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$CD4^+$ T-cell inhibitory ligands: a tool for characterizing dysfunctional $CD4^+$ T cells during chronic infection

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Summary

Activation of $CD4^+$ T cells helps to establish and maintain immune responses. During infection with lymphocytic choriomeningitis virus (LCMV) clone 13, the $CD4^+$ T-cell responses are lost. In this study, we were interested in the nature of the $CD4^+$ T-cell responses following infection with LCMV clone 13. To pursue this question, we infected C57BL/6 mice with LCMV clone 13. We used a GP66-80 MHC Class II tetramer to determine whether the $CD4^+$ T cells were present following infection with LCMV clone 13. We determined that the cells were present and antigen specific, but not functional. We attributed their dysfunction to the presence of $CD4^+$ T-cell inhibitory ligands. We further stained for the presence of $CD4^+$ T-cell inhibitory ligands. We found that the during chronic infection the number of $CD4^+$ T cells expressing programmed death-1 and CD160 were greater over the time–course study than the other $CD4^+$ T-cell inhibitory ligands. These data show that using $CD4^+$ T-cell inhibitory ligands as a reagent for characterization can help in understanding the complex immune responses associated with persistent infections.

Keywords: CD160; $CD4^+$; lymphocytic choriomeningitis virus; programmed death-1; tetramer.

Introduction

$CD4^+$ T cells play an important role in host defence against infection. $CD4^+$ T cells provide help to B cells, specifically those that produce neutralizing antibodies. $CD4^+$ T cells are also required for the activation, persistence and memory of $CD8^+$ T cells. $CD4^+$ T cells may also assist in viral clearance through the production of cytokines and cell-mediated cytotoxicity.[1]

Immune responses associated with pathogen clearance have been attributed to the activation and expansion of $CD4^+$ T cells that are virus specific. These virus-specific $CD4^+$ T cells are mediated through mechanisms by which priming and maintenance of cytotoxic T-lymphocyte (CTL) responses act to clear viral infection.[2–7] Certain viruses such as HIV, lymphocytic choriomeningitis virus (LCMV), and hepatitis C virus have developed mechanisms to establish persistent infections. Although there are potent responses early in infection, CTL responses have been shown to be ineffective in clearing these viral infections.[8–12] Several mechanisms have been shown to contribute to the ineffectiveness of the host in clearing these infections, which include clonal exhaustion, overexpression of programmed death 1 (PD-1), the rapid appearance of viral mutations resulting in escape variants yielding evasion from CTL responses and the lack of $CD4^+$ T-cell help early in infection.[6,10,13–20] In our study, we wanted to focus on the role of $CD4^+$ T-cell help in developing more functional CTL responses.

In our previous study, $CD4^+$ T-cell responses were characterized during acute infection with LCMV Armstrong, by measuring responses directed against the MHC class II restricted CTL epitopes in enzyme-linked immunospot (ELISPOT) and intracellular cytokine staining assays.[21,22] We have shown that in the H-2[d] setting, there were nine MHC class II-restricted epitopes directed against the NP, GP and Z proteins.[22] In the H-2[b] setting, we found that there were six MHC class II-restricted epitopes directed against the NP and GP proteins. In both of these settings, there were overlapping MHC class I-restricted epitopes. In both systems, a broad repertoire of MHC class II-restricted responses exists, which shows

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the complex nature of the helper responses. Other viruses such as vaccinia virus and influenza virus also have a broad repertoire of T helper responses, which suggests that the complex $CD4^+$ T-cell responses are not unique to LCMV.[21,22] Understanding the complex $CD4^+$ T-cell helper responses is vital to viral clearance.

In chronic LCMV infection, the immune system does not control viral replication effectively.[6,23–27] Little is known about $CD4^+$ T cells and the mechanism through which LCMV is able to persist during chronic infection with LCMV clone 13. During clone 13 infections, the immune system is suppressed. It has been shown that dendritic cells are overly infected and unable to stimulate a proper T-cell response.[28] The role of T helper responses has been insufficiently studied.

We were interested in whether $CD4^+$ T cells are present or deleted during infection with LCMV clone 13. Further, if the $CD4^+$ T cells are present and dysfunctional, we wanted to know what mechanism rendered them dysfunctional. Hence, in this study, we investigated the role of $CD4^+$ T-cell responses in chronic infection.

To address the question of whether $CD4^+$ T cells are present during chronic infection of LCMV, we infected C57BL/6 mice with LCMV Armstrong and clone 13 and determined the presence of $CD4^+$ T cells by surface staining for the presence of the $CD4^+$ marker. To further probe the specificity of the $CD4^+$ T cells, we used a tetramer specific for the GP66-80 epitope. The tetramer was tested after infection with LCMV Armstrong and clone 13 to determine the differences in the $CD4^+$ T-cell response between the two strains of LCMV.

Finally, we tested several different $CD4^+$ inhibitory ligands to characterize their differential expression during chronic infection. In an effort to characterize $CD4^+$ T-cell dysfunction, the expression of known inhibitory molecules was screened on the surface of $CD4^+$ T cells because of their previously observed presence on $CD8^+$ T cells.[13] The inhibitory molecules PD-1 and lymphocyte activation gene 3 (LAG-3) are over-expressed on $CD8^+$ T-cell lymphocytes during clone 13 infections and their subsequent blockade leads to a reversal of $CD8^+$ T-cell exhaustion.[29] It is also known that HIV-specific $CD4^+$ T cells co-express PD-1 and another inhibitory molecule cytotoxic T-lymphocyte antigen 4 (CTLA-4).[30] CD160 has been reported as an inhibitory molecule that is co-expressed along with PD-1, CTLA-4 and LAG-3 on $CD8^+$ T cells during chronic infection in the $H\text{-}2^b$ setting.[29] The presence of any inhibitory molecules on the surface of $CD4^+$ T cells has been vastly understudied. Therefore, we characterized the expression profile of $CD4^+$ T cells during acute and chronic infection and selected the mentioned inhibitory markers based on previous $CD8^+$ T-cell studies.[29] The profiling of $CD4^+$ T-cell inhibitory molecule expression was aimed at identifying key markers for chronic LCMV infection. The need to accurately identify the mechanism through which viral clearance can be facilitated, will aid in the future development of remedies for other viruses in other systems.

Materials and methods

Mice and infections

C57BL/6 (H-2b) mice were purchased from The Jackson Laboratory (Bar Harbor, ME). C57BL/6 (H-2b) mice were infected with 2×10^5 plaque-forming units of LCMV Armstrong intraperitoneally. Mice were killed by CO_2 inhalation, and spleens were harvested 8, 15 and 30 days after infection. For LCMV clone 13 experiments, C57BL/6 mice were infected with 2×10^6 plaque-forming units of LCMV clone 13 retro-orbitally. Mice were killed by CO_2 inhalation, and spleens were harvested 8, 15 and 30 days after infection. All studies were conducted at the La Jolla Institute for Allergy and Immunology, in facilities approved by the Association for Assessment and Accreditation of Laboratory Animal Care and according to Institutional Animal Care and Use Committee-approved animal protocols.

MHC-II tetramer, primary and secondary antibodies

MHC-II tetramer (15-mer) was developed by the National Institutes of Health tetramer core facility at Emory University (Atlanta, GA). GP66-80 Tetramer, presenting a previously identified peptide shown to elicit an immune response based on interferon-γ production,[21] was used to identify $CD4^+$ T-cell populations in samples from mice infected *ex vivo* with Armstrong and clone 13. Freshly harvested splenoctyes from naive and infected mice were incubated with tetramer at 1 : 100 (1 μl tetramer into 99 μl FACS buffer) (PBS–5% fetal bovine serum) for 1 hr at 37°. Cells were washed with PBS supplemented with 5% fetal bovine serum to remove unbound tetramer. Splenocytes were then incubated at 1 : 200 (1 μl tetramer into 199 μl FACS buffer) with mouse Biotin anti-CD160 (eBioscience, San Diego, CA) primary antibody on ice for 30 min. Cells were washed with PBS, supplemented with 5% fetal bovine serum, to remove unbound antibody. Splenocytes were then incubated with mouse $\alpha CD4^+$, streptavidin α−biotin (eBioscience), αLAG-3 (BioLegend, San Diego, CA), αCTLA-4 (Invitrogen/Life Technologies, Carlsbad, CA) and αPD-1 (BioLegend) antibodies on ice for 30 min. Cells were washed with PBS supplemented with 5% fetal bovine serum to remove unbound antibody. Splenocytes were then sorted using FACS with LSR and FACS DIVA software. Cell-sorted populations were analysed using FlowJo 9.1 and SPICE.

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Results

Antigen-specific CD4+ T-cell populations are present during chronic infection

To characterize responses during chronic infection, we proceeded with studies using the GP66-80 tetramer. Mice were infected and killed at days 8, 15 and 30 post-infection to accurately quantify antigen-specific CD4+ T-cell populations over the course of infection to observe functional lymphocyte exhaustion. Splenocytes from Armstrong-infected and clone 13-infected mice were sorted on αCD4 antibody and GP66 tetramer to determine the presence of CD4+ T cells during acute and chronic infection. FACS analysis indicated the presence of CD4+ GP66+ T cells at each time point (Fig. 1).

A total of 200 000 events were collected for each experimental sample. Total CD4+ T cells varied between 3383 (2%) and 60 589 (30%) among the different groups. Specifically, in the naive groups, CD4+ T cells ranged between 11 143 and 60 589 (average 20 211). In the infected animals, Armstrong-infected animals ranged between 3819 and 31 473 (average 16 762) and clone 13-infected animals ranged between 3383 and 33 732 (average 12 516). CD4+ GP66+ T cells varied between 40 and 929 among the different groups. Specifically, in the naive groups, CD4+ GP66+ T cells ranged between 51 and 657 (average 175). In the infected animals, Armstrong-infected animals ranged between 100 and 929 (average 314) and clone 13-infected animals ranged between 40 and 481 (average 187). There was not a significant difference in between the CD4+ GP66+ naive T cells and the infected clone 13 T cells over the time–course. However, there was a significant difference between the Armstrong-infected CD4+ GP66+ T cells and the naive CD+ GP66+ T cells ($P = 0{\cdot}0002$). This demonstrates the differences between the infected and naive animals as well as variability between the different viral infections.

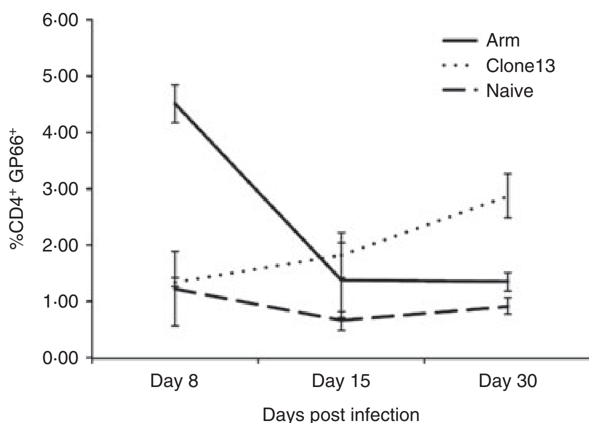

Figure 1. Quantification of antigen-specific CD4+ T cells over a 30-day infection period. C57BL/6 mice were infected intraperitoneally with 2×10^5 plaque-forming units (PFU) of lymphocytic choriomeningitis virus (LCMV) Armstrong, retro-orbitally with 2×10^6 PFU of LCMV clone 13, or left uninfected. Eight days post-infection, CD4+ T cells from splenocyte samples were stained and tested against phycoerythrin-conjugated MHC II tetramers. Samples were then sorted using flow cytometry and data were analysed using FlowJo 9.1. *$P < 0{\cdot}05$.

At day 8 post-infection, antigen-specific T cells were present at high levels during Armstrong infection (4·51%), but remain at low levels (1·35%) in clone 13-infected mouse groups (Fig. 1) ($P < 0{\cdot}001$, $t = 1{\cdot}94$). During Armstrong infection at day 15, GP66-specific T cells had returned to lower levels (1·39%). Compared with Armstrong-infected mouse groups, clone 13-infected groups showed an elevation in overall number by day 30 (2·88%), indicating that antigen-specific cells are present even at exhausted phases of chronic viral infection despite having a dysfunctional phenotype as previously shown (Fig. 2).[21]

CD4+ T cells express abnormal levels of PD-1 during chronic infection

In an effort to investigate lymphocyte deficiency on the basis of suppression via inhibitory molecules, we first examined surface expression of total CD4+ T cells during a 30-day course of infection and co-stained splenocyte samples using primary antibodies specific for a subset of co-expressed lymphocyte inhibitory-associated molecules. This pre-determined subset of molecules included LAG-3, CTLA-4, CD160 and PD-1.[29]

Of the inhibitory molecules screened, only PD-1 and CD160 showed any differential expression between infected and naive mice over the time–course (Fig. 3a,b). The mean fluorescence intensity (MFI) of PD-1 with regard to Armstrong-infected and naive mice differed at day 8 ($P < 0{\cdot}02$). The MFI of PD-1 with regard to clone 13-infected and naive mice differed at day 30 ($P < 0{\cdot}0001$; data not shown). The MFI of CD160 with regard to Armstrong-infected and naive mice differed at day 15 ($P < 0{\cdot}05$; data not shown). CD4+ T cells showed no difference in CTLA-4, or LAG-3 expression between infected and naive mouse groups over the time–course (Fig. 4a,b) ($P > 0{\cdot}05$). The MFI among the other groups were not significantly different from each other. In terms of the molecules differentially expressed between naive and infected animals, by day 15, CD4+ T cells from clone 13-infected mice expressed PD-1 at higher levels when compared with Armstrong-infected mouse groups (Fig. 3a) ($P < 0{\cdot}001$, $t = 1{\cdot}94$). The increased level of PD-1 expression was also maintained at 30 days post-infection when comparing clone 13 and Armstrong-infected mouse groups (Fig. 3a) ($P < 0{\cdot}001$, $t = 1{\cdot}94$). This shows that the elevated levels of PD-1 further limit T-cell function.

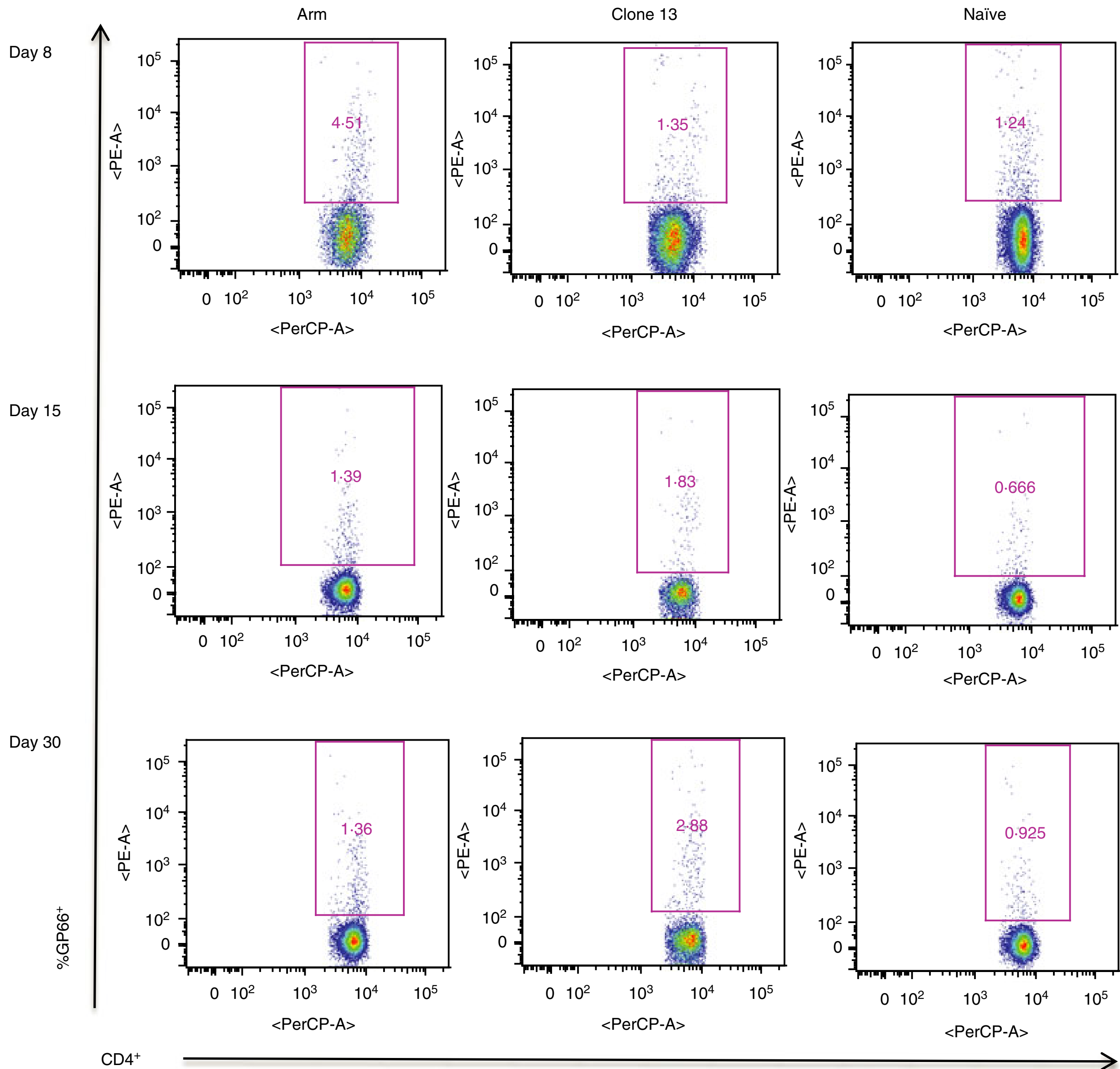

Figure 2. Quantification of antigen-specific CD4$^+$ GP66$^+$ T cells was assessed over a 30-day time–course of infection. Splenocytes were collected and sorted using flow cytometry and data were analysed using FlowJo 9.1.

Antigen-specific T-cell populations against GP66 express disproportionately higher levels of PD-1 during chronic infection

To properly characterize antigen-specific CD4$^+$ T-cell expression of the mentioned inhibitory molecules, splenocyte samples from infected mice were collected and stained with previously stated antibodies and GP66 tetramer. At day 30 post-infection, clone 13-infected mice show that PD-1 is more highly expressed on CD4$^+$ GP66$^+$ T cells compared with Armstrong-infected mouse groups (Fig. 5) ($P < 0{\cdot}01$, $t = 1{\cdot}94$). PD-1 is therefore a marker for CD4$^+$ T-cell dysfunction during chronic LCMV infection. GP66-specific CD4$^+$ T cells showed no difference in CD160, CTLA-4, or LAG-3 expression between infected and naive mouse groups (Fig. 6a,b,c) ($P > 0{\cdot}05$).

Antigen-specific CD4$^+$ T cells co-express multiple inhibitory ligands during infection

The co-expression of inhibitory ligands (PD-1, CD160, CTLA-4 and LAG-3) was analysed using flow cytometry. On day 8 post-infection, a small portion of Armstrong-infected cells expressed more than two inhibitory ligands,

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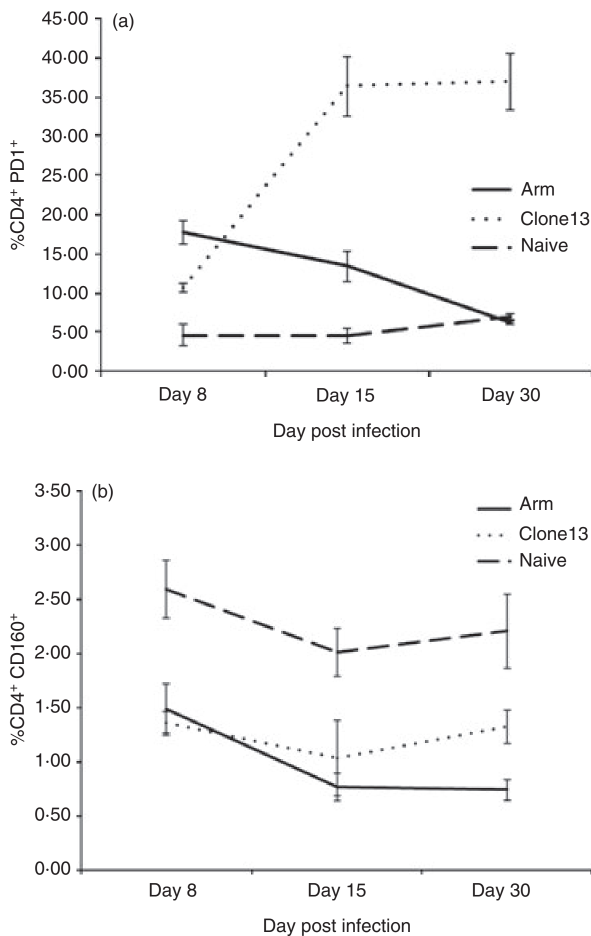

Figure 3. Quantification of $CD4^+$ T-cell expression of programmed death 1 (PD-1) and CD160 over a 30-day infection period. C57BL/6 mice were infected intraperitoneally with 2×10^5 plaque-forming units (PFU) of lymphocytic choriomeningitis virus (LCMV) Armstrong, retro-orbitally with 2×10^6 PFU of LCMV clone 13, or left uninfected. Eight, 15 and 30 days post-infection, $CD4^+$ T cells from splenocyte samples were stained with (a) αPD-1 (b) αCD160 murine antibodies. Samples were then sorted using flow cytometry and data were analysed using FlowJo 9.1. *$P < 0{\cdot}05$.

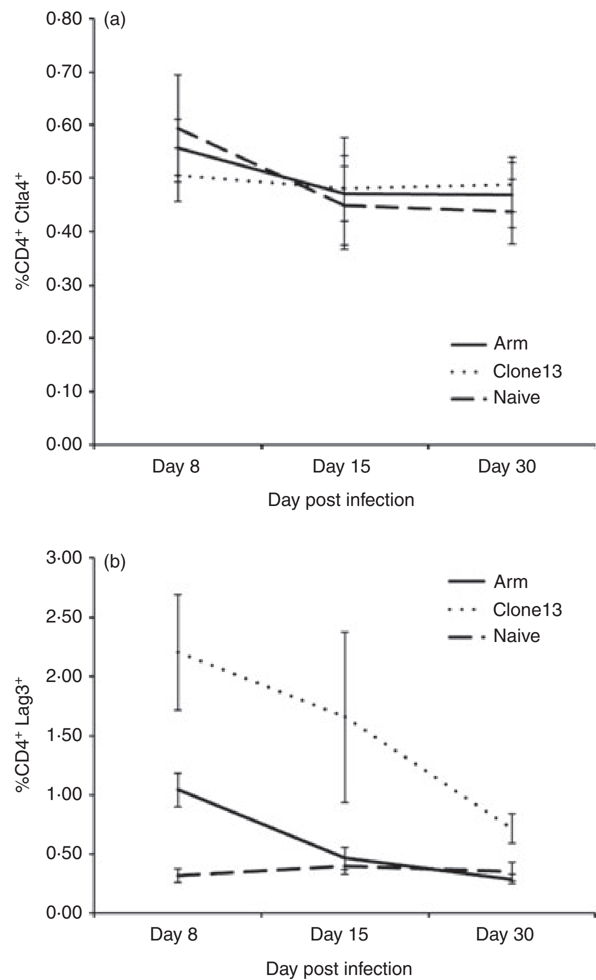

Figure 4. Quantification of $CD4^+$ T-cell expression of cytotoxic T-lymphocyte antigen 4 (CTLA-4) and lymphocyte activation gene 3 (LAG-3) expression over a 30-day infection period. C57BL/6 mice were infected intraperitoneally with 2×10^5 plaque-forming units (PFU) of lymphocytic choriomeningitis virus (LCMV) Armstrong, retro-orbitally with 2×10^6 PFU of LCMV clone 13, or left uninfected. Eight, 15 and 30 days post-infection, $CD4^+$ T cells from splenocyte samples were stained with (a) αCTLA-4 (b) αLAG-3 murine antibodies. Samples were then sorted using flow cytometry and data were analysed using FlowJo 9.1.

whereas, clone 13-infected cells had a greater number of inhibitory molecules being co-expressed (Fig. 7). Fifteen days post-infection, there was an increase in the co-expression of PD-1 and LAG-3 in clone 13-infected mice (Fig. 7). On day 30 post-infection, there was an additional increase in the Armstrong-infected and clone 13-infected mice co-expressing PD-1 and LAG-3 (Fig. 7).

There was a significant difference in the percentage of antigen-specific cells co-expressing two inhibitory molecules versus three or four inhibitory molecules, 8 days post-infection in Armstrong-infected mice ($P < 0{\cdot}05$). For clone 13, 8 days post-infection, there was also significant difference in the percentage of antigen-specific $CD4^+$ T cells co-expressing two inhibitory molecules versus three or four inhibitory molecules ($P < 0{\cdot}02$).

There was a significant difference in the percentage of antigen-specific $CD4^+$ T cells co-expressing two inhibitory molecules versus three or four inhibitory molecules, 15 days post-infection in Armstrong-infected mice ($P < 0{\cdot}03$). In clone 13, 15 days post-infection, there was also a significant difference in the percentage of antigen-specific $CD4^+$ T cells co-expressing three inhibitory molecules versus four inhibitory molecules ($P < 0{\cdot}04$).

There was a significant difference in the percentage of antigen-specific $CD4^+$ T cells co-expressing two inhibitory molecules versus four inhibitory molecules ($P < 0{\cdot}04$), and the co-expression of three inhibitory molecules versus

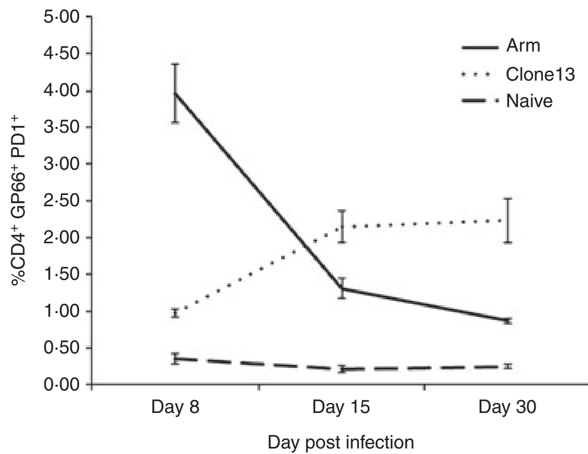

Figure 5. Characterization of antigen-specific CD4+ T-cell expression of programmed death 1 (PD-1) over a 30-day infection period. C57BL/6 mice were infected intraperitoneally with 2×10^5 plaque-forming units (PFU) of lymphocytic choriomeningitis virus (LCMV) Armstrong, retro-orbitally with 2×10^6 PFU of LCMV clone 13, or left uninfected. Eight, 15 and 30 days post-infection, CD4+ T cells from splenocyte samples were stained with αPD-1 murine antibody. Samples were then sorted using flow cytometry and data were analysed using FlowJo 9.1. $*P < 0{\cdot}05$.

four inhibitory molecules ($P < 0{\cdot}05$) 30 days post-infection in Armstrong-infected mice. In clone 13, 30 days post-infection, there was a significant difference in the percentage of antigen-specific CD4+ T cells co-expressing three inhibitory molecules verses four inhibitory molecules ($P < 0{\cdot}05$).

Discussion

CD8+ T cells have been characterized during infection with LCMV. During acute infection with LCMV viral clearance is obtained. During infection with LCMV clone 13, viral persistence occurs. It has been shown that CD8+ T cells become exhausted and are unable to clear the virus, which results in viral persistence. We were interested to understand the role of LCMV-specific CD4+ T-cell responses during chronic infection, including the effect of regulatory and inhibitory molecules.[19,20,31]

In an effort to further characterize CD4+ T-cell presence during chronic infection, an MHC class II tetramer was obtained to precisely quantify the number of antigen-specific CD4+ T cells present during LCMV infection. Time–course studies revealed that antigen-specific CD4+ T-cell populations are present at days 8, 15 and 30 post-infection with LCMV clone 13 (Fig. 1). The CD4+ T-cell populations that were observed 15 and 30 days post-infection were GP66 positive. These data not only confirm the presence of CD4+ T-cell populations, but they are also specific for the GP66 immunodominant epitope previously identified.[21,32]

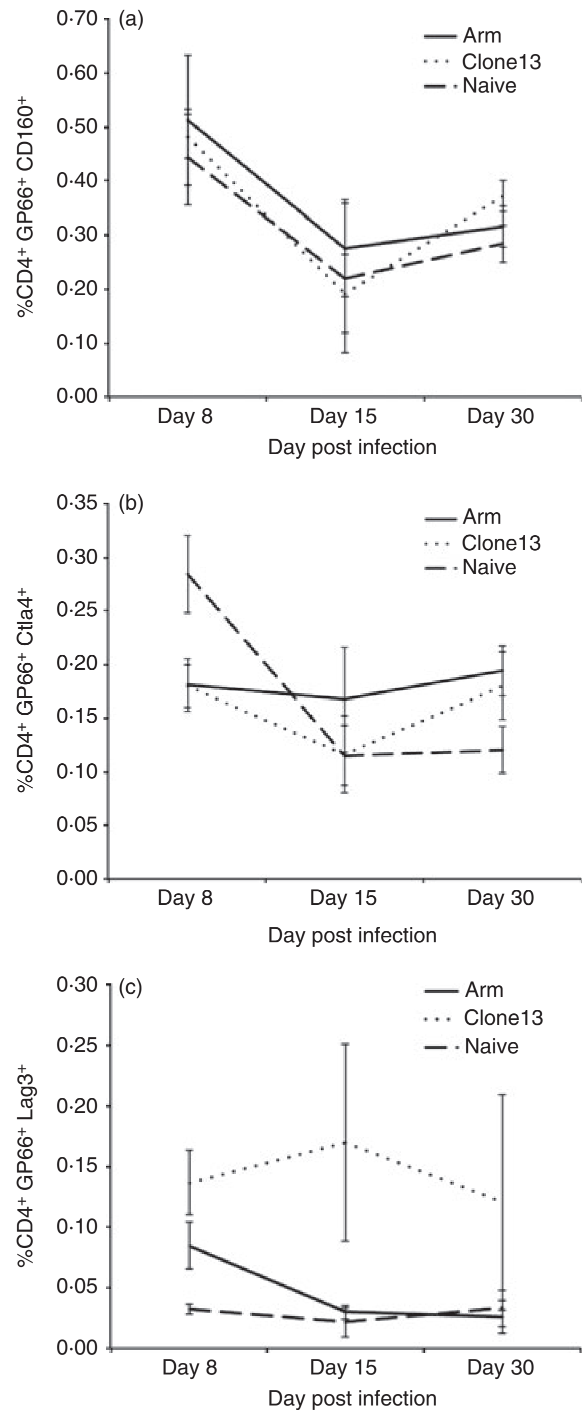

Figure 6. Characterization of antigen-specific CD4+ T-cell expression of (a) CD160, (b) cytotoxic T-lymphocyte antigen 4 (CTLA-4) and (c) lymphocyte activation gene 3 (LAG-3) over a 30-day infection period. C57BL/6 mice were infected intraperitoneally with 2×10^5 plaque-forming units (PFU) of lymphocytic choriomeningitis virus (LCMV) Armstrong, retro-orbitally with 2×10^6 PFU of LCMV clone 13 or left uninfected. Eight, 15 and 30 days post-infection, CD4+ T cells from splenocyte samples were stained with αCD160, αCTLA-4 and αLAG-3 murine antibodies. Samples were then sorted using flow cytometry and data were analysed using FlowJo 9.1.

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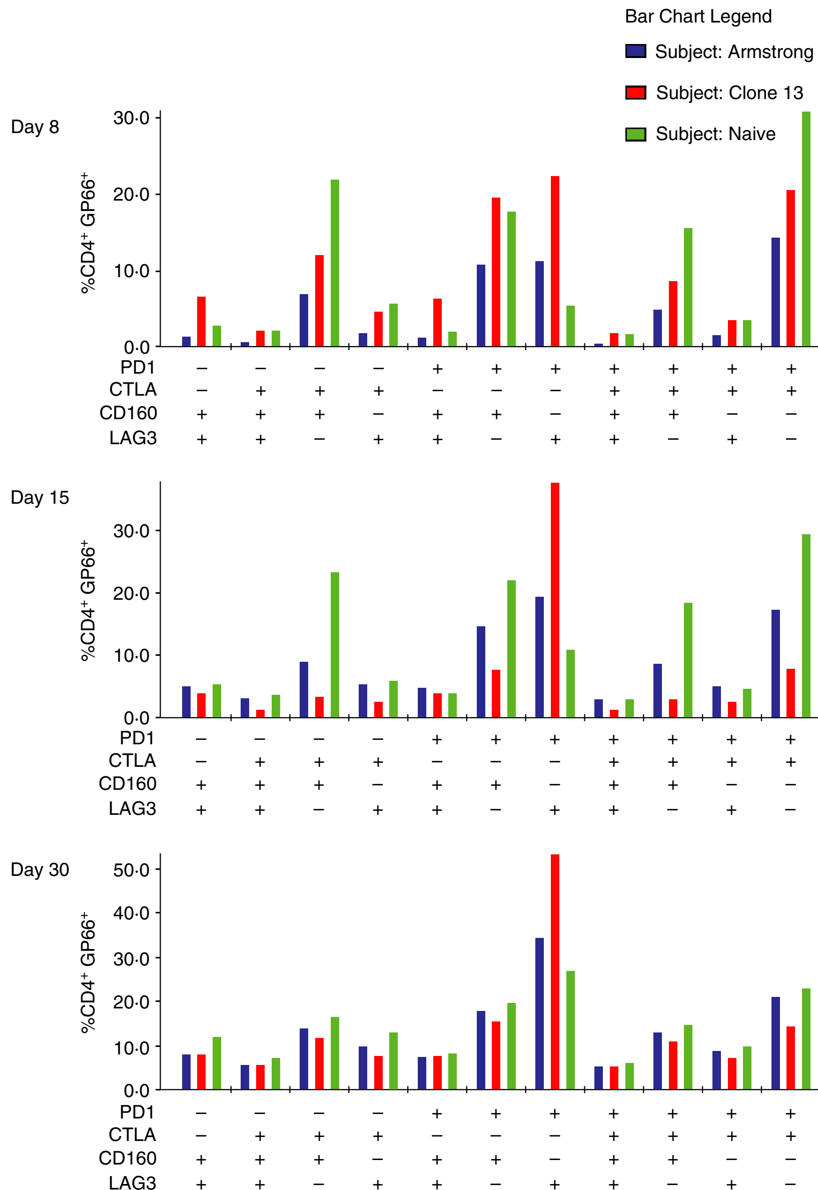

Figure 7. The concurrent expression of multiple inhibitory ligands [programmed death 1 (PD-1), cytotoxic T-lymphocyte antigen 4 (CTLA-4), lymphocyte activation gene 3 (LAG-3), CD160) on GP66$^+$ CD4$^+$ splenocytes from the three experimental groups at days 8, 15 and 30 post-infection. Individual populations were grouped based on total number of inhibitory ligands expressed using Boolean gating to analyse groups and SPICE for coexpression comparisons. The percentages of GP66$^+$ CD4$^+$ T-cells expressing combinations of inhibitory ligands are shown.

T-cell lymphocyte dysfunction has been observed in recent CD8$^+$ T-cell studies where a set of co-expressed inhibitory molecules were identified as contributors to the persistence of chronic infection.[29] Recently, more studies involving inhibitory molecules have become the focus of restoring function to dysfunctional T helper cells. These inhibitory molecules include PD-1, CTLA-4, CD-160 and LAG-3. Blocking these inhibitory molecules improves the immune system's ability to clear the virus.[13,29] The role that inhibitory molecules play is not fully understood. It has been observed that up-regulation of PD-1 is a contributor of CD8$^+$ T-cell exhaustion. By blocking the PD-1 receptor, partial function was restored to the CD8$^+$ T cells.[13] Little is known about the relationship of PD-1 and CD4$^+$ T cells during chronic infection. We were interested in whether inhibitory receptors were unique to CD8$^+$ T cells or if they have the same mechanism on CD4$^+$ T cells.

In our study, we looked at the expression of the PD-1 on antigen-specific CD4$^+$ T cells during infection with LCMV clone 13. After 8 days, CD4$^+$ T cells had relatively low PD-1 expression, but by days 15 and 30 post-infection,

over-expression of PD-1 was observed on the surface of these cells (Fig. 3a). Flow cytometry analysis of antigen-specific $CD4^+$ T cells showed a disproportionately high number of $CD4^+$ $GP66^+$ PD-1^+ T cells at the exhaustion stages of infection, namely day 30 (Fig. 5) ($P < 0{\cdot}01$, $t = 1{\cdot}94$). The over-expression of PD-1 on the surface of antigen-specific T cells indicates that $CD4^+$ T-cell dysfunction does correspond with an elevated PD-1 expression profile. The significantly elevated presence of PD-1 on $CD4^+$ T cells shows that PD-1 expression is not unique to an MHC class I $CD8^+$ T-cell model of LCMV clone 13 infection.

CD160 has been shown to act as an inhibitory ligand of $CD8^+$ T-cell activity when co-expressed with PD-1.[29] CD160 levels were shown to remain the same following clone 13 infection as in Armstrong-infected groups (Fig. 3b). Also, $CD4^+$ T cells sorted on LAG-3 and CTLA-4 showed no deviation in expression pattern among the mouse groups tested (Fig. 4a,b) ($P > 0{\cdot}05$). Hence, it appears that CD160, LAG-3 and CTLA-4 alone are not associated with $CD4^+$ T-cell dysfunction during chronic infection. CD160, LAG-3 and CTLA-4 showed no change in expression level on the surface of antigen-specific $CD4^+$ T cells during chronic infection over the time–course studies, even though they have been characterized as over-expressed inhibitory molecules in the $CD8^+$ T-cell setting. Over the time–course in Armstrong-infected mice, the expression of two inhibitory ligands on antigen-specific $CD4^+$ T cells were statistically significant. However, over the time–course in clone 13-infected mice, the number of antigen-specific $CD4^+$ T cells expressing three inhibitory ligands during the exhaustive stages of infection were statistically significant. It was observed that there was a direct correlation between the number of antigen-specific $CD4^+$ T cells and the number of co-expressed inhibitory ligands. The data showed that the percentage of antigen-specific $CD4^+$ T cells decreased as the number of co-expressed inhibitory ligands increased (Fig. 7). During the exhaustive stages of infection, days 15 and 30, the expression of PD-1 in combination with the other inhibitory molecules indicates the dysfunction of the $CD4^+$ T-cell response.

This study showed that mice that harbour a chronically persisting virus, LCMV clone 13, exhibit a dysfunctional cytokine phenotype and this disease-associated phenotype also corresponds to the over-expression of PD-1 on the surface of antigen-specific $CD4^+$ T-cell populations. The over-expression of PD-1 on the surface of antigen-specific $CD4^+$ T cells is a reliable marker for LCMV clone 13 infection and corresponds to dysfunctional MHC II-restricted responses.

Since $CD4^+$ T-cell responses are instrumental in establishing and sustaining CTL responses in viral infections, we have provided a profile for further characterizing the dysfunction of $CD4^+$ T cells.[33–36] Our study further demonstrates that $CD4^+$ T-cell responses are complex and highlights the need for accurate tools to characterize these mechanisms.

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Disclousures

The authors state that there are no financial or commercial conflicts of interest.

References

1 Sant AJ, Chaves FA, Krafcik FR, Lazarski CA, Menges P, Richards K, Weaver JM. Immunodominance in CD4 T-cell responses: implications for immune responses to influenza virus and for vaccine design. *Expert Rev Vaccines* 2007; **6**:357–68.

2 Battegay M, Moskophidis D, Rahemtulla A, Hengartner H, Mak TW, Zinkernagel RM. Enhanced establishment of a virus carrier state in adult $CD4^+$ T-cell-deficient mice. *J Virol* 1994; **68**:4700–4.

3 Cardin RD, Brooks JW, Sarawar SR, Doherty PC. Progressive loss of $CD8^+$ T cell-mediated control of a gamma-herpesvirus in the absence of $CD4^+$ T cells. *J Exp Med* 1996; **184**:863–71.

4 Gerlach JT, Diepolder HM, Jung MC *et al.* Recurrence of hepatitis C virus after loss of virus-specific $CD4^+$ T-cell response in acute hepatitis C. *Gastroenterology* 1999; **117**:933–41.

5 Kalams SA, Buchbinder SP, Rosenberg ES, Billingsley JM, Colbert DS, Jones NG, Shea AK, Trocha AK, Walker BD. Association between virus-specific cytotoxic T-lymphocyte and helper responses in human immunodeficiency virus type 1 infection. *J Virol* 1999; **73**:6715–20.

6 Matloubian M, Concepcion RJ, Ahmed R. $CD4^+$ T cells are required to sustain $CD8^+$ cytotoxic T-cell responses during chronic viral infection. *J Virol* 1994; **68**:8056–63.

7 Wherry EJ, Ahmed R. Memory CD8 T-cell differentiation during viral infection. *J Virol* 2004; **78**:5535–45.

8 Bailey J, Blankson JN, Wind-Rotolo M, Siliciano RF. Mechanisms of HIV-1 escape from immune responses and antiretroviral drugs. *Curr Opin Immunol* 2004; **16**:470–6.

9 Bowen DG, Walker CM. Mutational escape from $CD8^+$ T cell immunity: HCV evolution, from chimpanzees to man. *J Exp Med* 2005; **201**:1709–14.

10 Fuller MJ, Khanolkar A, Tebo AE, Zajac AJ. Maintenance, loss, and resurgence of T cell responses during acute, protracted, and chronic viral infections. *J Immunol* 2004; **172**:4204–14.

11 Hamer DH. Can HIV be cured? Mechanisms of HIV persistence and strategies to combat it. *Curr HIV Res* 2004; **2**:99–111.

12 Simmonds P. Genetic diversity and evolution of hepatitis C virus – 15 years on. *J Gen Virol* 2004; **85**:3173–88.

13 Barber DL, Wherry EJ, Masopust D, Zhu B, Allison JP, Sharpe AH, Freeman GJ, Ahmed R. Restoring function in exhausted CD8 T cells during chronic viral infection. *Nature* 2006; **439**:682–7.

14 Day CL, Kaufmann DE, Kiepiela P *et al.* PD-1 expression on HIV-specific T cells is associated with T-cell exhaustion and disease progression. *Nature* 2006; **443**:350–4.

15 Freeman GJ, Wherry EJ, Ahmed R, Sharpe AH. Reinvigorating exhausted HIV-specific T cells via PD-1–PD-1 ligand blockade. *J Exp Med* 2006; **203**:2223–7.

16 Fuller MJ, Zajac AJ. Ablation of CD8 and CD4 T cell responses by high viral loads. *J Immunol* 2003; **170**:477–86.

17 Grakoui A, John Wherry E, Hanson HL, Walker C, Ahmed R. Turning on the off switch: regulation of anti-viral T cell responses in the liver by the PD-1/PD-L1 pathway. *J Hepatol* 2006; **45**:468–72.

18 Moskophidis D, Lechner F, Pircher H, Zinkernagel RM. Virus persistence in acutely infected immunocompetent mice by exhaustion of antiviral cytotoxic effector T cells. *Nature* 1993; **362**:758–61.

19 Wherry EJ, Blattman JN, Murali-Krishna K, van der Most R, Ahmed R. Viral persistence alters CD8 T-cell immunodominance and tissue distribution and results in distinct stages of functional impairment. *J Virol* 2003; **77**:4911–27.

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20 Zajac AJ, Blattman JN, Murali-Krishna K, Sourdive DJ, Suresh M, Altman JD, Ahmed R. Viral immune evasion due to persistence of activated T cells without effector function. *J Exp Med* 1998; **188**:2205–13.

21 Dow C, Oseroff C, Peters B, Nance-Sotelo C, Sidney J, Buchmeier M, Sette A, Mothé BR. Lymphocytic choriomeningitis virus infection yields overlapping CD4+ and CD8+ T-cell responses. *J Virol* 2008; **82**:11734–41.

22 Mothe BR, Stewart BS, Oseroff C *et al.* Chronic lymphocytic choriomeningitis virus infection actively down-regulates CD4+ T cell responses directed against a broad range of epitopes. *J Immunol* 2007; **179**:1058–67.

23 Frohlich A, Kisielow J, Schmitz I *et al.* IL-21R on T cells is critical for sustained functionality and control of chronic viral infection. *Science* 2009; **324**:1576–80.

24 Yi JS, Du M, Zajac AJ. A vital role for interleukin-21 in the control of a chronic viral infection. *Science* 2009; **324**:1572–6.

25 Elsaesser H, Sauer K, Brooks DG. IL-21 is required to control chronic viral infection. *Science* 2009; **324**:1569–72.

26 Matloubian M, Kolhekar SR, Somasundaram T, Ahmed R. Molecular determinants of macrophage tropism and viral persistence: importance of single amino acid changes in the polymerase and glycoprotein of lymphocytic choriomeningitis virus. *J Virol* 1993; **67**:7340–9.

27 Fahey LM, Wilson EB, Elsaesser H, Fistonich CD, McGavern DB, Brooks DG. Viral persistence redirects CD4 T cell differentiation toward T follicular helper cells. *J Exp Med* 2011; **208**:987–99.

28 Borrow P, Evans CF, Oldstone MB. Virus-induced immunosuppression: immune system-mediated destruction of virus-infected dendritic cells results in generalized immune suppression. *J Virol* 1995; **69**:1059–70.

29 Blackburn SD, Shin H, Haining WN *et al.* Coregulation of CD8+ T cell exhaustion by multiple inhibitory receptors during chronic viral infection. *Nat Immunol* 2009; **10**:29–37.

30 Kaufmann DE, Walker BD. PD-1 and CTLA-4 inhibitory cosignaling pathways in HIV infection and the potential for therapeutic intervention. *J Immunol* 2009; **182**:5891–7.

31 Gallimore A, Glithero A, Godkin A, Tissot AC, Pluckthun A, Elliott T, Hengartner H, Zinkernagel R. Induction and exhaustion of lymphocytic choriomeningitis virus-specific cytotoxic T lymphocytes visualized using soluble tetrameric major histocompatibility complex class I-peptide complexes. *J Exp Med* 1998; **187**:1383–93.

32 Homann D, Lewicki H, Brooks D, Eberlein J, Mallet-Designe V, Teyton L, Oldstone MB. Mapping and restriction of a dominant viral CD4+ T cell core epitope by both MHC class I and MHC class II. *Virology* 2007; **363**:113–23.

33 Berger DP, Homann D, Oldstone MB. Defining parameters for successful immunocytotherapy of persistent viral infection. *Virology* 2000; **266**:257–63.

34 Janssen EM, Lemmens EE, Wolfe T, Christen U, von Herrath MG, Schoenberger SP. CD4+ T cells are required for secondary expansion and memory in CD8+ T lymphocytes. *Nature* 2003; **421**:852–6.

35 Shedlock DJ, Shen H. Requirement for CD4 T cell help in generating functional CD8 T cell memory. *Science* 2003; **300**:337–9.

36 Shedlock DJ, Whitmire JK, Tan J, MacDonald AS, Ahmed R, Shen H. Role of CD4 T cell help and costimulation in CD8 T cell responses during *Listeria monocytogenes* infection. *J immunol* 2003; **170**:2053–63.