0.3).

In contrast to memory CD8 T splenocytes, relatively few IEL made IL-2 upon antigenic restimulation. Virtually all spleen T_{CM} and T_{EM} produced the proinflammatory and antiviral cytokines TNF-α and IFN-γ (Fig. 1*F* and Ref. 13). In contrast, only a portion of IEL memory cells produced these cytokines (Fig. 1*F*).

Memory CD8 T cells undergo a modest level of Ag-independent division, termed homeostatic proliferation. This process is mediated by IL-15 and is required for memory T cell maintenance (28). As indicated by forward scatter, IEL are slightly larger, consistent with the fact that they contain lytic granules (Fig. 1*G*). However, IEL contained very few actively dividing cells. We confirmed this by adding BrdU to the drinking water of LCMV immune mice for 8 days. Dividing cells incorporate BrdU into their DNA, which can be detected by flow cytometry. As seen in Fig. 1*G*, LCMV-specific IEL were undergoing a much slower rate of division than LCMV-specific memory T cells present in spleen. As shown in Fig. 1*C*, IEL memory cells lacked expression of IL-15Rβ, which may explain their failure to undergo homeostatic division. IL-7 and IL-2 are other cytokines that have been implicated in lymphocyte survival, and may explain the persistence of IEL in the absence of functional IL-15R (28). However, IEL memory cells expressed lower levels of IL-7Rα than conventional memory CD8 T cells and did not express IL-2Rα (Fig. 1*C*). However, it should be noted that IEL memory cells expressed high levels of the antiapoptotic molecule Bcl2, a marker typically associated with long-lived resting memory CD8 T cells (26). These data suggest that IEL may depend on unique factors for survival.

Phenotypic changes gradually occur within gut

The data presented in Fig. 1 describe several biological properties of memory CD8 T cells that have previously been described in spleen, but not among virus-specific IEL. These data demonstrate that IEL undergo a unique and previously uncharacterized differentiation program, which results in a novel population of memory CD8 T cells. It remained unclear whether phenotypic differences were evident before migration into the intestinal mucosa or whether they developed in situ. As shown in Fig. 2, the phenotype of LCMV-specific CD8 T cells was similar in both locations early after infection. For instance, 4.5 days after infection both populations expressed similar levels of granzyme B, CD62L, CD69, Ly6C, CD103, and β_7. However, phenotypic differences between cells isolated from spleen and IEL accrued gradually throughout the course of the response. As early as 7 days postinfection, IEL had begun to down-regulate Ly6C, and up-regulate CD103 and CD69. Eighty-five days

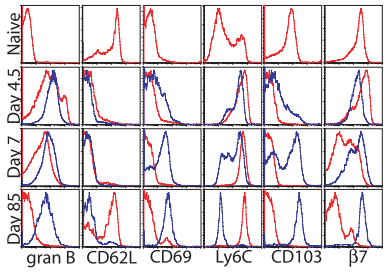

FIGURE 2. Phenotypic changes occur gradually in gut. P14 CD8 T cells isolated from spleen (red) and IEL (blue) were stained with the indicated Abs 0, 4.5, 7, or 85 days post-LCMV infection.

postinfection, spleen and IEL phenotype was even more distinct. These data favor the interpretation that IEL gradually adopt a unique differentiation program in situ within the intestinal mucosa.

Proliferation, homing, and phenotypic changes among splenic and IEL memory CD8 T cells after in vivo challenge

The phenotype of virus-specific IEL suggested that they might be terminally differentiated. To compare the proliferative potential of memory cells isolated from IEL vs spleen, we transferred 20,000 LCMV-specific memory cells isolated from each tissue to naive mice. The following day, recipient mice were challenged with LCMV, and the ensuing recall CD8 T cell response was monitored in blood. As shown in Fig. 3, IEL mounted a significant secondary response (resulting in approximately a 100-fold expansion), although their proliferative capacity was 8- to 20-fold less (among four different experiments) than that of memory CD8 T cells isolated from spleen. This secondary response was not due to the presence of a few contaminating conventional memory CD8 T cells within our IEL preparation. For example, $Ly6C^{low}$ cells (IEL phenotype) were recruited, and only gradually up-regulated Ly6C throughout the secondary response (Fig. 3*B*). Although the difference in expansion between spleen and IEL-derived memory cells was evident in recipient spleen and lung as well as blood, relatively

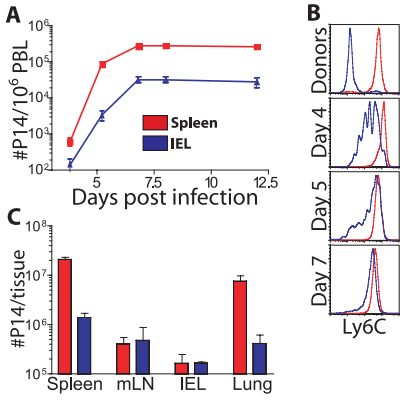

FIGURE 3. Proliferation and homing potential of splenic and IEL memory CD8 T cells upon in vivo challenge. *A*, Twenty thousand day 160 memory P14 isolated from spleen (red) or IEL (blue) were transferred i.v. to naive recipients. The following day, recipient mice were challenged with LCMV, and the frequency of P14 was monitored among peripheral blood lymphocytes (PBL). *B*, Expression of Ly6C on spleen and IEL donor cells in PBL following infection. *C*, Recovery of spleen (red) and IEL (blue) donors from various tissues 7 days following infection.

similar numbers of effectors were isolated from IEL and mesenteric lymph node (Fig. 3*C*). This result underscores that both spleen and IEL memory cells have the intrinsic capacity to disseminate throughout the organism upon reactivation, yet demonstrates that some differences in homing capacity may be epigenetically maintained.

We then examined the phenotype of splenocytes and IEL that had populated the spleen and IEL compartments upon secondary infection (Fig. 4*A*). Upon restimulation, the fraction of spleen-derived memory cells that disseminated into the gut gradually down-regulated Ly6C. Conversely, IEL up-regulated and maintained expression of Ly6C upon reactivation and localization to the spleen. In other words, both spleen and IEL memory populations largely adopted the phenotypic characteristics of their environment upon reactivation. Some markers, such as CD103, were more resistant to change. However, restimulated IEL that were isolated from spleen eventually adopted a spleen-like phenotype by all criteria tested (Fig. 4*B*). These data demonstrated that the phenotype of memory cells from different compartments was malleable, i.e., could be reprogrammed upon secondary activation, and was directly associated with anatomic location.

Discussion

In summary, we demonstrate that memory CD8 T cells residing within the intestinal mucosa differ from their clonotypic counterparts within spleen with regards to phenotype, function, cell cycle, and cytokine receptor expression (Fig. 1). In other words, memory IEL bear little resemblance to recirculating T_{CM} and T_{EM} and do not ascribe to the cardinal properties of CD8 T cell memory described previously.

Considerable attention has been paid to defining the specification events underlying the development of T cell memory subsets. Evidence suggests that developmental cues within lymphoid tissue, such as the degree and duration of TCR stimulation, impacts the progression to T_{CM} and T_{EM} subsets (15, 26, 29, 30). However, additional signals may contribute to memory CD8 T cell differentiation following emigration from secondary lymphoid tissue.

Memory phenotype is coupled to anatomic location (Fig. 1). We extend this observation by showing that acquisition of the IEL memory phenotype occurred gradually in situ within the gut (Fig. 2). In fact, following restimulation and migration, memory CD8 T cells from both spleen and gut gradually converted to a phenotype more characteristic of their new location (Fig. 4). For instance, IEL remained $CD62L^{-}$ indefinitely within the mucosa, but could be reprogrammed to form T_{CM} following restimulation and memory differentiation within spleen (Fig. 4). Based on these data, we propose that the tissue environment influences the memory CD8 T cell differentiation program. Indeed, the process of induction contributes to developmental programming in many cell types. During embryogenesis, neighboring cells influence cell fate decisions. In the adult organism stromal cells instruct stem cell differentiation, and location changes may even promote transdifferentiation from one cell type to another (31). It is unclear how the gut microenvironment could influence memory CD8 T cell differentiation. Preliminary evidence from our laboratory demonstrates that TGF-β, a cytokine that is highly expressed within the gut, induces CD103 expression among effector splenocytes in vitro (data not shown). Gut epithelial cells condition mucosal dendritic cells to acquire a noninflammatory phenotype (32), and future experiments will test their role in modulating CD8 T cell differentiation. Other nonlymphoid tissues may also affect T cell phenotype. For instance, the peritoneum and lung environments may influence T cell expression of α_4 and CD11a, respectively (33, 34). Perhaps several factors within distinct anatomical compartments contribute to unique memory T cell differentiation programs within each tissue.

It is tempting to speculate that the particular phenotype of mucosal memory cells has evolved to strike a balance between achieving immunological protection without compromising organ integrity. Specifically, the reduced ability of IEL memory cells to proliferate and produce inflammatory cytokines (Figs. 1 and 3) may limit immunopathology upon local Ag re-exposure. However, maintenance of a heightened cytolytic state may provide a greater degree of protection at the mucosal surface. In conclusion, these data argue that location within the gut specifies a population of memory CD8 T cells that do not conform to the cardinal properties of previously defined memory T cells. Thus, models of memory CD8 T cell differentiation based solely on analysis of recirculating lymphocyte populations will fail to capture the full complexity of cellular immunity. Given the prevalence of the gut as a site of infection, delineating the relationship between IEL phenotype and their role in immunological protection is of considerable importance.

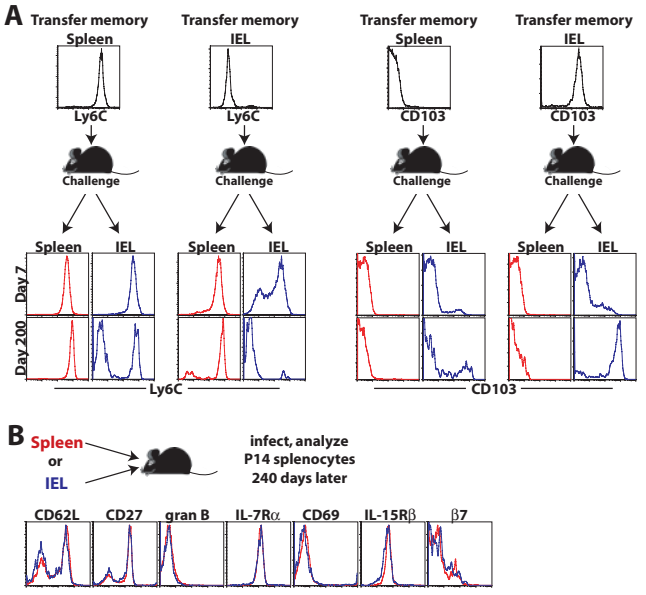

FIGURE 4. Spleen and IEL memory cells are reprogrammable and convert phenotype following transfer, restimulation, and migration to spleen or IEL. *A*, Expression of Ly6C and CD103 on spleen and IEL P14 donors recovered from spleen (red) and IEL (blue) following LCMV challenge. *B*, Expression of various phenotypic markers on spleen (red) and IEL (blue) P14 donors recovered from spleen 240 days following transfer and LCMV challenge.

Disclosures

The authors have no financial conflict of interest.

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