Antigen-Independent Differentiation and Maintenance of Effector-like Resident Memory T Cells in Tissues

Kerry A. Casey, Kathryn A. Fraser, Jason M. Schenkel, Amy Moran, Michael C. Abt, Lalit K. Beura, Philip J. Lucas, David Artis, E. John Wherry, Kristin Hogquist, Vaiva Vezys and David Masopust

*J Immunol* 2012; 188:4866-4875; Prepublished online 13 April 2012; doi: 10.4049/jimmunol.1200402

http://www.jimmunol.org/content/188/10/4866

---

**References**

This article cites 53 articles, 24 of which you can access for free at: http://www.jimmunol.org/content/188/10/4866.full#ref-list-1

---

**Why The JI?** Submit online.

- **Rapid Reviews! 30 days** from submission to initial decision
- **No Triage!** Every submission reviewed by practicing scientists
- **Fast Publication!** 4 weeks from acceptance to publication

*average

---

**Subscription**

Information about subscribing to *The Journal of Immunology* is online at: http://jimmunol.org/subscription

**Permissions**

Submit copyright permission requests at: http://www.aai.org/About/Publications/JI/copyright.html

**Email Alerts**

Receive free email-alerts when new articles cite this article. Sign up at: http://jimmunol.org/alerts
Antigen-Independent Differentiation and Maintenance of Effector-like Resident Memory T Cells in Tissues

Kerry A. Casey,*†§,1 Kathryn A. Fraser,*†§,1 Jason M. Schenkel,*†§,1 Amy Moran,† Michael C. Abt,‡‡ Lalit K. Beura,*† Philip J. Lucas,‡ David Artis,‡ E. John Wherry,‡ Kristin Hogquist † Vaiva Vezys,*† and David Masopust*†

Differentiation and maintenance of recirculating effector memory CD8 T cells (TEM) depends on prolonged cognate Ag stimulation. Whether similar pathways of differentiation exist for recently identified tissue-resident effector memory T cells (TRM), which contribute to rapid local protection upon pathogen re-exposure, is unknown. Memory CD8αβ+ T cells within small intestine epithelium are well-characterized examples of TRM, and they maintain a long-lived effector-like phenotype that is highly suggestive of persistent Ag stimulation. This study sought to define the sources and requirements for prolonged Ag stimulation in programming this differentiation state, including local stimulation via cognate or cross-reactive Ags derived from pathogens, microbial flora, or dietary proteins. Contrary to expectations, we found that prolonged cognate Ag stimulation was dispensable for intestinal TRM ontogeny. In fact, chronic antigenic stimulation skewed differentiation away from the canonical intestinal T cell phenotype. Resident memory signatures, CD69 and CD103, were expressed in many nonlymphoid tissues including intestine, stomach, kidney, reproductive tract, pancreas, brain, heart, and salivary gland and could be driven by cytokines. Moreover, TGF-β-driven CD103 expression was required for TRM maintenance within intestinal epithelium in vivo. Thus, induction and maintenance of long-lived effector-like intestinal TRM differed from classic models of TEM ontogeny and were programmed through a novel location-dependent pathway that was required for the persistence of local immunological memory. The Journal of Immunology, 2012, 188: 4866–4875.

Effectors memory CD8 T cells (TEM) comprise a heterogeneous population that is excluded from lymph node entry through high endothelial venules due to the absence of CD62L or CCR7 expression (1, 2). Numerous studies have related memory T cell quality and homing potential to protective immunity, thus driving efforts to understand the regulation of memory T cell differentiation state. The regulation of TEM ontogeny has been extensively studied on recirculating TEM populations isolated from blood, the red pulp of spleen, or nonlymphoid organs such as the liver. This work has revealed a central role for prolonged or potent cognate Ag stimulation in the programming of TEM (3). For instance, acute infections that are characterized by repetitive or extended durations of Ag stimulation favor TEM differentiation (4–7). In this context, recirculating TEM may be transient and either convert to central memory T cells (TCM) or die weeks to months after Ag clearance (8–11). In contrast, persistent infections that result in repetitive Ag exposure result in recirculating TEM that may be maintained indefinitely at the population level, although maintenance may be dependent upon Ag persistence (10, 12).

Recent work has revealed the existence of stable populations of TEM that are nonrecirculating within intestinal epithelium, the CNS, the skin epidermis, and potentially the salivary gland and a subset of cells within the respiratory mucosa (13–21). This subset of TEM has been designated tissue-resident effector memory T cells (TRM). TRM populations may be very long-lived; for example, mouse and non-human primate studies reported minimal decay over ~1–2 y (11, 22). In addition to lacking CD62L expression, TRM populations are characterized by CD69 expression and typically CD103 expression (15, 16, 18, 19, 23). CD69 expression is often interpreted as a surrogate for recent TCR engagement, and recent studies have proposed that CD103 expression by TRM is induced or maintained by local cognate Ag (15, 19). CD8αβ+ TRM that reside within the epithelium of the small intestine (intraepithelial lymphocytes [IELs]) have been particularly well characterized and uniformly bear numerous other indications of recent or even ongoing activation, including constitutive retention of lytic function and impaired proliferation potential, and appear similar to CD8 T cells in spleen that are specific for chronic viral infections (23–26). This phenotype is compatible with Ag-driven models of TEM differentiation, although the potential source of Ag is unclear (27–31). One explanation is that the intestine-specific differentiation state is regulated by continued local stimulation via cognate or cross-reactive Ags derived from pathogens, microbial flora, or dietary proteins. For instance, the human gut lumen is colonized with up to 1014 microbes, and the average American consumes >30 kg of protein per year; thus, intestinal T cells might be exposed to a tremendous diversity of...
antigenic peptides (32, 33). Reports indicate that IELs are reduced in number and exhibit a restricted TCRβ repertoire when raised under germ-free conditions (34, 35) or ectopically transplanted and rendered free from luminal Ags (36). Mice reared on an Ag-minimized diet also exhibit reductions in TCRαβ small intestine (SI) IELs, suggesting that bacterial and food-derived Ags influence the intestinal immune system (37). When splenocytes are transferred to SCID mice, CD8 T cells populate the small intestinal epithelium and acquire effector-like properties, which was interpreted to add additional support to the hypothesis that IELs are undergoing a response to luminal Ags (38). Likewise, radio-labeling experiments indicated that blasting, not resting, thoracic duct lymphocytes migrated most efficiently to the intestinal mucosa (39). Collectively, these observations are consistent with the hypothesis that recent or constitutive stimulation by Ag may contribute to the activated-like differentiation state of SI IELs.

This study used a variety of complementary approaches to test the requirement for prolonged Ag stimulation in programming or maintaining TRM within the intestine. In contrast to expectations, we found that prolonged cognate Ag stimulation was dispensable for TRM differentiation among intestinal IELs and provide evidence that the characteristic CD103+/CD69+ TRM phenotype was present among a subset of TEM distributed within numerous host tissues, including pancreas, stomach, kidney, and heart. In contrast to classical models of recirculating TEM ontogeny, these findings support the hypothesis that TRM differentiation and maintenance is dependent on tissue microenvironment rather than prolonged Ag stimulation.

Materials and Methods

Mice and infections

All mice were used in accordance with the guidelines of the Institutional Animal Care and Use Committees at the University of Minnesota or the University of Pennsylvania. For assessment of lymphocytic choriomeningitis virus (LCMV)-specific P14 responses, splenocytes containing 9 × 10^6 naive Thy1.1+ transgenic P14 CD8 T cells were isolated from naive Thy1.1+ P14 transgenic mice (40) maintained in our colony and transferred i.v. into C57BL/6J recipients. The following day, recipient mice were infected with 2 × 10^7 PFU LCMV Armstrong i.p. Where indicated, C57BL/6J mice were infected with 2 × 10^7 PFU LCMV Armstrong i.p. or 2 × 10^5 PFU LCMV clone 13 i.v. without transfer of P14 CD8 T cells.

To assess the role of TGF-β in the adoption of canonical IEL phenotype, 5 × 10^5 wild-type (WT) and 5 × 10^5 dnTGFβRII OT-I cells (41, 42) were cotransferred into naive C57BL/6J recipients to be infected the following day with 1 × 10^6 PFU recombinant vesicular stomatitis virus expressing chicken ovalbumin (VSVova) i.v. Alternatively, 5 × 10^5 WT and 5 × 10^5 dnTGFβRII P14 cells were cotransferred into naive C57BL/6J recipients to be infected the following day with 2 × 10^5 PFU LCMV (Armstrong strain) i.p.

To assess the role of CD103 in the generation and maintenance of IEL memory, 5 × 10^6 WT and 5 × 10^6 CD103-deficient P14 cells were cotransferred into naive sex-matched C57BL/6J recipients (43). The following day, recipient mice were infected with 2 × 10^5 PFU LCMV (Armstrong strain) i.p. For histological analysis, experiments were carried out in a similar fashion, however WT and CD103-deficient P14 cells were transferred into separate recipients.

To assess the role of Ag in expression of the IEL phenotype, we used transgenic mice expressing GFP from the gene Nrl41 (Nur77) locus (44). For anti-CD3 stimulation, Nur77 mice were given 50 μg of anti-CD3 Ab i.v. and analyzed 16 h later. Alternatively, OT-I were transferred into 232-4 transgenic mice, which express OVA under the control of the IFABP promoter (45).

For assessment of phenotype in RAG−/− mice, 1 × 10^5 Thy1.1+ P14 or CD45.1+ OT-I cells (41, 42) were transfected into conventionally housed or germ-free C57BL/6J mice, as indicated. Experiments involving germ-free mice were performed at the University of Pennsylvania using mice obtained from the Penn Gnotobiotic Mouse Facility. Germ-free mice were sterilizedly maintained in plastic isolator units and fed autoclaved LabDiet5021 mouse chow (LabDiet) and autoclaved water.

Isolation and phenotyping of donor CD8 T cells

For isolation of SI IELs, the small intestine was removed, Peyer’s patches were excised, and the intestine was cut longitudinally and then laterally

FIGURE 1. Virus-specific CD8αβ T cells adopt a recently stimulated phenotype after migration to SI epithelium. (A) Phenotype of gp33-specific Thy1.1+ P14 CD8 T cells in spleen and SI IELs on the indicated days after LCMV infection. All plots are gated on Thy1.1+ lymphocytes. (B) Kinetics of CD62L expression among P14 in spleen and SI IELs after LCMV infection. Error bars indicate SEM; n = 5 per time point. ***p < 0.001 (Student unpaired t test). (C) Phenotype of gp33-specific P14 CD8 T cells (top row is spleen, bottom row is SI IEL) 37 d after LCMV Armstrong infection. Phenotypes are representative of >10 mice. (D and E) Mice were infected with the Armstrong or CI-13 strains of LCMV, and 35 d later, the phenotype of H-2Dα/gp33 MHC class I tetramer+ splenocytes was compared. (D) Gated on lymphocytes and (E) gated on CD8+ H-2Dα/gp33 MHC class I tetramer+ lymphocytes.
into 1-cm pieces. Gut pieces and stomachs were incubated with 15.4 mg/ml dithioerythritol in 10% HBSS/HEPES bicarbonate (30 min at 37°C, stirring at 200 rpm) to remove IELs. For lamina propria lymphocyte (LPL) isolation, gut and stomach pieces were further treated with 100 U/ml type I collagenase (Worthington) in 5% RPMI 1640/2 mM MgCl₂/2 mM CaCl₂ (45 min at 37°C, stirring at 200 rpm). Brains, pancreata, hearts, and kidneys were resected and cut into small pieces prior to treatment with 100 U/ml type I collagenase (Worthington) in 5% RPMI 1640/2 mM MgCl₂/2 mM CaCl₂ (45 min at 37°C, stirring at 200 rpm). For isolation of the female reproductive tract, the uterine horns, cervix, and vaginal tissue were resected and cut into small pieces prior to treatment with 100 U/ml type IV collagenase (Sigma) 5% RPMI 1640/2 mM MgCl₂/2 mM CaCl₂ (45 min at 37°C, stirring at 200 rpm). Lymphocytes from intestine, stomach, brain, and female reproductive tract were purified on a 44/67% Percoll gradient (800 x g at 23°C for 20 min).

In vitro stimulation assays. The medium used for all cell cultures was RPMI 1640 supplemented with 10% FCS, 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, 0.25 mg/ml Amphotericin B, and 50 μM 2-mercaptoethanol (RP-10). Single-cell splenic suspensions from intact naive P14, day 4.5 or memory P14/B6 chimeras were placed in flat-bottom microtiter wells in 200 μl of RP-10 at 7 x 10⁵ cells/well. Where indicated, cultures were supplemented with the following cytokines: human TGF-β1 at 10 ng/ml, mouse IL-23 and IL-33 at 100 ng/ml (R&D Systems); murine type I IFN at 5000 U/ml (PBL Biomedical Laboratories); mouse TNF-α at 125 ng/ml, mouse IL-6 and IL-1β at 100 ng/ml, and IFN-γ at 2000 U/ml (eBioscience). Where indicated, gp 33–41 peptide was added at 0.1 mg/ml. Cells were then incubated for 40–60 h as described and stained with Live/Dead Fixable Aqua per the manufacturer’s directions (Invitrogen) prior to surface staining to exclude dead cells from the analysis.

Cryopreservation, immunofluorescence, and microscopy. Tissues were snap frozen in OCT, cut to 7- to 8-μm thicknesses, and fixed for 20 min in acetone. Sections were then stained with directly conjugated or Ab pairs purchased from BioLegend, Invitrogen, or Acris and counterstained with DAPI. Immunofluorescence microscopy was performed using a Leica DM5500 B microscope. Separate images taken with a 20X objective were collected for each channel and overlaid to obtain a multicolor image. Final image processing was performed using Fiji and Adobe Photoshop software.

In situ quantification of virus-specific donor CD8 T cells and nucleated cells. P14 cells were identified as Thy1.1+ single-positive cell-shaped events of lymphocyte size surrounding a demarcated DAPI-positive nucleus. P14 cells containing nuclei on the luminal side or lamina propria side of the collagen IV + basement membrane were assigned as IEL or LPL, respectively. E-cadherin and collagen IV staining was used to confirm the luminal side of the intestinal basement membrane. Raw TIFF files were imported into Fiji, background was subtracted using a 10-μm rolling ball radius, and DAPI-filled nuclei were enumerated using the find maxima function over an average of three threshold settings empirically set to reproduce with best fidelity hand-counted results within a series of spot- 4868 TISSUES PROGRAM MEMORY T CELL DIFFERENTIATION

![FIGURE 2](http://www.jimmunol.org/) Induction and maintenance of activated phenotype is Ag independent. (A) Naive CD45.1+ OT-I/RAG-2/- CD8 T cells were transferred to RAG-2/- C57BL/6 mice. Sixty days later, OT-I were isolated from spleen and SI IELs and analyzed for the indicated markers (plots gated on CD45.1+ lymphocytes). One representative of five mice; experiment was performed three times. (B) OT-I were transferred to conventionally housed specific pathogen-free (SPF) or germ-free RAG-2/- mice. Twenty-seven days later, spleen and SI IELs were harvested and analyzed for the indicated markers (plots gated on H-2Kb/SIINFEKL tetramer+ lymphocytes). One representative of five mice. (C) PD-1 expression among transferred P14 isolated from spleen and SI IELs 2 mo after LCMV Armstrong infection. One representative of three mice per experiment; experiment was performed twice. (D) Polyclonal CD8αβ+ lymphocytes were isolated from spleen and SI IEL of Nur77–GFP transgenic mice or C57BL/6 mice were examined for GFP and CD69 expression with or without systemic treatment with anti-CD3 Ab (Plots gated on CD8α+CD8β+ lymphocytes). One representative of three mice per experiment; experiment was performed twice. (A–D) Spleen, solid gray; SI IEL, black line.
checked frames. Cells residing within Peyer’s patches were masked out of the analysis.

**Results**

**Phenotype of intestinal resident TEM**

To investigate the phenotype of CD8αβ T cells that are first primed against pathogens in secondary lymphoid organs and then disseminate to SI epithelium, monoclonal P14 Thy1.1⁺ naïve CD8 T cells specific for LCMV were transferred to naïve C57BL/6J mice. The next day, mice were infected with the Armstrong strain of LCMV, and lymphocytes were isolated from spleen and SI epithelium at various time points. Our previous studies indicated that migration of P14 to SI IEL occurs most efficiently during the early stages of the response (day 4.5 through day 7), after which they become resident with minimal evidence for recirculation (13). As shown in Fig. 1A–C, virus-specific CD8αβ⁺ SI IELs contrasted starkly from their clonal counterparts in the spleen because they gradually acquired and maintained an effector-like phenotype and did not upregulate CD62L.

To determine whether prolonged Ag stimulation could induce or maintain effector-like SI IEL phenotypic signatures (Fig. 1A) among CD8 T cells within the spleen, C57BL/6 mice were infected with the CI-13 strain of LCMV, which causes a persistent infection. As a control, mice were infected with the Armstrong strain of LCMV, which is cleared from spleen within ~1 wk. Thirty-five days later, endogenous LCMV-specific CD8 T cells were identified by H-2D⁺/gp33 tetramer staining (Fig. 1D). In contrast to acute LCMV Armstrong infection, persistent LCMV CI-13 infection results in a subset of virus-specific CD8 T splenocytes that express decreased levels of CD44, low levels of Ly6C, and elevated levels of CD69 and granzyme B (Fig. 1E). These data confirm and extend earlier observations that SI IELs adopt phenotypic signatures indicative of ongoing Ag stimulation in situ and suggest that many of these markers can be induced outside of the intestinal mucosa in the context of a chronic viral infection (23, 26).

**Role of Ag in the induction and maintenance of IEL phenotype**

To interrogate the role of foreign Ag in programming or maintaining phenotypic signatures of SI IELs, we wished to localize Ag-specific CD8 T cells to the SI epithelium in the absence of viral infection. However, neither naïve nor memory CD8 T cells migrate to SI epithelium upon i.v. transfer (13). To localize Ag-specific CD8 T cells to the SI epithelium in the absence of infection, we tested whether the “emptiness-driven” proliferation induced among naïve monoclonal CD8 T cells upon lymphopenic mice would be sufficient to induce CD8 T cell migration to the SI epithelium. Thy1.1⁺ OT-I/RAG⁻/⁻ CD8 T cells (1 × 10⁶) (specific for the SIINFEKL peptide from chicken OVA) were transferred to RAG⁻/⁻ C57BL/6 mice (Fig. 2A). It should be noted that mouse chow did not contain chicken OVA. Sixty days later, spleens and IELs were harvested and analyzed for the presence of donor OT-I CD8 T cells via staining with MHC class I tetramers and confirmed with Thy1.1 staining. Remarkably, homeostatic proliferation in lymphopenic mice was sufficient to induce intestinal homing, and OT-I IEL adopted all the cardinal features of effector-like virus-specific IEL (as in Fig. 1). Similar results were observed after transfer of naïve P14 CD8 T cells to RAG⁻/⁻ mice, suggesting that local induction of an effector-like phenotype was not restricted to OVA-specific CD8 T cells (data not shown). These data ruled out the possibility that occult intestinal infection with LCMV Armstrong was responsible for regulating the SI IEL differentiation state observed in Fig. 1.

However, the lumen of the small intestine is populated with a complex commensal flora, which could provide a diverse source of Ags that may have stimulated transferred T cells, resulting in migration to the intestinal epithelium and constitutive maintenance of an activated phenotype. To address this possibility, we transferred OT-I into gnotobiotic RAG⁻/⁻ mice that lacked microbial exposure. We found no difference compared with conventionally housed mice, suggesting that IELs adopted an activated phenotype independently of the flora (Fig. 2B).

To confirm this interpretation, we assessed markers that serve as surrogates for recent antigenic stimulation. PD-1 is upregulated upon TCR stimulation and is often maintained on CD8 T cells during chronic infection (46). However, P14 IELs isolated from LCMV immune chimeras did not express PD-1 (Fig. 2C). Nur77 is a transcription factor that is transiently expressed upon TCR stimulation (44). To assess Nur77 among polyclonal CD8αβ⁺ IELs, we used mice that expressed GFP under the Nur77 promoter. In resting mice, IELs were GFP negative, indicating that they did not constitutively transduce a TCR-dependent Nur77-inducing signal (Fig. 2D). As a control, forced stimulation via injection of anti-CD3 promoted GFP expression. Collectively, these results supported the hypothesis that induction and maintenance of the effector-like phenotype among IEL memory CD8 T cells was not dependent on persistent cognate Ag stimulation.

**Persistent Ag alters canonical IEL phenotype**

Thus far, we failed to find positive evidence that cognate Ag promoted differentiation and maintenance of the canonical effector...
memory IEL phenotype. For that reason, we interrogated IEL differentiation state under conditions of purposeful persistent antigenic stimulation. First, we compared endogenous CD8 T cell responses in spleen and SI IELs by staining isolated lymphocytes with H-2D\(^{b}\)/gp33 MHC class I tetramers 3 wk after acute LCMV Armstrong and persistent LCMV Cl-13 infections. Within 3 wk of acute Armstrong strain infection, most LCMV-specific CD8 T cells in SI IELs had upregulated CD103. In contrast, persistent Cl-13 strain infection prevented this upregulation (Fig. 3A, 3B). To address this further, we exploited a mouse transgenic model of chronic local self-antigen exposure. OT-I were transferred to 232-4 mice, which express OVA under the IFABP promoter that targets expression to epithelial cells within the small intestine (45). This results in activation of OT-I in associated lymphoid tissues and accumulation of OT-I within the epithelium where they respond to Ag, but does not induce detectable pathology (45). As was the case with chronic LCMV Cl-13 infection, persistent self-Ag recognition prevented CD103 upregulation (measured 150 d after OT-I transfer, Fig. 3C). These observations provide further evidence that maintenance of the canonical SI IEL phenotype, even though it bears markers of recent activation, is not due to persistent Ag-stimulation.

Cytokine milieu can induce canonical IEL phenotype

Because SI IEL phenotype has indirectly been shown to be regulated by local environment (23), and the experiments presented in Figs. 1–3 excluded a demonstrable role for local Ag, we examined the ability of cytokines to drive the SI IEL differentiation program. TGF-\(\beta\) has been associated with CD103 regulation on regulatory, anti-tumor, and allograft-specific T cells (47, 48). Moreover, TGF-\(\beta\) is constitutively expressed in SI, for instance, as measured by in situ hybridization to detect mRNA in rat (49). For this reason, we cultured early CD8 T cell effectors, isolated from spleen 4.5 d postinfection, for 40–60 h with TGF-\(\beta\). As shown in Fig. 4A, TGF-\(\beta\) induced CD103 upregulation among effector CD8 T cells. Fully differentiated resting memory CD8 T cells were less likely to undergo TGF-\(\beta\)-induced CD103 expression. Consistent with our interpretation of Fig. 3, inclusion of peptide Ag with TGF-\(\beta\)-cultured effector cells prevented upregulation of CD103 (Fig. 4B), further suggesting that CD103\(^{+}\) SI IELs are not chronically Ag stimulated.

We then tested whether culture with TGF-\(\beta\) and various other cytokines could recapitulate cardinal phenotypic signatures of SI IELs among splenocytes (Fig. 4C). We found that certain cytokines, particularly TNF-\(\alpha\) and IL-33, could synergize with TGF-\(\beta\).
and resulted in splenocytes that adopted a CD103+/Ly6C−/CD69+ phenotype (Fig. 4D). Because culture experiments were short-term (40–60 h) and depended on an effector state of differentiation, granzyme B and other markers that distinguish memory SI IELs from splenocytes were not addressed. Nevertheless, these data demonstrate that the cytokine milieu can induce splenocytes to mimic the SI IEL phenotype.

To test the role of TGF-β in vivo, we bred transgenic mice with a dominant negative mutation in TGFβRII (41) with Thy.1.1+ OT-I mice, resulting in Thy.1.1/Thy.1.2 heterozygous OT-I mice expressing a single copy of the dominant negative mutation. CD44b (naive) OT-I were FACs sorted from these mice and mixed in a 1:1 ratio with WT Thy.1.1+ OT-I. Cells were transferred to WT C57BL/6J mice (Fig. 5A). In summary, recipient mice were WT, half of the transferred OT-I population was WT, and half of transferred OT-I were refractory to TGF-β-mediated signaling. The following day, mice were infected with VSVova, and lymphocytes were isolated from spleen and SI IELs 7 d later. As shown in Fig. 5B and 5C, dnTGFβRII OT-I failed to develop the CD103+/CD69− subset upon migration to SI epithelium. Similar results were observed when P14 cells and LCMV were substituted for OT-I cells and VSVOva in an identical experimental design (Fig. 5D). Although this defect in CD103 and CD69 expression was not absolute, it should be noted that effector dnTGFβRII P14 splenocytes upregulated CD103 in response to TGF-β in vitro (albeit suboptimally; data not shown), consistent with the fact that this dominant-negative mutation did not render T cells completely insensitive to TGF-β; likewise, dnTGFβRII P14 that disseminated to IEL upon in vivo activation eventually upregulated CD103 (data not shown).

CD103 expression is required for maintenance of virus-specific memory IEL

CD103 binds E-cadherin, expressed by SI epithelial cells, and has been reported to play a role in retention in several contexts (43, 50). CD103 expression is required for maintenance of virus-specific memory IELs that disseminated to IEL upon in vivo activation eventually resulted in the CD103+/CD69+ subset upon migration to SI epithelium. However, CD103+/− P14 were specifically underrepresented within the small intestine by day 30 postinfection. Together with previous work defining the kinetics of P14 migration to SI IEL after LCMV infection (13), these data suggest that there is no requirement for CD103 in migration to SI, but a defect in maintenance or survival. As this result differed from the previous study analyzing the requirement of CD103 in SI IEL maintenance after an acute viral infection, we confirmed our analysis histologically. C57BL/6J mice received either Thy.1.1+ WT or CD103+/− P14 and were then infected with LCMV Armstrong. Thirty days later, the entire length of the SI was frozen in pinwheels, which allowed a single region (indicated in Fig. 6B) to capture portions along the entire length of the SI. IEL (Fig. 6C) and LPL (Fig. 6D) could be discriminated on the basis of orientation to the basement membrane (defined by collagen IV staining). Thy.1.1+ IEL and LPL were counted throughout the SI region of mice that received WT or CD103+/− P14 and enumerated as the number of each subset per 10⁷ SI nuclei (Fig. 6E), or as a ratio of SI IEL to SI LPL (Fig. 6F). These data confirmed that induction of CD103 is required for the maintenance of resident pathogen-specific memory CD8 T cells within small intestine epithelium.

Induction of T_{RM} phenotypic signatures outside of intestine

We next tested the distribution of T_{RM} phenotypic signatures among subsets of T cells present outside of the intestinal mucosa.

![FIGURE 5. TGF-β induces CD103+/CD69− SI IEL population in vivo. (A and B) Thy.1.1+ OT-I and Thy.1.1/Thy.1.2 dnTGFβRII OT-I were mixed in a 1:1 ratio and cotransferred to C57BL/6J mice. The following day, recipient mice were infected with VSVOva, and lymphocytes were isolated from spleen and SI IELs 7 d later and analyzed for CD103 and CD69 expression by flow cytometry. Plots gated on WT OT-I (WT) or dnTGFβRII OT-I (DN), as indicated. (C) Number of recovered WT (black) and dnTGFβRII OT-I (gray) SI IELs among the indicated subsets. (D) Identical experimental design as in (A)–(C), however WT and mutant P14 were substituted for OT-I, and mice were infected with LCMV Armstrong. Error bars indicate SEM. **p < 0.01 (Student unpaired t test). Flow plots are representative of five mice per experiment. Each experiment was performed at least three times.](http://www.jimmunol.org/ by guest on February 1, 2021 http://www.jimmunol.org/ Downloaded from)
as well as the role of persistent cognate Ag stimulation in their induction and maintenance. P14 CD8 T cells were transferred to naïve mice prior to infection with LCMV Armstrong. Three months later, lymphocytes were harvested from spleen, SI IEL, SI LPL, female reproductive tract, and brain. As shown in Fig. 7A and 7B, each of these nonlymphoid tissues contained a subset of CD103+ memory CD8 T cells that expressed higher levels of granzyme B compared with those of splenocytes and was invariably CD69+. We then further expanded our analysis to include the kidney, pancreas, stomach, and heart, all of which had a subset of CD62L−/CD103+/CD69+ memory CD8 T cells (Fig. 7B). These data provide further evidence that intestine-specific sources of persistent Ag, such as proximity to the commensal flora or dietary Ags, were not responsible for the maintenance of the stereotypic T<sub>R<sub>M</sub></sub> phenotype embodied by SI IEL.

To exclude a role for local or prolonged stimulation with cognate viral-derived Ag in the induction and maintenance of T<sub>R<sub>M</sub></sub> in these nonlymphoid tissues, we localized Ag-specific CD8 T cells to the SI epithelium in the absence of infection by transferring naive monoclonal CD8 T cells to lymphopenic mice. CD45.1+ OT-I/RAG<sup>2−/−</sup> CD8 T cells (1×10<sup>6</sup>) were transferred to RAG<sup>2−/−</sup> C57BL/6 mice. We found that “emptiness-driven” proliferation was sufficient to drive dissemination of OT-I into all nonlymphoid tissues tested. Furthermore, the proportion of CD62L−/CD103+/CD69+ CD8 T cells was similar between models of LCMV infection versus “emptiness-induced” proliferation-driven migration and differentiation. It should be noted that brain was an exception; in the absence of infection bearing cognate Ag, CD8 T cells that migrated to brain exhibited a paucity of CD103+ CD8 T cells, consistent with a proposed role for local Ag in inducing this T<sub>R<sub>M</sub></sub>
phenotype within this tissue (15). Nevertheless, these data demonstrate that the T RM phenotypic signature was broadly distributed in many nonlymphoid tissues and was largely independent of persistent or local cognate Ag interaction.

**Discussion**

This study clearly demonstrates that the maintenance of the resident TEM differentiation state that typifies SI IEL memory CD8 T cells is regulated independently of Ag and also provides evidence that cytokines induce location-specific T cell properties required for the maintenance of local immunity. These findings precipitate consideration of the relationship between SI IEL, other tissue-resident T RM subsets, and recirculating T EM pools. Implications for protective immunity and existing models of T cell differentiation are discussed.

SI IELs were rapidly incorporated into the emerging TCM/TEM classification scheme because constitutive maintenance of cytotoxic potential was originally a defining feature of TEM (22, 52). In mice, the definition of TEM has since broadened to include all T cells that lack expression of CD62L and/or CCR7. Given the growing evidence of a relationship between memory CD8 T cell subsets and protective immunity, there is considerable interest in understanding the regulation of commitment to TEM lineage(s) (1). Existing models largely focus on the role of Ag and indicate that high levels of Ag during priming, repetitive stimulation events, or chronic infections all favor TEM development (1). Compared to TEM, various studies provide evidence that recirculating TEM may be relatively short-lived in the absence of continued Ag stimulation (8, 9). However, it has remained unclear whether these conclusions should be conflated with memory CD8 T regulation within the intestinal mucosa, particularly because SI IELs show little evidence of recirculation (13). Given the potential exposure of the intestinal mucosa to numerous foreign peptides, we examined the mechanisms regulating SI IEL phenotype.

This study provides several complementary lines of evidence indicating that foreign Ag-dependent signals are not required for the induction or maintenance of “effector-like” CD8 T SI IELs. Homeostatic proliferation induced among naive transgenic CD8 T cells upon transfer into lymphopenic recipients was sufficient to induce migration to the intestinal mucosa and acquisition of SI IEL effector-like phenotypic signatures (Fig. 2A). This experiment demonstrated that “programming” fully differentiated SI IEL was not LCMV-infection dependent, and it ruled out a role of persistent stimulation by chronic occult LCMV Armstrong infection. This result was recapitulated in germ-free RAG2−/− mice, indicating that cross-reactivity with microbial Ags was also not required (Fig. 2B). Key features of the SI IEL phenotype were also displayed by CD103+ memory CD8 T cells isolated from the
female reproductive tract, kidney, brain, and so forth, suggesting that a “resting effector” phenotype might extend to other tissue-resident memory CD8 T cell populations and further ruling out a requirement for both intestinal flora and also dietary Ags (Fig. 7). Absence of PD-1 or Nur77 expression further supported the hypothesis that SI IELs were not persistently stimulated with cognate Ag (Fig. 2C, 2D). Indeed, when a source of constitutive Ag was provided via infection with a chronic strain of LCMV or by expression of cognate Ag by intestinal epithelial cells, SI IELs deviated from their conventional phenotype and failed to acquire normal levels of CD103 expression (Fig. 3).

Notably, ex vivo culture of effector CD8 T splenocytes with combinations of TGF-β and IL-33 or TNF-α promoted acquisition of SI IEL phenotypic signatures, as measured by CD103, CD69, and Ly6C expression. The presence of cognate Ag prevented the ability of TGF-β to induce CD103 expression among effector splenocytes. In this light, it will be interesting to determine whether CD103 expression may serve as a useful marker to distinguish resting versus recently Ag-stimulated CD8αβ T cells in certain nonlymphoid compartments.

The results of this study do not diminish the central role of Ag in regulating T cell differentiation. However, our data do indicate that anatomic location, independently of Ag-stimulation history, also significantly and directly affects CD8 T cell differentiation state and optimize the maintenance of host protective immunity at common portals of pathogen entry.

We thank Marion Pepper, Botond Igyarto, Stephen Jameson, and Michael Germer for helpful discussions.

Acknowledgments

References


