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Heterogeneity among Viral Antigen-Specific CD4+ T Cells and Their De Novo Recruitment during Persistent Polyomavirus Infection

Eugene Lin, Christopher C. Kemball,¹ Annette Hadley, Jarad J. Wilson, Amelia R. Hofstetter, Christopher D. Pack, and Aron E. Lukacher

Virus-specific CD4+ T cells optimize antiviral responses by providing help for antiviral humoral responses and CD8+ T cell differentiation. Although CD4+ T cell responses to viral infections that undergo complete clearance have been studied extensively, less is known about virus-specific CD4+ T cell responses to viruses that persistently infect their hosts. Using a mouse polyomavirus (MPyV) infection model, we previously demonstrated that CD4+ T cells are essential for recruiting naive MPyV-specific CD8+ T cells in persistently infected mice. In this study, we defined two dominant MPyV-specific CD4+ T cell populations, one directed toward an epitope derived from the nonstructural large T Ag and the other from the major viral capsid protein of MPyV. These MPyV-specific CD4+ T cells vary in terms of their magnitude, functional profile, and phenotype during acute and persistent phases of infection. Using a minimally myeloablative-mixed bone marrow chimeraism approach, we further show that naive virus-specific CD4+ T cells, like anti-MPyV CD8+ T cells, are primed de novo during persistent virus infection. In summary, these findings reveal quantitative and qualitative differences in the CD4+ T cell response to a persistent virus infection and demonstrate that naive antiviral CD4+ T cells are recruited during chronic polyomavirus infection. The Journal of Immunology, 2010, 185: 1692–1700.

CD4+ T cells shape and regulate host immune responses to microbial pathogens (1). Virus-specific CD4+ T cells drive B cell differentiation to memory B cells and plasma cells bearing isotype-switched, affinity-matured Abs (2, 3). A sizeable body of literature documents the critical contribution of CD4+ T cell help for generating memory CD8+ T cells capable of mounting strong anamnestic responses (4). CD4+ T cells may also elaborate antiviral cytokines (5–7) or directly kill infected MHC class II-expressing cells (8–10). In the setting of acute viral infections that are completely resolved, virus-specific CD4+ T cells, like their antiviral CD8+ T cell counterparts, give rise to phenotypically and functionally mature memory T cells that are maintained by Ag-independent self-renewal and are capable of robust recall responses to challenge infection (1, 11, 12).

In the face of high-level persistent virus infection, however, CD4+ T cells are handcapped in their effector activities. In viremic individuals, HIV-specific CD4+ T cells fail to proliferate or produce IL-2 (13) but upregulate inhibitory receptors that mitigate their functional integrity (14, 15). Using the lymphocytic choriomeningitis virus (LCMV) clone 13 chronic infection mouse model, Brooks et al. (16) recently showed that virus-specific CD4+ T cells acquire functional defects early in the course of infection. In contrast, antiviral CD4+ T cells maintained in the setting of low-level persistent virus infections (e.g., human and mouse CMV and EBV) generally retain their functionality (17–20).

Maintenance of memory virus-specific CD8+ T cells in persistent infection, as in acute infection, depends on a functional virus-specific CD4+ T cell response, which is affected by the magnitude and duration of infection and, likely, cell types harboring the infection. For LCMV clone 13 infection, functional deterioration and deletion of virus-specific CD8+ T cells are exacerbated by the absence of CD4+ T cells, with recovery of CD8+ T cell numbers and function associated with resolution of viremia (21–23). For low-level persistent infections, CD4+ T cell deficiency likewise dramatically affects the size of the memory antiviral CD8+ T cell compartment. Maintenance of stable virus-specific CD8+ T cell numbers during persistent viral infection depends on extensive proliferation of memory CD8+ T cells and/or de novo priming of naive virus-specific CD8+ T cells (24–26). For persistent mouse polyomavirus (MPyV) infection, virus-specific CD8+ T cells fail to divide and suffer rapid attrition, putting the onus for their maintenance on naive Ag-specific CD8+ T cell recruitment (25, 27). Moreover, we recently demonstrated that CD4+ T cell help is required for recruitment of naive antiviral CD8+ T cells during persistent MPyV infection (28). Whether or not MPyV-specific CD4+ T cells conserve their effector activities, are stably maintained, and are resupplied via naive CD4+ T cell recruitment during persistent infection is not known.

Efforts to characterize CD4+ T cells in persistent infections have been hampered by the small numbers of defined epitopes, the low magnitude of their response, and until recently, few reagents to track Ag-specific populations compared with CD8+ T cells. To assess the evolution of the antiviral CD4+ T cell response through acute and persistent phases of MPyV infection, we have identified two MPyV-specific CD4+ T cell populations in B6 mice, one directed toward an epitope derived from the nonstructural large T (LT) Ag and the
other from the major viral capsid protein of MPyV (VP1). These epitope-specific CD4+ T cells differ in magnitude, phenotype, functional profile, and TCR repertoire. However, CD4+ T cells of both specificities are stably maintained during persistent infection despite exhibiting minimal proliferation. We show in this study that naive progenitors of each specificity are primed de novo in persistently infected mice. These findings highlight the heterogeneity of the CD4+ T cell response during this low-level systemic persistent viral infection and suggest that recruitment of naive virus-specific T cells contributes to the maintenance of virus-specific CD4+ T cell populations.

Materials and Methods

Mice
C57BL/6NCr (B6) and B6/CD45.1 female mice were purchased from the Frederick Cancer Research and Development Center of the National Cancer Institute (Frederick, MD). Mice were housed and bred in accordance with the guidelines from the Institutional Animal Care and Use Committee and the Department of Animal Resources of Emory University (Atlanta, GA).

MPyV preparation and inoculation
MPyV strain A2 was molecularly cloned and plaque purified, and virus stocks were prepared on primary baby mouse kidney cells as described previously (29). Each mouse received 2 × 10^6 PFU of virus s.c. Mice were inoculated at 6–12 wk of age.

Cell isolation and flow cytometry
Spleens were digested in a solution of 125 U/ml collagenase type XI, 60 U/ml hyaluronidase type I-s, and 60 U/ml DNase I in RPMI 1640 supplemented with 5% FCS plus Ca^2+ and Mg^2+ for 1 h at 37°C, then passed through cell strainers followed by RBC lysis. Allotypiccoinconjugated I-A^b/LT678–690 and I-A^b/VP1221–235 tetramers were constructed by the National Institutes of Health Tetramer Core Facility at Emory University (Atlanta, GA). Tetramer staining was performed at 37°C for 1.5 h in the RPMI 1640 containing 2% FCS at a concentration of 8 μg/ml per 1 × 10^6 cells, followed by surface staining for CD4, CD44, and other indicated surface markers at 4°C for 30 min. In some experiments, cells were stained with 7-aminoactinomycin D (7-AAD, BD Pharmingen, San Diego, CA) per manufacturer’s instructions. For CTLA-4, Bcl-2, Ki-67, and TCR VB, cells were permeabilized with Cytotix/Cytoperm buffer (BD Pharmingen) after I-Ab tetramer and surface staining then stained intracellularly for the indicated marker. For Annexin V staining, I-Ab tetramer and surface marker-stained cells were stained with FITC Annexin V (BD Pharmingen) followed by 7-AAD.

The following mAbs were purchased from BD Pharmingen: FITC-conjugated anti-CD44 and anti–Ki-67; allotypiccoinconjugated anti–IFN-γ; PE-conjugated anti-CD62L, Bcl-2, IL-2, TNF-α (TN3-19.12), IL-10, IL-17, and IL-18; V450-conjugated anti-CD44; and isotype controls. Pacific blue-conjugated anti-CD4 (RM4-5); PerCP-Cy5.5-conjugated mAb to CD45.1 and CD4 (RM4-5); PE-conjugated mAb to CD27 (L27.7P9), CD127 (AATR34), programmed death 1 (PD-1) (RMP1-30), LAG-3, TIM-3 (RMT3-23), CD154, CTLA-4, ICOS (7E.17G9), OX-40, CD28, and isotype controls were purchased from eBioscience (San Diego, CA). Samples were acquired either on a FACSCalibur or LSR-II (both from BD Biosciences, San Jose, CA). Data were analyzed using FlowJo software (Tree Star, Ashland, OR).

Brdu incorporation assay
MPyV-infected B6 mice were administered 1 mg Brdu (Sigma-Aldrich, St. Louis, MO) i.p. once daily for 7 d. On the day after the last injection of Brdu, mice were sacrificed, and splenocytes were stained with I-A^b/LT678 or I-A^b/VP1221 tetramers followed by surface staining for CD4 and CD44. Brdu incorporation was then assessed using the FITC Brdu flow kit (BD Biosciences) per manufacturer’s instructions.

Intracellular cytokine staining
Cells were stimulated with peptide (10–50 μM) for 5.5 h in the presence of GolgiPlug (brefeldin A; BD Pharmingen) or GolgiStop (monensin; BD Pharmingen), then surface stained with PerCP-Cy5.5-conjugated anti-CD4 (RM4-5). After washing, cells were permeabilized with Cytofix/Cytoperm buffer (BD Pharmingen) and stained for intracellular IFN-γ and CD154. For multicytokine analysis, cells were stained intracellularly with anti–IFN-γ and PE-conjugated Abs to IL-2, TNF-α, IL-10, IL-17, or IL-4. Intracellular staining for IL-21 was performed as described by Suto et al. (30).

In vivo programmed death ligand 1 blockade
B6 mice persistently infected by MPyV (35–50 d postinfection [p.i.]) received 200 μg rat anti-mouse programmed death ligand 1 (PD-L1) Ab (10F.9G2) or rat IgG control i.p. every 3 d over 2 wk.

Quantification of PyV genomes
DNA isolation and TaqMan PCR were performed as described previously (27). MPyV DNA quantity is expressed in genome copies per milligram of tissue and is calculated based on a standard curve of known MPyV genome copy number versus threshold cycle of detection. The detection limit with this assay is 10 copies of genomic viral DNA.

Generation of CD45 congenic bone marrow chimeras
A minimal myeloablation and bone marrow transplantation protocol was performed as previously described, with the following modifications (25, 27). Naive or persistently MPyV-infected B6 mice were given 600 μg busulfan i.p. (Busulfex; Otsuka America Pharmaceutical, Rockville, MD). Twenty-four hours later, these mice received 2 × 10^6 nucleated cells i.v. from the bone marrow of naive B6/CD45.1 mice. Establishment of chimerism was confirmed by flow cytometric analysis of whole-blood cells for CD45.1 expression (data not shown). We have previously shown that the minimally myeloablative dose of busulfan that we use (25 mg/kg) does not impact the number of PyV genomes per milligram tissue (27). Spleens of chimeric mice were analyzed by flow cytometry >90 d after bone marrow transplantation.

IFN-γ ELISPOT assay
The single-cell ELISPOT assay was performed as described previously (27).

Statistics
Statistical significance was determined by a two-tailed nonparametric Mann-Whitney U test using Prism software (GraphPad, La Jolla, CA). A p value <0.05 was considered statistically significant.

Results

Identification of MPyV-specific CD4+ T cell epitopes
As a first step to empirically map MPyV-specific CD4+ T cell epitopes, we used IFN-γ ELISPOT assays to screen overlapping synthetic peptides covering sequences for each of the six MPyV proteins for their ability to stimulate spleen cells from acutely infected B6 mice. Overlapping peptides yielding positive wells were then assayed by IFN-γ intracellular staining and CD4 surface staining, and minimum length peptides stimulating optimal responses were determined. From these analyses, one 13-mer and one 15-mer CD4+ T cell epitope for MPyV were identified: LT678–690 (NEYLLPQTVW ARF) from the nonstructural LTAg and VP1221–235 (GMYPVEIWHPDPAKN) from the VP1 major capsid protein. Fig. 1A shows a representative example of LT678–690- and VP1221-induced intracellular IFN-γ production by splenic CD4+ T cells from B6 mice at day 8 p.i. To facilitate detection of Ag-reactive CD4+ T cells, we performed intracellular co-staining for CD154 (CD40L), which is transiently expressed by CD4+ T cells following Ag stimulation (31); nearly all of the Ag-reactive CD4+ T cells coexpressed CD154.

To enumerate and phenotype MPyV-specific CD4+ T cells directly ex vivo, we used MHC class II tetramers complexed to each of these antigenic peptides. As shown in Fig. 1B, I-A^b/LT678 and I-A^b/VP1221 tetramers bound Ag-experienced (i.e., CD44hi) splenic CD4+ T cells, which were not stained by control I-A^b/HCLIP tetramers; in addition, I-A^b/LT678 and I-A^b/VP1221 tetramers did not stain CD4+ T cells from uninfected B6 mice. Using these tetramers, we found that >2-fold more VP1221-specific CD4+ T cells than LT678-specific cells were detected by tetramers in spleens of day 8-infected mice (Fig. 1C). The larger number of I-A^b/VP1221 tetramer+ cells was evident as early as 6 d p.i. and continued well into the persistent phase of infection. Notably, both CD4+ T cell populations underwent a relatively small contraction and were then maintained at steady
levels, response profiles that stand in contrast to the progressive attrition of memory CD4+ T cell populations seen in acute LCMV and *Listeria monocytogenes* infections (32–34).

**VP1221- and LT678-specific CD4+ T cells differ in functional competence**

In Fig. 1A we noted that fewer VP1221-specific CD4+ T cells at day 8 p.i. produced IFN-γ than were stained by I-A^b/VP1221 tetramers, a difference not seen with the LT678-specific CD4+ T cells. By peptide titration, we first determined that 50 μM peptide stimulated maximal intracellular IFN-γ production by both epitope-specific CD4+ T cell populations and did not resolve the apparent absence of IFN-γ production by VP1221-specific CD4+ T cells (data not shown). LT678-specific CD4+ T cells were also found to express equivalent levels of surface CD3 and slightly higher levels of CD4 than naive T cells (data not shown), as previously reported for Ag-stimulated CD4+ T cells (35). In addition, the frequency of CD4+ T cells induced to produce IFN-γ by VP1221 peptide stimulation closely approximates those that upregulate CD40L (Fig. 1A and data not shown), suggesting that a proportion of the VP1221-specific CD4+ T cell population is refractory to T cell activation. We then asked whether this early decline in functionality by VP1221-specific CD4+ T cells was a transient defect that resolved over time and whether LT678-specific CD4+ T cells preserved their cytokine effector function during persistent MPyV infection. As shown in Fig. 2A, only 40–50% of the I-A^b/VP1221 tetramer+CD4+ T cells produced IFN-γ at all acute and persistent infection time points examined, whereas LT678-specific CD4+ T cells retained function well into persistent infection. Moreover, when one takes into account this selective dysfunction by the VP1221-specific CD4+ T cells, the numerical advantage of these cells over the LT678-specific CD4+ T cells nearly vanishes during acute infection and narrows by approximately half of that based on tetramer binding during persistent infection (Fig. 2B). Finally, these analyses reveal that this IFN-γ deficiency by the VP1221-specific CD4+ T cells is evident as early as day 6 p.i., suggesting that factors early in acute infection affect the cytokine effector profile or long-term fitness of the CD4+ T cell response.

We next asked whether this lack of IFN-γ production by nearly half of the VP1221-specific CD4+ T cells extended to other effector functions. Using spleen cells from acutely (day 8 p.i.) and persistently (day 60 p.i.) infected mice, we determined the frequency of IFN-γ+ cells that coproduced either TNF-α or IL-2 after stimulation by VP1221 or LT678 peptides. The data in Fig. 3A show that only a fraction of either the VP1221-specific or LT678-specific CD4+ T cell populations in acutely infected mice coproduce TNF-α and IL-2 along with IFN-γ, although the inability to produce these effector cytokines is greater for the VP1221-specific CD4+ T cells (especially when taking into account their lack of IFN-γ-producing capability). Moreover, the deficiency in coproducing these cytokines was maintained into the persistent phase of MPyV infection.

We then investigated the possibility that these VP1221-specific CD4+ T cells may have modulated their effector cytokine profiles. Although neither the VP1221- nor LT678-specific CD4+ T cell populations produced IL-4 or IL-17 (data not shown), we observed that a fraction of IFN-γ+ cells of each specificity coproduced IL-21 or IL-10 (Fig. 3B). Moreover, in contrast to the stability of IFN-γ/TNF-α/IL-2 coproducers over the course of MPyV infection, for both VP1221- and LT678-specific CD4+ T cells, there was a doubling of those making IL-21 but a halving of those making IL-10 between acute and persistent stages of infection. In addition, <5% of either VP1221- or LT678-specific CD4+ T cells express the regulatory T cell transcription factor Foxp3 in either acutely or persistently infected mice (data not shown). As recently reported for influenza virus-specific CD8+ T cells (36), it is tempting to speculate that these virus-specific CD4+ T cells may adjust secretion of IL-10 to counter immune-mediated pathology, the potential for which would be higher in acute than persistent infection. The shift toward IL-21 production by persistent infection-phase MPyV-specific CD4+ T cells also fits with recent reports showing that this cytokine contributes to maintenance of virus-specific CD8+ T cells during chronic LCMV infection (37–39).
CD4+ T cells during acute infection is in line with the interpretation of IFN-γ. Differences between frequency of IFN-γ and I-Aβ tetramer+CD4+ T cells is statistically significant by the nonparametric Mann-Whitney U test (p < 0.05) at each time point for VP1221-specific CD4+ T cells and days 10 and 100 p.i. for LT678-specific cells. Enumeration of splenic LT678- and VP1221-specific CD4+ T cells might exhibit differences in cytokine effector function between LT678- and VP1221-specific CD4+ T cells in absence of peptide subtracted) over the course of MPyV infection. Numbers and frequencies are of CD4+ T cells that are I-Ab tetramer+CD44hi or IFN-γ+CD154+. Values for each time point represent the mean ± SD of six to nine mice from two to three independent experiments.

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TCR repertoire and phenotypic heterogeneity of MPyV epitope-specific CD4+ T cells

Differences in efficiency of T cell activation by Ag in vivo have been correlated with changes in the level of clonotypic diversity by the responding T cells (40). We thus asked whether the VP1221-specific and LT678-specific CD4+ T cells might exhibit differences in TCR repertoire diversity early in the course of MPyV infection. By co-staining I-Ab tetramer+CD4+ T cells from individual B6 mice with a panel of Vβ mAbs, we observed that the LT678-specific T cells express a diverse, and mouse-to-mouse variable, Vβ usage profile. In sharp contrast, 80–90% of the VP1221-specific CD4+ T cells in individual B6 mice predominantly express TCRs using Vβ8.1/8.2 and Vβ14 domains (Fig. 4). This constrained Vβ expression profile by the VP1221-specific CD4+ T cells during acute infection is in line with the interpretation that only a subset of the naive T cell population directed to this epitope is recruited.

T cells maintained in the setting of persistent Ag often express surface receptors that dampen their effector function (15, 41). Recent studies using the LCMV infection model have shown that the extent of T cell dysfunction parallels both the level of expression and diversity of inhibitory receptors expressed by virus-specific CD8+ T cells, which correlates with the severity and duration of persistent infection (42). We therefore asked whether differences in cytokine effector function between LT678- and VP1221-specific CD4+ T cells were reflected in differences in T cell functionality and diversity of inhibitory receptors expressed by virus-specific CD8+ T cells. Although PD-1 on its own does not appear to play a role in limiting the function of VP1221-specific CD4+ T cells, other inhibitory mechanisms may contribute. Thus, it will be of interest in future studies to see whether CTLA-4 blockade singly or in combination with PD-1 blockade bolsters the expansion and function of MPyV-specific CD4+ T cell functionality was similar between anti–PD-L1-treated and control mice. In addition, no differences between these groups were seen in the dominant D9/LT359-specific CD8+ T cell response or in MPyV viral levels (data not shown). Although PD-1 on its own does not appear to play a role in limiting the function of VP1221-specific CD4+ T cells, other inhibitory mechanisms may contribute. Thus, it will be of interest in future studies to see whether CTLA-4 blockade singly or in combination with PD-1 blockade bolsters the expansion and function of MPyV-specific T cells and results in a drop of the persistent MPyV infection set point.

The likelihood that these two MPyV-specific CD4+ T cell populations differentially engage Ag is further supported by other phenotypic differences. Although VP1221-specific and LT678-specific CD4+ T cell populations in persistently infected mice are CD62Llo and express decreased levels of Bcl-2 compared with CD44hiCD62lo cells, indicative of an effector/effector-memory differentiation state, all of the LT678-specific CD4+ T cells and most of the VP1221-specific CD4+ T cells are CD127hi and CD27hi (Fig. 5B, 5C). An explanation for this mixed effector-
memory/central-memory phenotype may lie in the slower pace for re-expression of surface CD62L than CD127 and CD27 by T cells when cognate Ag is limiting (43). Yet, VP1221-specific CD4+ T cells also have sizeable CD127lo and PD-1hi populations, whereas LT678-specific CD4+ T cells are uniformly CD127hi and PD-1int (Fig. 5A,5B), suggesting that VP1221-specific CD4+ T cells are more chronically stimulated.

Recruitment of MPyV-specific CD4+ T cells during persistent infection

We previously demonstrated by adoptive transfer that MHC class Ia-restricted MPyV-specific CD8+ T cells during persistent infection are short-lived (25). However, because of the small frequency of MPyV-specific CD4+ T cells in persistently infected mice, we were unable to detect them by tetramer staining and follow their fate after adoptive transfer to infection-matched recipients. Instead we assessed expression of both the prosurvival molecule Bcl-2 and the apoptotic marker Annexin V on LT678- and VP1221-specific CD4+ T cells in persistently infected mice (day 60 p.i.). Compared with CD44hiCD4+ cells, both MPyV-specific CD4+ T cell populations expressed lower levels of Bcl-2 and higher levels of surface Annexin V staining (Fig. 5C). These data suggest that MPyV-specific CD4+ T cells are short-lived.

Because the LT678- and VP1221-specific CD4+ T cell populations are stably maintained during persistent infection (Figs. 1C, 2B), we then asked whether these T cells were capable of self-renewal and/or were resupplied by de novo priming of naive Ag-specific T cells. We monitored levels of DNA synthesis of persistently infected LT678- and VP1221-specific CD4+ T cells based on their incorporation of the thymidine analog BrdU. We observed little anti-BrdU costaining of either I-Ab tetramer+CD4+ T cell population in contrast to the strong BrdU costaining of these cells in acutely infected mice (Fig. 5D). Consistent with this finding, few splenic I-Ab tetramer+CD4+ T cells from persistently infected mice expressed the nuclear proliferation marker Ki-67 (data not shown).

FIGURE 3. Variations in effector cytokine profiles of LT678-specific and VP1221-specific CD4+ T cells over the course of MPyV infection. At acute (day 8 p.i.) and persistent (day 60 p.i.) infection time points, splenocytes were stimulated with LT678 or VP1221 peptides for 5.5 h then stained for surface CD4 followed by costaining for intracellular IFN-γ and TNF-α, IL-2, IL-10, or IL-21. A, Representative plots of IFN-γ/TNF-α or IFN-γ/IL-2 (left panels) and frequency of IFN-γ+CD4+ cells that express TNF-α or IL-2 (right graphs). B, Frequency of IFN-γ+CD4+ cells that express IL-10 or IL-21. Data in all graphs represent mean ± SD of six mice in two independent experiments. The p values were calculated using the nonparametric Mann-Whitney U test.
ual mice showed variability in the frequency of I-Ab tetramer+ CD4+ T cells that are donor-derived (Fig. 6B). Notably, despite having similar levels of chimerism, individual mice showed variability in the frequency of I-A^b tetramer+ CD4+ T cells that are donor-derived (Fig. 6B). This mouse-to-mouse variability may be a reflection of differences in frequency of encounter by naive virus-specific CD4+ T cells with low numbers of epitope-expressing MHC class II+ cells in persistent MPyV-infected mice; moreover, it is conceivable that the range of viral epitopes presented by APCs during persistent infection may vary among individual mice as a function of those that are productively (i.e., expressing both structural and nonstructural viral proteins) and nonproductively (i.e., expressing only nonstructural viral proteins) infected. Taken together, these data demonstrate that MPyV-specific CD4+ T cells are recruited during persistent infection.

**Discussion**

The natural MPyV provides a valuable model for studying T cell responses to viruses that establish low-level systemic persistent infection. In this study, we defined two I-A^b–restricted viral protein epitopes, one recognized by CD4+ T cells directed toward the nonstructural LT Ag and the other toward the VP1 capsid protein. Using MHC class II tetramers and intracellular cytokine assays, we found that virus-specific CD4+ T cells to these epitopes differed quantitatively and qualitatively over the course of infection. Most notably, the VP1-specific CD4+ T cell response dominated that directed toward LT but differed from the latter in terms of its profound early and sustained effector cytokine dysfunction, which was not offset by blockade of the PD-1:PD-L1 pathway. Despite this difference, the LT-specific and VP1-specific CD4+ T cell populations are stably maintained during persistent infection. Data presented in this paper showing that MPyV-specific CD4+ T cell populations exhibit little proliferation in persistently infected hosts with a substantial number suffering apoptosis, coupled with the finding that naive CD4+ T cells of both specificities are primed in persistent infection, suggest that de novo recruitment contributes to maintenance of MPyV-specific CD4+ T cells in chronically infected hosts.

CD4+ T cell expansion and differentiation are governed by multiple factors, including level and duration of epitope availability, sustained colocalization with APCs, costimulation, and proinflammatory cytokines (44). For persistent viruses that make up a host’s “virome,” additional factors merit consideration, such as viral tropism, kinetics of viral gene expression, state of the viral genome during persistent infection, and tactic(s) orchestrated by the virus to evade host surveillance (45). Characterization of MPyV-specific CD4+ T cell responses to epitopes from a nonstructural and structural viral protein may offer insights into the relationship between polyomaviruses and their hosts. For example, although MPyV DNA is detectable long term at low levels in select tissues, whether it is maintained latently in an episomal state or whether infectious virus is continuously produced at low yield by semipermissive cells remains an open question. As VP1 protein production is generally taken as evidence of productive infection, PD-1 and CTLA-4 upregulation by VP1221-specific CD4+ T cells in persistently infected mice, suggestive of repetitive Ag stimulation, favors the infectious state scenario. This possibility is further supported by evidence presented in this paper that naive VP1-specific CD4+ T cells are primed de novo during persistent infection.

Differences in functional competence by the LT678-specific and the VP1221-specific CD4+ T cells could stem from differences in availability of MHC class II-restricted epitopes derived from nonstructural and structural viral proteins. Extending from studies showing a positive association between Ag levels and CD8+ T cell dysfunction (46, 47), the effector cytokine defect of VP1221-specific, but not LT678-specific, CD4+ T cells suggests more frequent TCR engagement by the former. This functional disparity may lie in differences in the range of potential APCs and the ability of these viral proteins to access the MHC class II processing machinery. Because polyomaviruses bind to sialylated glycolipids and glycoproteins, viropions adsorb to a broad array of cells, irrespective of their capacity to support infection (48). Bound viropions will thus undergo receptor-mediated endocytosis, which would facilitate shunting of capsid proteins into degradative and MHC class II peptide-loading compartments. In addition, the ratio of infectious to physical particles for MPyV in vitro is ~1:100, raising the possibility that defective viropions (including empty capsids) could also contribute VP1-derived epitopes for MHC class II presentation. In contrast, as expression of nonstructural viral T proteins requires synthesis by infected cells, access to MHC class II compartments would most likely be relegated to less efficient nonreceptor-mediated uptake of protein debris from lytically infected cells.

Recent reports detailing Ag-specific CD4+ T cell expansion, maintenance, and function to different experimental viral infections
highlight variability, but also commonalities, among these responses. For example, infection by acutely resolved LCMV-Armstrong gives rise to virus-specific memory CD4+ T cells that then suffer progressive attrition, but without appreciable functional impairment (32). In contrast, virus-specific CD4+ T cells to the persistently infecting mouse CMV are stably maintained during latent infection and retain polyfunctionality (17, 20). Examination of the I-Ab/GP61–80–specific CD4+ T cell response to LCMV clone 13, which establishes high-level persistent infection, revealed functional inactivation of these T cells as early as day 9 of infection (16). Analysis of the MPyV-specific CD4+ T cell response yielded a composite of these quantitative and qualitative behaviors, with stable maintenance of virus-specific CD4+ T cell populations during persistent infection yet with variations in functional integrity among epitope-specific responses. Reminiscent of the GP61–80-specific CD4+ T cell response to LCMV clone 13 infection, dysfunction of the MPyV VP1221–specific CD4+ T cell response was also evident during acute infection. Thus, events at the level of priming (e.g., epitope presentation by nonprofessional APCs) or soon thereafter (e.g., high epitope density and high TCR avidity) may handicap...
May be suboptimally primed by productively infected APCs, (Control I-Ab/hCLIP tetramer staining of donor and host CD4+ T cell population expressing equivalent TCR levels by surface anti-CD3ε population). To support the above possibility, we found that I-Aα/VP1221 class II tetramer MFI at optimal concentrations have been taken as a sufficient high avidity are recruited (40). Differences in MHC TCR Vb structural T Ags, because these are also expressed by abortively infected cells. As a result, naive VP1221-specific CD4+ T cells may be shorter than for T cells recognizing epitopes from non-viral capsid proteins is a harbinger for virus-induced cell death, the MPyV life cycle. Because expression of VP1221 of the human BK polyomavirus are also detected in seropositive fraction of the VP1221-specific CD4+ T cell population.

Early dysfunction by the VP1221-specific CD4+ T cells may also be a consequence of the MPyV life cycle. Because expression of viral capsid proteins is a harbinger for virus-induced cell death, the duration of antigenic stimulation for capsid epitope-specific T cells may be shorter than for T cells recognizing epitopes from non-structural T Ags, because these are also expressed by abortively infected cells. As a result, naive VP1221-specific CD4+ T cells may be suboptimally primed by productively infected APCs, whereas naive LT678-specific CD4+ T cells may continue to receive stimulation by nonproductively infected APCs. This alternative scenario may account not only for the early functional deficiencies of the VP1221-specific CD4+ T cells but also for their restricted TCR Vβ usage if only a subset of the naive T cells having TCRs of sufficiently high avidity are recruited (40). Differences in MHC class II tetramer MFI at optimal concentrations have been taken as a measure of differential TCR binding affinity (49, 50). Thus, in support of the above possibility, we found that I-Aβ/VP1221 tetramers stain splenic CD4+ T cells from day 8 p.i. mice with significantly higher gMFI than I-Aβ/LT678 tetramers, with each population expressing equivalent TCR levels by surface anti-CD3ε staining (data not shown). Stronger TCR signaling by higher-affinity VP1221-specific CD4+ T cells may also account for their higher level of expression of PD-1 and CTLA-4 receptors.

A central finding of this study is that naive MPyV-specific CD4+ T cells are recruited in persistently infected mice. This result is in line with recent reports demonstrating emergence of novel clonotypes of virus-specific CD4+ T cells during persistent CMV infection in humans and rhesus macaques and evidence for de novo generation of virus-specific CD4+ T cells in baboons persistently infected by baboon CMV and an EBV-like virus (51–53). Given the variability in priming conditions and history of antigenic exposure of individual virus-specific T cells over the course of a persistent infection, it is not surprising that a phenotypic snapshot of these cells in chronically infected hosts reveals heterogeneity in markers indicative of effector/effector-memory/central memory differentiation, as seen for MPyV-specific CD4+ and CD8+ T cells (Fig. 5) (25). Kinetic profiling of the MPyV-specific CD4+ T cell response (Fig. 1C) is notable for its exceedingly shallow contraction phase, suggesting that naive antiviral CD4+ T cells may be vigorously recruited even at this early time point. Because more LT678-specific than VP1221-specific CD4+ T cells bind the Annexin V marker of apoptosis (Fig. 5C), even though both populations maintain stable numbers, it is also tempting to speculate that the efficiency for recruitment during persistent infection is higher for the LT678-specific CD4+ T cells. In addition, our data offer a possible explanation for the deterioration of the memory CD4+ T cell pool in mice infected by LCMV-Armstrong (i.e., lack of I-Aβ/GP61–80 Ag for priming naive CD4+ T cells to replenish the virus-specific memory CD4+ T cell compartment).

As we recently reported, CD4+ T cells, although dispensable for expansion of MPyV-specific CD8+ T cells during acute infection, are essential for de novo priming of antiviral naive CD8+ T cells in persistently infected hosts (28). The low-level inflammatory milieu in persistent infection may shift the burden to CD4+ T cells for licensing APCs to drive naive virus-specific CD8+ T cell differentiation. Collectively, our data favor the concept that ongoing recruitment of naive virus-specific CD4+ T cells is linked to priming of naive antiviral CD8+ T cells during persistent infection.

In summary, we have found marked differences between two viral epitope-specific CD4+ T cell responses to MPyV infection. Interestingly, despite overall differences in magnitude, which is conventionally used to denote level of immunodominance, the LT678- and VP1221-specific CD4+ T cell responses are nearly co-dominant based on IFN-γ function. Thus, at least with respect to persistent infections, functional rather than numerical attributes may be a more meaningful guide to positioning epitope-specific T cell populations within immunodominance hierarchies. It is also interesting to note that CD4+ T cells directed to epitopes in LT and VP1 of the human BK polymavirus are also detected in seropositive healthy individuals (54). Infections by BK and the related human polymavirus, JC, are associated, respectively, with kidney transplant rejection and progressive multifocal leukoencephalopathy, a demyelinating disease seen in patients immunocompromised by HIV/AIDS or by agents that block T cell entry into the CNS (55, 56). Another implication of our data, then, is that immunomodulatory regimens designed to interdict T cell priming may interfere with recruitment of virus-specific naive T cells, shrinking the antiviral effector/memory T cell pool needed to keep otherwise silent persistent viral infections in check.

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Disclosures

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