

Short Communication

Upregulation of CD94/NKG2A receptors and Qa-1^b ligand during murine cytomegalovirus infection of salivary glands

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Following acute infection, murine cytomegalovirus (MCMV) replicates persistently in the salivary glands, despite the vigorous response of activated CD8 T cells that infiltrate this gland. Virus-specific CD8 T lymphocytes isolated from this organ were found to express the inhibitory CD94/NKG2A receptor that, in some virus models, confers an inhibitory response to cytotoxic T lymphocytes (CTLs). In response to MCMV infection, expression of the CD94/NKG2A ligand, Qa-1^b, increased dramatically in the submandibular gland (SMG) prior to upregulation of H-2D^d. However, there was no net negative impact on virus-specific T-cell function, as virus titres were similar in CD94⁻ and CD94⁺ mice. CD94/NKG2A expression, also known to inhibit apoptosis, did not influence the numbers of accumulated T, NK and NK T cells. These data indicate that expression of inhibitory CD94/NKG2A receptors does not account for the failure of MCMV-specific CTLs to clear the SMG of infection.

Received 2 August 2006

Accepted 17 January 2007

Cytomegaloviruses (CMVs) are ubiquitous betaherpesviruses that persist in their host for life following acute infection. In the immunocompetent host, this lifelong infection is typically asymptomatic; however, virus is excreted in mucosal secretions intermittently and for extended periods of time in the absence of clinical disease. Murine CMV (MCMV) infection within the salivary gland serves as a model for prolonged mucosal infection and shedding. In MCMV-infected mice, virus replicates to high titres in the salivary gland long after it is cleared from systemic organs, including the spleen, lung, liver, kidney, adrenal glands and lymph nodes (Jonjic *et al.*, 1989). CD8 T cells are necessary and sufficient for clearing infectious virus from all target organs except the salivary gland (Jonjic *et al.*, 1989; Pavic *et al.*, 1993), where persistent virus replication is confined to the acinar epithelial cells (Jonjic *et al.*, 1989). The eventual elimination of MCMV from acinar epithelial cells requires CD4 T cells (Jonjic *et al.*, 1989), as well as the production of gamma interferon (IFN- γ) and tumour necrosis factor alpha (Pavic *et al.*, 1993).

A previous study from our laboratory demonstrated a vigorous and sustained immune response to MCMV in the submaxillary (submandibular) gland (SMG) (Cavanaugh *et al.*, 2003). At the peak of virus replication (14–21 days post-infection), infiltrating CD8 T cells predominated overwhelmingly. Notably, these CD8 T cells were highly functional, expressing IFN- γ and readily lysing virus-infected target cells *ex vivo*. Compared with the robust

number of CD8 T cells, other potential effector cells increased moderately (NK T), minimally (CD4⁺ T) or not at all (NK). It remains unclear how virus replication within the acinar epithelial cells resists the antiviral effects of the activated CD8 T cells. In support of the theory that infected SMG epithelial cells are relatively resistant to cytotoxic T-lymphocyte (CTL)-mediated lysis, the MCMV-encoded immunoevasion genes that inhibit CTL recognition of infected target cells (m04, m06 and m152) have minimal impact upon replication in the SMG (Lu *et al.*, 2006).

Further characterization of MCMV-specific CTLs isolated from infected SMGs revealed that 93% of tetramer-positive, IE1-specific T cells expressed the CD94/NKG2 family of NK-cell receptors on their surface (Fig. 1). The antibody used recognizes NKG2A, C and E. However, recent studies indicate that, of these three receptors, only the inhibitory CD94/NKG2A isoform is expressed on the surface of CD8 T cells (Gunturi *et al.*, 2004; Miller *et al.*, 2002). Commitment to CD94/NKG2A expression is a clonal attribute acquired following T-cell receptor (TCR) expression during development (Jabri *et al.*, 2002). Its surface expression depends on TCR engagement and occurs during the first encounter with antigen (Arlettaz *et al.*, 2004; Jabri *et al.*, 2002; McMahan *et al.*, 2002). Indeed, because surface expression of this receptor is induced upon infection with numerous pathogens (McMahan *et al.*, 2002; Miller *et al.*, 2002), it is regarded as a common marker for CD8 T-cell activation (Gunturi *et al.*, 2004). In

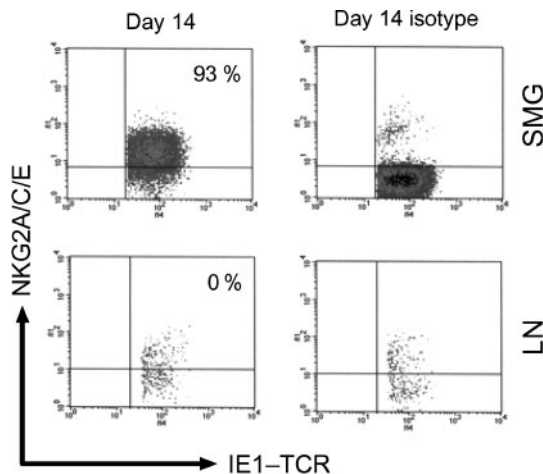


Fig. 1. Most MCMV-specific CD8 T cells in the SMG of infected mice express NKG2A/C/E. Leukocytes isolated from the SMG and draining lymph nodes (LN) of BALB/c mice on day 14 following MCMV infection were analysed by cytofluorometric analysis as described previously (Cavanaugh *et al.*, 2003), using antibodies to CD3 ϵ , CD8 α , NKG2A/C/E (rat clone 20d5; BD Biosciences) and a tetrameric complex of mouse H-2L^d and a nonapeptide from MCMV IE1 (pp89; m122/123). The data are represented as density plots of gated CD3⁺/CD8⁺/tetramer⁺ T cells expressing IE1-TCR (*x*-axis) and NKG2A/C/E (*y*-axis). An isotype-matched control for the NKG2A/C/E antibody is shown, and the percentages represent those of tetramer⁺ CTLs expressing NKG2A/C/E corrected for this non-specific staining.

fact, expression of NKG2A on MCMV-specific T cells has been used as a sensitive marker for antigen-experienced T cells (Gold *et al.*, 2004).

Although CD94/NKG2A is known as an inhibitory receptor, the effects of engagement of this receptor on CD8 T-cell effector function are variable, depending on the virus system. For example, upregulation of CD94/NKG2A on polyomavirus-specific CTLs results in downregulation of their antigen-specific cytotoxicity (Moser *et al.*, 2002). Neurons latently infected with herpes simplex virus are protected from cytotoxicity by CD94/NKG2A expressed on virus-specific memory CD8 T cells (Suvas *et al.*, 2006). However, expression of this receptor has no effect on the cytotoxic or cytokine-producing functions of lymphocytic choriomeningitis virus-specific CTLs (McMahon *et al.*, 2002; Miller *et al.*, 2002). More consistent data indicate that CD94/NKG2A expression reduces apoptotic cell death dramatically in CD8 T cells, thus permitting survival and contributing to clonal expansion and their memory pool *in vivo* (Gunturi *et al.*, 2004). Collectively, these data suggest that expression of CD94/NKG2A on CTLs within a specific tissue environment may provide a mechanism to regulate an antiviral immune response. This prompted us to determine the extent to which CD94/NKG2A expression contributes to the persistence of virus and/or T cells in MCMV-infected salivary glands.

The CD94/NKG2A receptors on MCMV-specific CTLs must engage the non-classical major histocompatibility complex (MHC) class Ib molecule Qa-1^b with its presented peptide (Qdm) if they are to deliver regulatory signals to these effector cells. Therefore, we assessed the relative expression of Qa-1^b, compared with classical MHC class I, in infected salivary-gland parenchymal tissue (Fig. 2a). Male BALB/c mice (Harlan Laboratories) were infected with MCMV as described previously (Cavanaugh *et al.*, 2003) and SMG tissues were harvested (days 0, 7 and 14 days post-infection) and crushed with a mortar and pestle under liquid nitrogen. Lysates were prepared and 20 μ g total protein was loaded per lane for Western blot analysis as described previously (Karabekian *et al.*, 2005) using anti-Qa-1^b (rabbit polyclonal; kindly provided by James Forman, University of Texas Southwestern Medical Center, Dallas, TX, USA) or anti-H-2D^d (mouse clone 34-2-12; BD Biosciences) antibodies. Although the 45 kDa Qa-1^b heavy chain was undetectable in the SMG of uninfected mice, its expression increased dramatically by day 7 after infection and then decreased precipitously by day 14. In contrast, H-2D^d heavy chain within the SMG was below the level of detection until day 14 post-infection. Both of these heavy chains were also expressed in mononuclear cells within lymphoid tissues (Fig. 2b), including CD8 T cells, NK cells and NK T cells from MCMV-infected mice (data not shown). Because the number of mononuclear cells infiltrating the gland is in a vast minority compared with the parenchymal cells, expression of Qa-1^b and H-2D^d heavy chains in Fig. 2(a) is probably representative of salivary-gland parenchymal cells. We conclude that, at early times post-infection of the SMG (7 days), there was a strong potential for engagement of inhibitory CD94/NKG2A receptors on virus-specific T cells.

Upregulation of Qa-1^b in MCMV-infected salivary-gland tissue was quite striking. However, these experiments did not address the question of whether virus-infected epithelial cells, the targets of the CTLs, upregulated Qa-1^b or whether non-infected cells exposed to IFN- γ within the infected SMG accounted for the enhanced levels. In order to prove that isolated SMG epithelial cells are capable of expressing Qa-1^b upon infection, we used flow cytometry to quantify this ligand on the surface of a clonal population of BALB/c salivary-gland epithelial cells in culture (SGC1; kindly provided by Marie Piechocki, Wayne State University, Detroit, MI, USA). These glandular epithelial cells are highly permissive for MCMV replication, with titres as high as or exceeding those typically produced in fibroblasts (data not shown). BALB/c fibroblasts or salivary-gland epithelial cells were seeded with or without 50 U IFN- γ ml⁻¹ for 48 h. Half of the cultures receiving IFN- γ were infected with MCMV (m.o.i. of 5) for the last 24 h. The data shown in Fig. 2(c) demonstrate that the epithelial cells, but not fibroblasts, express low but somewhat enhanced levels of surface Qa-1^b in response to MCMV infection alone, and upregulated levels in response to infection and IFN- γ treatment, which mimics the SMG

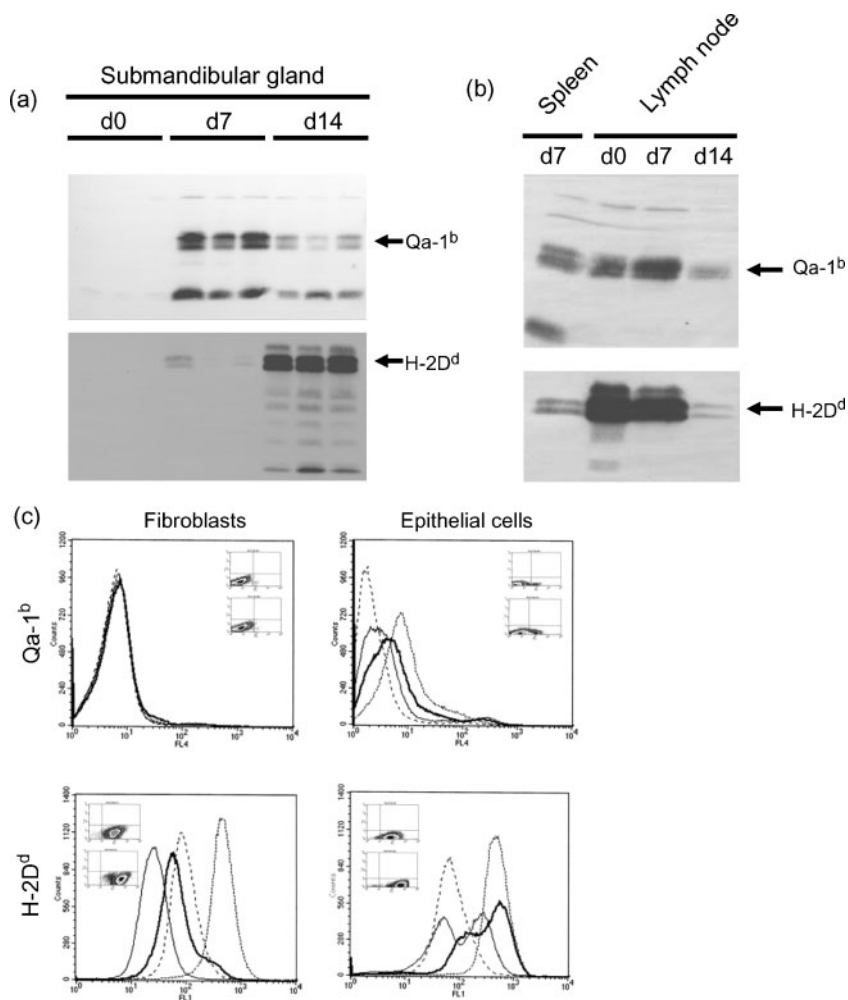


Fig. 2. Expression of Qa-1^b is increased prior to the upregulation of MHC class I in the SMG following MCMV infection. Lysates were prepared from the SMG (a) and spleen or draining cervical and periglandular lymph nodes (b) of MCMV-infected BALB/c mice on days 0 (uninfected), 7 and 14 after infection, and 20 μ g total protein was loaded per lane for Western blot analysis using antibodies to Qa-1^b or H-2D^d. The heavy chains of each molecule are indicated by arrows. (a) Lysates were prepared from the SMGs of three individual animals at each time point; (b) the draining lymph nodes of three mice were pooled at each time point and a single lysate was prepared. (c) Expression of Qa-1^b and MHC class I was analysed on the surface of mKSA fibroblasts and salivary-gland epithelial cells (SGC1 cell line) by flow cytometry using anti-Qa-1^b (mouse clone 6A8.6F10.1A6; BD Biosciences) or anti-H-2D^d (mouse clone 34-2-12; BD Biosciences) antibodies. Cells were mock-infected (long dashed line), treated with recombinant murine IFN- γ for 24 h (50 U ml⁻¹, short dashed line), infected with MCMV alone (5 p.f.u. per cell, thin solid line) or infected with MCMV (5 p.f.u. per cell) and treated with IFN- γ for 24 h (bold solid line). Within each histogram, the upper dot-plot represents mock-infected cells and the lower plot represents cells treated with IFN- γ .

environment during a natural infection (Cavanaugh *et al.*, 2003).

The fact that Qa-1^b levels were lower on infected, IFN- γ -treated cells than on those subjected to IFN- γ treatment alone could be explained by an MCMV-induced down-regulation of MHC class I, the source of the Qdm peptide, which is the ligand for Qa-1^b. Therefore, we also assessed surface expression of MHC class I (H-2D^d) on the same cell populations. As expected, infected fibroblasts downregulated class I molecules significantly in either the presence or absence of IFN- γ (Fig. 2c). Epithelial cells upregulated MHC class I molecules in response to IFN- γ , and MCMV infection reduced these levels on only a subpopulation of the cells. Interestingly, MCMV infection in the absence of IFN- γ treatment upregulated class I molecules on a significant portion of cells. In summary, Qa-1^b expression increased on the surface of MCMV-infected and IFN- γ -treated epithelial cells in a manner that reflected the relative levels of MHC class I molecules. This is in contrast to fibroblasts, which did not upregulate Qa-1^b despite elevated MHC class I in response to IFN- γ .

To determine whether the CD94/NKG2A receptor expressed on the virus-specific CTLs has a negative impact on the immune response, and thereby contributes to MCMV persistence in salivary glands, virus titres in the SMG were quantified in mice devoid of this receptor, compared with those expressing this dimer. The DBA/2 strain of mice from Jackson Laboratories, but not from several other commercial vendors, does not express the CD94 gene naturally and is therefore devoid of cell-surface CD94/NKG2A receptors (Vance *et al.*, 2002). This phenotype is associated with a functional defect in the ability of NK cells to lyse Qdm/Qa-1^b-expressing target cells *in vivo* (Jia *et al.*, 2000). Accordingly, male DBA/2 mice from Jackson Laboratories (CD94⁻) and from Charles River Laboratories (CD94⁺) were infected with MCMV and virus titres were quantified in the SMG on days 4, 7, 14, 21 and 28 after infection as described previously (Cavanaugh *et al.*, 2003). The presence of CD94/NKG2A receptors in Charles River mice and their absence in Jackson mice was verified in three representative animals of each strain by cytofluorometric analysis of whole-blood leukocytes (Vance *et al.*, 2002) (Fig. 3a). MCMV titres differed

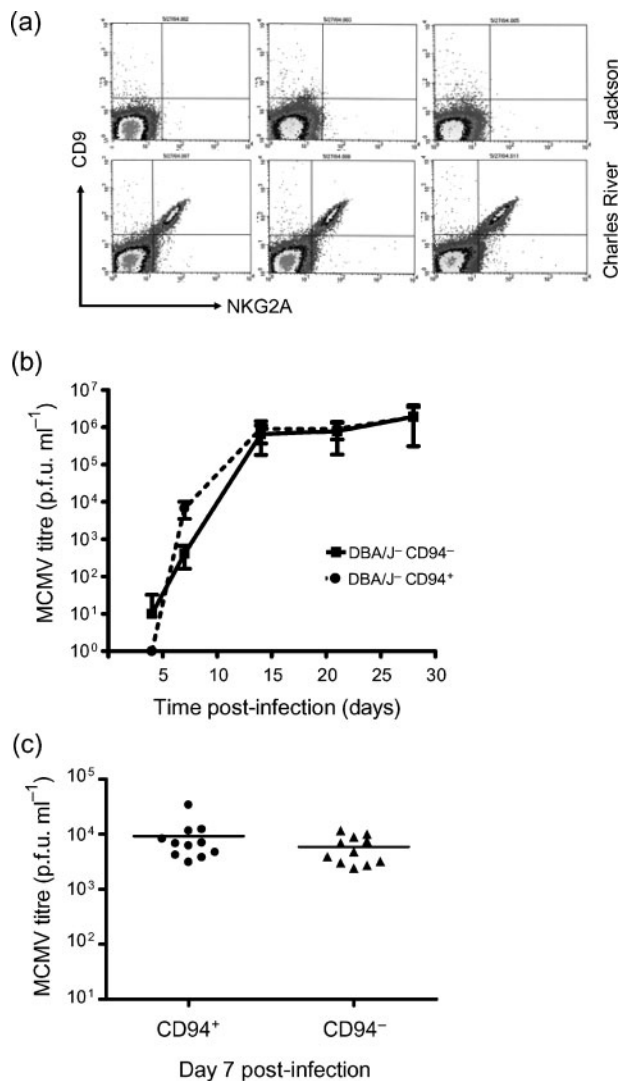


Fig. 3. MCMV titres are not significantly different in the SMGs of infected CD94⁻ and CD94⁺ mice. (a, b) DBA/2 mice from Jackson Laboratories (CD94⁻) and from Charles River Laboratories (CD94⁺) were infected with MCMV and their SMGs and whole blood were harvested on days 4, 7, 14, 21 and 28 after infection. (a) Blood leukocytes collected from three representative animals from each strain were analysed for surface expression of CD3 ϵ , DX5 (a pan-NK marker), CD94 (rat clone 18d3; BD Biosciences) and NKG2A/C/E by cytofluorometry on day 7 after infection as described previously (Vance *et al.*, 2002). The data are represented as density plots of gated CD3⁺/DX5⁺ cells expressing the CD94/NKG2A heterodimer. (b) Infectious MCMV was quantified in the SMG by preparing tissue homogenates (20%, w/v) and titrating them by standard plaque assay as described previously (Cavanaugh *et al.*, 2003) for five mice at each time point. The results are presented as log₁₀ MCMV (ml tissue homogenate)⁻¹; standard error bars are shown. (c) MCMV titres were quantified in the SMGs of Jackson mice with an introduced CD94 transgene (●) and in their CD94⁻ littermates (▲) on day 7 after infection. The results are represented as log₁₀ MCMV (ml SMG homogenate)⁻¹ in a scatter plot of 11 animals in each group. The means of each group are shown as a horizontal line.

between the two strains only on day 7 following infection, where the titres of CD94⁻ Jackson mice were approximately 1 log₁₀ lower than those of the CD94⁺ Charles River mice (Fig. 3b). By day 14, virus titres in both strains were nearly identical and remained similarly elevated through day 28 post-infection. From these data, it appears doubtful that the absence of the CD94/NKG2A receptor played a significant role in MCMV persistence in the SMG.

The 1 log₁₀ difference in virus titre on day 7 post-infection could be due to unknown variations in the backgrounds of the Jackson and Charles River mice, in addition to the CD94 genotype difference. Therefore, to determine specifically the significance of this difference, a similar experiment was carried out in a transgenic mouse lineage created from DBA/2 Jackson mice (Vance *et al.*, 2002). In this experiment, MCMV titres in Jackson mice were compared with those in their transgenic littermates with an introduced CD94 gene. As shown in Fig. 3(a), the presence or absence of surface CD94/NKG2A receptors was confirmed in each animal by flow-cytometric analysis of whole blood (Vance *et al.*, 2002) (data not shown). CD94⁻ and CD94⁺ littermates were infected with MCMV, and virus titres in the SMG were compared on day 7. Titres were nearly identical between the two groups of mice, at approximately 1 × 10⁴ p.f.u. (ml tissue homogenate)⁻¹ (Fig. 3c). These data indicate that the absence or presence of the CD94/NKG2A receptor has no consequence on MCMV replication within salivary glands, even at this early time post-infection.

Another function ascribed to the CD94/NKG2A receptor is that of inhibiting apoptosis and enhancing the survival of immune cells (Gunturi *et al.*, 2003, 2004), which could explain the plethora of CTLs within the infected SMG during MCMV infection. To examine the role of CD94/NKG2A expression in cell survival, the accumulations of CD8 T cells, NK cells and NK T cells were compared in CD94⁻ and CD94⁺ mouse strains at the peak of MCMV infection in salivary glands. Leukocytes were isolated from the SMGs of DBA/2 Jackson mice (CD94⁻) and DBA/2 Harlan mice (CD94⁺) and were analysed by flow cytometry. The relative proportions of CD8 : CD4 T cells before infection or on day 14 after infection were not significantly different between the two mouse strains. In Jackson mice, 51 % of gated CD3⁺ T cells were CD8⁺ and 44 % were CD4⁺, and in Harlan mice, 57 % were CD8⁺ and 38 % were CD4⁺. Both strains also displayed similar proportions of NK- and NK T-cell populations following MCMV infection, where the SMGs of Jackson mice contained 5 % NK cells and 19 % NK T cells, and those of Harlan mice contained 8 % NK cells and 18 % NK T cells. Importantly, significant proportions of these cell populations in the Harlan strain expressed cell-surface CD94/NKG2A receptors (78 % of CD8 T cells, 41 % of NK cells and 54 % of NK T cells), whereas none of them did in the Jackson mice, as expected. These results indicate that expression of the CD94/NKG2A receptor has no effect on the accumulation of these cell populations and is therefore

not responsible for the abundance of immune cells within the infected SMG.

Our data demonstrate that expression of CD94/NKG2A does not contribute significantly to MCMV replication or persistence *in vivo*, despite the potential for this inhibitory receptor to impact negatively upon CTL effector function in the environment of the SMG in MCMV-infected mice. Inhibitory CD94/NKG2A receptors are expressed on the vast majority of at least IE1-specific CD8 T cells in the SMG during an acute primary immune response, and expression of its ligand, Qa-1^b, is increased dramatically 1 week prior to the upregulation of activating H-2D^d molecules. However, at the peak of Qa-1^b expression, MCMV titres were not significantly different in the tissues of mice genetically devoid of CD94/NKG2A compared with those expressing this surface molecule. In addition, this receptor did not influence the accumulation of immune-cell populations within the SMG during MCMV infection.

For interpretation of those experiments comparing virus titres in CD94⁺ versus CD94⁻ mice, it is important to consider that NKG2C also dimerizes with CD94 and, as such, recognizes Qa-1^b as an NK-activating receptor. However, in murine NK cells, NKG2C is expressed minimally. Ninety-five per cent of NKG2 mRNA in mouse NK cells is NKG2A and, functionally, Qa-1^b delivers a net inhibitory signal (Vance *et al.*, 1999). Therefore, virus titres in CD94⁻ mice compared with those in their CD94⁺ littermates reflected the degree of immunoregulation by NKG2A selectively.

Thus, control of persistent MCMV replication and sequestration of immune effector cells in the SMG are regulated by mechanisms refractory to the influence of inhibitory CD94/NKG2A receptors. As reported for other antiviral T cells (Moser *et al.*, 2002), at the cellular level, individual CTLs may receive an inhibitory signal by engaging NKG2A and fail to deliver a lethal hit to infected epithelial cells. However, this is the first report to demonstrate that this mechanism does not account for the failure of virus-specific CTLs as a population to clear a persistent virus infection. This may be due to differences in expression of the ligand and receptor molecules on individual target and effector cells, to the presence of compensatory mechanisms that operate in the absence of NKG2A to retain inhibition of cytolysis or to viral immune-evasion strategies that prevent CTL lysis in the absence of NKG2A. In conclusion, NKG2A receptors on virus-specific CTLs within MCMV-infected salivary-gland tissue do not in themselves deliver a net inhibitory signal and, therefore, other regulatory molecules prevent CTL-mediated lysis of infected target cells.

Acknowledgements

We thank Ellen Jing and Yuping Deng of the Glennan Center for Geriatrics and Gerontology Flow Cytometry Laboratory, Eastern Virginia Medical School, for excellent technical assistance. We appreciate the expertise of Sylvia Singletary and Suzanne Hahto of the Division of Comparative Medicine, Eastern Virginia Medical School, for the care of and blood collection from CD94 transgenic

mice. We also acknowledge Julie Kerry of the Department of Microbiology and Molecular Cell Biology, Eastern Virginia Medical School, for technical advice and computer programming assistance. We are grateful to the NIAID Tetramer Facility and the NIH AIDS Research and Reference Reagent Program (Emory University Vaccine Center, Yerkes Regional Primate Research Center, Atlanta, GA, USA) for production of the tetramer used in this study. This work was supported by the Department of Health and Human Services through the National Institute of Allergy and Infectious Disease grants R03 AI45084 to A. E. C. and R01 AI35021 to D. H. R., and partially through the National Cancer Institute grant R01 CA41451 to A. E. C.

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