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Innate immunity defines the capacity of antiviral T cells to limit persistent infection

Daniel M. Andrews,[1,3] Marie J. Estcourt,[1,3] Christopher E. Andoniou,[1,3] Matthew E. Wikstrom,[1,3] Andrea Khong,[1,3] Valentina Voigt,[1,3] Peter Fleming,[1,3] Hyacinth Tabarias,[1,3] Geoffrey R. Hill,[4] Robbert G. van der Most,[2] Anthony A. Scalzo,[1,3] Mark J. Smyth,[5] and Mariapia A. Degli-Esposti[1,3]

[1]Immunology and Virology Program, Centre for Ophthalmology and Visual Science, [2]University Department of Medicine, University of Western Australia, Nedlands, Western Australia 6009, Australia
[3]Centre for Experimental Immunology, Lions Eye Institute, Nedlands, Western Australia 6009, Australia
[4]Bone Marrow Transplantation Laboratory, Queensland Institute of Medical Research, Herston, Queensland 4006, Australia
[5]Cancer Immunology Program, Sir Donald and Lady Trescowthick Laboratories, Peter MacCallum Cancer Centre, East Melbourne, Victoria 3002, Australia

Effective immunity requires the coordinated activation of innate and adaptive immune responses. Natural killer (NK) cells are central innate immune effectors, but can also affect the generation of acquired immune responses to viruses and malignancies. How NK cells influence the efficacy of adaptive immunity, however, is poorly understood. Here, we show that NK cells negatively regulate the duration and effectiveness of virus-specific CD4$^+$ and CD8$^+$ T cell responses by limiting exposure of T cells to infected antigen-presenting cells. This impacts the quality of T cell responses and the ability to limit viral persistence. Our studies provide unexpected insights into novel interplays between innate and adaptive immune effectors, and define the critical requirements for efficient control of viral persistence.

CORRESPONDENCE
Mariapia A. Degli-Esposti:
mariapia@cyllene.uwa.edu.au

Abbreviations used: CTL, cytotoxic T lymphocyte; HCMV, human cytomegalovirus; HCV, hepatitis C virus; IE1, immediate early 1; MCMV, murine cytomegalovirus; pi, postinfection.

The development of effective therapies to prevent and treat persistent infections is of the highest priority, as they cause considerable clinical challenges and ongoing health care costs. Efforts to improve the treatment and prevention of chronic viral infections, such as those elicited by HIV, hepatitis C virus (HCV), and human cytomegalovirus (HCMV), require a better understanding of the immune responses needed to achieve optimal control of persistent viruses in the long term. Although innate and adaptive immune responses have historically been thought to be nonoverlapping, recent evidence clearly indicates that interplays between components of the immune system occur frequently and form the basis of effective immunity. Infection with murine cytomegalovirus (MCMV) is a well-established experimental system to study host–pathogen interactions and to dissect the relevance of different arms of the immune response. Because of the similarities in structure and biology between HCMV and MCMV, and because MCMV is a natural mouse pathogen, MCMV infection provides a unique model to study a medically important virus in vivo after infection of its biological host. Like HCMV, MCMV causes persistent infection, and thus the model can be exploited to gain a better understanding of the requirements for effective prevention and treatment of chronic viral infections.

Early control of MCMV infection is dependent on NK cell responses and is associated with a single dominant locus that encodes the NK cell–activating receptor Ly49H (Brown et al., 2001; Daniels et al., 2001; Lee et al., 2001). In mouse strains like C57BL/6J, NK cells express Ly49H and efficiently limit early viral replication (Bancroft et al., 1981; Shellam et al., 1981; Bukowski et al., 1984; Scalzo et al., 1990). Ly49H binds to the MCMV-encoded MHC class I like glycoprotein m157 to deliver

D.M. Andrews and M.J. Estcourt contributed equally to this paper.

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activating signals to NK cells (Arase et al., 2002; Smith et al., 2002). In contrast to C57BL/6J ($Ly49H^+$) mice, BALB/c mice, whose NK cells lack the Ly49H-activating receptor, show increased susceptibility to MCMV during the early phase of infection (Allan and Shellam, 1984). In BALB/c ($Ly49H^-$) mice, activation of NK cells is limited further by viral interference with the expression of ligands for the NKG2D NK cell–activating receptor (Lodoen et al., 2003; Lodoen et al., 2004; Hasan et al., 2005; Krmpotic et al., 2005), and early control of MCMV infection requires a CD8 T cell–mediated response. The antiviral $CD8^+$ T cell response commences within 4 d after infection and is directed predominantly against the nonameric peptide YPHFMPTNL (Del Val et al., 1991), which is derived from the virus immediate early 1 (IE1) nonstructural protein (Volkmer et al., 1987). The IE1 epitope, specifically recognized by BALB/c $CD8^+$ T cells in an H-$2L^d$–restricted manner, represents the best-studied MCMV antigenic determinant. Despite the large body of evidence demonstrating a role for $CD8^+$ T cells in limiting CMV, virus-specific $CD4^+$ T cells are also important. Evidence from both HCMV and MCMV studies suggests that $CD4^+$ T cell responses are a critical component of immunity to these viruses. In humans, virus-specific $CD4^+$ T cells are required to control HCMV-induced disease (Hsieh et al., 2001; Gamadia et al., 2003). In MCMV infection, $CD4^+$ T cells participate in limiting viral replication in salivary glands (Jonjic et al., 1989).

Our recent study demonstrated that interplays between DCs and NK cells during the very early phase of infection are important to achieve maximal control of the virus (Andoniou et al., 2005). Functional interrelationships between DCs and $Ly49H^+$ NK cells occur during the late stage of acute MCMV infection in vivo (Andrews et al., 2003). Although $Ly49H^+$ NK cells are undoubtedly important for the early control of viral infection in visceral organs (Brown et al., 2001; Daniels et al., 2001; Lee et al., 2001), it has been postulated that they may also regulate ensuing adaptive antiviral immune responses (Dokun et al., 2001; Su et al., 2001; Andrews et al., 2003). Recent studies have shown that NK cells promote early activation of MCMV-specific $CD8^+$ T cells through the ability to regulate the production of IFN-$\alpha\beta$ (Robbins et al., 2007). These effects however are transient, and it is unclear whether they impact on controlling virus replication. Here, we exploited the differences in NK cell activation that occur in $Ly49H^-$ (e.g., BALB/c) versus $Ly49H^+$ (e.g., C57BL/6) mice during MCMV infection to investigate the impact of differences in NK cell responses on the generation, maintenance, and in vivo effectiveness of antiviral $CD4^+$ and $CD8^+$ T cell responses.

RESULTS

NK cells affect antigen-specific CD8 T cell responses

To assess the interplay between NK cell–mediated activity and antiviral T cell responses we used BALB/c congenic mice (BALB.B6-CT8 or BALB.B6-Cmv1r), whose Natural Killer Complex (NKC) encodes the NK1.1 (NKR-P1C) and Ly49H NK cell receptors (Scalzo et al., 1999). Like C57BL/6J ($NK1.1^+$, $Ly49H^+$) mice, BALB.B6-CT8, and BALB.B6-Cmv1r mice express the NK1.1 and Ly49H receptors. Mice from these three strains are herein collectively referred to as $Ly49H^+$ mice and, because of the presence of the activating Ly49H receptor, they exhibit improved control of MCMV replication in the spleen during the early stage of acute infection (Scalzo et al., 1999). Importantly, the congenic strains allowed us to test how NK cell activation mediated by Ly49H affects subsequent antiviral responses on a common genetic background. The BALB/c background of these strains also enabled us to investigate virus-specific $CD8^+$ T cell responses using the well characterized H-$2L^d$–restricted IE1 epitope. In addition to the prototype $Ly49H^-$ strain BALB/c, in our comparative studies we also used a BALB/c congenic strain carrying NK1.1, but lacking Ly49H, known as BALB.B6-CT6 ($NK1.1^+$, $Ly49H^-$; Scalzo et al., 1999). BALB/c and BALB.B6-CT6 strains are herein collectively referred to as $Ly49H^-$ mice.

Antiviral cytotoxic T lymphocyte (CTL) responses were measured at 2, 4, 6, 8, and 10 d after infection. The in vivo CTL assay used in our studies measures the specific loss of IE1 peptide-pulsed targets in comparison to targets that have not been pulsed with peptide. Anti-IE1–specific CTL activity could not be detected in either $Ly49H^-$ (BALB/c) or $Ly49H^+$ (BALB.B6-Cmv1r) mice at 2 d post-infection (pi), and was minimal at day 4 pi (Fig. 1 A). In both $Ly49H^-$ and $Ly49H^+$ mice, maximal and equivalent CTL activity was observed at day 6 after MCMV infection (Fig. 1 A). However, in $Ly49H^+$ mice, the CTL response was significantly reduced compared with $Ly49H^-$ mice at both day 8 ($P < 0.005$) and day 10 ($P < 0.0001$) pi (Fig. 1 A). To determine whether the reduction in IE1-specific CTL killing was influenced by NK cell–mediated activities, $Ly49H^+$ mice were depleted of NK cells. Maximal CTL activity was observed at day 6 pi in both untreated $Ly49H^-$ mice and $Ly49H^+$ mice depleted of NK cells (Fig. 1 B). At 10 d pi, $Ly49H^+$ mice depleted of NK cells showed antiviral CTL activity equivalent to that observed in $Ly49H^-$ mice (Fig. 1 B). In contrast, and as expected, the CTL activity was reduced at 10 d pi in control Ig-treated $Ly49H^+$ mice (Fig. 1 B). In $Ly49H^+$ mice, NK cells are activated by engagement of the Ly49H receptor by the viral protein m157. The reduced CTL activity observed in $Ly49H^+$ mice was increased to $Ly49H^-$-like levels by infection with an MCMV virus lacking a functional m157 gene product, and thus unable to activate $Ly49H^+$ NK cells (Fig. 1 C). Significant differences in antiviral CTL activity between $Ly49H^-$ (BALB/c) and $Ly49H^+$ (BALB.B6-CT8) mice were detected out to day 40 pi (Fig. 1 D). Despite early similarities in the kinetics of CTL response, these data provide evidence that there are ongoing differences in CTL function in $Ly49H^-$ versus $Ly49H^+$ mice which are NK cell mediated and Ly49H dependent.

Having found that NK cells regulate the ongoing efficacy of cytolytic antiviral $CD8^+$ T cell responses, the activation of MCMV-specific $CD8^+$ T cells, as defined by IE1-tetramer binding, was compared by flow cytometry in infected $Ly49H^+$ and $Ly49H^-$ mice. A higher proportion of $IE1^+CD8^+$ T cells were positive for the early activation marker CD69 in $Ly49H^-$

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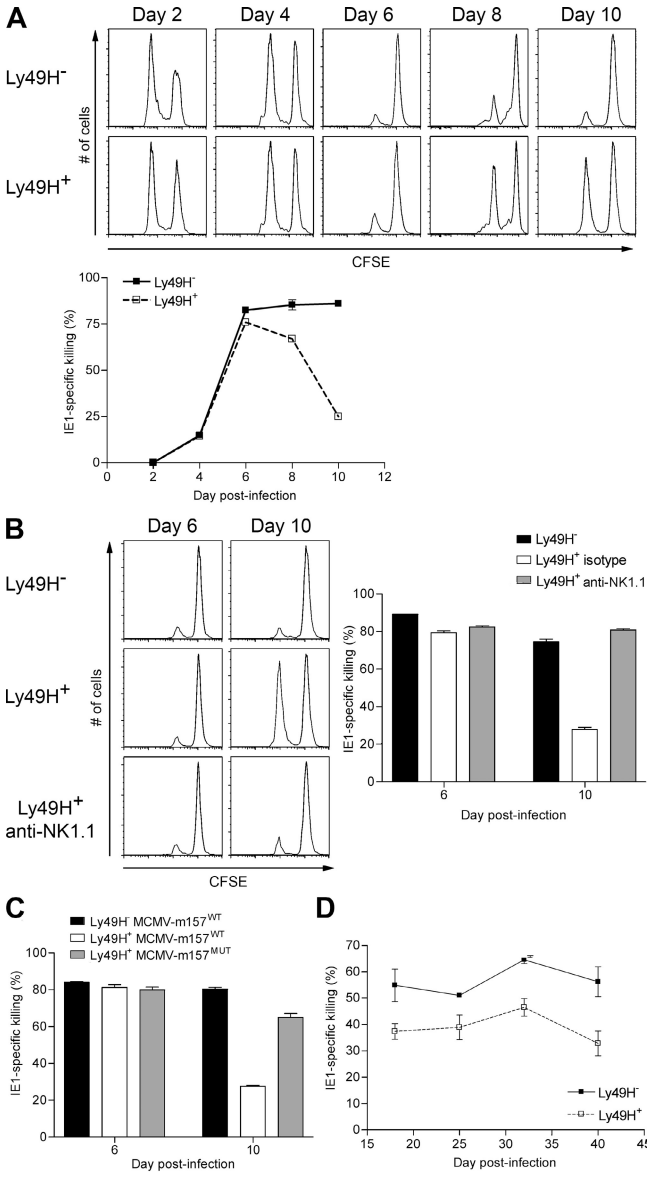

Figure 1. NK cells affect antiviral CTL function. Ly49H$^-$ and Ly49H$^+$ mice were infected with MCMV (5×10^3 PFU K181-Perth). (A) Virus-specific CTL activity was assessed in vivo at days 2, 4, 6, 8, and 10 pi by measuring the elimination of adoptively transferred CFSE low targets pulsed with the IE1 viral peptide. Unpulsed (CFSE high) targets are used for comparison. The histograms represent the number of CFSE high (unpulsed) and CFSE low (IE1 pulsed) targets in the spleens of relevant mice. The data are representative of at least two experiments each using three mice per time point. (bottom) CTL data are shown as percentage of IE1-specific cytotoxicity determined as described in the Materials and methods section. Arithmetic means and standard errors were plotted using the results from two independent experiments each using three mice per time-point. (B) Ly49H$^-$, Ly49H$^+$ isotype-matched control Ig-treated mice, and Ly49H$^+$ anti-NK1.1–treated mice were infected with MCMV (5×10^3 PFU). Virus-specific CTL activity was measured in vivo at days 6 and 10 pi. Data are representative of at least five independent experiments each using three mice per time point. (right) CTL data are shown as percentage of IE1-specific cytotoxicity. Arithmetic means and standard errors were plotted using the results from two independent experiments each using three mice per time point. (C) Ly49H$^+$ mice were infected with wild-type MCMV (MCMV-m157^{wt}) or a mutant MCMV virus with a nonfunctional m157 glycoprotein (MCMV-m157^{mut}). CTL data are plotted as percentage of IE1-specific cytotoxicity. CTL data from Ly49H$^-$ mice infected with wild-type MCMV are shown for comparison. Arithmetic means and standard errors are plotted. The data are representative of at least three independent experiments each with at least three mice per group (D) Virus-specific CTL activity was measured at days 18, 25, 32, and 40 after infection in ■ (Ly49H$^-$) and □ (Ly49H$^+$) mice. Data are representative of two independent experiments each using 3 mice per group. Arithmetic means and standard errors are plotted. The differences were significant at all times. $P < 0.0005$. Experiments described in A–C were performed in BALB/c (Ly49H$^-$) and BALB.B6-Cmv1r (Ly49H$^+$) mice. Experiments described in D were performed in BALB/c (Ly49H$^-$) and BALB.B6-CT8 (Ly49H$^+$) mice.

(BALB.B6-CT6) mice compared with Ly49H$^+$ (BALB.B6-CT8) mice on both 6 and 10 d pi (Fig. 2 A). In contrast, early CD8$^+$ T cell activation was equivalent in both strains, with 80–90% of CD8$^+$ T cells positive for CD69 at 2 d pi (CT6, 93.6 ± 1.56%; CT8, 83.95 ± 3.7%). The frequency of CD8 T cells producing both IFN-γ and TNF-α, an indicator of functional competence, was also examined and found to be higher in Ly49H$^-$ mice compared with Ly49H$^+$ mice at both day 6 and 10 pi (Fig. 2 B). Therefore, the activation and effector function of virus-specific CD8$^+$ T cells, as assessed by measuring cytotoxicity and cytokine production, is greater in Ly49H$^-$ mice compared with Ly49H$^+$ mice.

NK cells affect antiviral CD4 T cell responses

Next, we examined whether virus-specific CD4$^+$ T cell responses were similarly affected by NK cells. Given that CD4-restricted MCMV epitopes have not been defined for BALB/c mice, we measured the frequency of CD4$^+$ T cells able to produce IFN-γ after culture of lymphocytes with MCMV-infected DC as a source of antigen. At day 10 pi, the proportion of CD4$^+$ T cells secreting IFN-γ$^+$ was significantly higher ($P = 0.0022$) in Ly49H$^-$ (BALB/c) mice compared with Ly49H$^+$ (BALB.B6-CT8) mice (Fig. 3 A). Depletion of NK cells in Ly49H$^+$ mice increased the frequency of CD4$^+$ IFN-γ$^+$ T cells to a level comparable to that observed in Ly49H$^-$ mice, and significantly different to that observed in undepleted Ly49H$^+$ mice ($P = 0.045$ at day 10 pi; Fig. 3 A). The increased proportions of CD4$^+$ IFN-γ$^+$ T cells translated into increased numbers in Ly49H$^-$ mice compared with Ly49H$^+$ mice ($P = 0.026$). Similarly, NK depletion increased the numbers of CD4$^+$ IFN-γ$^+$ T cells in Ly49H$^+$ mice to levels similar to those observed in Ly49H$^-$ mice (Fig. 3 B).

NK cells determine the extent of DC/APC infection

Having shown that in Ly49H$^+$ mice NK cells impair antiviral CD4 and CD8 T cell responses, we sought to define the relevant underlying mechanisms. DCs play the most critical role in the generation of adaptive T cell responses (Steinman and Witmer, 1978; Jung et al., 2002). Viral infection of DCs allows for the generation of epitopes that can be presented to CD4$^+$ and CD8$^+$ T cells. Early antiviral immune responses,

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such as those mediated by NK cells, may affect the extent to which DCs are infected or may lead to their elimination soon after infection. Thus, NK cell–mediated responses may reduce the number of DCs able to present viral antigens, and consequently affect the generation and/or maintenance of virus-specific $CD4^+$ and $CD8^+$ T cells. We have previously shown that MCMV targets DCs in vivo (Andrews et al., 2001) and that there is a phenotypic difference in the DC compartment of MCMV-infected $Ly49H^-$ and $Ly49H^+$ mice (Andrews et al., 2003). To define the extent and duration of DC infection in $Ly49H^-$ and $Ly49H^+$ mice, we used a recombinant MCMV expressing LacZ, so that infected cells could be identified and enumerated. Infection of DCs was monitored at 2, 4, and 6 d pi using fluorescein di-galactosidase (FDG), as previously described (Andrews et al., 2001), in combination with CD11c, CD11b, MHC-II, and CD8. A significantly higher frequency of infected $CD11c^+$ DCs was observed in $Ly49H^-$ mice compared with $Ly49H^+$ (BALB.B6-CT8) mice at all times pi ($P = 0.0153$ at day 2; $P = 0.0006$ at day 4; $P = 0.0202$ at day 6 pi; Fig. 4 A). To assess the role of NK cells in limiting DC/APC infection, $Ly49H^+$ mice were depleted of NK cells before infection with MCMV-LacZ. Analysis of infected splenocytes ($LacZ/FDG^+$) revealed a difference in the number of infected $MHC\text{-}II^+$ APC/DC in $Ly49H^-$ versus $Ly49H^+$ mice over the course of infection; this difference was not observed in $Ly49H^+$ mice depleted of NK cells (Fig. 4 B). To determine the mechanism by which NK cells affect the frequency of infected APCs present over the course of infection, we examined the frequency of these cells in $Ly49H^+$ mice lacking the cytolytic effector molecule perforin (pfp). In BALB.B6-Cmv1r.$pfp^{-/-}$ mice, the frequency of $CD11c^+$ $LacZ^+$ cells was increased at all times pi compared with wild-type $Ly49H^+$ BALB.B6-Cmv1r mice (Fig. 4 C). At day 4 after infection, the frequency of infected DCs in $Ly49H^+$ perforin-deficient mice was equivalent to that observed in $Ly49H^-$ mice or in $Ly49H^+$ mice depleted of NK cells (Fig. 4, A and C). Thus, elimination of infected DCs/APCs is NK cell–mediated and perforin dependent. Unlike $Ly49H^+$ BALB.B6-Cmv1r.$pfp^{-/-}$ mice, $Ly49H^+$ BALB.B6-Cmv1r mice depleted of NK cells did not show a further increase in infected DCs at day 6 pi (Fig. 4), suggesting that perforin-dependent CTL activity may be involved in eliminating infected DCs from day 6 onwards.

One explanation for the longevity of the CTL response in $Ly49H^-$ mice is an increased viral load. Thus, we challenged $Ly49H^-$ mice with a viral dose (10^2 PFU) that results in viral titers below the limit of detection (Andrews et al., 2008). On day 10 pi, this dose of virus produced a CTL response ($89.0 \pm 1.2\%$) as robust as that induced by infection with 5×10^3 PFU of virus ($86.0 \pm 1.3\%$). Thus, the differences in CTL activity observed in $Ly49H^-$ versus $Ly49H^+$ mice were not caused by differences in viral loads (Table S1).

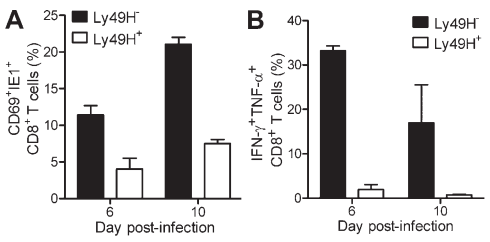

Figure 2. Differences in $CD8^+$ T cells from $Ly49H^-$ and $Ly49H^+$ mice. $Ly49H^-$ and $Ly49H^+$ mice were infected with 5×10^3 PFU MCMV and splenocytes harvested at day 6 and 10 pi. (A) Expression of CD69 was assessed on live $TCR^+CD8^+IE1\text{-}tetramer^+$ cells. The frequency of $CD69^+$ $CD8^+IE1\text{-}tetramer^+$ cells is plotted. Frequencies are shown as arithmetic means with standard errors. The data are representative of two independent experiments each with three mice per group. (B) The frequencies of IFN-γ^+TNF-α^+ $CD8^+IE1^+$ cells are plotted. The data are representative of two independent experiments each with three mice per group. Frequencies are shown as arithmetic means with standard errors. Experiments were performed in BALB/c ($Ly49H^-$) and BALB.B6-CT8 ($Ly49H^+$) mice.

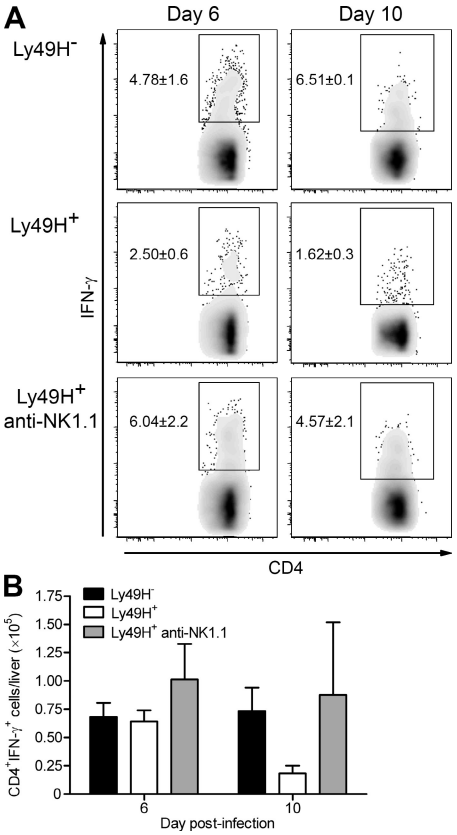

Figure 3. NK cells affect the frequency of activated $CD4^+IFN\text{-}\gamma^+$ T cells. $Ly49H^-$, $Ly49H^+$, and $Ly49H^+$ NK1.1-depleted mice were infected with MCMV (5×10^3 PFU). (A) The frequencies of $CD4^+IFN\text{-}\gamma^+$ T cells in the liver at days 6 and 10 pi are shown. The frequencies of IFN-γ^+ cells are shown as a percentage of TCR^+CD4^+ cells. Density plots are representative of results from three independent experiments using three mice per time point, except the NK depletion which was performed once. Frequencies of cells in each boxed region are shown as arithmetic means with standard errors. (B) The numbers of $CD4^+IFN\text{-}\gamma^+$ T cells in the liver at days 6 and 10 pi are shown as arithmetic means with standard errors. The experiments were performed in BALB/c ($Ly49H^-$) and BALB.B6-CT8 ($Ly49H^+$) mice.

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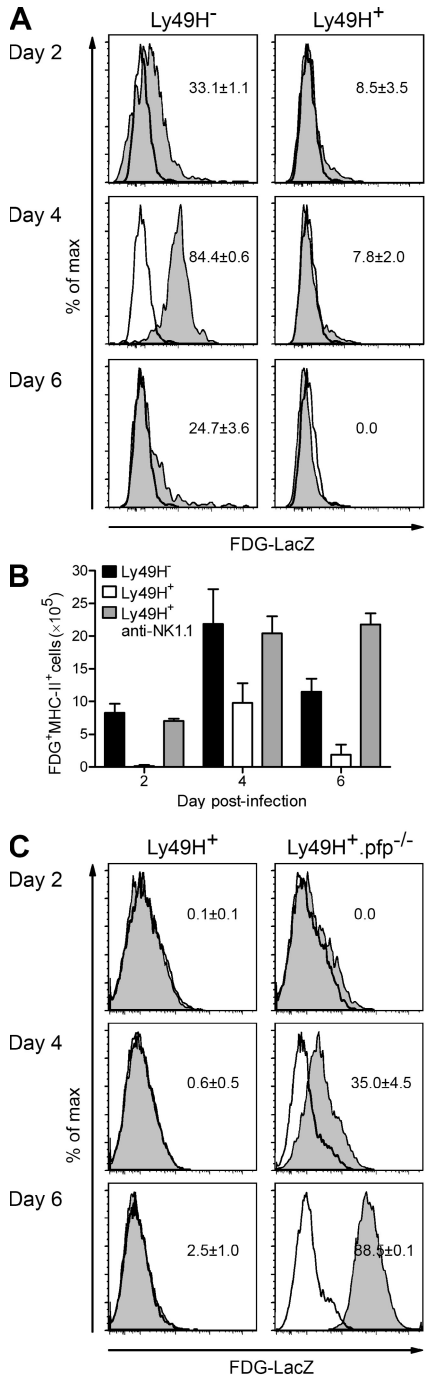

Figure 4. Sustained APC infection in Ly49H⁻ mice, but not in Ly49H⁺ mice. (A) Ly49H⁻ (BALB/c) and Ly49H⁺ (BALB.B6-CT8) mice were infected with 5×10^3 PFU of MCMV-K181-LacZ, and the frequency of infected APCs in the spleen was measured 2, 4, and 6 d pi using FDG. Histograms represent the expression of FDG-LacZ in CD11c⁺ MHC-II⁺ cells from the spleen. The proportion of FDG⁺ cells was first corrected for background fluorescence by subtracting the FDG⁺ values measured in uninfected controls. Arithmetic means and standard errors are shown. Uninfected controls are shown as a solid black line. Infected mice are shown as gray-filled histograms. The frequencies of infected FDG⁺ cells are shown and represent data from three mice. The data are representative of two independent experiments. (B) The number of infected MHC-II⁺ APC in the spleens of MCMV infected mice at days 2, 4, and 6 d pi are shown. The numbers of infected MHC-II⁺ APC were determined using FDG after correction for background fluorescence. Arithmetic means and standard errors are shown. (C) Ly49H⁺ BALB.B6-Cmv1r and BALB.B6-Cmv1r. pfp$^{-/-}$ mice were infected with 5×10^3 PFU of MCMV-K181-LacZ. Histograms represent the expression of FDG-LacZ in CD11c⁺ MHC-II⁺ cells purified from the spleen. Uninfected controls are shown as a solid black line. Infected mice ($n = 3$) are shown as gray filled histograms. The data are representative of 3 independent experiments.

Because viral load could not explain the longevity of the CTL response in Ly49H⁻ mice, we examined the amount of viral peptide–MHC complex available to T cells. To formally determine whether NK cells affect the amount of viral peptide–MHC complex available to T cells, and hence are responsible for the differences in antiviral T cell responses, we used a functional assay that measures the proliferation of naive T cells in an antigen-specific manner. This assay specifically quantifies the amount of viral peptide antigen presented by DC/APC to T cells, rather than total amount of antigen/virus. In this assay, purified T cells from TCR-transgenic mice are cultured with DCs enriched from MCMV-infected mice and CFSE labeling is used to measure the extent of T cell proliferation, which is directly proportional to the amount of antigen presented by the infected APCs. Ly49H⁻ (BALB.B6-CT6) and Ly49H⁺ (BALB.B6-CT8) mice were infected with a recombinant MCMV expressing the HA epitope of influenza (MCMV-K181-HA), and APC populations enriched by density gradient and negative antibody enrichment on days 0, 2, 4, and 6 pi. The phenotype of the enriched APC populations was analyzed to determine the frequency of cDCs and pDCs (Fig. 5 A). Enriched APC populations were subsequently co-cultured with CFSE-labeled HA-specific T cells isolated from the lymph nodes of HA-transgenic CL4 mice and the proliferation of CD8⁺ T cells measured. Naive antigen-specific CD8⁺ T cells proliferated equally well when incubated with APCs isolated from either Ly49H⁻ or Ly49H⁺ mice at day 2 pi (Fig. 5 B, top). In contrast, proliferation was observed in naive antigen-specific T cells when these were primed by APCs isolated at 4 or 6 d pi from infected Ly49H⁻ mice, but not when they were primed by APC isolated from infected Ly49H⁺ mice (Fig. 5 B, middle and bottom). These data provide evidence that the molecular basis for the observed differences in antiviral T cell responses in the presence of effective NK cell responses is the difference in the frequency and number of infected APCs/DCs that can prime naive T cells and recruit them to the effector population. These results further validate our hypothesis that the impaired T cell responses observed in Ly49H⁺ mice are caused by the elimination of infected DCs and the consequent reduction in the amount of viral peptide–MHC complexes available to T cells.

The longevity of antiviral T cell responses determines the extent of viral persistence

Control of viral replication in visceral organs (spleen, liver, and lung) of Ly49H⁻ mice is dependent on IE1-specific CTL

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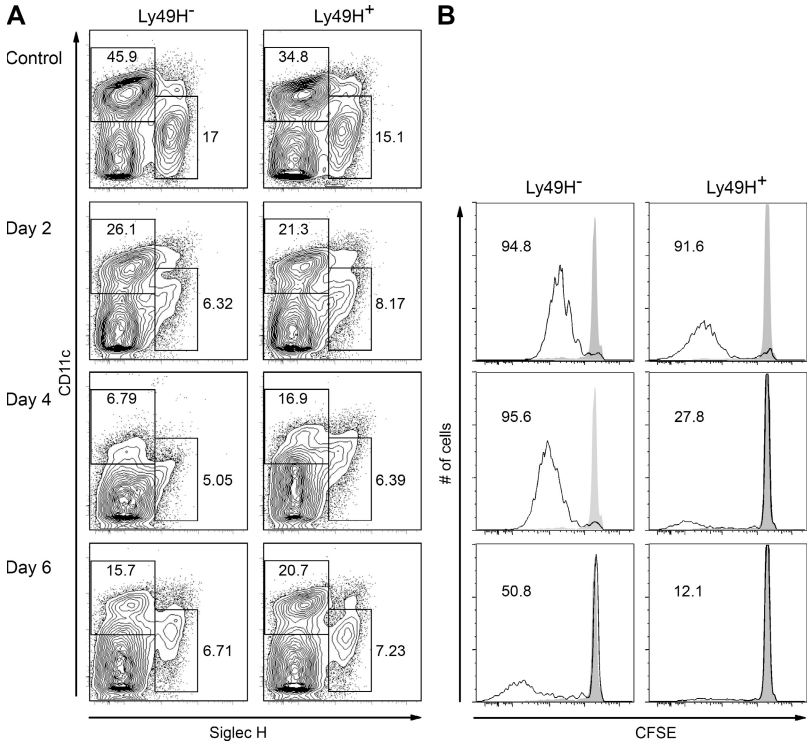

Figure 5. More viral peptide–MHC complex available to T cells in Ly49H⁻ mice sustains ongoing T cell proliferation. Ly49H⁻ and Ly49H⁺ mice were infected with 5×10^3 PFU of MCMV-K181-HA and APC populations enriched by density gradient and negative antibody enrichment on day 2, 4, and 6 pi. (A) The phenotype and frequency of the enriched populations is shown. Density plots (gated on live lymphocytes) depict the CD11c and Siglec H expression in Ly49H⁻ and Ly49H⁺ mice. (B) APCs were enriched from MCMV-K181-HA infected Ly49H⁻ and Ly49H⁺ mice, as shown in A, at day 0, 2, 4, and 6 pi, and incubated in vitro with CFSE-labeled Tg-HA CD8⁺ T cells for 5 d. Histograms show proliferation of CD8⁺ T cells (gated on live, TCR⁺, CD8⁺, and CD4⁻). CD8⁺ T cells incubated with uninfected APCs are shown as gray-filled histograms. CD8⁺ T cells incubated with APC from 2, 4, and 6 d pi are shown as solid black lines. The frequencies of cells with diluting CFSE are shown. Data are representative of two independent experiments performed in triplicate. All experiments were performed in BALB.B6-CT6 (Ly49H⁻) and BALB.B6-CT8 (Ly49H⁺) mice.

function (Volkmer et al., 1987). In the salivary glands, a site of viral persistence and transmission, control of virus is thought to require the combined activities of $CD8^+$ and $CD4^+$ T cells (Jonjic et al., 1989; Lucin et al., 1992). How interplays between different arms of the host immune response affect viral persistence in different organs is unknown. We were specifically interested in understanding whether the NK cell–mediated modulation of T cell responses could affect viral persistence in salivary glands. Persistent virus in salivary secretions is the major source of horizontal transmission for both murine and human CMV.

A comparison of infectious viral titers in the salivary glands of Ly49H⁻ and Ly49H⁺ mice revealed significantly higher titers in Ly49H⁻ mice at 10 d ($P < 0.01$), 18 d ($P < 0.0005$), and 25 d ($P < 0.0005$) pi. Viral titers remained constant at $>10^4$ PFU per organ in Ly49H⁺ mice 10–60 d after infection. In contrast, in Ly49H⁻ mice, viral titers declined sharply by day 32, and no virus was detected in the salivary glands of these mice from day 40 onward (Fig. 6, A and B). These findings indicate that the capacity to limit viral persistence differs in Ly49H⁻ and Ly49H⁺ mice, and suggest that the NK cell regulated differences in T cell functions observed in these mice may contribute to the ability of the virus to persist in salivary glands.

Further independent evidence that NK cells regulate the responses required to limit viral persistence was obtained using viruses lacking the m157 viral glycoprotein. In Ly49H⁻ mice, wild-type and m157 mutant viruses replicated to the same levels in spleen, liver, lung, and salivary glands at all time points tested (unpublished data). In contrast, the persistent infection and sustained viral loads observed in salivary glands of Ly49H⁺ mice after infection with wild-type MCMV were significantly reduced after infection with m157 mutant viruses ($P < 0.05$ d 40 MCMV $m157^{MUT}$ versus MCMV $m157^{REV}$; Fig. 6 C). Thus, activation of NK cells, through engagement of Ly49H by the m157 viral protein, favors MCMV persistence in salivary glands. These data demonstrate that Ly49H-mediated NK cell activation regulates the responses required to limit viral persistence in salivary glands.

Although a role for $CD4^+$ T cells in controlling virus replication in salivary gland has been reported (Jonjic et al., 1989; Lucin et al., 1992), virus-specific $CD8^+$ T cells are detected in this organ in high numbers (Cavanaugh et al., 2003). Furthermore, vaccination protocols that induce $CD8^+$IFN-γ^+ T cells have been reported to effectively reduce viral persistence in the salivary gland (Rouvio et al., 2005). To determine the relative importance of $CD4^+$ versus $CD8^+$ T cells in limiting persistent MCMV infection, we compared the numbers of $CD4^+$IFN-γ^+ and $CD8^+IE1^+$ T cells in the salivary glands of Ly49H⁻ (BALB/c and BALB.B6.CT6) and Ly49H⁺ (BALB.B6-CT8) mice. Significantly higher numbers of both $CD8^+IE1^+$ (Fig. 7 A) and $CD4^+$IFN-γ^+ T cells (Fig. 7 B) were detected in the salivary glands of infected Ly49H⁻ mice at days 10, 18, and 25 pi. These data suggest that differences in virus-specific $CD4^+$ and $CD8^+$ T cells may contribute to the differences in the ability to limit persistent MCMV infection observed between Ly49H⁻ and Ly49H⁺ mice.

To formally define the specific role of $CD4^+$ and $CD8^+$ T cells in limiting persistent viral infection, we performed timed depletion of these cell subsets from Ly49H⁻ mice late in infection. Depletions were commenced 16 d before measuring viral replication in salivary glands. Depletion of $CD8^+$ T cells did not significantly affect viral titers at any of the time points tested (Fig. 7 C). In contrast, depletion of $CD4^+$ T cells resulted in significantly ($P < 0.05$) increased and sustained viral replication in salivary glands compared with undepleted

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mice (Fig. 7 C). Together, these results provide evidence that $CD4^+$, but not $CD8^+$ T cells, play a critical role in limiting viral persistence in salivary glands.

DISCUSSION

Using a model of persistent viral infection, we have defined a novel link between components of the innate and adaptive immune system, whereby NK cells limit the ongoing efficacy of virus-specific $CD4^+$ and $CD8^+$ T cell responses. We have shown that the effects of NK cells are mediated by controlling the frequency and duration of infection of DCs/APCs. In the face of effective NK cell responses, the proportion and number of antigen-bearing APCs is reduced, and the extent of T cell activation is consequently limited. MCMV infection of DCs leads to decreased expression of MHC and co-stimulatory molecules, and functional impairment characterized by the inability to stimulate alloreactive T cell responses (Andrews et al., 2001). MCMV-induced DC impairment, however, is preceded by a phase of DC activation (Andrews et al., 2001), and indeed MCMV-infected DCs can efficiently prime autologous naive T cells (Mathys et al., 2003). Importantly, MCMV-infected DCs are a major source of infectious viral progeny, and immature DCs are a principal target of MCMV infection

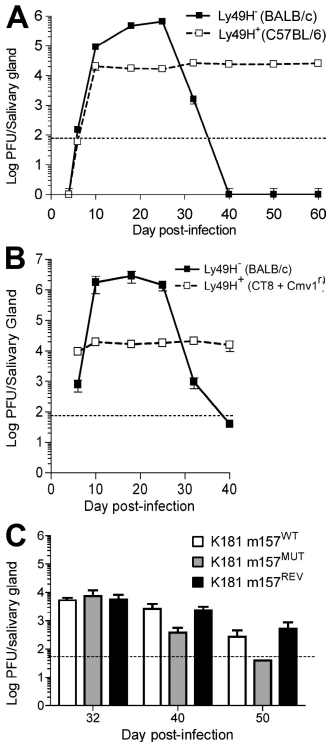

Figure 6. Viral persistence differs in Ly49H⁻ and Ly49H⁺ mice. (A) Titers of replicating virus in salivary glands of infected BALB/c and C57BL/6J mice are shown over a course of infection. Arithmetic means and standard errors are plotted. Data are combined results from at least three independent experiments each using three mice per time point. (B) Viral titers equivalent to those observed in C57BL/6 mice were obtained in Ly49H⁺ BALB/c congenic mice. Arithmetic means and standard errors are plotted. Each data point represents six mice (three BALB.B6-Cmv1r and three BALB.B6-CT8). Data from BALB.B6-Cmv1r and BALB.B6-CT8 were combined because both strains are identical in respect to MCMV growth characteristics in vivo. Day 32 and day 40, P = 0.0022 when comparing Ly49H⁻ with Ly49H⁺ mice. (C) Viral persistence in the salivary glands of Ly49H⁺ mice infected with a mutant virus lacking m157 is reduced, and is equivalent to that observed in Ly49H⁻ mice. Ly49H⁺ C57BL/6 mice were infected with wild-type virus (MCMV-$m157^{WT}$) or a virus with a nonfunctional m157 glycoprotein (MCMV-$m157^{MUT}$). A revertant virus (MCMV-$m157^{REV}$), generated by inserting a functional m157 glycoprotein in the MCMV-$m157^{MUT}$, was also used. Organs were collected at days 32, 40, and 50 pi. Titers of replicating virus in the salivary glands are shown as arithmetic means and standard errors. $n = 7$. Day 40, P = 0.0313 when comparing MCMV-$m157^{MUT}$ with MCMV-$m157^{REV}$.

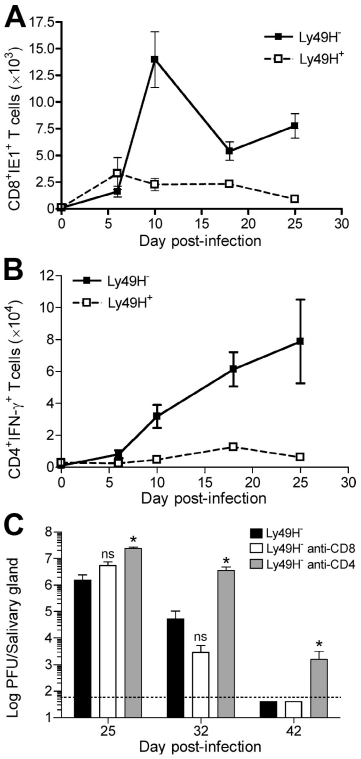

Figure 7. Viral persistence differs in Ly49H⁻ and Ly49H⁺ mice because of differences in CD4⁺ T cell responses. (A) Absolute numbers of $CD8^+IE1^+$ T cells from three mice and representative of four independent experiments and (B) absolute numbers of $CD4^+IFN\text{-}\gamma^+$ T cells from data pooled from five experiments ($n = 15$) at various times pi in the salivary glands of Ly49H⁻ and Ly49H⁺ mice infected with MCMV (5×10^3 PFU). Absolute numbers are shown as arithmetic means and standard error. (C) Viral titers in the salivary glands of Ly49H⁻ mice depleted of either $CD4^+$ or $CD8^+$ T cells after infection with 10^4 PFU MCMV are shown. Titers in infected, undepleted Ly49H⁻ mice are shown for comparison. Arithmetic means and standard errors were plotted from one representative experiment with three mice per group and are representative results obtained in four independent experiments. *, P < 0.05. BALB/c (Ly49H⁻) and BALB.B6-CT8 (Ly49H⁺) mice were used in the experiments described in A, except one where BALB.B6-CT6 (Ly49H⁻) mice were used instead of BALB/c (Ly49H⁻) mice. The experiments described in B were performed three times in BALB/c (Ly49H⁻) and twice in BALB.B6-CT6 (Ly49H⁻) in comparison with BALB.B6-CT8 (Ly49H⁺) mice. BALB/c (Ly49H⁻) mice were used for all experiments described in C.

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(Andrews et al., 2001). Based on these findings and our current results, we propose the following model. Immediately after infection, MCMV-infected DCs are activated and prime naive T cells in both Ly49H$^-$ and Ly49H$^+$ mice. In Ly49H$^-$ mice, where the NK cell response is suboptimal, MCMV-infected DCs are not eliminated. Despite the fact that infected DCs become functionally impaired some time after infection, they remain a major source of progeny virus which can in turn infect immature DCs, including those repopulating the spleen, and thus maintain a pool of effective APCs over a long period of time. In contrast, the NK cell–mediated elimination of infected DCs in Ly49H$^+$ mice limits the ongoing priming of antiviral T cells by removing the cells that are the source of infectious virus soon after infection. To our knowledge, this is the first physiological characterization of the mechanism whereby NK cells affect the ongoing effectiveness of T cell responses by limiting exposure to professional APCs. Importantly, we have also shown that the mechanisms by which NK cells limit the exposure of naive T cells to infected APCs is perforin-mediated elimination of infected cells.

Our observations show that antiviral CTL responses are modulated by NK cells, and specifically by Ly49H$^+$ NK cells. Furthermore, we found that the interaction of the m157 viral glycoprotein with the Ly49H NK cell activating receptor favors viral persistence in salivary glands. Previous studies have analyzed the role of the m157 viral protein during acute viral infection. Binding of m157 to host Ly49H NK cell receptors delivers activating signals to NK cells (Arase et al., 2002; Smith et al., 2002). The involvement of a viral protein in activating antiviral responses was partly reconciled by the finding that m157 can rapidly mutate in response to repeated passage through Ly49H$^+$ mice, and consequently lose the capacity to activate NK cells (Voigt et al., 2003). These studies provided a clear example of how MCMV evolves in response to strong repeated selection pressure, such as the one imposed by NK cells during acute infection. Such pressure is likely to compromise the ability of the virus to replicate, and hence viruses with mutated m157 are selected as they have a growth advantage over parent virus. We were prompted to analyze the long-term role of m157 because it seemed peculiar that a viral protein would activate antiviral responses without any ultimate benefit to the virus. The effects of m157 we present in the current study relate to transmission and evolutionary survival and the relevant mechanisms represent manipulation of host immune responses, rather than blocking, as afforded by mutating the m157 sequence. Developing means to specifically favor viral transmission and evolutionary survival as opposed to ensuring early viral replication are very different challenges, but both are dealt with by MCMV very efficiently. The results presented here resolve the apparent incongruity as to the role of a viral protein that apparently favors early antiviral immunity mediated by NK cells, and present exciting evidence of a new strategy of viral immune evasion.

Several studies have highlighted the role of innate immunity in promoting the adaptive response (for review see Degli-Esposti and Smyth, 2005) and the potential for exploiting these interplays therapeutically. Two previous studies have demonstrated that NK cells are able to temporally accelerate (Robbins et al., 2007) or enhance (Krebs et al., 2009) adaptive immune responses, particularly the CD8$^+$ T cell response. Although these results appear to be at odds with those of our studies, we believe they point to a complex set of interplays between the innate and adaptive immune systems that are slowly being unraveled. Our current data demonstrate that caution needs to be taken when considering approaches that activate NK cells therapeutically, because, in some circumstances, an over effective NK cell response may be detrimental to the quality of ongoing T cell responses.

In the context of our current findings, it is worth noting a study that analyzed the impact of polymorphisms in the inhibitory NK cell receptor KIR3DL1 on HIV-1 disease progression in a large cohort of >1,500 HIV-1–infected individuals (Martin et al., 2007). The data showed that high expression of the inhibitory NK cell receptor KIR3DL1 and its HLA ligand (Bw4-801) confers protection from AIDS progression. This unexpected result has been explained "by a model in which greater dependency on the expression of specific KIR3DL1 + Bw4 receptor–ligand pairs for NK cell inhibition in the resting state (where *KIR3DL1*h > KIR3DL1*l*) results in more pronounced NK cell responses when that inhibition is abrogated in the face of infection" (Martin et al., 2007). In view of our current results, an alternative explanation may be that inhibition of NK cell activities allows HIV-infected DCs to be maintained longer, resulting in better ongoing HIV-specific T cell responses and, ultimately, protection from AIDS progression.

Notably, our data have shown that impairment of antiviral T cell responses contributes significantly to viral persistence, and have clearly demonstrated that ongoing CD4$^+$ T cell responses, independently of CD8$^+$ T cell responses, are critical for this effect. This was somewhat unexpected given that both CD4$^+$ and CD8$^+$ T cells are present in greater numbers in the salivary glands of mice displaying reduced viral persistence. Recent studies have demonstrated that the efficacy of antiviral CD8$^+$ T cells is limited by their capacity to maintain contact with virally infected targets long enough to result in their elimination (unpublished data). Most MCMV-infected targets down-regulate MHC-I, and many of the pathogens that, like CMV, cause a persistent infection have evolved elaborate strategies to avoid immune recognition. We postulate that in chronic infections control of persistent virus will require activities mediated by CD4$^+$ T cells. In HIV infection, although an impairment in the function of CD8$^+$ T cells has been postulated to be responsible for the inability to control persistent infection (Appay et al., 2000; Champagne et al., 2001), additional evidence suggests that successful control of viral persistence requires efficient CD4$^+$ T cell responses (Rosenberg et al., 1997; Pitcher et al., 1999; Boaz et al., 2002; Harari et al., 2004; D'Souza et al., 2007; Kaufmann et al., 2007). Similarly, suboptimal CD4$^+$ T cell responses are associated with persistent HCV infection and chronic disease (for review see Semmo and Klenerman, 2007).

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Thus, as originally proposed (Matloubian et al., 1994), accumulating evidence suggests that $CD4^+$ T cell responses are a critical component of effective antiviral responses and are particularly important to limit persistent infection. The mechanisms that account for the failure of these responses in chronic infection however have, until now, remained unclear. Our results define the impact of NK cells and APCs in shaping the quality of CD4 T cell responses in MCMV, and their ability to limit viral persistence in salivary glands, the main site of viral transmission. These findings provide new insights in the management of persistent viral pathogens, but also demonstrate the general utility of exploiting the basic biology of viral infection to better understand how the immune system works and how to interfere with some of its specific functions. This type of analysis defines the key molecular events and checkpoints that need to be targeted to alter the outcome of immune responses. Our current data provide grounds to reassess the interplay between innate and adaptive immune responses, not only in the context of infectious diseases, but also tumor immunology and transplantation.

MATERIALS AND METHODS

Mice. Inbred C57BL/6J ($H\text{-}2^b$, $I\text{-}A^b$, $NK1.1^+$ $Ly49H^+$) and BALB/c ($H\text{-}2^d$, $I\text{-}A^d$, $NK1.1^-$ $Ly49H^-$) mice of 8 wk of age were obtained from the Animal Resources Centre (Perth, Western Australia). The $Ly49H^+$ congenic strains BALB.B6-CT8 ($H\text{-}2^d$, $I\text{-}A^d$, $NK1.1^+$, and $Ly49H^+$), BALB.B6-Cmv1r ($H\text{-}2^d$, $I\text{-}A^d$, $NK1.1^+$, and $Ly49H^+$), BALB.B6-CT13 ($H\text{-}2^d$, $I\text{-}A^d$, $NK1.1^-$, and $Ly49H^+$) and BALB.B6-Cmv1r.$pfp^{-/-}$, and the congenic $Ly49H^-$ strain BALB.B6-CT6 ($H\text{-}2^d$, $I\text{-}A^d$, $NK1.1^+$, and $Ly49H^-$; Scalzo et al., 1999) were bred in the Animal Services Facility of the University of Western Australia. All mice were maintained in this facility during the course of infection. All animal experimentation was performed with the approval of the Animal Ethics and Experimentation Committee of the University of Western Australia, according to the guidelines of the National Health and Medical Research Council.

MCMV infection. Mice were infected by intraperitoneal administration of 5×10^3 or 10^2 PFU of salivary gland propagated MCMV diluted in PBS-0.05% FCS. The viruses used were as follows: MCMV-K181-Perth (referred to as MCMV); MCMV-K181-Perth-LacZ, which is a virus genetically modified to encode the LacZ gene in the intron of the m129-m131 open reading frame; or MCMV-K181-Perth-HA, which is a virus genetically modified to carry the CD8 CL4/H2Kd-restricted HA (512–520) IYSTVASSL epitope from mouse influenza at the end of the MCMV ie1 gene. Control mice received PBS/0.05% FCS.

m157 mutant viruses. Three MCMV mutants (C2, C6, and E4) derived from the wild-type MCMV-K181 contain mutations in the m157 ORF; these viruses are referred to as MCMV-$m157^{mut}$. The derivation of the C2 and C6 MCMV-$m157^{mut}$ viruses is described in our previously published study (Voigt et al., 2003). In addition, we purified additional independent m157 mutants, including the E4 used here, from the salivary glands of C57BL/6 mice sequentially infected two times with virus, as described (Voigt et al., 2003). The E4 MCMV-m157 mutant carries a 1-bp deletion at nucleotide position 216,833 (relative to the nucleotide assignment in the prototypic Smith MCMV strain sequence) resulting in a frame shift and premature stop. The MCMV-$m157^{mut}$ E4 clone was confirmed incapable of activating Ly49H, as previously described (Voigt et al., 2003). A wild-type MCMV clone was purified concurrently, and designated MCMV-$m157^{wt}$. The MCMV-$m157^{wt}$ and MCMV-$m157^{mut}$ E4 viruses were confirmed to be clonal by serial dilution, sequencing, and functional analyses. An m157 mutant virus (K181-m157-MUT) was also generated using a K181 BAC pARK25 backbone (K181-m157-WT). K181-m157-MUT carries the previously described C2 mutation which results in a premature stop codon in the m157 sequence and a lack of m157 expression (Voigt et al., 2003). A revertant virus (K181-m157-REV) was generated by replacing the mutated m157 sequence in the K181-m157-MUT virus with a wild-type full-length m157 sequence.

In vivo CTL assay. Virus-specific, CTL-mediated cytotoxicity was assessed by measuring the elimination of targets pulsed with the IE1 viral peptide YPHFMPTNL. The in vivo CTL assay quantifies CTL activity by measuring the loss of specific peptide-pulsed targets in comparison to targets that have not been pulsed with peptide. Here, target cell lysis was measured using a modification of a previously described assay (Barber, 2001). IE1 peptide–pulsed (0.02 μg/ml) splenocyte targets were labeled with a low concentration (final, 0.025 μM) of CFSE (Invitrogen), whereas control targets, not pulsed with peptide, were labeled with a high concentration (final, 0.25 μM) of CFSE. Labeled cells were washed to remove free CFSE and differential labeling confirmed by flow cytometry. CFSE-low IE1–pulsed targets and CFSE-high–unpulsed control targets were resuspended in PBS, mixed in equal proportions, and a total of 4×10^7 cells per mouse transferred intravenously into syngeneic mice that had been infected with MCMV for various periods of time. Mice were sacrificed 18 h later, and single-cell suspensions from spleen, lung, and cervical and parotid lymph nodes analyzed by flow cytometry. Transferred cells serve as targets for peptide-specific CTL. The frequency of unpulsed targets serves as an internal control for trafficking, recovery, and nonspecific cell loss. The loss of specific peptide-pulsed targets is a measure of CTL activity against targets pulsed with the IE1 viral peptide. Specific lysis was determined using the following formula: percentage specific lysis of CFSE-labeled target cells in each mouse is calculated as follows: $(1 - [\text{r uninfected control mouse/r infected test mouse}]) \times 100$ where r = (frequency of unpulsed targets/frequency of peptide-pulsed targets). The assay is a very sensitive and specific method to measure MHC class I–restricted, CD8-dependent lysis in vivo.

NK cell depletion. NK cells were depleted using the NK1.1-specific mAb PK136 at days −2, 0, and 2 relative to MCMV infection, as previously described (Scalzo et al., 1992). Depletion was confirmed using ^{51}Cr-labeled YAC cells as targets in a standard 4-h NK cell lysis assay.

DC enrichment. Spleens were cut into small fragments and incubated in IMDM, 2% FCS medium containing collagenase (1 mg/ml, type IV; Invitrogen) for 25 min at room temperature with continuous agitation. EDTA was added at a concentration of 0.01 M and incubation continued for 5 min. Undigested material was removed by filtering through a stainless steel sieve and cells pelleted by centrifugation. Cells were then resuspended in PBS and overlaid with isoosmotic OptiPrep (Nyegaard Diagnostics) solutions for DC purification, as described by the manufacturer. The low-density fraction was obtained by centrifugation at 600 *g* for 15 min. Cells in the low-density fraction, accounting for ∼1% of the starting cell population, were washed and used for further analysis.

Flow cytometry. Single-cell suspensions were preincubated on ice for 30 min with EDTA-SS-FCS containing 20% normal goat serum. To detect IE1-specific T cells, APC-conjugated TCRβ (H57-597; BioLegend) was used together with APC-Cy7–conjugated CD8α (53–6.7; BioLegend) and PE-conjugated H-2Ld-168-YPHFMPTNL-176 MCMV IE1 tetramer (ImmunoID Tetramers). To assess the activation status of CD8 T cells, APC-Cy7–conjugated CD8α (53–6.7; BioLegend) and FITC-conjugated TCR (H57-597; BioLegend) were used together with PE-Cy7–conjugated CD69 (H1-2F3; BD). To detect MCMV-infected DCs, PE-conjugated CD11c (HL-3), PE-Cy7–conjugated CD11b (M1/70), APC-Cy7–conjugated CD8α (53–6-7), and APC-conjugated I-A/I-E (M5/114-15-2) from BioLegend were used together with FDG (Invitrogen). 7-amino-actinomycin D was incorporated into the final wash at 2 μg/ml to exclude dead cells from the analysis. The fluorescence-labeled preparations were analyzed on a FACSCanto (BD).

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Appropriately stained controls were used to check compensation for all fluorochromes used. Files of at least 1000 events in the gate of interest were collected and analyzed using FlowJo software (Tree Star, Inc.).

Measuring virus-specific CD4$^+$ T cells. Single-cell suspensions were stimulated directly ex vivo by co-culture with MCMV-infected bone marrow–derived DC or on CD3-coated 96 well plates for 4 h at 37°C in 200 µl complete RPMI+10%FCS. Cells were then washed and incubated on ice in PBS/2% FCS containing 10% normal goat serum before staining with PE-Cy7–conjugated CD4 (RM4-5; BD), APC-Cy7–conjugated CD8α, and FITC-conjugated TCRβ antibodies for 30 min on ice. Cells were washed two times with PBS/2% FCS and once with PBS. Cells were fixed for 20 min in Cytofix/Cytoperm (BD), washed, and stained with APC-conjugated IFN-γ (XmG1-2; BioLegend) in Permwash (BD) for 30 min on ice. Cells were washed three times and analyzed on a FACSCanto. Files of 20,000 CD4$^+$ cells were collected and analyzed using the FlowJo software.

T cell proliferation assay. CD8$^+$ T cells were isolated from the lymph nodes of CL4-HA TCR transgenic mice. Cells were enriched using BD IMag CD8 T lymphocyte enrichment kit (BD) to a purity of 86–90%. Cells were then stained with 0.025 mM CFSE for 10 min at 37° C, washed, and cultured for 5 d with DCs from MCMV-infected mice at a ratio of 10:1. DCs were isolated from BALB.B6-CT6 and BALB.B6-CT8 mice. In brief, 0, 2, 4, and 6 d after infection, 3 spleens were harvested and pooled from mice infected with 5×10^3 PFU MCMV-K181-Perth-HA. Organs were processed and cells were purified over a Nycodenz gradient. DCs were isolated by negative selection using anti-CD3 (KT3-1.1), anti-Thy1 (T24/31.7), anti-Ly6G (1A8), anti-CD19 (ID3), and anti-erythrocyte (TER119). After co-culture, T cell proliferation was measured after staining with CD4, CD8, TCR, and PI, and assessing CFSE dilution.

Measuring virus in salivary glands after depletion of CD4$^+$ or CD8$^+$ T cells. Mice were infected by intraperitoneal administration of 10^4 PFU MCMV. CD4$^+$ T cells were depleted using 500 µg of the CD4-specific mAb GK1.5 per delivery. CD8$^+$ T cells were depleted using 250 µg of the CD8β specific mAb 53.5.8 per delivery. Mice were (a) depleted on days 9, 11, 16, and 20 pi and salivary glands were harvested on day 25; (b) depleted on days 16, 18, 23, and 27 pi and salivary glands harvested on day 32; or (c) depleted on days 24, 26, 31, and 35 pi and salivary glands harvested on day 40. Depletion was confirmed by flow cytometry. Salivary glands were harvested and homogenized, and virus titers were determined (Andrews et al., 2003).

Statistical analysis. Two-tailed Mann-Whitney tests were performed using InStat (GraphPad Software). All data are shown as mean ± SE.

Online supplemental material. Table S1 summarizes the CTL response in Ly49H$^-$ and Ly49H$^+$ mice after MCMV infection. Online supplemental material is available at http://www.jem.org/cgi/content/full/jem.20091193/DC1.

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