CD94-NKG2A receptors regulate antiviral CD8+ T cell responses

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CD8+ T lymphocytes mediate immunosurveillance against persistent virus infections and virus-induced neoplasia. Polyoma virus, a highly oncogenic natural mouse DNA virus, establishes persistent infection, but only a few mice are highly susceptible to tumors induced by the virus. Mature antiviral CD8+ T cells expand in tumor-susceptible mice, but their cytotoxic effector activity is nonfunctional *in vivo*. Here we show that the natural killer cell inhibitory receptor, CD94-NKG2A, is up-regulated by antiviral CD8+ T cells during acute polyoma infection and is responsible for down-regulating their antigen-specific cytotoxicity during both viral clearance and virus-induced oncogenesis.

Systemic viral infections drive the marked expansion and differentiation of antigen-specific CD8+ T cells, which have many antiviral cytotoxic and cytokine effector activities. With the eradication of infected cells, the population of antiviral CD8+ T cells contracts with a concomitant loss in antigen-specific cytotoxic activity; however, the rate of decline in *ex vivo* cytotoxic activity of antiviral CD8+ T cells on a per cell basis during viral clearance often exceeds the rate of reduction in cell number[1–3]. The dissociation between this effector activity and cell number points toward an important *in vivo* mechanism that mitigates potential bystander cytopathology as antiviral CD8+ T cells differentiate from effector to memory states. The premature activation of such an inhibitory process in virus-specific CD8+ T cells might lead to virus-induced cytopathology and the establishment of persistent viral infection.

The extent of T cell activation depends on the integration of positive signaling events initiated by the T cell receptor (TCR) and negative signals mediated by other T cell surface proteins[4]. Discrete subpopulations of antiviral CD8+ αβ T cells can express natural killer (NK) cell markers and receptors that impair the cytotoxic and cytokine activities of NK cells[5]. Inhibitory NK cell receptors (NKRs) fall into two main families of molecules: type I transmembrane proteins that belong to the immunoglobulin (Ig) superfamily, such as the human killer cell Ig-like receptors (KIRs) and leukocyte Ig-like receptors (LIRs); and type II transmembrane proteins that contain a C-type lectin domain, such as Ly49 homodimers in mice and CD94-NKG2A heterodimers in both mice and humans[6].

Similar to TCRs, these inhibitory NKRs have major histocompatibility complex (MHC) class I molecules as ligands[4]. The Ig-like NKRs and Ly49 family interact with particular allelic variants of classical MHC class I molecules. In contrast, the ligand for the CD94-NKG2A receptor is the nonclassical MHC class I molecule HLA-E, or its murine ortholog Qa-1^b, complexed to a nonapeptide, Qdm, which is derived from the leader sequence of particular classical MHC class I heavy chains[7,8]. Inhibitory NKRs possess intracytoplasmic immunoreceptor tyrosine inhibition motifs that, on tyrosine phosphorylation, recruit Src homology domain 2 (SH2)-containing protein tyrosine phosphatases, which are thought to dephosphorylate the immunoreceptor tyrosine activation motifs of NK cell–activating receptors[9,10].

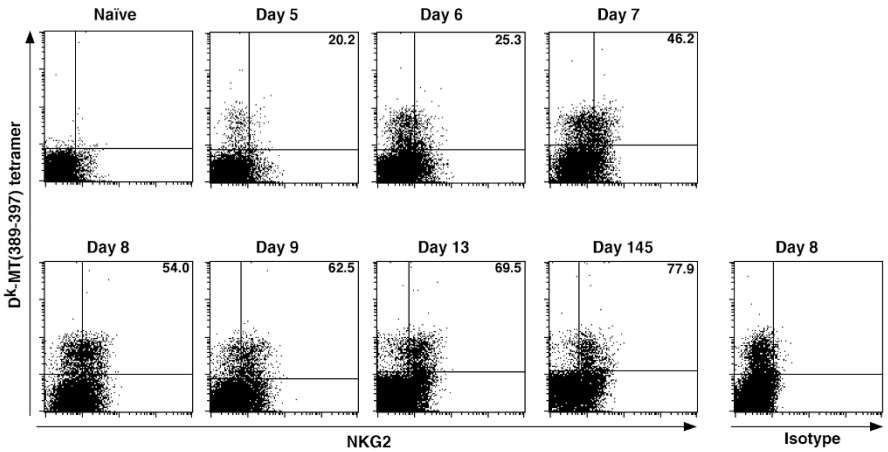

Figure 1. Kinetic analysis of CD94-NKG2 receptor expression by MT(389–397)-specific CD8+ T cells. Spleen cells from naïve and polyoma-infected adult C3H/HeN mice on each of the indicated days after infection were stained with the D^k-MT(389–397) tetramer and CD8α and NKG2 mAbs, and analyzed by flow cytometry. Plots are gated on CD8+ T cells, and values shown are the percentage of MT(389–397)-specific T cells that express NKG2 in the indicated quadrant. A rat IgG2a mAb was used as an isotype control for the NKG2 mAb. Results shown are representative of three experiments.

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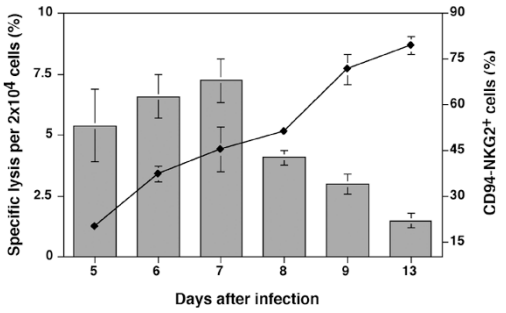

Figure 2. Comparison of *ex vivo* MT(389–397)-specific cytotoxicity with CD94-NKG2A expression during infection with polyoma virus. On the indicated day after infection, spleen cells from adult-inoculated C3H/HeN mice were analyzed for their lysis of unpulsed and MT(389–397)-pulsed syngeneic target cells. The percentage of specific lysis was normalized to 2 10^4 MT(389–397)-specific $CD8^+$ T cells, as determined by staining with the D^k-MT(389–397) tetramer, for each of the indicated days after infection, and is shown on the left *y* axis (bar graph). The percentage of D^k-MT(389–397) $tetramer^+$ $CD8^+$ T cells that express CD94-NKG2A, as determined by costaining with Qa-1^b–Qdm tetramer, is shown on the right *y* axis (line graph). Each value represents the mean±s.e.m. percentage of specific lysis or the mean±s.e.m. percentage of MT(389–397) $tetramer^+$ $CD8^+$ T cells that express CD94-NKG2A in the spleens of three mice. No lysis of unpulsed target cells by spleen cells from mice at any timepoint after infection was observed (data not shown). Results shown are representative of two experiments.

Similar mechanisms might account for how inhibitory NKRs finely regulate the thresholds for TCR activation[11].

Mounting evidence suggests that inhibitory NKRs regulate $CD8^+$ T cell functionality. Because $CD8^+$ T cells that express inhibitory KIR isoforms are oligoclonal populations found in the peripheral blood of normal individuals and exhibit a memory T cell phenotype, they are probably a result of antigen-specific expansion *in vivo*[12]. In HIV-infected individuals KIR^+CD8^+ T cells are detected at high frequencies, and *in vitro*–expanded HIV-specific $CD8^+$ T cells exhibit KIR-dependent inhibition of antigen-specific effector activities[13]. Diminished $CD8^+$ T cell–mediated antiviral and anti-tumor responses are seen in Ly49A-transgenic mice[14,15]; however, low expression of endogenous Ly49A only inhibits early events of TCR activation[16]. $CD8^+$ T cells that infiltrate primary melanomas and metastatic lymph nodes express predominantly CD94-NKG2A[17]. An *in vitro*–expanded CD94-NKG2A$^+$ subpopulation of melanoma-specific $CD8^+$ T cells exhibits only weak cytotoxic activity that is amplified by blockade with CD94 monoclonal antibodies (mAbs)[18]. Taken together, these observations raise the possibility that $CD8^+$ T cells that are chronically exposed to cognate antigen—derived from either tumors or persistent viral infections—are not anergic, but are functionally inhibited by NKR–MHC class I molecule interactions. However, there is no direct evidence that inhibitory receptors regulate antigen-specific $CD8^+$ T cell function *in vivo*.

Infection by the highly oncogenic mouse polyoma virus induces a marked expansion of $CD8^+$ T cells directed to an epitope derived from the viral middle T (MT) oncoprotein[2]. In mice that are susceptible to tumors induced by polyoma virus, this MT-specific $CD8^+$ T cell response lacks antigen-specific cytotoxic effector activity; in contrast, tumor-resistant mice generate potent cytotoxicity against this epitope[19]. Because these dysfunctional $CD8^+$ T cells are indistinguishable from antipolyoma cytotoxic lymphocyte (CTL) effectors in terms of intracellular concentrations of perforin and expression of T cell effector surface markers, and because they rapidly acquire antigen-specific cytotoxicity after *in vitro* restimulation, we proposed previously that their cytotoxic effector activity was restrained *in vivo*[19]. Here we show that the CD94-NKG2A receptor is induced in polyoma virus–specific $CD8^+$ T cells during acute infection and is responsible for blocking their cytotoxic effector activity in mice susceptible to tumors induced by polyoma virus.

Results

Expression of CD94-NKG2 by antipolyoma $CD8^+$ T cells

By day 6–9 of primary polyoma virus infection, adult C3H/HeN mice generate potent *ex vivo* cytotoxicity to the dominant H-$2D^k$–restricted polyoma virus epitope, amino acids 389–397 of the MT oncoprotein (MT(389–397)); this cytotoxicity coincides with a peak expansion of D^k-MT(389–397) $tetramer^+$ $CD8^+$ T cells[2]. With clearance of the infectious virus by day 13 of infection, MT(389–397)-specific *ex vivo* cytotoxicity falls sharply despite the presence of large numbers of antigen-specific $CD8^+$ T cells[2]. Thus, we wanted to determine whether this apparent shut-off in the antiviral cytotoxic effector function of $CD8^+$ T cells correlates with up-regulated expression of an inhibitory NKR during acute infection with polyoma virus.

At each timepoint after infection, less than 10% of D^k-MT(389–397) $tetramer^+$ $CD8^+$ T cells were stained by mAbs to either D^k-reactive Ly49 inhibitory molecules (Ly49A, Ly49C or Ly49I) or the DX5 NK cell surface marker (data not shown). In contrast, the proportion of MT(389–397)-specific $CD8^+$ T cells that stained with either a mAb specific for the mouse NKG2A, NKG2C and NKG2E isoforms (**Fig. 1**) or a Qa-1^b–Qdm tetramer (which binds specifically to heterodimers of CD94 and NKG2A, NKG2C or NKG2E[20], data not shown) increased progressively through acute infection. By day 13, nearly 70% of the D^k-MT(389–397) $tetramer^+$ $CD8^+$ T cells expressed a CD94-NKG2 receptor. Of the three CD94-NKG2 receptors, only the CD94-NKG2A is inhibitory.

Few $CD8^+$ T cells from naïve adult C3H/HeN mice expressed any of these NKRs (**Fig. 1** and data not shown). Most of the memory MT(389–397)-specific $CD8^+$ T cells expressed CD94-NKG2 with a mean fluorescence intensity equivalent to that expressed by $NKG2^+$ D^k-MT(389–397) $tetramer^+$ $CD8^+$ T cells in acutely infected mice (**Fig. 1** and data not shown); thus, once expressed, this inhibitory receptor was maintained stably by these antiviral $CD8^+$ T cells. The NKG2 mAb also stained a population of D^k-MT(389–397) $tetramer^-$ $CD8^+$ T cells, whose frequency increased coordinately with that of MT(389–397)-specific $CD8^+$ T cells during the course of acute infection. These D^k-MT(389–397) $tetramer^-$ $CD8^+$ T cells might recognize subdominant polyoma epitopes and/or arise through bystander activation, an effect that is thought to account for only a minor component of the overall virus-driven expansion of $CD8^+$ T cells[3]. As seen typically for antigen-specific

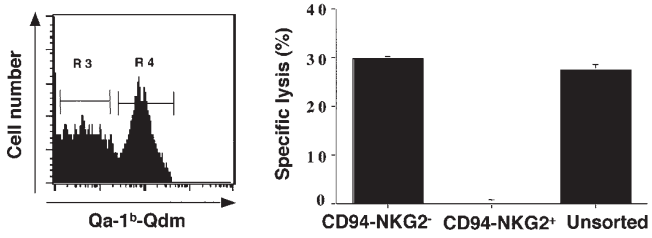

Figure 3. *Ex vivo* MT(389–397)-specific cytotoxicity is inversely correlated with expression of CD94-NKG2A. On day 8 after infection, spleen cells from three adult C3H/HeN mice were stained with the D^k-MT(389–397) tetramer, the Qa-1^b–Qdm tetramer or a CD8α mAb, and then MT(389–397)-specific $CD8^+$ T cells were sorted by FACS into Qa-1^b–Qdm $tetramer^+$ (region 4) and Qa-1^b–Qdm $tetramer^-$ (region 3) subpopulations. Pooled sorted (effector:target of 4:1) and unsorted (effector:target of 100:1) spleen cells were analyzed for their lysis of unpulsed and MT(389–397)-pulsed syngeneic target cells. Each value represents the mean±s.e.m. percentage of specific lysis. No lysis of unlabeled target cells was detected (data not shown). Results shown are representative of two experiments.

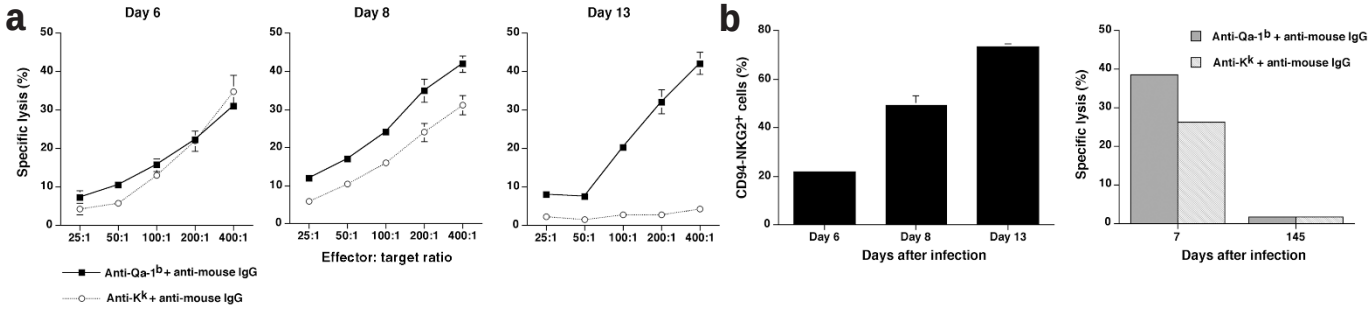

Figure 4. Blockade with Qa-1^b mAb restores *ex vivo* MT(389–397)-specific cytotoxicity in acutely infected but not immune mice. On the indicated day after infection, spleen cells from adult C3H/HeN mice were analyzed for their lysis of MT(389–397)-pulsed target cells pretreated with either Qa-1^b mAb plus anti-mouse secondary antibody or K^k antibody plus anti-mouse secondary antibody and brefeldin A. (**a**) The mean±s.e.m. percentage of specific lysis is shown against MT(389–397)-pulsed target cells pretreated with Qa-1^b mAb plus anti-mouse secondary antibody or K^k antibody plus anti-mouse secondary antibody. No lysis of similarly treated, unpulsed target cells was detected (data not shown). (**b**) The mean±s.e.m. percentage of D^k-MT(389–397) tetramer$^+$ CD8$^+$ T cells expressing CD94-NKG2A is shown for each of the indicated days after infection. Results shown are representative of two experiments. (**c**) The experiment in **a** was repeated with adult immune C3H/HeN mice at day 145 after infection, with adult C3H/HeN mice at day 7 after infection included as a control. No lysis of similarly treated, unpulsed target cells was detected (data not shown). Results shown are representative of two experiments.

CD8$^+$ T cells that express this and other NKR types[5], all of the NKG2$^+$CD8$^+$ T cells that expanded during polyoma infection bore the CD11a^{hi}CD44^{hi} surface phenotype of antigen-experienced T cells (data not shown).

There was a strong association between the extinction of antigen-specific cytotoxicity and expression of the CD94-NKG2 receptor during acute polyoma virus infection (**Fig. 2**). We normalized the MT(389–397)-specific lytic activity at each timepoint after infection to the number of D^k-MT(389–397) tetramer$^+$ CD8$^+$ T cells, and compared it with the number of cells that costained with Qa-1^b–Qdm tetramers. During days 6–9 of acute infection, when *ex vivo* MT(389–397)-specific cytotoxicity is maximal on the basis of the numbers of splenic mononuclear cells, the cytotoxic activity of MT(389–397)-specific CD8$^+$ T cells plummets as virus is cleared[2]. The finding that expression of the CD94-NKG2 increased rapidly during this period implicates the CD94-NKG2A inhibitory receptor in restraining the cytopathic effector function of MT(389–397)-specific CD8$^+$ T cells. Antiviral CD8$^+$ T cells undergo a marked increase in functional avidity early in virus infection[21], which raises the possibility that virus-specific CD8$^+$ T cells may become progressively less sensitive to CD94-NKG2A–mediated inhibition over the course of acute infection; this might necessitate increased expression of this receptor late in infection to achieve functional inhibition.

Regulation of antipolyoma cytotoxicity by CD94-NKG2A

To determine directly whether the CD94-NKG2A receptor impaired the cytotoxicity of MT(389–397)-specific CD8$^+$ T cells, we sorted D^k-MT(389–397) tetramer$^+$ CD8$^+$ T cells from day 8 infected mice into discrete subpopulations on the basis of CD94-NKG2 expression. We then analyzed them for their recognition of target cells pulsed with MT(389–397). The antigen-specific cytotoxic activity of MT(389–397)-specific CD8$^+$ T cells matched their pattern of expression of the CD94-NKG2A receptor, with *ex vivo* antigen-specific killing only manifested by cells lacking surface CD94-NKG2A (**Fig. 3**).

We next determined whether interrupting the interaction between CD94-NKG2A and Qa-1^b–Qdm could restore *ex vivo* polyoma-specific cytotoxic activity from acutely infected mice. Target cells pulsed with MT(389–397) were pretreated with mouse Qa-1^b mAb and anti-mouse secondary antibody to provide maximal blocking and/or promote the internalization of surface Qa-1^b molecules. We included brefeldin A to prevent re-expression of Qa-1^b; brefeldin A also blocks *de novo* surface expression of other molecules that are potentially up-regulated by cross-linking Qa-1^b (ref. 21). Compared with the progressive loss of MT(389–397)-specific killing of target cells pretreated with a control isotype-matched primary anti-K^k mAb (**Fig. 4a**), Qa-1^b blockade restored MT(389–397)-specific cytotoxic activity. The capacity of Qa-1^b blockade to restore this effector activity paralleled the proportion of MT(389–397)-specific CD8$^+$ T cells that expressed CD94-NKG2A at different stages of acute infection (**Fig. 4b**). The 400:1 spleen cell to target cell ratio used for each of the three timepoints after infection corresponded to MT(389–397)-specific CD8$^+$ T cell to target cell ratios of 3:1, 5:1 and 3:1, respectively. Qa-1^b blockade failed to reveal MT(389–397)-specific *ex vivo* cytotoxicity in mice that are immune to polyoma virus (**Fig. 4c**), although the MT(389–397)-specific CD8$^+$ T cell to target cell ratio was 3:1. This

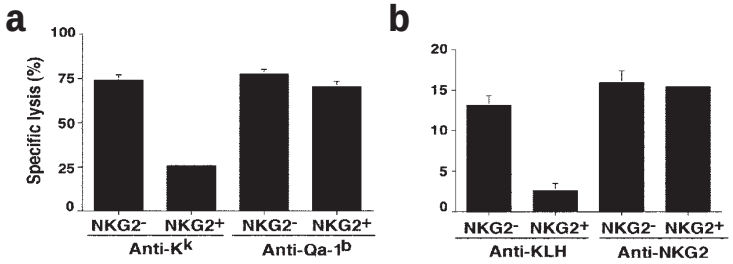

Figure 5. CD94-NKG2A–mediated inhibition of MT(389–397)-specific CD8$^+$ T cell cytotoxic effector activity. FACS-sorted NKG2$^+$ and NKG2$^-$ subpopulations of D^k-MT(389–397) tetramer$^+$ CD8$^+$ T cells were pooled from the spleen cells of four adult C3H/HeN mice on day 8 after infection. (**a**) Each subpopulation was analyzed at an effector:target ratio of 4:1 for their lysis of MT(389–397)-pulsed target cells pretreated with either Qa-1^b or K^k mAb, followed by treatment with an anti-mouse secondary antibody. Analysis after sorting showed that there was approximately 15% contamination of the NKG2$^+$ population by the NKG2$^-$ population. (**b**) Each subpopulation was analyzed at an effector:target ratio of 0.5:1 for their lysis of MT(389–397)-pulsed target cells in the presence of either NKG2 or isotype-matched keyhole limpet hemocyanin (KLH) mAb. Analysis after sorting showed that there was approximately 10% contamination of the NKG2$^+$ population by the NKG2$^-$ population. Data represent the mean±s.e.m. percentage of specific lysis of triplicate wells. No lysis of unpulsed target cells was detected (data not shown).

Table 1. MT(389–397)-specific CD8⁺T cell responses in immune resistant and susceptible mice infected by recombinant vaccinia viruses

	C3H/HeN			CBA/J		
	Immune	Vac.MT(389–397)	Vac.GP99	Immune	Vac.MT(389–397)	Vac.GP99
Total CD8⁺ T cells (10^6)	7.27±0.37	14.27±1.2	20.4±2.2	8.2±1.2	11.1±0.22	19.1±6.9
Total D^k-MT(389–397) tetramer⁺ cells (10^5)	1.35±0.15	7.17±0.5	1.76±0.65	1.32±0.3	0.69±0.07	1.8±0.8

Spleen cells from newborn-inoculated polyoma-infected immune mice, either unchallenged or 5 days after challenge with vaccinia virus, were stained with PE-conjugated anti-CD8α and D^k-MT(389–397) tetramer and analyzed by flow cytometry. Data are the mean±s.e.m. of two separate experiments.

finding suggests that these immediate post-effector CD8⁺ T cells and memory CD8⁺ T cells represent functionally different stages of antigen-specific T cell differentiation.

We tested whether Qa-1^b blockade could reveal *ex vivo* antigen-specific cytotoxicity by NKG2⁺ D^k-MT(389–397) tetramer⁺ CD8⁺ T cells sorted from the spleens of acutely infected adult mice. Target cells pulsed with MT(389–397) and pretreated with Qa-1^b mAb but not K^k mAb markedly boosted target cell lysis by the NKG2⁺ population (**Fig. 5a**). D^k-MT(389–397) tetramer⁺ CD8⁺ T cells that lacked surface NKG2 expression exhibited strong *ex vivo* cytotoxicity regardless of Qa-1^b blockade. In addition, mAb-mediated NKG2 blockade restored full antigen-specific cytotoxic activity to NKG2⁺ D^k-MT(389–397) tetramer⁺ CD8⁺ T cells (**Fig. 5b**). These studies support the conclusion that CD94-NKG2A receptors that are induced on antigen-specific CD8⁺ T cells during acute infection regulate cytotoxic effector function.

Antipolyoma CD8⁺ T cells in tumor-susceptible mice

Although MT(389–397)-specific CD8⁺ T cells expand during the neonatal infection of mice from H-2^k strains that are resistant and susceptible to polyoma virus–induced tumors, *ex vivo* MT(389–397)-specific cytotoxicity is selectively absent in susceptible mice[19]. To investigate whether this effector function deficit is associated with expression of the CD94-NKG2A receptor, D^k-MT(389–397) tetramer⁺ CD8⁺ T cells from newborn-inoculated susceptible and resistant mice were costained with Qa-1^b–Qdm tetramers. At day 13, when MT(389–397)-specific CD8⁺ T cell expansion is maximal[19], approximately 40% of the D^k-MT(389–397) tetramer⁺ CD8⁺ T cells from mice of the resistant strain C3H/HeN failed to bind Qa-1^b–Qdm tetramers. In contrast, nearly all of the MT(389–397)-specific CD8⁺ T cells in mice of the susceptible CBA/J strain bound Qa-1^b–Qdm tetramers and therefore expressed CD94-NKG2 receptors (**Fig. 6a**). D^k-MT(389–397) tetramer⁺ CD8⁺ T cells from mice of either strain did not bind Qa-1^b–Qdm.R5K tetramers, in which an arginine to lysine substitution in Qdm prevents binding to CD94-NKG2 receptors[22].

We determined whether CD94-NKG2A receptors were responsible for negating *ex vivo* cytotoxic activity by MT(389–397)-specific CD8⁺ T cells in susceptible mice. Blockade and/or internalization of Qa-1^b molecules from the surface of target cells pulsed with MT(389–397) restored potent *ex vivo* antigen-specific cytotoxicity in spleen cells from newborn-inoculated CBA/J mice at day 13 after infection (**Fig. 6b**). Thus, the CD94-NKG2A receptor clearly compromises the cytotoxic effector function of the dominant antipolyoma CD8⁺ T cell response in acutely infected mice that are susceptible to polyoma virus–induced tumorigenesis.

Memory antipolyoma CD8⁺ T cell responses

Polyoma virus infection induces efficient virus-neutralizing antibody responses in both resistant and susceptible mice[23,24]. Therefore, to investigate the impact of re-exposure to the MT(389–397) epitope on expansion and CD94-NKG2 expression by memory antipolyoma CD8⁺ T cells in resistant and susceptible mice, we infected newborn-inoculated, immune C3H/HeN and CBA/J mice with a recombinant vaccinia virus carrying a MT(389–397) minigene. This recombinant vaccinia virus, Vac.MT(389–397), efficiently expanded MT(389–397)-specific CTL effectors in adult-inoculated C3H/HeN and CBA/J mice (data not shown). Vac.MT(389–397) infection triggered a fivefold expansion of MT(389–397)-specific CD8⁺ T cells in immune C3H/HeN mice, whereas infection by a control recombinant vaccinia virus carrying a minigene for the H-$2K^d$–restricted GP(99–108) epitope of lymphocytic choriomeningitis virus (LCMV), Vac.GP(99–108), did not (**Table 1**); however, Vac.GP(99–108) infection elicited a threefold expansion in total splenic CD8⁺ cells as compared with unchallenged immune mice. The antigen-specific CD8⁺ T cell expansion in Vac.MT(389–397)-challenged immune C3H/HeN mice was accompanied by strong MT(389–397)-specific *ex vivo* cytotoxicity (data not shown). Nearly 60% of the D^k-

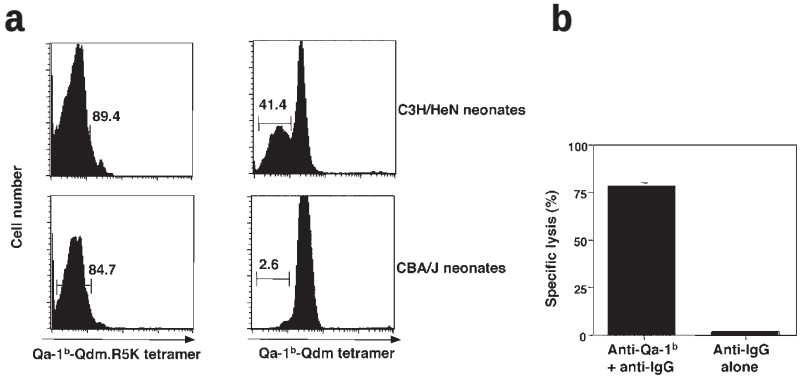

Figure 6. CD94-NKG2A inhibits *ex vivo* cytotoxicity by MT(389–397)-specific CD8⁺ T cells in mice susceptible to polyoma virus–induced tumors. (**a**) Spleen cells from neonatally infected C3H/HeN and CBA/J mice on day 13 after infection were stained with D^k-MT(389–397) tetramer, CD8α mAb and either Qa-1^b–Qdm tetramer or a control Qa-1^b–Qdm.R5K tetramer, and analyzed by flow cytometry. Plots are gated on D^k-MT(389–397) tetramer⁺ CD8⁺ T cells and values shown are the percentages of D^k-MT(389–397)⁺ CD94-NKG2A⁻ T cells in the indicated region. (**b**) Spleen cells from day 13 neonatally infected CBA/J mice were analyzed (effector:target ratio of 400:1) for lysis of unpulsed or MT(389–397)-pulsed target cells pretreated with Qa-1^b mAb and anti-mouse secondary antibody or anti-mouse secondary antibody alone. Each value represents the mean±s.e.m. percent specific lysis by spleen cells of three mice. No lysis of unlabeled target cells was detected (data not shown). Results shown are representative of two experiments.

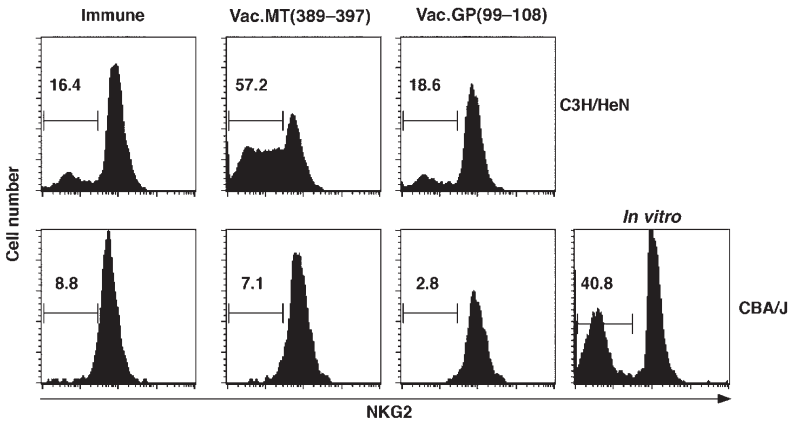

Figure 7. Regulation of CD94-NKG2A expression by MT(389–397)-specific CD8⁺ T cells through recombinant vaccinia virus infection of immune polyoma tumor–resistant and polyoma tumor–susceptible mice. Adult C3H/HeN and CBA/J mice inoculated at birth with polyoma virus were infected for 5 days with recombinant vaccinia viruses carrying minigenes for MT(389–397) or LCMV GP(99–108) epitopes. Spleen cells from these mice, polyoma-immune mice and polyoma-immune mice stimulated for 5 days *in vitro* with polyoma-infected irradiated syngeneic spleen cells were stained with D^k-MT(389–397) tetramer, and CD8α and NKG2 mAbs. Plots are gated on CD8⁺ D^k-MT(389–397) tetramer⁺ cells. Data represent the percentage of MT(389–397)-specific CD8⁺ T cells that lack NKG2 expression

MT(389–397) tetramer⁺ CD8⁺ T cells in these mice were found to express low amounts of CD94-NKG2 receptors (**Fig. 7**).

Immune susceptible CBA/J mice showed a different response to infection with Vac.MT(389–397), which failed either to induce expansion of MT(389–397)-specific CD8⁺ T cells or to alter their high expression of CD94-NKG2 (**Table 1** and **Fig. 7**). As seen in immune C3H/HeN mice, however, vaccinia virus infection could induce expansion of splenic CD8⁺ T cells in immune CBA/J mice (**Table 1**). In contrast, *in vitro* restimulation of spleen cells from neonatally infected immune CBA/J mice led to a 30-fold expansion of D^k-MT(389–397) tetramer⁺ CD8⁺ T cells, of which 40% lacked expression of CD94-NKG2 (**Fig. 7** and data not shown). As seen with *in vitro* restimulated spleen cells from 13-day infected CBA/J neonates[19], *in vitro* expansion in immune CBA/J spleen cells generated potent MT(389–397)-specific cytotoxic activity (data not shown).

These findings suggest that a unique microenvironment is established in polyoma tumor–susceptible mice that not only restrains the cytotoxic effector function of antipolyoma CD8⁺ T cells during acute infection, but also blocks the expansion and differentiation of polyoma-specific memory CD8⁺ T cells.

Discussion

We have shown here that the CD94-NKG2A receptor down-regulated the antigen-specific cytotoxic effector function of antiviral CD8⁺ T cells during acute infection. The maximal expansion of antiviral CD8⁺ T cells responding to systemic infection tends to overshoot clearance of infectious virus[2,3], probably because activation of individual naïve CD8⁺ T cells initiates a fixed, antigen-independent program of cell division and differentiation to CTL effectors[25,26]. As a result, mechanisms must be engaged to restrain T cell effector activity as numbers of infected cells become limiting. Inhibition of antiviral effector T cells is also essential to prevent widespread immunopathology against viruses that establish persistent infection, such as polyoma virus, while maintaining control over the extent of infection. We found that CD94-NKG2A expression is up-regulated by polyoma-specific CD8⁺ T cells in parallel with the resolution of virus infection and impairs their antigen-specific cytotoxicity before differentiation into memory T cells.

Regulation of CD94-NKG2A expression by polyoma-specific CD8⁺ T cells probably represents a primary determinant of host resistance to polyoma virus–induced tumors. Inherited susceptibility to polyoma virus–induced tumors results from the deletion of polyoma-specific CD8⁺ thymocytes that express TCR V_β domains that react with the endogenous Mtv-7 superantigen[27]. As a consequence of this constrained antipolyoma CD8⁺ T cell repertoire, infectious polyoma virus attains high titers and is cleared at a substantially slower rate in susceptible than in resistant mice[19]. Because TCR activation up-regulates the expression of KIR in CD8⁺ T cells[28], the rapid high-titer infection in polyoma tumor–susceptible mice might accelerate the expression of CD94-NKG2A by antiviral CD8⁺ T cells and prematurely terminate their cytotoxic effector capability. In connection with this, it should be noted that CD69, an early transient marker of TCR activation, was expressed *in vivo* by the MT(389–397)-specific CD8⁺ T cells that expressed the highest amounts of CD94-NKG2A (data not shown).

Alternatively, because specific cytokines such as interleukin 15 (IL-15), IL-10 and transforming growth factor-β can up-regulate the expression of CD94-NKG2A on CD8⁺ T cells *in vitro*[29], and because virus infection can induce the production of IL-15[30,31], the cytokine milieu in polyoma tumor–susceptible mice might also favor high stable expression of this inhibitory NKR. Functionally compromised antipolyoma CD8⁺ T cells at an early stage of acute infection would leave the host with a high residual viral burden. Because polyoma virus–induced cellular transformation is an infrequent event and tumorigenesis preferentially targets tissues carrying high numbers of replicating viral genomes[32], a lack of CD8⁺ T cell–mediated control of persistent polyoma virus would promote tumor incidence. Conversely, efficient elimination of infected cells by antiviral CD8⁺ T cells during acute infection would limit the number of persistently infected cells, and in turn reduce the probability of viral oncogenesis.

Inhibitory NKRs may also impair the proliferation of antigen-specific CD8⁺ T cells. Constitutively high expression of Ly49A in transgenic mice limits both the virus-specific expansion of CD8⁺ T cells and effector function[14]. The murine KLRG1 inhibitory receptor is induced in antiviral CD8⁺ T cells and inhibits their antigen-specific proliferation[33]. Similarly, high expression of CD94-NKG2A by polyoma-specific CD8⁺ T cells in susceptible mice might present a barrier that fully blocks the ability of the MT(389–397) minigene recombinant vaccinia virus to drive their expansion. Because *in vitro* restimulation promoted efficient expansion of fully competent antipolyoma CTL effectors from immune susceptible mice, *in vivo* factors must account for the dysfunctional phenotype of these CD8⁺ T cells. Thus, strategies aimed at reducing the amount of persistent infection or neutralizing particular cytokines might impair negative regulation by CD94-NKG2A receptors and promote expansion of antigen-specific CTL secondary effectors.

Qa-1 has a broad tissue distribution, although its expression is generally lower than that of classical MHC class I molecules[34]. Interferon-γ (IFN-γ) up-regulates the expression of $Qa\text{-}1^b$ on pancreatic β cell lines[35] and increases surface expression of functional $Qa\text{-}1^b$ molecules by the AG104A fibrosarcoma target cell line used here (J. M. Moser

and L. Reed, unpublished data). As a dominant cytokine induced during viral infection, IFN-γ may similarly up-regulate expression of Qa-1 by antigen-presenting cells in infected tissues, and thereby promote signaling through CD94-NKG2 receptors expressed by NK cells and T cells. It has been shown that IFN-γ up-regulates expression of HLA-E and renders cells resistant to lysis mediated by NK cells[36]. Other cytokines, such as IFN-α and IFN-β, which induce expression of classical MHC class I molecules[37], may similarly up-regulate the expression of nonclassical MHC class I molecules. Although Qa-1^b optimally binds Qdm, this nonclassical MHC class I molecule can be loaded with many different peptides[22]. The CD94-NKG2A receptor, however, is highly specific for Qa-1^b–Qdm complexes[22]. This raises the possibility that the proportion of Qa-1^b molecules occupied by Qdm may vary among cell types, or be altered by direct virus infection or the virus-induced cytokine microenvironment, and in turn affect the extent of CD94-NKG2A receptor activation.

Because polyoma virus is a persistent mouse pathogen[24,38,39], antipolyoma CD8$^+$ T cells must maintain surveillance against virus-induced neoplasia in tumor-resistant mice. Approximately 20% of the MT(389–397)-specific CD8$^+$ T cells in resistant mice retain the CD94-NKG2A$^-$ phenotype in memory cells. Whether these T cells are capable of direct *ex vivo* cytotoxicity is difficult to ascertain given their small numbers *in vivo*; however, *in vivo* exposure of immune resistant mice to the MT(389–397) epitope presented *via* recombinant vaccinia virus infection led to the efficient expansion of MT(389–397)-specific CD8$^+$ T cells that possessed strong antigen-specific *ex vivo* cytotoxicity, coupled with the emergence of a large CD94-NKG2A$^-$ subpopulation. Studies on other inhibitory NKRs suggest that these receptors may promote survival of memory CD8$^+$ T cells, possibly by blocking activation-induced cell death[40,41]. By extension, CD94-NKG2A expression may be essential to maintain stable populations of polyoma-specific memory CD8$^+$ T cells in the face of persistent antigen. We favor a linear differentiation pathway that involves conversion from CD94-NKG2A$^+$ to CD94-NKG2A$^-$ memory CD8$^+$ T cells, which subsequently differentiate into secondary effector CTLs. In this model, the small CD94-NKG2A$^-$ subpopulation of polyoma-specific memory CD8$^+$ T cells in resistant mice represents a transition state for rapid differentiation into CTL effectors to eradicate cells that are persistently infected and/or transformed by polyoma virus.

Our study also points toward the existence of a distinct transient stage of antigen-specific CD8$^+$ T cell differentiation that is interposed between effector CTL and memory stages. Interrupting the binding of CD94-NKG2A to its ligand, Qa-1^b–Qdm, fully restored antigen-specific *ex vivo* cytotoxicity by polyoma-specific CD8$^+$ T cells during acute infection, but failed to do so once these cells entered the memory stage. Thus, despite their phenotypic similarity, these post-CTL effectors are qualitatively different from memory CD8$^+$ T cells in their potential for *ex vivo* cytotoxicity. Whether these "restrained" CTL effectors are a necessary precursor to memory differentiation is an important issue. Another possibility is that these CD8$^+$ T cells may protect against an early resurgence of a primary or secondary virus infection, in which strong stimulation of the TCR might overwhelm CD94-NKG2A–mediated inhibition.

In summary, our results provide evidence for the functional inhibition of antiviral CD8$^+$ T cells *in vivo* by an endogenous NKR. A general picture is emerging in which the magnitude and diversity of antigen-specific T cell effector activity are controlled by an integration of positive signals transduced by the TCR and negative signals originating from inhibitory cell surface molecules[6,11]. An imbalance in favor of inhibitory signaling may contribute to the dysfunction of antigen-specific CD8$^+$ T cells in chronic viral infections and in tumor-bearing hosts[42]. Precise titration of antigen-specific cytotoxic effector activity by CD8$^+$ T cells is likely to be critical for maintaining effective immunosurveillance, without immunopathologic consequences, against persistent virus infections.

Methods

Mice. We purchased C3H/HeNCr and CBA/JCr mice from the Frederick Cancer Research and Development Center of the National Cancer Institute (Frederick, MD). Mice were housed and bred in accordance with the guidelines of the Institutional Animal Care and Use Committee, and the Department of Animal Resources at Emory University. We housed pregnant mice in individual microisolator cages and monitored them daily for births. Adult mice were used at 6–9 weeks.

Viruses. The wild-type polyoma virus strain A2 was molecularly cloned and plaque purified, and virus stocks were prepared on primary baby mouse kidney cells as described[43]. We inoculated adult mice subcutaneously in their hind footpads with 2 10^6 plaque-forming units (PFU) of virus. Newborn mice were inoculated subcutaneously in their hind footpads with 2 10^5 PFU of virus within 12 h of birth. We made recombinant vaccinia viruses containing minigenes for the H-2K^d–restricted MT(389–397) polyoma CD8$^+$ T cell epitope[43] or the H-2K^d–restricted GP(99–108) LCMV epitope[44] as described[45,46]. Adult mice were given an intraperitoneal inoculation of 5 10^5 PFU of virus.

^{51}Cr-release assay. We prepared ^{51}Cr-labeled AG104A target cells (H-2^k, MHC class II$^-$, spontaneous fibrosarcoma[47], maintained in Dulbeccos' modified Eagle's medium containing 5% fetal bovine serum (FBS)) as described[43]. For Qa-1^b blocking experiments, ^{51}Cr-labeled AG104A cells were pulsed with 10 M peptide for 1 h at 37 °C in the presence of brefeldin A (10 g/ml), and then incubated in the presence of brefeldin A (1 g/ml) and FcBlock (BD Pharmingen, San Diego, CA) with mouse Qa-1^b mAb (BD Pharmingen) or mouse H-2K^k mAb (Caltag, Burlingame, CA) at 20 g/ml for 20 min at 22 °C in medium containing 1% serum, followed by goat anti-mouse antibody (20 g/ml; Jackson ImmunoResearch, West Grove, PA) for 20 min at 22 °C in medium containing 1% serum. For NKG2 blocking experiments, untreated target cells or target cells treated with 10 M MT(389–397) were cultured with D^k-MT(389–397) tetramer$^+$ CD8$^+$ T cells that had been sorted for NKG2 expression in the presence of mAb to mouse NKG2A, NKG2C and NKG2E (43 g/ml, clone 20d5; BD Pharmingen) or an isotype-matched keyhole limpet hemocyanin (KLH) mAb (43 g/ml, clone A110-2; BD Pharmingen). We resuspended target cells in Iscove's modified Dulbecco's medium (Life Technologies, Grand Island, NY) containing 10% FBS, and aliquoted 4,000 cells per well into V-bottom 96-well microtiter plates (Costar, Cambridge, MA). Spleen cells were treated with RBC lysing buffer (Sigma, St Louis, MO), and viable mononuclear cells were aliquoted at the indicated effector:target ratios into U-bottom 96-well microtiter plates (Costar) containing 5,000 target cells. After a 4–5 h incubation at 37 °C, half of the volume of each well was removed and counted in a Quantum γ-counter (Packard, Downers Grove, IL). We calculated the percentage of specific lysis as described[43].

Flow cytometry. Erythrocyte-lysed spleen cells were surface stained with fluorescein isothiocyanate (FITC), phycoerythrin (PE) or Tricolor-conjugated CD8α mAb (clone CT-CD8a; Caltag), and then FITC-conjugated mAb 20d5, CD11a mAb, or Ly-49A or Ly-49C/Ly-49I mAb (BD PharMingen), and PE-conjugated Qa-1^b–Qdm tetramer, or allophycocyanin-conjugated D^k-MT(389–397) tetramer. We prepared H-2D^k tetramers of MT(389–397) and the Qa-1^b tetramer containing either Qdm or the Qdm.R5K peptide, and verified their specificity as described[22]. Samples were acquired on a FACSCalibur (Becton-Dickinson, San Jose, CA).

For flow cytometry sorting, erythrocyte-lysed spleen cells were stained in phenol red–free RPMI 1640 medium (Life Technologies, Gaithersburg, MD) supplemented with 2% FBS, which contained allophycocyanin-conjugated D^k-MT(389–397) tetramer, FITC- or PE-conjugated anti-CD8α, and mAb 20d5 with PE-conjugated goat anti-mouse secondary antibody (Jackson ImmunoResearch) or PE-conjugated Qa-1^b–Qdm tetramer. Cells were acquired and sorted on a FACSVantage (Becton Dickinson).

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Competing interests statement

The authors declare that they have no competing financial interests.

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