Insights into CD8 T Cell Activation and Exhaustion from a Mouse Gammaherpesvirus Model

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Abstract

(S.R.S.) I was introduced to viral immunology while working in Peter Doherty's laboratory in the early stages of my research career, inspiring a lifelong interest in this area. During those early years under Peter's mentorship, we studied a mouse gammaherpesvirus model (murine gammaherpesvirus-68 [MHV-68]) that provided a useful small animal model for investigating the immunological control of gammaherpesvirus infection. Interestingly, while CD4 T cells were not required for acute control of MHV-68 in the lung, CD8 T cell-mediated control was progressively lost in the absence of CD4 T cell help, leading to viral recrudescence. This was one of several early studies showing that CD8 T cell control of persistent viral infections was lost in the absence of CD4 T cell help, preceding the concept of CD8 T cell exhaustion. Further studies showed that MHV-68 infection of mice offered a unique model for comparing the mechanisms of acute and long-term control of a persistent viral infection and developing strategies for reversing T cell exhaustion. Here, we provide a brief review of the literature on CD8 T cell activation and exhaustion in this model, focusing on the role of CD40 and B7 family members and including some previously unpublished data.

Keywords: mouse gammaherpesvirus, CD8 T cell exhaustion, CD40, CD80/86, CD28/CTLA4, PD-1

Introduction

MURINE GAMMAHERPESVIRUS-68 (MHV-68) is a naturally occurring rodent pathogen (11) that is closely related to Epstein Barr Virus (EBV) and Kaposi's sarcoma-associated herpesvirus (KSHV) (27,76). EBV and KSHV are associated with a range of human diseases, including infectious mononucleosis, lymphoproliferative disease, nasopharyngeal carcinoma, Kaposi's sarcoma, and lymphoma.

Intranasal administration of MHV-68 to mice results in acute productive infection of lung epithelial cells and a latent infection in various cell types including B lymphocytes, dendritic cells, epithelia, and macrophages (28,29,65,67,73,79). MHV-68 pathogenesis resembles that of EBV in humans, including lymphadenopathy, splenomegaly, and mononucleosis, although structurally the virus is more similar to KSHV (44,66,68,71,72). MHV-68 has also been reported to affect the vasculature, accelerating atherosclerosis in ApoE^{-/-} mice (1) and inducing vasculitis in several types of immune-compromised mice (78).

Infectious MHV-68 is cleared from the lungs by a T cell-dependent mechanism that can be mediated by either CD4 or CD8 T cells, while lifelong latency is established in immunocompetent mice (28,66,69). While CD4 T cells are not essential for primary clearance of replicating virus, they are required for effective long-term control and virus reactivates in the lungs of CD4 T cell-deficient mice, from approximately day 25 after infection onward. This eventually leads to death, probably due to lung damage caused by long-term viral replication (18,58). Similarly, in AIDS patients, CD4 T cell counts are predictive of the incidence and severity of gammaherpesvirus-related disease, including malignancies (22).

Although an effect of CD4 T cells on CD8 T cell function was initially suspected in the MHV-68 model, several studies failed to find any difference in the number, cytolytic activity, tumor necrosis factor (TNF), or interferon (IFN)- γ production of virus specific CD8 T cells from wild-type and CD4 T cell-deficient mice (7,18,64). Furthermore, postexposure vaccination against MHV-68 epitopes known to be protective in a primary response failed to prevent viral

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reactivation, despite massive expansion of virus specific CD8 T cells, suggesting that the inability of CD8 T cells to mediate long-term control of MHV-68 in CD4 T cell-deficient mice was not due to a quantitative defect (8).

Closer examination of the expanded T cell population showed that the relative prevalence of polyfunctional T cells making both TNF and IFN γ and cytotoxic T lymphocyte (CTL) activity on a per cell basis was reduced in spleens of CD4 T cell-deficient mice following secondary expansion *in vitro* (45,51). However, it is unclear how this would affect viral reactivation, when the overall levels of cytokine production or CTL activity were unchanged in CD4 T cell-deficient mice.

B cell-deficient mice or mice with B cells that are unable to produce virus-specific antibody (77), clear MHV-68 with normal kinetics and do not show viral reactivation in the lungs (65) (S. Sarawar, Unpublished Data). However, T cell depletion during the latent phase of infection, following clearance of infectious MHV-68 in B cell-deficient mice results in viral reactivation (65). Viral reactivation did not occur unless both CD4 and CD8 T cells were depleted, showing that CD8 T cells could control MHV-68 in the absence of CD4 T cells, provided that they had been primed in the presence of CD4 T cells (65). These data suggest that CD4 T cells, CD8 T cells, and B cells play overlapping roles in preventing or controlling reactivation of MHV-68 during the latent phase of infection. However, the B and CD8 T cell-mediated control mechanisms do not develop in the absence of an early effect of CD4 T cells.

Costimulatory Molecules in the Immune Response to MHV-68

Activation of T cells requires interaction with antigen-presenting cells (APCs). Naïve T cells require two signals for activation—one from the interaction of the T cell receptor with peptide presented by major histocompatibility complex (MHC) molecules and a second via costimulatory molecules (35,37,38). In some cases a third signal may be required and is often provided by interleukin (IL)-12 (74). However, the sum of multiple positive and negative signals to both the APC and T cells determines the overall response and may change over time in chronic infections.

CD4 T cells are thought to provide “help” in part by conditioning APCs to activate CD8 T cells (9,19,57,61), while there is some evidence that viral infection of APC can bypass this requirement in the generation of CTL (57). This may explain the ability of CD8 T cells to clear some primary viral infections in the absence of CD4 T cell help. However, as exemplified by MHV-68 or lymphocytic choriomeningitis virus (LCMV) infection of mice and human AIDS patients, CD4 T cells are often required for control of persistent or recurrent viral infections (18,22,50).

CD40/CD40L

CD40 is expressed on APCs such as dendritic cells, B cells, and macrophages while its ligand is expressed on activated CD4 T cells (48). Stimulation via CD40 on APCs results in upregulation of numerous surface and secreted molecules, initiating cross-talk between APCs and T cells at the immunological synapse.

We showed that stimulation via CD40 could substitute for CD4 T cell function in the long-term control of MHV-68.

Thus, an agonistic antibody to CD40 prevented reactivation of MHV-68 in MHC Class II $^{-/-}$ (CII $^{-/-}$) mice, which lack CD4 T cells (59). Injection of the antibody 1 and 15 days after infection had a long-term effect and prevented viral reactivation for at least 100 days (24). In addition, it prevented death in the CII $^{-/-}$ mice. These data confirm that “help” is not required continuously to maintain CD8 T cell function in this model, but appears to be necessary for priming a robust response.

Treatment with an agonistic antibody to CD40 did not appear to stimulate MHV-68-specific antibody, cytolytic activity, or IFN- γ production in CII $^{-/-}$ mice, but was ineffective when CD8 TCR $^{+}$ cells were depleted after initial viral clearance (59). CD8 T cell depleted CII $^{-/-}$ mice showed somewhat higher levels of viral reactivation than control CII $^{-/-}$ mice, suggesting that the “unhelped” CD8 T cells were exerting some level of viral control, although it was inadequate to prevent viral recrudescence (18,59). Since no change in the activity of the CD8 T cells was detected *in vitro*, a key question was whether the anti-CD40 treatment resulted in a change in the function of the CD8 T cells or of another cell type that was required in addition to CD8 T cells. To address this question, we showed that adoptive transfer of CD8 T cells from MHV-68 infected wild-type or anti-CD40 treated, but not from control, CII $^{-/-}$ mice caused

FIG. 1. Anti-CD40 treatment induces a change in CD8 T cell function. Groups of donor or recipient MHC Class II $^{-/-}$ or donor WT mice were infected with MHV-68 (5×10^4 PFU intranasal). Donor Class II $^{-/-}$ mice were treated with either 100 μ g/mouse anti-CD40 or isotype control antibody intravenously on days 1 and 15 postinfection. At day 18 after infection, donor mice were killed and CD8 T cells were purified by negative selection from pooled lymph node and spleen cells from each group. 3×10^6 purified CD8 cells were administered intravenously to the recipient mice, which had been lightly irradiated (300 rads) 1 day earlier. Lung virus titers were determined by plaque assay at day 42 after infection. Data are pooled from two independent experiments and represent titers for individual mice. There was a highly significant difference between the lung virus titers in mice that had received cells from control antibody-treated and anti-CD40 treated CII $^{-/-}$ donors (*** $p < 0.001$, Mann-Whitney Rank Sum test) or WT donors (** $p < 0.01$). Reprinted with permission from Dias *et al.* (24). MHV-68, murine gammaherpesvirus-68; WT, wild-type. MHC, major histocompatibility complex; PFU, plaque-forming units.

a significant reduction in lung virus titers in recipient virus-infected CII^{-/-} (24) (Fig. 1). Thus, anti-CD40 treatment changed CD8 T cell function, resulting in effective viral control.

Brooks *et al.* (15) showed, using CD40L^{-/-} mice, that CD40L is essential for long-term control of MHV-68. Consistent with these findings, we showed that, like CD4 T cell-deficient mice, CD40^{-/-} mice were able to control acute infection with MHV-68, but showed viral reactivation in the lungs at late times after infection (43). Thus, CD40 ligation is both essential and sufficient for long-term control of MHV-68 and there are no redundant pathways.

CD40 is expressed by a number of different cell types including mature dendritic cells and B cells and activated T cells. CD40-stimulated dendritic cells or B cells have been shown to act as a "conditioned bridge" mediating T cell activation in some models (9,19,57,61), whereas others have reported that CD40 expression on CD8 T cells themselves was essential for activation of this cell type (13). Therefore, it was unclear which CD40+ cell type mediated the effect of the agonistic antibody. To address this question, adoptive transfer experiments were performed. Adoptive transfer of anti-CD40-treated dendritic cells, but not anti CD40-treated B cells, at days 1 and 15 after infection, significantly reduced lung virus titers in CII^{-/-} mice, although both cell types expressed CD40 and upregulated CD80 and 86 in response to anti-CD40 treatment (34). We observed that the anti-CD40 treatment upregulated CD70 on dendritic cells, but not on B cells. Athymic nude or rag^{-/-} mice reconstituted with T cells from CD40^{+/+} or ^{-/-} mice maintained effective long-term control of MHV-68, showing that CD40 on T cells was not essential for this function, even in the absence of B cells (34).

CD28

CD40 stimulation upregulates CD80 (B7-1) and 86 (B7-2) on dendritic cells (19) and these receptors interact with positive and negative costimulatory ligands, CD28 and CD152 (CTLA-4) respectively (4). CD28 is present on resting T cells and its expression is upregulated following activation. Therefore, it was of interest to determine whether interaction of CD28 with CD80 or CD86 played a role in the effect of CD40 stimulation of dendritic cells on CD8 T cell function in the long-term control of MHV-68. However, two independent studies showed that CD28^{-/-} mice were able to clear infectious MHV-68 from the lungs and showed effective long-term control of the virus (42,43). Both studies reported that peak infectious virus titers were somewhat higher in CD28^{-/-} mice. However, latent virus burden was lower in CD28^{-/-} mice (42).

Although recall interferon gamma responses developed more slowly in CD28^{-/-} mice, CTL activity was similar in CD28^{-/-} and wild-type mice, consistent with the ability to clear replicating virus from the lungs by day 15 after infection. However, CD28^{-/-} mice failed to form germinal centers and virus-specific antibody responses were dramatically reduced, with aberrant class switching. Interestingly, Kim *et al.* (42) showed that the compromised antibody response in CD28^{-/-} mice was further reduced over time and alone was ineffective in maintaining long-term control of MHV-68. Thus, depletion of both CD4 and CD8 T cells

during the latent phase of infection in CD28^{-/-} mice resulted in viral recrudescence, whereas T cell depleted wild-type mice could maintain effective viral control. The studies on MHV-68 infection of CD28^{-/-} mice also suggested that some CD40-mediated functions in viral control are independent of both CD28 and antibody.

Protein kinase C-theta

Stimulation of T cells with antigen and CD28 induces translocation of protein kinase C-theta (PKC θ), a T cell-specific isoenzyme of PKC, into the plasma membrane lipid rafts where it co-localizes with the TCR in the immune synapse (10,52). PKC θ mediates activation of several transcription factors resulting in T cell activation. We observed that PKC θ ^{-/-} mice infected with MHV-68 have a similar profile to CD28^{-/-} mice. Thus, PKC θ ^{-/-} mice cleared lytic virus and maintained effective long-term control of latency (33). CD8 T cell expansion, recruitment to the lungs and CTL activity were similar in PKC θ ^{+/+} and ^{-/-} mice, whereas antiviral antibody production and T helper cell cytokine production were significantly lower in PKC θ ^{-/-} mice (33). This suggested that the pathway by which CD4 T cells act to induce effective long-term control of MHV-68 is not dependent on PKC θ .

Further studies showed that acute control of infectious MHV-68 could be mediated by alternative CD4 T cell-dependent and PKC θ -dependent pathways of CD8 T cell activation (25). Thus, adoptive transfer of PKC θ ^{-/-} CD8 T cells that had developed in the presence of CD4 T cells reduced lung virus titers in CD4 T cell-depleted PKC θ ^{-/-} hosts significantly more than the transfer of PKC θ ^{-/-} CD8 T cells from CD4 T cell-deficient donors. In addition, CD8 CTL activity from PKC θ ^{-/-} CD4 T cell-deficient hosts was dramatically reduced compared with that from PKC θ ^{-/-} control mice. In contrast, CD4 T cell depletion in WT mice did not affect CD8 CTL function. However, long-term control of MHV-68 was dependent on CD4 T cells, even in PKC θ -sufficient mice (18,25).

CD80 and CD86

Several studies have reported that the effects of CD80 and CD86 do not always mirror those of CD28 and CTLA4 (49,81) and this is also true in the MHV-68 model. We, and others, reported that CD80/86^{-/-} mice failed to maintain effective long-term control of MHV-68 and showed viral reactivation in the lungs (32,47). However, mice deficient in either CD80 or CD86 alone did not show viral reactivation in the lungs, indicating that these molecules played overlapping roles in the long-term control of MHV-68 (47). Antiviral antibody responses were dramatically reduced in CD80/86^{-/-} mice, while CD8 CTL activity was not significantly affected (32,47).

IFN- γ production by virus-specific CD8 T cells was reduced to a similar extent in CD28^{-/-} and CD80/86^{-/-} mice (32), suggesting that, although this cytokine has been shown to play an important role in the control of latency in MHV-68 infection (63), differential production of IFN- γ at late time points after infection could not explain the difference in viral reactivation between CD80/86^{-/-} and CD28^{-/-} mice. Similar to CD28^{-/-} mice, CD80/86^{-/-} mice showed a reduction in splenic latency in the first few weeks after infection (32). This may relate to the requirement of germinal center

formation for the establishment of splenic latency (30), as CD28 and CD86 are required for germinal center formation.

Although usually inhibitory, CTLA4 has been reported to also have a positive costimulatory role (60,80). However, Fuse *et al.* (32) showed that a CTLA4 specific blocking antibody did not induce MHV-68 reactivation in the lungs or increase latent viral load in the spleens of CD28^{-/-} mice. Furthermore, we demonstrated that CD28/CTLA4^{-/-} mice did not show viral reactivation in the lungs (24) (Fig. 2). This ruled out an unusual costimulatory role for CTLA4. Taken together, these data suggest that there could potentially be a novel receptor for CD80 and 86 other than CD28 or CTLA4.

A negative costimulatory interaction between PD-L1 (which also binds to the inhibitory receptor, PD-1) and CD80 has been described (16,17). This was unexpected as both were previously thought to be ligands, although the crystal structure suggests the capability of signaling (16). Our data also argue against a role for CD80-PD-L1 interactions in this model, as the interaction of the proposed novel costimulatory receptor with CD80/86 has a stimulatory effect, resulting in decreased viral titers, while blocking PD-L1 reduced viral reactivation (see below) (24), whereas lack of CD80/86 increases viral titers (47). Furthermore,

Butte *et al.* (16) reported that PD-L1 only interacts with CD80 and not CD86, whereas CD80 and CD86 have similar functions in our model (47).

If there is a molecule other than CD28 that has a positive costimulatory interaction with CD80 and 86, it has not yet been identified. However, Mandelbrot *et al.* (49) showed that CD28/CTLA4^{-/-} T cell proliferation was inhibited by the use of antigen presenting cells lacking both CD80 and CD86, but could be induced by CHO cells expressing CD80 or CD86, suggesting that, as in our model, something other than CD28 or CTLA4 provides a positive costimulatory signal via CD80 or CD86.

CTLA4Ig

CTLA4 binds with higher affinity than CD28 to CD80 and 86 and CTLA4Ig fusion proteins such as belatacept are used clinically to prevent transplant rejection by blocking T cell costimulation via CD80 and 86. Gammaherpesviruses, such as EBV and KSHV, can present problems in transplant recipients receiving immunosuppressive therapy and can cause serious and sometimes fatal post-transplant lymphoproliferative disease (PTLD) or Kaposi's sarcoma. EBV seronegative patients are particularly at risk from primary

FIG. 2. PD-1 expression on CD8 T cells and lung virus titers in WT, Class II^{-/-}, CD40^{-/-}, CD80/86^{-/-}, and CD28/CTLA4^{-/-} mice. Groups of 3–5 mice WT, MHC Class II^{-/-}, CD40^{-/-}, CD80/86^{-/-}, and CD28/CTLA4^{-/-} mice were infected with MHV-68. Infiltrating cells were harvested from the lung by BAL via the trachea at day 50 postinfection and dual-stained with FITC-conjugated anti-CD8 and PE conjugated anti-PD-1. (A). Representative dot plots of PD-1 and CD8 expression in BAL. (B). Mean percentages + standard error of mean of CD8 T cells that were positive for PD-1 in the BAL of CII^{-/-}, CD40^{-/-}, and CD80/86^{-/-} mice compared with that of WT mice ****p* < 0.001, ***p* < 0.01. There was no significant difference in the percentages for WT and CD28/CTLA4^{-/-} mice. (C). Lung virus titers were determined at day 50 after infection by plaque assay. Data represent titers for individual mice. Reprinted with permission from Dias *et al.* (24). BAL, bronchoalveolar lavage; FITC, fluorescein isothiocyanate.

infections acquired from the transplanted tissue, although PTLTD also occurs as a consequence of reactivated disease (3). In clinical trials, kidney transplant recipients treated with belatacept (CTLA4Ig) showed a higher incidence of PTLTD compared with those treated with cyclosporine, resulting in a black box warning for belatacept (5,26,75).

CTLA4Ig could block T cell costimulatory functions of both CD28 and the putative novel receptor. The putative novel receptor for CD80 and CD86 could function either at early time points in the generation of a robust immune response or later in the maintenance of the response. To further investigate this, we treated CD28/CTLA4^{-/-} mice with a noncytolytic form of CTLA4-Ig (which blocks all interactions with CD80 and 86 without depleting cells bearing these molecules) from days 0 to 15, 35 to 50, or 1 to 50. We were able to provoke viral replication in the lungs with each of these regimens (Fig. 3). This is consistent with the presence of an alternative receptor for CD80 and CD86 and suggests that the putative receptor is important throughout the course of infection.

A study by Pinelli *et al.* (55) also showed that treatment with CTLA4Ig increased the burden of latent virus in the spleen at day 20 after infection with MHV-68 and reduced the expansion and cytokine production by virus-specific CD8 T cells. Interestingly, rapamycin, which is normally immunosuppressive, was able to reverse the effect on T cell function and viral burden. Thus, rapamycin increased the number of virus-specific T cells and IFN- γ production (55). In a later study, the same group showed that, consistent with studies in CD28^{-/-} mice, both CTLA4Ig and a CD28-specific antibody decreased the burden of latent virus in the spleen to the same extent at day 14 (probably due to an effect on germinal centers) (21). Titers of infectious virus in the lung were not assessed in either study, as an intraperitoneal route of infection was used, which does not lead to viral replication in the lung.

However, one caveat to studies using CTLA4Ig treatment is that ligation of CD80 and 86 by CTLA4-Ig can trigger indoleamine 2,3 dioxygenase (IDO) activity in dendritic

cells by reverse signaling, which allows them to suppress T cell function (12,54). While initial reports suggested that the effect of IDO was restricted to CD4 T cells, subsequent reports showed that it could also mediate inhibition of CD8 T cells, during infection with several viruses, including EBV (46). However, T cell suppression by IDO, induced by reverse signaling through CD80 and 86, clearly cannot explain either viral reactivation in CD80/86^{-/-} mice or the absence of viral reactivation in mice lacking both known ligands for CD80 and 86.

Inhibitory Molecules and T Cell Exhaustion During MHV-68 Infection of Immunocompromised Mice

Our adoptive transfer experiments showed that there was a qualitative difference in CD8 T cell from wild-type and CII^{-/-} mice, but it was not clear exactly what that difference was. Therefore, we compared the expression and role of potential inhibitory molecules on the surface of CD8 T cells from CII^{-/-} and wild-type mice (24).

NKG2A

The heterodimeric molecule NKG2A has been shown to inhibit CD8 T cell function in a mouse polyoma virus model (53). We noticed a significant reduction in the percentage of CD8 T cells expressing the inhibitory receptor NKG2A in bronchoalveolar lavage (BAL) from wild-type or anti-CD40-treated CII^{-/-} mice compared to that in control CII^{-/-} mice (24). In addition, the level of expression was significantly lower in CD8 T cells from wild-type or anti-CD40 treated CII^{-/-} mice than in control CII^{-/-} mice. To address the role of this molecule in the response to MHV-68, we compared the long-term control of MHV-68 in DBA/1 and DBA/2 mice that had been depleted of CD4 T cells. DBA/1 mice are deficient in CD94, which is required for surface expression of NKG2A. However, we saw no difference in the level of viral recrudescence in DBA/1 and DBA/2 mice (24).

PD-1 and PD-L1

The inhibitory molecule PD-1 is expressed on the surface of CD8 T cells and has been implicated in CD8 T cell exhaustion in infections with several viruses including LCMV and HIV (6,20,23,70,82). We found that PD-1 expression was significantly upregulated on the surface of CD8 T cells from Class II^{-/-}, CD40^{-/-}, and CD80/86^{-/-} mice (which all show viral reactivation in the lungs), compared with that on CD8 T cells from either wild-type or CD28/CTLA4^{-/-} mice (that show effective control of the virus) (Fig. 2) (24). Furthermore, the level of PD-1 expression on CD8 T cells correlated with the level of viral reactivation in the lungs in the knockout mouse strains, suggesting that PD-1 plays a role in CD8 T cell exhaustion in persistent MHV-68 infection (Fig. 2).

An alternative explanation is that upregulation of PD-1 was a result of viral reactivation, rather than a cause. To distinguish between these possibilities, we blocked PD-1-PD-L1 interactions using monoclonal antibodies to either molecule in CII^{-/-} mice. The results showed that blocking PD-1-PD-L1 interactions significantly reduced viral reactivation (Fig. 4) (24). In contrast, antibodies to PD-L2 had no significant effect (24). Blocking both PD-1 and PD-L1 has

FIG. 3. Viral reactivation induced by CTLA4-Ig in MHV-68-infected CD28/CTLA4^{-/-} mice. Groups of 3–4 CD28/CTLA4^{-/-} mice were infected with MHV-68 and treated with 0.1 mg/mouse CTLA4-Ig (Chimerigen) i.p. every 2 days for the indicated time periods. Lung virus titers were determined by plaque assay at day 50 after infection. There was a significant difference between titers in control and CTLA4Ig-treated groups of mice (* p < 0.05, ** p < 0.01, t -test). i.p., intraperitoneal; Ig, immunoglobulin.

no greater effect than blocking PD-1 alone. Fluorescence-activated cell sorting analysis of BAL cells from mice treated with anti-PD-1, anti-PD-L1, or anti-PD-L2 showed that the antibodies were nondepleting and that the reduction in viral titers did not significantly reduce the expression of either PD-1 or PD-L1 (24). Thus, upregulation of PD-1 on CD8 T cells in Class II^{-/-} mice appears to be a cause, rather than solely a result, of viral reactivation. However, anti-PD-1 only partially blocked viral reactivation, leading us to postulate that other inhibitory receptors might also be involved.

Freeman *et al.* (31) showed that PD-1 was upregulated on all MHV-68-specific CD8 T cells, in the absence of CD4 T cell help. CD8 T cells of a subdominant specificity increased in prominence at late times after infection and showed enhanced functionality in that a higher proportion expressed TNF, IFN- γ , and CD107a, a marker of degranulation. It is possible that these subdominant CD8 T cells are responsible for some of the residual viral control observed in CD4 T cell-deficient mice, although they are unable to completely block viral reactivation.

Interleukin-10

Molloy *et al.* (51) found that levels of IL-10 were higher in the sera of CD4 T cell-depleted MHV-68 infected mice than those from undepleted mice. Similarly, splenocytes from CD4 depleted MHV-68 infected mice produced more IL-10 than those from undepleted mice when restimulated *in vitro* with MHV-68 peptides. Furthermore, CD4 T cell-depleted IL-10R^{-/-} mice showed lower levels of MHV-68 reactivation than those from CD4 T cell-depleted wild-type mice. Using bone marrow chimeras, the authors showed that cell intrinsic production of IL-10 by CD8 T cells compromised long-term control of MHV-68.

Combined effect of blocking IL-10R, PD-1, and TIM-3

Neither PD-1 nor IL10 blockade alone could completely ablate viral reactivation in CD4 T cell-deficient mice, leading us to investigate additional inhibitory receptors and the effect of blocking combinations of inhibitory receptors.

FIG. 4. Increased expression of the inhibitory receptors PD-1, TIM-3, and LAG-3 on the surface of CD8 T cells in the lungs of mice lacking CD4 T cells and effect of blocking inhibitory receptors on lung virus titers. Groups of four CII^{-/-} mice or WT were infected with MHV-68. BAL cells were harvested at day 50 postinfection and dual-stained with FITC-conjugated anti-CD8 and phycoerythrin-conjugated anti-PD-1, anti-LAG-3, or anti-TIM-3, followed by flow cytometric analysis. Aliquots were also restimulated with MHV-68 p56 and p79 peptides before surface staining for CD8 and intracellular staining for IL-10. (A) Representative dot plots of PD-1, LAG-3, TIM-3, or IL-10 and CD8 staining. (B) Mean percentages $\pm$ standard deviation of CD8 T cells that were positive for PD-1, LAG-3, TIM-3, or IL-10 in BAL of WT and MHC Class II^{-/-} mice. There was a significant difference in the expression of all four molecules between the two groups. *** $p < 0.001$, * $p < 0.05$, t test. (C). Lung virus titers at day 50 after infection in mice were treated with 0.25 mg/mouse i.p. of RMP1-14 anti-PD-1, 1B1.3a anti-IL-10R, RMT3-23 anti-TIM-3, combinations of these antibodies, or control rat Ig every 2–3 days from day 35 postinfection onward. In some groups, 277 mM lactose was added to the drinking water. There was a statistically significant difference between lung viral titers in control Ig and anti-PD-1 ($p < 0.01$), anti-IL-10R ($p < 0.001$), or anti-PD-1 + anti-IL-10R ($p < 0.0001$) treated Class II^{-/-} mice. In contrast there was no significant difference between control and anti-TIM-3 or lactose-treated Class II^{-/-} mice. Class II^{-/-} mice treated with anti-PD-1 + anti-IL-10R had significantly lower lung viral titers than those treated with anti-PD-1 alone ($p < 0.01$) or anti-IL-10R alone ($p < 0.01$). There was no significant difference in groups treated with anti-PD-1 + anti-IL-10R versus anti-PD-1, anti-IL-10R + lactose, or anti-TIM-3. In addition, there was no significant difference in virus titers in mice treated with anti-TIM-3 + anti-PD-1 versus, those treated with anti-PD-1 alone. ** $p < 0.01$, *** $p < 0.001$, NS, not significant. IL, interleukin.

We found that multiple inhibitory receptors were, in fact, upregulated on the surface of CD8 T cells in CII-deficient mice. In addition to PD-1, LAG-3, and TIM-3 were also upregulated on CD8 T cells in the BAL of CII^{-/-} versus WT mice (Fig. 4). The majority of exhausted CD8 T cells expressed all three molecules. IL-10 production by CD8 T cells was also modestly increased, consistent with a previous report (51). Recent reports have suggested that the transcription factor, thymocyte selection-associated high mobility group box protein (TOX), is responsible for the transcriptional programming of CD8 T cell exhaustion (2,41,62) and we have also observed that this transcription factor is expressed at much higher levels in CD8 T cells from CII^{-/-} mice than in those from wild-type mice (L. Lee and S. Sarawar, Unpublished Data).

While blockade of either PD-1 or IL-10R reduced reactivation of MHV-68 in CD4 T cell-deficient mice, combined blockade of both of these molecules had a significantly greater effect than blocking either alone, suggesting that they operate via different mechanisms in this model, as previously reported by Brooks *et al.* (14) in an LCMV model of T cell exhaustion. In contrast, neither anti-TIM-3 nor lactose (which blocks the interaction of TIM-3 with its receptor, galectin 9) had any effect on viral titers, either alone or in combination with anti-IL-10R and anti-PD-1. This contrasts with the reported effects of TIM-3 in other viral models of T cell exhaustion, (36,39,40,56), in which blocking TIM3's interaction with its receptor resulted in improved viral control, highlighting the fact that, while

multiple inhibitory receptors are coordinately upregulated on exhausted T cells in many different viral models, the relative roles of each receptor in suppressing T cell activity and viral control may vary.

Although LAG3 was also upregulated, it is unlikely that it plays an inhibitory role in the MHV-68 model that we used, as its receptor is MHC Class II, although it could potentially play a role in MHV-68 reactivation in CD4 T cell-depleted wild-type mice. Interestingly, we observed that many of the inhibitory molecules were also upregulated on CD8 T cells during acute infection, when MHV-68 was controlled effectively. Continued expression of inhibitory molecules over several weeks appears to be associated with lack of viral control, although it is not clear whether the same subsets of CD8 T cells are involved at early and late time points.

Summary

In summary, CD40L on CD4 T cells activates antigen presenting cells, such as dendritic cells, which then upregulate CD80 and 86 (Fig. 5). CD80 and 86 function in a currently unspecified way that allows programming of a functional change in CD8 T cells, enabling them to maintain effective long-term control of MHV-68. In the absence of CD4 T cell help, antigen presenting cells do not upregulate CD80 and CD86 and fail to adequately prime CD8 T cells. As a consequence, multiple inhibitory molecules are upregulated on the CD8 T cells by a mechanism that is likely

FIG. 5. Model for T cell activation and exhaustion during MHV-68 infection in the presence and absence of CD4 T cell help. CD40L on CD4 T cells activates APCs, which then upregulate CD80 and 86. The activated APCs program a functional change CD8 T cells, enabling them to maintain effective long-term control of MHV-68, by a mechanism that is dependent on CD80/CD86. This change results in reduced expression of multiple inhibitory molecules. In the absence of CD4 T cell help, multiple inhibitory molecules are upregulated by a mechanism that is likely coordinated by TOX. This results in reduced antiviral activity and viral recrudescence.

coordinated by TOX. This results in reduced antiviral activity and viral recrudescence.

During acute infection, either PKC θ or CD4 T cell-dependent pathways can mediate viral control, suggesting that the PKC θ -dependent CD4 T cell-independent pathway of CD8 T cell activation was either short-lived or somehow incapable of maintaining long-term control of the virus.

There are several outstanding questions arising from the work described in this review including whether a novel receptor for CD80 and 86 plays a role in viral control, how inhibitory receptors depress CD8 T cell function, what is the precise mechanism of viral control, and whether inhibitory molecules differentially affect CD8 T cell function in acute and long-term control of MHV-68.

Acknowledgments

We thank Deanna Leon and Igor Maricic for excellent technical assistance, Dr. Arlene Sharpe for the CD28/CTLA4^{-/-} mice, and Drs. Hisaya Akiba and Hideo Yagita for providing critical reagents.

Author Disclosure Statement

No competing financial interests exist.

Funding Information

We gratefully acknowledge support from the National Institutes of Health grants AI050810 and AI057599.

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