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 excretion of infectious virus from all target organs except the salivary gland (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/NKG2A 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; McMahon et al., 2002). Indeed, because surface expression of this receptor is induced upon infection with numerous pathogens (McMahon et al., 2002; Miller et al., 2002), it is regarded as a common marker for CD8 T-cell activation (Gunturi et al., 2004).
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 receptors on virus-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-1b, with its presented peptide (Qdm) if they are to deliver regulatory signals to these effector cells. Therefore, we assessed the relative expression of Qa-1b, 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 μg total protein was loaded per lane for Western blot analysis as described previously (Karabekian et al., 2005) using anti-Qa-1b (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-1b 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-1b 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-1b 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-1b 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-1b 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-1b in response to MCMV infection alone, and upregulated levels in response to infection and IFN-γ treatment, which mimics the SMG...
environment during a natural infection (Cavanaugh et al., 2003).

The fact that Qa-1\(^{b}\) levels were lower on infected, IFN-\(\gamma\)-treated cells than on those subjected to IFN-\(\gamma\) treatment alone could be explained by an MCMV-induced downregulation 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-\(\gamma\) (Fig. 2c). Epithelial cells upregulated MHC class I molecules in response to IFN-\(\gamma\), and MCMV infection reduced these levels on only a subpopulation of the cells. Interestingly, MCMV infection in the absence of IFN-\(\gamma\) 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-\(\gamma\)-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-\(\gamma\).

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
between the two strains only on day 7 following infection, where the titres of CD94\(^{-}\) Jackson mice were approximately 1 log\(_{10}\) 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 log\(_{10}\) 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\(\times\)10\(^4\) 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 CD4\(^{+}\) 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

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**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\(_{a}\), 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 log\(_{10}\) 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 log\(_{10}\) 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.
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 H-2Dd-specific CD8+ T cells in the SMG during an acute primary immune response, and expression of its ligand, Qa-1b, is increased dramatically 1 week prior to the upregulation of activating H-2Dd molecules. However, at the peak of Qa-1a expression, MCMV titres in CD94+ versus CD94− mice, it is important to consider that NKG2C also dimerizes with CD94 and, as such, recognizes Qa-1b 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-1b 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.

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