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SHORT COMMUNICATION

Maintenance of PD-1 on brain-resident memory CD8 T cells is antigen independent

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Infection of the central nervous system (CNS) by murine polyomavirus (MuPyV), a persistent natural mouse pathogen, establishes brain-resident memory CD8 T cells (bT_{RM}) that uniformly and chronically express programmed cell death protein 1 (PD-1) irrespective of the expression of α_E integrin CD103, a T_{RM} cell marker. In contrast, memory antiviral CD8 T cells in the spleen are PD-1$^-$, despite viral loads being similar in both the brain and spleen during persistent infection. Repetitive antigen engagement is central to sustained PD-1 expression by T cells in chronic viral infections; however, recent evidence indicates that expression of inhibitory receptors, including PD-1, is part of the T_{RM} differentiation program. Here we asked whether PD-1 expression by CD8 bT_{RM} cells during persistent MuPyV encephalitis is antigen dependent. By transferring MuPyV-specific CD8 bT_{RM} cells into the brains of naive mice and mice infected with cognate epitope-sufficient and -deficient MuPyVs, we demonstrate that antigen and inflammation are dispensable for PD-1 maintenance. *In vitro* and direct *ex vivo* analyses indicate that CD103$^-$ MuPyV-specific CD8 bT_{RM} retain functional competence. We further show that the *Pdcd-1* promoter of anti-MuPyV bT_{RM} cells is epigenetically fixed in a demethylated state in the brain. In contrast, the *PD-1* promoter of splenic antiviral memory CD8 T cells undergoes remethylation after being demethylated during acute infection. These data show that PD-1 expression is an intrinsic property of brain T_{RM} cells in a persistent CNS viral infection.
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Programmed cell death protein 1 (PD-1) expression has been proposed to constitute a facet of the resident memory CD8 T cells (T_{RM}) differentiation program to prevent inadvertent deployment of poised mRNAs for effector molecules.[1] In chronic lymphocytic choriomeningitis virus (LCMV) infection, T-cell receptor (TCR) signaling upregulates PD-1 expression at the effector stage of the splenic CD8 T cell response, with sustained PD-1 driving differentiation of exhausted T (T_{EX}) cells to prevent immunopathology.[2,3] The state of PD-1 expression and its dependence on antigen by tissue T_{RM} during persistent viral infection remains to be defined. For example, CD8 brain T_{RM} (bT_{RM}) cells from mice with acutely resolved vesicular stomatitis virus (VSV) encephalitis express PD-1 transcripts but not PD-1 receptors, whereas bT_{RM}s from mice persistently infected with mouse cytomegalovirus are PD-1$^+$.[4–6] This discrepancy in PD-1 expression by bT_{RM} cells raised the question whether antigen and/or inflammation is involved in maintenance of PD-1 expression by bT_{RM} cells during central nervous system (CNS) infection. Tissue-intrinsic factors are also dominant determinants of the dependence on antigen for CD8 T_{RM} cell generation and/or maintenance. Antigen is required for T_{RM} cell formation and CD103 upregulation in the brain and dorsal root ganglion[5,7,8] but not in the skin, small intestine, female reproductive tract and salivary glands.[7,9–12] The role of antigen in maintenance of the expression of PD-1 and CD103 by CD8 T_{RM} cells in the brain remains to be determined.

The PD-1 promoter of virus-specific CD8 T cells undergoes dynamic epigenetic reprogramming during development of memory T cells and T_{EX} cells.[13] In acutely resolved LCMV Armstrong infection, virus clearance was associated with remethylation of the *Pdcd-1* promoter and loss of PD-1 expression; however, in the high-level chronic LCMV clone 13 infection model, the *Pdcd-1* promoter remained unmethylated in T_{EX} cells even after virus levels fell below detection.[13,14] Notably, these epigenetic analyses were only performed on splenic LCMV-specific CD8 T cells in an infection where PD-1 is expressed by antiviral CD8 T cells in all nonlymphoid organs.[15] This led us to investigate the epigenetic programming of bT_{RM} cells during persistent viral encephalitis.

Murine polyomavirus (MuPyV) is a natural mouse pathogen that establishes a low-level persistent infection. CNS infection with MuPyV yields a stable population of virus-specific bT_{RM} cells.[16] Here we show that, during persistent MuPyV infection, PD-1 is expressed by bT_{RM}

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cells but not splenic memory anti-MuPyV CD8 T cells, despite virus loads being similar in both organs, suggesting dissociation between the viral load and PD-1 expression. We further show that maintenance of PD-1 expression by bT_{RM} cells is independent of cognate viral antigen and inflammation. As seen for splenic virus-specific CD8 T cells in chronic LCMV infection, the *pdcd-1* promoter of bT_{RM} cells from MuPyV-infected mice remains demethylated. However, the *PD-1* locus in splenic anti-MuPyV CD8 T cells undergoes partial remethylation. Collectively, these findings indicate that PD-1 expression is part of the developmental program of bT_{RM} cells to a persistent CNS viral infection.

RESULTS AND DISCUSSION

MuPyV-specific bT_{RM} cells express PD-1 during persistent infection

Naive B6 mice received a 'physiological' number (200 cells per mouse) of Thy1.1-congenic TCR-I cells and were inoculated intracerebrally (i.c.) the next day with MuPyV.LT206 virus. At day 9 postinfection (p.i.), the magnitude of the endogenous LT206-specific CD8 response in the brain, kidney and spleen was similar to that of the donor TCR-I cells (Supplementary Figure S1). Both the endogenous D^bLT206-specific CD8 T cells and the TCR-I cells in acutely infected mice expressed PD-1 and CD69, with those in the brain having the highest level of expression (Figure 1a); notably, virus levels in these organs were similar at day 9 and also at day 30 p.i. (Figure 1b). In addition to CD69, CD103 (α_E integrin) is often used identify T_{RM} cells.[17] At day 9 p.i., D^bLT206-specific CD8 T cells in both lymphoid and nonlymphoid organs did not express CD103; however, by day 30 p.i., a fraction of $CD103^+$ endogenous and donor CD8 T cells were seen in the brain but not in the kidney or spleen. Despite a decline in virus levels by day 30 p.i. (Figure 1b), PD-1 expression was sustained on both endogenous D^bLT206-specific CD8 T cells and TCR-I cells in the brain and kidney but not those in the spleen (Figure 1a), as was CD69 (Figure 1a). Analysis of PD-1 transcript levels in TCR-I cells from the spleen at days 8 and 30 p.i. showed that splenic memory TCR-I cells expressed lower PD-1 mRNA levels than those from the spleens of acutely infected mice, albeit this difference was not statistically significant (Figure 1c). Discordance between PD-1 mRNA levels and surface expression by CD8 T_{RM} cells was similarly observed in the VSV acute encephalitis infection model.[4] To determine whether PD-1 functionally inhibited MuPyV-specific CD8 T cells, we assayed intracellular interferon (IFN)-γ production by TCR-I cells after stimulation by LT206 peptide-pulsed $PD\text{-}L1^+$ DC2.4 cells in the presence of a PD-L1 blocking monoclonal antibody (Ab). As shown in Figure 1d, PD-L1 blockade resulted in a significant increase in frequency of IFN-γ expressing TCR-I cells from brains, kidney and spleen of mice at day 9 p.i. At day 30 p.i., PD-1 blockade increased the frequency of $IFN\text{-}\gamma^+$ cells only of brain-resident TCR-I cells, which expressed the highest levels of PD-1 among T cells from all the organs examined.

Maintenance of PD-1 expression by brain T_{RM} cells is antigen and inflammation independent

We found that $PD\text{-}1^{hi}$ expression by MuPyV-specific b_{TRMs} cells was a stable phenotype and dissociated from virus infection levels

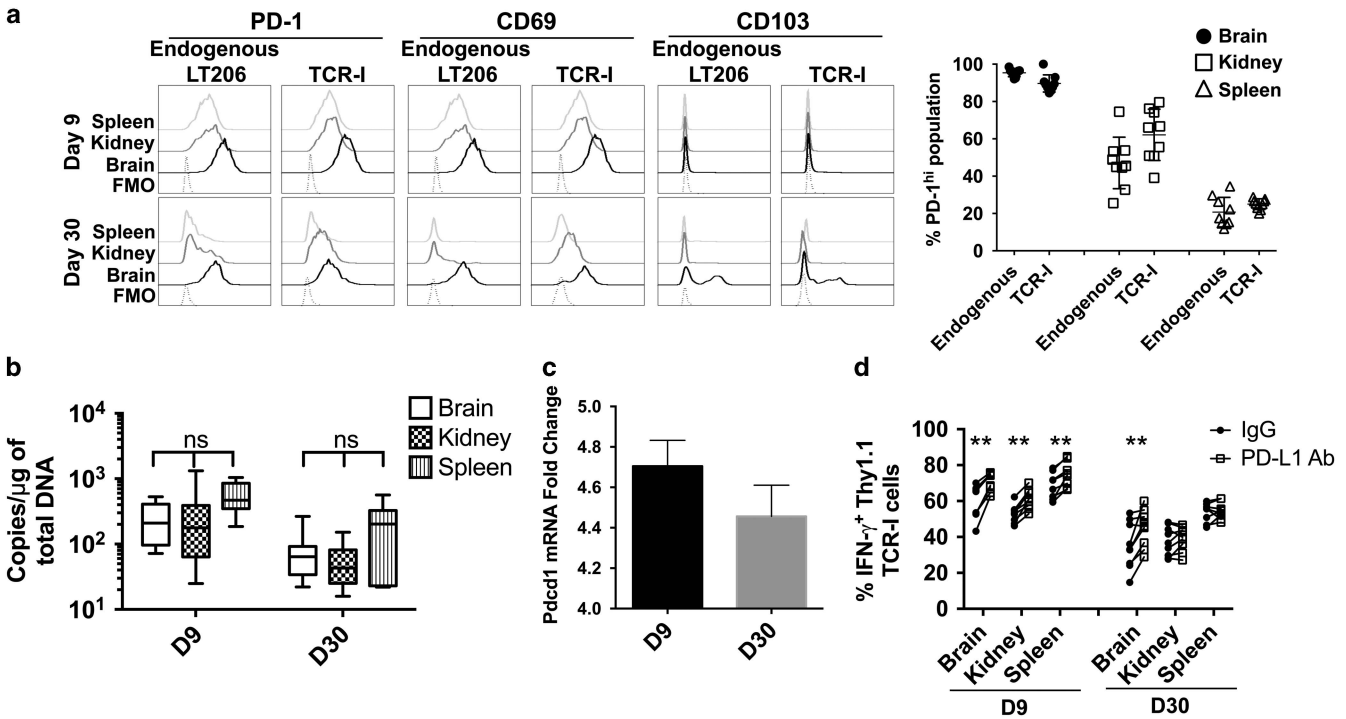

Figure 1 Expression and function of PD-1 on MuPyV-specific CD8 T cells. (**a**) Surface expression of PD-1, CD69 and CD103 by D^bLT206-specific endogenous and Thy1.1 TCR-I CD8 T cells from the indicated organs at days 9 and 30 p.i. (left panels), and frequency of $PD\text{-}1^{hi}$ cells at day 30 p.i. on endogenous D^bLT206-specific CD8 T cells and TCR-I cells (right panel). (**b**) Real-time PCR assay of MuPyV genome copy numbers from total genomic DNA isolated from the brain, spleen and kidney at days 9 and 30 p.i. One-way analysis of variance, Kruskal–Willis test with Dunn's multiple comparisons test was used to determine statistical significance. (**c**) Real-time PCR analysis for the expression of PD-1 on cDNA prepared from FACS-purified GFP^+ TCR-I cells from spleens of naive, day 8- and day 30-infected mice. (**d**) Mononuclear cells from the brain, kidney and spleen of TCR-I-recipient mice at days 9 and 30 p.i. were stimulated with LT206 peptide-pulsed DC2.4 cells in the presence of control rat IgG (–●–) or PD-L1 blocking Ab (–▣–). A nonparametric paired Student's *t*-test with Wilcoxon matched-pairs signed rank test was used to determine statistical significance between control rat IgG and PD-L1 blocking Ab-treated samples for each organ at a single time point. Each data point represents each mouse in that group. Data are from two independent experiments with 4–5 mice each group. Values are mean ± s.d.; $^{**}P \leqslant 0.01$, not significant (ns) $P > 0.05$.

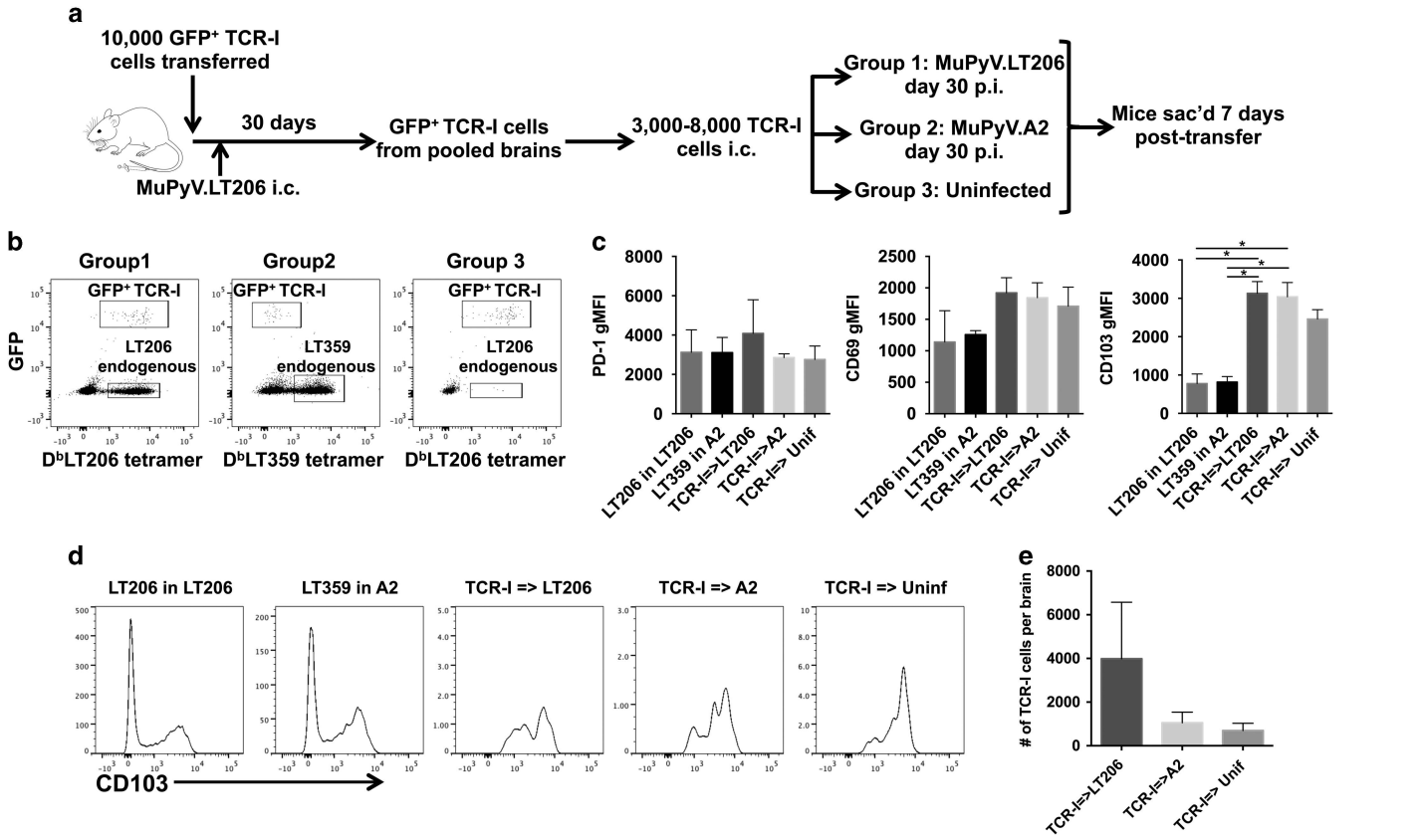

Figure 2 Analysis of PD-1, CD103 and CD69 expression on brain T_{RM} CD8 cells in the absence of cognate epitope and/or inflammation. (**a**) Experimental setup. (**b**) Representative dot plots for endogenous D^bLT206- or D^bLT359-specific and transgenic GFP$^+$ TCR-I cells in respective groups of mice. (**c**) Expression of PD-1, CD69 and CD103 on endogenous D^bLT206- or D^bLT359-specific and GFP$^+$ TCR-I cells in groups of mice as indicated, represented as gMFI of the population. One-way analysis of variance, Kruskal–Willis test with Dunn's multiple comparisons test was used to determine statistical significance. (**d**) Representative histograms showing the expression of CD103 D^bLT206- or D^bLT359-specific and GFP$^+$ TCR-I cells. 'LT206 in LT206' indicates CD8 T cells specific to D^bLT206 epitope in Group 1, 'LT359 in A2' indicates CD8 T cells specific for D^bLT359 epitope in Group 2, 'TCR-I = >LT206/A2/Uninf' indicates the transferred GFP$^+$ TCR-I cells in Groups 1, 2 and 3, respectively. Data are cumulative from two independent experiments, with 2–3 mice each group. Values are mean ± s.d.; $*P<0.05$. (**e**) Number of GFP$^+$ TCR-I donor cells recovered from the brains of recipient mice. Data are representative from one of the two experiments performed where 8000 GFP$^+$ TCR-I cells were transferred i.c. into the recipient brains.

(Figure 1b); that is, splenic antiviral memory CD8 T cells were PD-1$^-$ but spleen and brain virus loads were similar. In contrast, PD-1 expression by memory, TCR transgenic P14 CD8 T cells from LCMV clone13-infected mice declines following transfer to LCMV-immune mice or mice infected with a epitope-null mutant LCMV.[14] To directly ask whether cognate antigen and/or virus-associated inflammation was required for PD-1 expression by brain T_{RM} cells, we sorted GFP$^+$ TCR-I cells from the brains of B6 mice infected 30 days earlier with MuPyV.LT206 and reintroduced i.c. in B6 at day 30 p.i. with MuPyV. LT206 (Group1: infection, cognate Ag), MuPyV.A2 virus (Group 2: infection, no cognate Ag) or into uninfected mice (Group 3) (Figure 2a). Seven days posttransfer, CD8 T cells from the brains were co-stained with D^bLT206 or D^bLT359 tetramers (Figure 2b). As shown in Figure 2c, PD-1 expression was similar among donor TCR-I T_{RM} cells in the brains of mice regardless of expression of cognate epitope and/or virus-associated inflammation. CD69 and CD103 expression by the TCR-I cells in each recipient cohort was also similar and unchanged from the original donor TCR-I cells in Group 1 (Figure 2c). High expression of PD-1, CD103 and CD69 by the donor TCR-I T_{RM} cells in naive recipients was maintained up to day 25 posttransfer (Supplementary Figure S1B). The possibility of MuPyV. LT206 virus carryover with the donor TCR-I cells is unlikely given the absence of detectable MuPyV DNA by quantitative PCR in the brains of uninfected recipients (Supplementary Figure S2A) and by the absence of D^bLT206 tetramer binding the endogenous CD8 T cells either in the brain (Figure 2b) or in the spleen (Supplementary Figure S2B) of uninfected recipient mice. Naive TCR-I cells transferred into mice infected by MuPyV.A2 failed to proliferate and did not upregulate CD44, indicating that maintenance of PD-1 expression by TCR-I T_{RM} cells in MuPyV.A2-infected recipients could not be attributed to cross-recognition of the D^bLT359 epitope (Supplementary Figure S2C). These data suggest that TCR stimulation and the inflammatory environment of persistent MuPyV CNS infection are dispensable for maintenance of PD-1 expression by antiviral brain T_{RM} cells.

As shown in Figure 2d, donor TCR-I bT_{RM} cells were predominantly CD103$^+$ irrespective of the presence of antigen or inflammation in the recipient brains, suggesting preferential survival of the CD103$^+$ population. Although fewer donor TCR-I bT_{RM} cells were recovered from the brains of naive and MuPyV.A2-infected recipients than from MuPyV.LT206-infected mice at day 7 posttransfer (Figure 2e), these CD8 bT_{RM} cells were found to persist in the brains of naive recipients for 25 days (Supplementary Figure S1). To ask whether PD-1 expression was an intrinsic property of MuPyV-specific bT_{RM} cells,

TCR-I cells were isolated from the brains of mice at day 30 p.i. and transferred intravenously to either naive or MuPyV.LT206 i.c. infected Thy1.2 congenic recipients. No donor cells, however, were detected at day 7 posttransfer in either cohort (data not shown), a finding mirroring that of Wakim *et al.*[5] using the VSV acute encephalitis mouse model and supporting the concept that CNS-specific factors are required for maintenance of bT_{RM} cells.

To ask whether the brain environment *per se* induced PD-1, memory GFP^+ TCR-I cells from the spleen were transferred i.c. into naive or acutely i.c. infected (MuPyV.LT206) mice. We found that the donor splenic memory TCR-I cells ($CD69^-$ $PD\text{-}1^-$, Figure 1a) 7 days posttransfer i.c. into naive mice or mice given MuPyV.LT206 virus i.c. 8 days earlier upregulated PD-1 and CD69 expression but not that of CD103 (Supplementary Figure S1C). Although splenic donor memory cells upregulated PD-1 expression in both naive and acutely infected recipients, it merits noting that PD-1 expression is lower in the naive than in the infected mice, consistent with a role for antigen in driving PD-1 expression. Taken together, these data confirm the ability of the brain environment to upregulate PD-1 and CD69 on infiltrating MuPyV-specific CD8 T cells.

$CD103^-$ MuPyV-specific CD8 bT_{RM} cells are functionally competent

CD8 T_{RM} cell populations defined by differential CD103 expression have been reported to vary in proliferative potential, transcriptome, antigen dependence and tissue distribution.[4,5,18,19] CD103 expression by donor TCR-I cells, however, was higher than on the endogenous D^bLT359 or D^bLT206 CD8 T cells (Figures 2c and d), a difference that may be linked to the higher survival potential of $CD103^+$ cells in the brain.[5]

We thus asked whether MuPyV-specific $CD103^-$ CD8 T cells infiltrating the brain express phenotypic and functional characteristics of T-cell exhaustion. Both $CD103^+$ and $CD103^-$ D^bLT359-specific CD8 T cells were maintained in the absence of circulating CD8 cells (Figure 3a), suggesting that each of these populations is comprised of T_{RM} cells. $CD103^+$ and $CD103^-$ D^bLT359-specific CD8 T cells also expressed similar levels of PD-1 and Tim3 (Figure 3b). Of note, the $CD103^-$ subset expressed higher TCF-1 levels and had higher proportion of $CXCR5^{hi}$ $TCF\text{-}1^{hi}$ cells compared with the $CD103^+$ cells. Elevated TCF-1 expression as well as the presence of a $CXCR5^{hi}$ $TCF\text{-}1^{hi}$ population by memory CD8 T cells in chronic infection models have been reported to exhibit improved functional capability.[20,21] BLIMP-1 and Eomesodermin (EOMES) T-box transcription factors are highly expressed by exhausted CD8 T cells;[22,23] however, $CD103^-$ D^bLT359-specific CD8 T cells were found to express lower BLIMP-1 and higher EOMES levels than the $CD103^+$ cells. These populations also showed similar Ki67 expression levels, indicating their comparable rates of proliferation during persistent MuPyV CNS infection (Figure 3c). Taken together, the pattern of expression of these molecules indicates that $CD103^-$ cells lack a phenotype associated with T-cell exhaustion. Moreover, $CD103^-$ and $CD103^+$ LT359 peptide-stimulated CD8 T cells from the brains of persistent MuPyV-infected mice exhibited similar frequencies of intracellular IFN-γ production (Figure 3d). To assess IFN-γ production directly *ex vivo* by $CD103^-$ and $CD103^+$ MuPyV-specific CD8 T cells in the brains of persistently infected mice, IFN-γ EYFP reporter mice were inoculated with MuPyV.A2 virus i.c. As shown in Figure 3e, a higher fraction of $CD103^+$ than $CD103^-$ D^bLT359-specific CD8 T cells were $EYFP^+$, a pattern paralleled by the expression of IRF4, a transcription factor upregulated by TCR stimulation.[24] The discrepancy between similar frequencies of IFN-γ-producing $CD103^+$ and $CD103^-$ bTRM cells following LT359 peptide stimulation vs lower $EYFP^+$ and $IRF4^+$ by the CD103- population may reflect regional differences between the $CD103^+$ and $CD103^-$ cells in engaging viral antigen-expressing cells and/or differences in TCR affinity.[16]

Although the CD103 integrin tethers T_{RM} cells to E-Cadherin and is important for their preferential localization at epithelial tissues, $CD103^-$ CD8 T_{RM} cells exist in particular organs, including the brain.[5,25] CD103 expression by CD8 T_{RM} cells may also be influenced by the nature of the viral infection, as lung CD8 T_{RM} cells to influenza virus and respiratory syncytial virus express CD103, whereas those specific for human cytomegalovirus are $CD103^-$.[26]

Demethylation of the PD-1 promoter by MuPyV-specific bT_{RM} cells

Using the mouse model of chronic LCMV infection, it has been previously demonstrated that the *Pdcd1* promoter is extensively demethylated at early stages of the immune response. This locus was remethylated in the acutely resolved LCMV Armstrong infection but remained demethylated in chronic LCMV clone13 infection even after host control of infection, a result tying PD-1 to the exhaustion state of differentiation.[13,14] Stability of the demethylated *Pdcd1* promoter was further demonstrated in human EBV, CMV and HIV-specific CD8 T cells.[13,27] It is yet to be confirmed whether the epigenetic changes observed at peripheral T_{EX} cells holds true for bT_{RM} cell population as well. The marked difference in PD-1 expression by memory MuPyV-specific CD8 T cells in the spleen and brain led us to ask whether cells in these organs differed in level and stability of *Pdcd1* promoter demethylation. *Pdcd1* methylation status was analyzed on Thy1.1 TCR-I CD8 cells sorted from the brains and spleens of acute (day 8 p.i.) and persistent (day 30 p.i.) infected recipient mice (Figure 4a). As expected, the *Pdcd1* promoter in naive splenic TCR-I cells was almost entirely methylated (Figures 4b and e). At day 8 p.i., TCR-I cells in both the spleen and brain exhibited extensive *Pdcd1* promoter demethylation (Figures 4c and e). By day 30 p.i., however, TCR-I cells in the spleen showed increased methylation of the *Pdcd1* promoter, while brain TCR-I cells retained a demethylated *Pdcd1* promoter (Figures 4d and f). This intermediate level of methylation in TCR-I cells from day 30 infected mice was intermediate between cells from a naive or day 8-infected mice. This observation is consistent with the lower trending PD-1 transcript levels seen at day 30 p.i. (Figure 1c). The $PD\text{-}1^{hi}$ phenotype of the brain TCR-I T_{RM} cells further suggests that a minimum threshold of *Pdcd1* promoter demethylation facilitates upregulation of PD-1 gene expression. Thus these data show that the *Pdcd1* promoter of CD8 bT_{RM} cells remains more extensively demethylated than splenic memory CD8 T cells despite having similar persistent MuPyV infection levels.

Recent studies document expression of PD-1 by memory CD8 T cells in inflamed immune-privileged organs, such as the eye and brain.[6,28] Expression of PD-1 on brain CD8 T cells has been shown to limit axonal bystander damage in a mouse coronavirus CNS infection model and mediate protection from experimental autoimmune encephalomyelitis.[29–33] Our finding that PD-1 blockade elevates effector function by anti-MuPyV CD8 bT_{RM} cell is in line with the concept that PD-1 expression by tissue-resident T cells may serve to restrain immunopathology, which would prove especially deleterious in the infected CNS. The divergence in PD-1 expression between brain and spleen MuPyV-specific CD8 T cells during persistent infection, with bT_{RM} cells maintaining PD-1 in the absence of cognate antigen and inflammation, supports the concept that the brain microenvironment favors PD-1 expression.

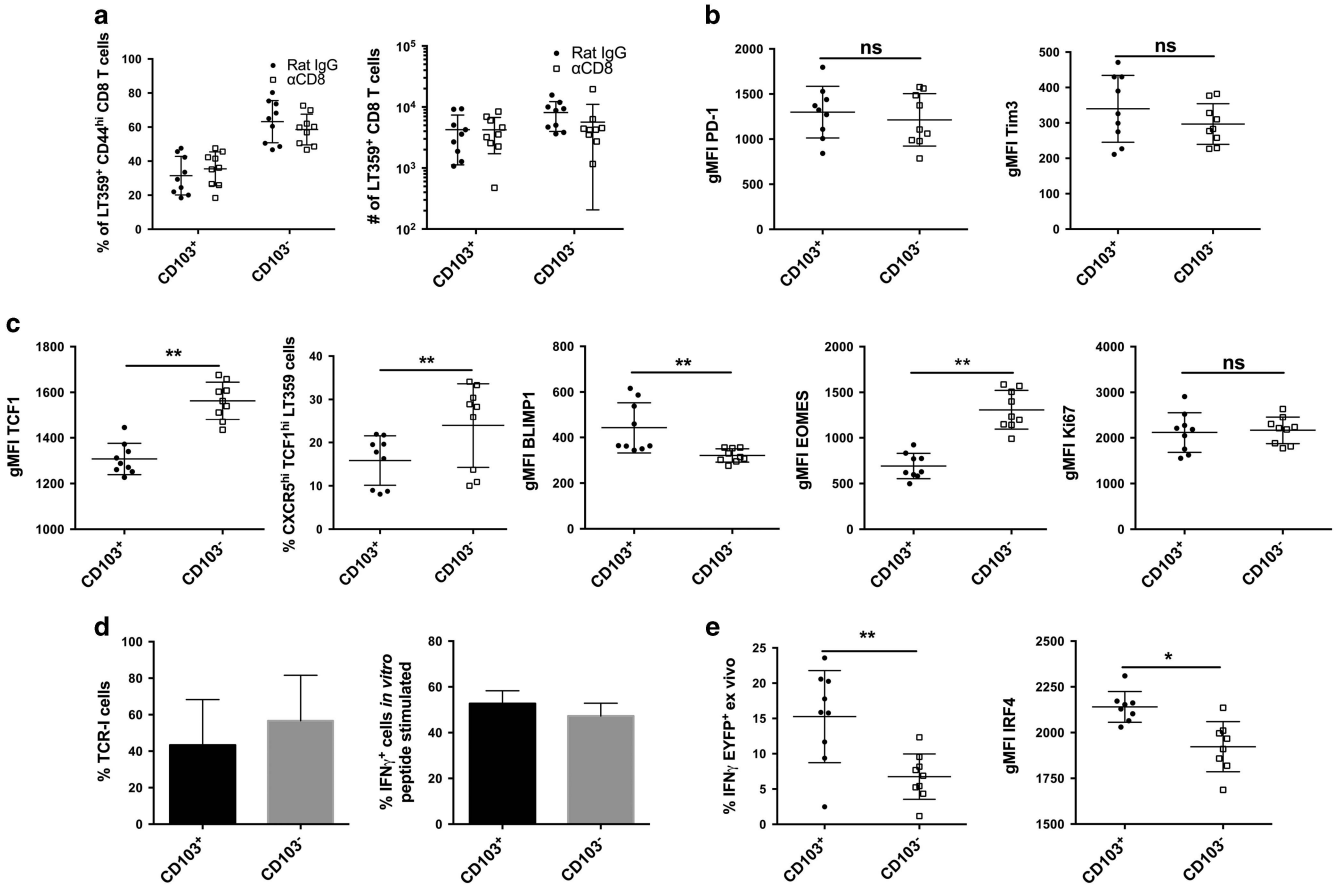

Figure 3 Functional and phenotypic characterization of CD103$^+$ and CD103$^-$ bT$_{RM}$ cells. (**a**) Frequency and number of CD103$^+$ and CD103$^-$ D^{b}LT359 tetramer-specific CD8 T cells from the brains of mice at day 30 p.i. after i.c. inoculation with MuPyV.A2 and treated weekly with anti-CD8α or control rat IgG from day 10 to day 30 p.i. (**b** and **c**) Surface expression of PD-1, Tim3 and CXCR5 and intracellular expression of TCF-1, BLIMP1, EOMES and Ki67 on CD103$^+$ and CD103$^-$ D^{b}LT359 tetramer-specific CD8 T cells from the brains of mice day 30 p.i. after i.c. inoculation with MuPyV.A2. (**d**) Frequency of CD103$^+$ and CD103$^-$ cells Thy1.1 TCR-I cells and IFN-γ-producing cells upon *in vitro* LT359 peptide stimulation from the brains of mice at day 30 p.i. after i.c. inoculation with MuPyV.LT206 virus. (**e**) Direct *ex vivo* intracellular staining for EYFP and IRF4 on D^{b}LT359 tetramer-specific CD8 T cells from the brains of mice at day 30 p.i. after i.c. inoculation with MuPyV.A2. Data are from two independent experiments with 4–5 mice per group. A nonparametric paired Student's *t*-test with Mann–Whitney test was used to determine statistical significance. Values are mean ± s.d.; $^*P \leqslant 0.05$, $^{**}P \leqslant 0.01$, not significant (ns) $P > 0.05$.

METHODS

Mice and viruses

C57BL/6NCr female mice were purchased from the National Cancer Institute (Frederick, MD, USA). B6.PL (Thy1.1) and C57BL/6-Tg (UBC-GFP) 30Scha/J mice ('GFP mice') and B6.129S4-Ifng$^{tm3.1Lky}$/J, IFN-γ reporter 'IFN-γ EYFP' mice were purchased from The Jackson Laboratory (Bar Harbor, ME, USA). TCR-I mice are transgenic for a TCR for the D^b-restricted peptide corresponding to amino acids 206–215 of the SV40 Large T Ag ('LT206').[22] The D^b-restricted LT359–368 epitope in MuPyV Large T Ag (strain A2; 'MuPyV.A2') was replaced by the D^{b}LT206 epitope ('MuPyV.LT206').[22] In all, 6.5×10^4 plaque-forming units per mouse of MuPyV.A2 or MuPyV.LT206 were injected i.c.[16] Mice were bred and housed in accordance with the guidelines of the IACUC of The Penn State College of Medicine.

Depletion of CD8 cells

CD8 Ab (clone YTS 169.4; BioXCell, West Lebanon, NH, USA) or rat IgG Ab (Jackson ImmunoResearch, West Grove, PA, USA) was injected via intraperitoneal route at 250 μg Ab per mouse once per week starting from day 10 p.i. to day 30 p.i. Efficiency of depletion of CD8$^+$ cells in blood was 90% (data not shown).

Adoptive cell transfer

Naive CD8 T cell (CD44^{low}CD62L^{high}) were purified from TCR-I mice using a negative selection isolation kit (Miltenyi Biotec, Ausburn, CA, USA), and unstained or carboxyfluorescein succinimidyl ester-labeled cells were injected intravenously per tail vain in 100 μl phosphate-buffered saline or by i.c. injection in 30 μl phosphate-buffered saline.

Quantitation of MuPyV genomes

Quantitative TaqMan-based PCR was performed using 10 ng template DNA purified from tissues, as described.[22]

Quantitative real-time PCR for transcript analysis

Fluorescence-activated cell sorter (FACS)-purified GFP$^+$ TCR-I cells were lysed in TRIzol (Ambion, Carlsbad, CA, USA) and total RNA was isolated per the manufacturer's instruction. cDNA was prepared using random primers and RevertAid H Minus Reverse Transcriptase enzyme (ThermoFisher Scientific, Leesport, PA, USA). Taqman primer probe set for *Pdcd1* and *Tbp* from IDT Technologies (Coralville, IA, USA) was used to perform quantitative PCR using the ABI StepOnePlus Real-Time PCR System (ThermoFisher Scientific). Sequences of the primer probe sets are as follows: PD-1, 5′-ATTTGCTCCCTCTGACACTG-3′; 5′-GTACCCTGGTCATTCACTTGG-3′; 5′-/56-FAM/TCCCTCACC/ZEN/TTCTACCCAGCCT/3IANKFQ/-3′ and TBP: 5′-TGTATCTACCGTGAATCTTGGC-3′; 5′-CCAGAACTGAAAATCAACGC AG-3′; 5′-/56-FAM/ACTTGACCT/ZEN/AAAGACCATTGCACTTCGT/3IABk FQ/-3′. Relative fold change over naive control was determined using the threshold cycle ($2^{-\Delta\Delta Ct}$) method.

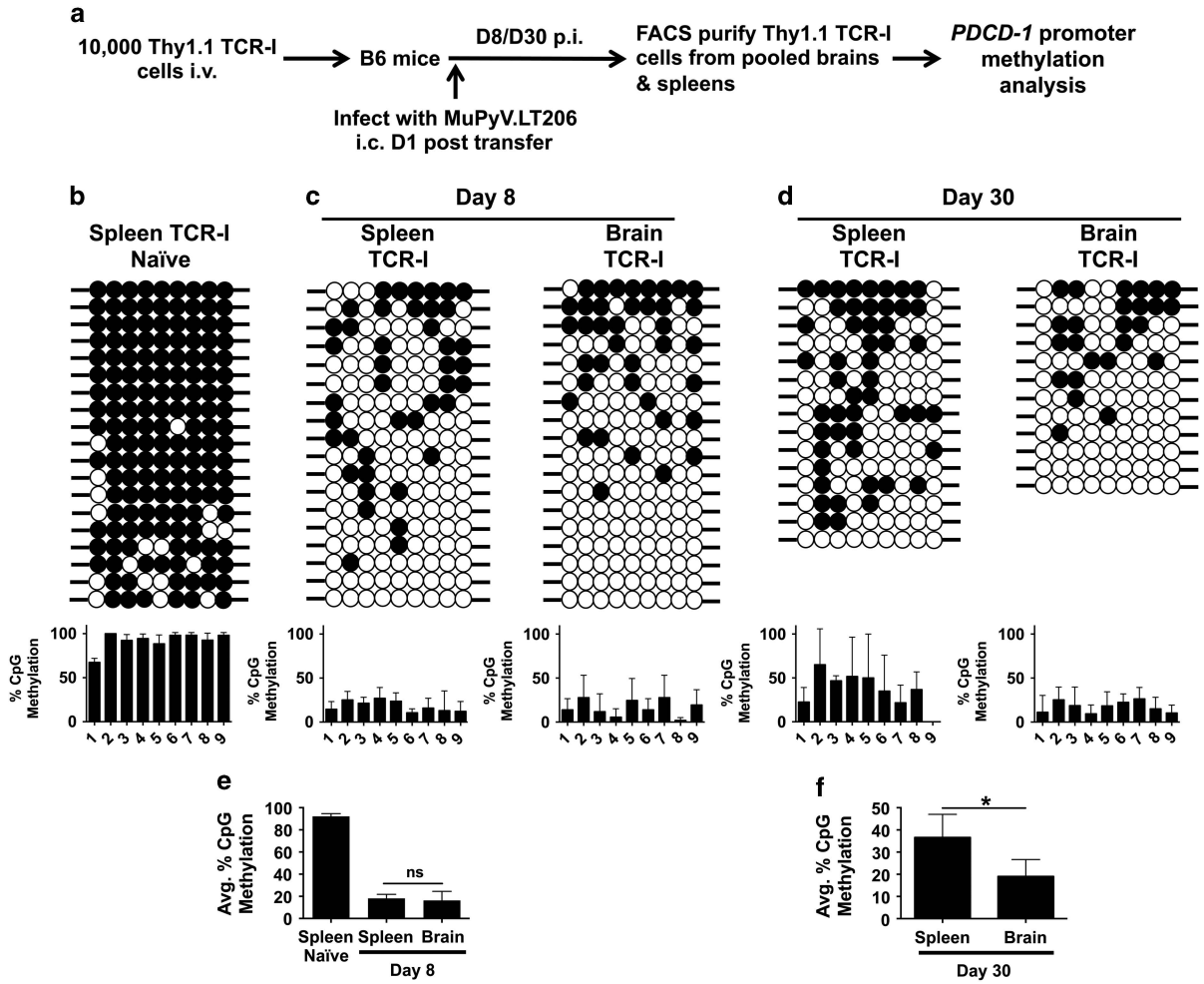

Figure 4 Methylation of the *Pdcd1* promoter from TCR-I cells in uninfected and MuPyV acutely infected mice. (**a**) Experimental setup. (**b**–**d**) Bisulfite sequencing of nucleotides −778 to −465 upstream of the *Pdcd1* promoter from genomic DNA of naive TCR-I mice (**b**) and of Thy1.1 FACS-sorted TCR-I cells from the spleens and brains of MuPyV-infected mice at day 8 p.i. (**c**) and day 30 p.i. (**d**). Quantification of methylation of nine CpGs sites (closed circles) in the *Pdcd1* promoter, as described previously.[13] (**d** and **e**) Combined quantification of percentage of methylation of the *Pdcd1* promoter for day 8 p.i. (**d**) and day 30 p.i. (**e**) (pooled cells from three to five mice, two independent experiments). A nonparametric paired Student's *t*-test with Mann–Whitney test was used to determine statistical significance. Values are mean ± s.d.; $*P \leqslant 0.05$, not significant (ns) $P > 0.05$.

Cell isolation, intracellular cytokine staining and flow cytometry

Mononuclear cells from brain and kidney were isolated from transcardially perfused mice by collagenase-DNase digestion and percoll gradient centrifugation, then treated with Fixable Viability Dye (eBioscience, San Diego, CA, USA) and stained with APC-D^{b}LT359 or BV421-D^{b}LT206 tetramers (NIH Tetramer Core Facility, Atlanta, GA, USA) and antibodies, as described.[16] Samples were acquired on a BD LSRII or BD LSRFortessa (BD Bioscience, San Jose, CA, USA) and analyzed using the FlowJo software (FlowJo, LLC, Ashland, OR, USA). GFP$^+$ cells stained with anti-CD8α and 4,6-diamidino-2-phenylindole were sorted using a BD FACSAria SORP Cell Sorter (BD Bioscience).

In vitro PD-L1 blockade

DC2.4 cells were exposed to IFN-γ to upregulate MHC I and PD-L1 (data not shown) and then pulsed with 10 μM LT206 peptide. Cells were added in the presence of 50 μg ml^{-1} control rat IgG or PD-L1 blocking Ab (10F.9G2; BioXCell). After 5 h incubation with BD GolgiPlug (BD Bioscience), cells were stained with Fixable Viability Dye, anti-CD8α and anti-Thy1.1 and then stained for intracellular IFN-γ.

Epigenetic analysis of PD-1 promoter

Thy1.1 TCR-I cells isolated from the spleens or brains of naive mice or from mice at days 8, 30 or 45 p.i. were FACS-sorted to >95% purity. Genomic DNA was isolated from the purified cells and subjected to bisulfite treatment. Bisulfite-modified DNA was PCR-amplified using PD-1 promoter-specific primers and cloned, and sequences were analyzed using the BISMA software (Bremen, Germany), as described.[13]

Statistical analysis

P-values were determined by an unpaired Student *t*-test with Mann–Whitney posttest or one-way analysis of variance with Kruskal–Wallis posttest using the GraphPad Prism software (GraphPad Software, La Jolla, CA, USA). *P*-values ⩽ 0.05 were considered significant.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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