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CD94/NKG2A Expression Is Associated with Proliferative Potential of CD8 T Cells during Persistent Polyoma Virus Infection

Anthony M. Byers, Nicolas P. Andrews, and Aron E. Lukacher

Memory CD8 T cells comprise a critical component of durable immunity because of their capacity to rapidly proliferate and exert effector activity upon Ag rechallenge. During persistent viral infection, memory CD8 T cells repetitively encounter viral Ag and must maintain a delicate balance between limiting viral replication and minimizing immunopathology. In mice infected by polyoma virus, a natural mouse pathogen that establishes long-term persistent infection, the majority of persistence-phase antiviral CD8 T cells express the inhibitory NK cell receptor CD94/NKG2A. In this study, we asked whether CD94/NKG2A expression is associated with Ag-specific recall of polyoma virus-specific CD8 T cells. During the persistent phase of infection, polyoma virus-specific CD8 T cells that express CD94/NKG2A were found to preferentially proliferate; this proliferation was dependent on cognate Ag both in vitro and in vivo. In addition, CD94/NKG2A+ polyoma-specific CD8 T cells have a markedly enhanced capacity to produce IL-2 upon ex vivo Ag stimulation compared with CD94/NKG2A− polyoma-specific CD8 T cells. Importantly, CD94/NKG2A+ anti-polyoma virus CD8 T cells appear to be essential for Ag-specific recall responses in mice persistently infected by polyoma virus. Because of its higher proliferative potential and capacity to produce IL-2, we propose that the CD94/NKG2A+ subpopulation represents a less differentiated state than the CD94/NKG2A− subpopulation. Identification of proliferation-competent subpopulations of memory CD8 T cells should prove valuable in designing therapeutic vaccination strategies for persistent viral infections. The Journal of Immunology, 2006, 176: 6121–6129.

Viral infection initiates a program of Ag-specific CD8 T cell expansion and differentiation that eventually leads to a stable population of memory CD8 T cells. Recent evidence suggests that the quality of the memory CD8 T cell response is determined during the initial priming of the naive T cell (1–3). Because this encounter is a multifactorial process (e.g., type and number of APC, duration of APC-T cell contact, presence of costimulatory molecules, and T cell help), it is not surprising that memory T cells vary greatly in trafficking properties, effector capabilities, and proliferative potential (4). Classification of these heterogeneous memory T cell populations into discrete subsets based on either phenotypic (e.g., CD62L and CCR7) or functional (e.g., cytotoxicity, cytokine profile, proliferative capacity) differences has become increasingly complex as greater numbers of cell surface molecules and different infection models are studied (5–11). Moreover, development and maintenance of memory CD8 T cells, as well as their functional and phenotypic heterogeneity, are distinctly different for persistent and acutely cleared viral infections (10, 12). Thus, canonical markers segregating and defining relationships between memory T cell subsets are not likely to be broadly applicable across microbial infections (13, 14).

Inhibitory NK cell receptors (iNKR)s, whose expression on NK cells determine whether an NK cell engages its effector functions, are present on subsets of antiviral CD8 T cells during effector and memory differentiation states in a variety of human and murine infections (15). The interaction of iNKR on CD8 T cells with MHC class I ligands on APCs provides a mechanism for fine-tuning TCR-mediated activation of CD8 T cells (16). iNKRs are grouped into two families: type I transmembrane Ig superfamily proteins, including human killer cell Ig-like receptors (KIRs) and leukocyte Ig-like receptors; and type II transmembrane proteins containing a C-type lectin domain, which include Ly49 homodimers in mice and CD94/NKG2A heterodimers in both mice and humans (17). The Ig-like NK cell receptors and Ly49 molecules interact with multiple classical MHC class Ia molecules (18). In contrast, the ligand for CD94/NKG2A is the nonclassical MHC class Ib molecule HLA-E or Qa-1B (mouse) complexed to a nonapeptide derived from the leader sequence of particular MHC class Ia H chains; in mice, this peptide is designated Qdm (Qa-1 determinant modifier) (19, 20). These receptors contain intracytoplasmic ITIMs, which recruit and activate Src homology 2-domain-containing protein tyrosine phosphatase 1 (SHP-1) molecules that mitigate NK and T cell activation signals by inhibiting protein kinase pathways activated by TCR ligation (16).

Viral infection induces iNKR exclusively on CD8 T cells that express surface molecules indicative of effector/memory differentiation (e.g., CD11a, CD44, and CD122) (21). A considerable amount of data indicates that iNKR engagement on antiviral CD8 T cells is associated with dampened effector activity (e.g., cytotoxicity, cytokine production) (17). A role for iNKR in regulating...
noneffecter functions of CD8 T cells has also been shown in several systems. Cross-linking of Ly49A or CD49/NKG2A receptors has been shown to decrease apoptosis of CD8 T cells expressing these iNKR (22, 23), and memory CD8 T cells accumulate in mice transgenic for both an inhibitory KIR and its cognate HLA class I ligand (24).

In mice infected by polyoma virus (PyV), CD49/NKG2A expression is rapidly induced on antiviral CD8 T cells (25–27). The majority of PyV-specific CD8 T cells express CD49/NKG2A during resolution of the acute phase of infection and coordinately exhibit reduced Ag-specific cytotoxicity; blockade of CD49/NKG2A binding to its ligand, Qa-1b/Qdm, relieves this inhibition (27). Work, using different pathogens and mouse strains, suggests that CD49/NKG2A-mediated inhibition of cytotoxic activity may be dependent on host-virus interactions and viral pathogenesis (25, 26). The frequency of CD49/NKG2A-expressing PyV-specific CD8 T cells increases throughout acute infection, with ~75% of these cells displaying this iNKR by 2 wk after infection; this level of CD49/NKG2A expression is maintained throughout persistent infection (27). These sustained elevated expression levels could provide a mechanism for down-regulating the cytopathic effector activity of PyV-specific CD8 T cells as infection becomes limiting (15). Moreover, because PyV establishes a persistent infection, preferential expansion of this CD49/NKG2A + population may be beneficial to the host by diminishing immunopathology while preserving surveillance for persistently infected cells. In this study, we analyzed whether CD49/NKG2A receptors were associated with the recall response of Ag-specific memory CD8 T cells in mice persistently infected by PyV. We found that CD49/NKG2A-expressing PyV-specific CD8 T cells preferentially proliferate and produce IL-2 during the persistent phase of infection and upon antigenic challenge. These results suggest that expression of CD49/NKG2A identifies a subpopulation of proliferation-competent antiviral memory CD8 T cells during persistent viral infection.

Materials and Methods

Mice
C3H/HeNCr female mice were purchased from the National Cancer Institute and inoculated with PyV at 6–10 wk of age. Mice were cared for in accordance with the guidelines of the Institutional Animal Care and Use Committee and the Department of Animal Resources at Emory University.

Viruses
PyV strain A2 was molecularly cloned and plaque purified, and virus stocks were prepared on baby mouse kidney cells, as previously described (28). PCR-based site-directed mutagenesis (Stratagene) was used to change the wild-type middle T (MT) protein 389–397 sequence RRLGRTLLL (M5I6). This mutant virus, A2.M5I6, was the wild-type middle T (MT) protein 389–397 sequence RRLGMLL (M5I6). This mutant virus, A2.M5I6, was stocks were prepared on baby mouse kidney cells, as previously described in Ref. 30) and the following RBC-lysed spleen cells were stained with allophycocyanin-conjugated Dk MT389 tetramers (as described in Ref. 31). After 5 days, cells were stained using anti-CD8α, anti-CD49/NKG2A/C/E, and D6 MT389 tetramers, and proliferation assessed based on extent of CFSE dilution by flow cytometry. For in vivo proliferation, 5 × 105 of FACS-sorted, CFSE-labeled CD49/NKG2* or CD49/NKG2*CD8 T cells, adjusted to 2 × 106 MT389-specific T cells by postsort D6 MT389 tetramer staining, were cultured with 1 × 106 MT389 peptide-pulsed (1 μM) naive C3H/HeN spleen cells, in the presence of IL-7, IL-15 (each 5 ng/ml), or TGFB-β (10 ng/ml) (R&D Systems) as indicated. CD8 T cells were labeled with CFSE as previously described (31).

Flow cytometry and intracellular cytokine staining
RBC-lysed spleen cells were stained with allophycocyanin-conjugated D6 MT389 tetramers (prepared as described in Ref. 30) and the following mAbs: PerCP-conjugated anti-CD8α (BD Biosciences); FITC-conjugated or purified anti-NKG2A/C/E (clone 2D4; BD Biosciences); and PE-conjugated mAbs to goat anti-rat IgG (Caltag Laboratories), CD25, CD122, and CD127 (BD Biosciences). For intracellular cytokine staining, spleen cells were cultured for 4 h in 96-well round-bottom microtiter plates in IMDM (Invitrogen Life Technologies) containing 10% FBS, penicillin/streptomycin, 50 μM 2-ME, synthetic peptides, and 1 μg/ml brefeldin A (BFA), unless otherwise noted. Cells were then stained for surface markers, washed, permeabilized with Cytofix/Cytoperm (BD Biosciences), and stained intracellularly using allophycocyanin-conjugated anti-IFN-γ (XMG 1.2; BD Biosciences) and PE-conjugated anti-IL-2 (JES6-5H4; BD Biosciences). Intracellular staining using PE-conjugated anti-Bcl-2 (BD Biosciences), its isotype control (Armenian hamster IgG), and PE-conjugated anti-activated caspase 3 (CPP-32; BD Biosciences) was performed as described above. Samples were acquired on a FACSCalibur (BD Biosciences) and data were analyzed using CellQuest (BD Biosciences) and FlowJo (Tree Star) software.

For FACS sorting, RBC-lysed spleen cells were stained with the following mAbs: anti-NKG2A/C/E followed by PE-conjugated goat anti-rat IgG (Caltag Laboratories) and FITC-conjugated anti-CD8α. Cells were resuspended in phenol red-free RPMI 1640 (Invitrogen Life Technologies) supplemented with 1% FCS, 25 μg/ml HEPEs, 25 μg/ml DNase, and 5 μM MgCl2, and sorted on a FACSVantage (BD Biosciences) flow cytometer. Purity of FACS-sorted samples was 91–93% for CD49/NKG2* and 95–98% for CD49/NKG2+ populations.

Proliferation assays
One hundred microliters of 10 mg/ml BrdU in PBS was administered i.p. and mice were sacrificed 5–8 h after injection; no differences were observed in BrdU uptake by PyV-specific CD8 T cells over this time frame. Intracellular staining with FITC-conjugated anti-BrdU was performed according to manufacturer’s instructions (BD Biosciences). For proliferation assays, FACS-sorted, CFSE-labeled CD49/NKG2* or CD49/NKG2*CD8 T cells, adjusted to 2 × 105 MT389-specific T cells by postsort D6 MT389 tetramer staining, were cultured with 1 × 106 MT389 peptide-pulsed (1 μM) naive C3H/HeN spleen cells, in the presence of IL-7, IL-15 (each 5 ng/ml), or TGFB-β (10 ng/ml) (R&D Systems) as indicated. D6 MT389 tetramers were labeled with CFSE as previously described (31).

Statistical significance was determined by the Mann-Whitney U test. A value of p < 0.05 was considered statistically significant.

Results

TCR engagement induces CD49/NKG2A expression on MT389-specific CD8 T cells
CD49/NKG2A heterodimer expression is rapidly induced on human and murine CD8 T cells by TCR engagement and/or cytokine stimulation in vitro, as well as by viral infection (16, 17, 31). In PyV-infected C3H/HeN mice, the frequency of CD8 T cells specific for the dominant D6-restricted MT389 epitope (28) that express CD49/NKG2A progressively increases during the acute phase of infection, with ~75% of MT389-specific CD8 T cells expressing this iNKR by 2 wk postinfection (p.i.); this expression level is maintained during persistent infection (27). Although the mAb used to detect NKG2A (clone 2D4) also recognizes the NKG2 isoforms NKG2C and NKG2E, it is the only mAb available to detect NKG2A in C3H mice; however, nearly all 2D4 mAb-sorted Ag-specific CD8 T cells predominantly express NKG2A transcripts (25, 31, 32) and, in C57BL/6 mice, an allele-specific NKG2A-specific mAb (33) stains ~90% of 2D4 + PyV-specific CD8 T cells (C. Kemball and A. Lukacher, unpublished observations). In addition, CD49/NKG2C has not been shown to mediate activation of murine NK or CD8 T cells, possibly because the transmembrane domain of murine (unlike human) NKG2C lacks a charged residue (typically a lysine) to enable association to an ITAM-containing adaptor protein, such as DAP-12 (34). For simplicity, we will refer to 2D4 mAb+ cells as CD49/NKG2A+.

To determine whether cognate Ag per se induces CD49/NKG2A surface expression on PyV-specific CD8 T cells, freshly explanted spleen cells from C3H/HeN mice at day 7 p.i., the peak of the

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CD94/NKG2A + T cells preferentially proliferate

CD94/NKG2A + T cells preferentially proliferate in response to TCR engagement. CD49/NKG2A heterodimer expression is rapidly induced on human and murine CD8 T cells by TCR engagement and/or cytokine stimulation in vitro, as well as by viral infection (16, 17, 31). In PyV-infected C3H/HeN mice, the frequency of CD8 T cells specific for the dominant D6-restricted MT389 epitope (28) that express CD49/NKG2A progressively increases during the acute phase of infection, with ~75% of MT389-specific CD8 T cells expressing this iNKR by 2 wk postinfection (p.i.); this expression level is maintained during persistent infection (27). Although the mAb used to detect NKG2A (clone 2D4) also recognizes the NKG2 isoforms NKG2C and NKG2E, it is the only mAb available to detect NKG2A in C3H mice; however, nearly all 2D4 mAb-sorted Ag-specific CD8 T cells predominantly express NKG2A transcripts (25, 31, 32) and, in C57BL/6 mice, an allele-specific NKG2A-specific mAb (33) stains ~90% of 2D4 + PyV-specific CD8 T cells (C. Kemball and A. Lukacher, unpublished observations). In addition, CD49/NKG2C has not been shown to mediate activation of murine NK or CD8 T cells, possibly because the transmembrane domain of murine (unlike human) NKG2C lacks a charged residue (typically a lysine) to enable association to an ITAM-containing adaptor protein, such as DAP-12 (34). For simplicity, we will refer to 2D4 mAb+ cells as CD49/NKG2A+.

To determine whether cognate Ag per se induces CD49/NKG2A surface expression on PyV-specific CD8 T cells, freshly explanted spleen cells from C3H/HeN mice at day 7 p.i., the peak of the
MT389-specific CD8 T cell response, were stimulated with MT389 peptide and the CD94/NKG2A expression of intracellular IFN-γ+ CD8 T cells was assessed. In the presence of the protein transport inhibitor BFA, approximately one-half of the IFN-γ+ CD8 T cells expressed CD94/NKG2A by 4 h of culture; this frequency matches the percentage of MT389-specific CD8 T cells that are positive for NKG2A directly ex vivo (Fig. 1, A and B, and data not shown). In contrast, when BFA was omitted during peptide stimulation, >85% of the IFN-γ-producing CD8 T cells expressed CD94/NKG2A. Kinetic analysis revealed that cognate peptide induces CD94/NKG2A expression as early as 2 h after stimulation, with maximal expression achieved by 4 h (data not shown). In the absence of BFA, D6 MT389 tetramers also induced CD94/NKG2A expression by purified CD8 T cells from acutely (day 7 p.i.) infected mice (data not shown). Previous studies using human PBMCs showed that IL-7, IL-12, IL-15, or TGF-β was capable of inducing expression of CD94/NKG2A on CD8 T cells in the absence of TCR engagement, whereas another study using OT-I TCR transgenic CD8 T cells reported that TGF-β selectively augmented CD94/NKG2A expression only when triggered by cognate Ag (17, 31). We found that inclusion of each of these cytokines, either alone or in the presence of MT389 peptide, for 4 h did not affect CD94/NKG2A expression levels (data not shown). In addition, unlike the pronounced increase in CD94/NKG2A expression induced by Ag for CD8 T cells in acutely infected mice, MT389 peptide only minimally changed CD94/NKG2A expression levels for CD8 T cells from mice at day 102 p.i. (compare BFA and no BFA in Fig. 1A); addition of IL-7, IL-15, or TGF-β did not increase CD94/NKG2A expression on MT389-specific CD8 T cells from persistently infected mice (data not shown). These findings suggest that antiviral CD8 T cells during acute and persistent PyV infection differ in their sensitivity to Ag-driven CD94/NKG2A up-regulation.

Differences in the Ag-driven CD94/NKG2A up-regulation of acute and persistent phase MT389-specific CD8 T cells led us to ask whether CD94/NKG2A+ and CD94/NKG2A− populations were derived from common precursors. The TCRs of MT389-specific CD8 T cells are heavily skewed toward expression of Vβ6 and Vβ8.1 domains (30); this same Vβ profile is seen in both acutely and persistently infected mice, irrespective of CD94/NKG2A expression (data not shown). To further probe the relationship between these populations, we compared the fine antigenic specificities of the CD94/NKG2A+ and CD94/NKG2A− MT389-specific CD8 T cells using a panel of MT389 analog peptides having consecutive single alanine substitutions. Based on the frequency of IFN-γ+ CD8 T cells elicited by these analog peptides compared with wild-type MT389 peptide, the CD94/NKG2A+ and CD94/NKG2A− MT389-specific CD8 T cells exhibited overlapping “TCR functional fingerprints” during acute infection and similar alanine scan profiles in persistently infected mice (Fig. 1C). These data strongly suggest that CD94/NKG2A+ and CD94/NKG2A− MT389-specific CD8 T cells, at both effector and memory stages, share cell lineage.

CD94/NKG2A+ MT389-specific CD8 T cells preferentially cycle during persistent PyV infection and recall responses

Given the relationship between CD94/NKG2A+ and CD94/NKG2A− MT389-specific CD8 T cell populations, accumulation of the CD94/NKG2A+ population during persistent PyV infection may be attributable to differential responses to antigenic stimulation between these cells and their CD94/NKG2A− counterparts. For example, CD94/NKG2A engagement may regulate sensitivity...
FIGURE 2. BrdU incorporation by MT389-specific CD8 T cells during persistent PyV infection and after VV-MT389 antigenic rechallenge. A, Spleen cells from persistent PyV-infected (≥50 days p.i.) C3H/HeN or VV-MT389 challenged mice at the indicated day p.i. were stained with D^8 MT389 tetramers and mAb to surface CD8^\alpha, NKG2A/C/E, and intracellular BrdU. Plots are gated on MT389-specific CD8 T cells. Values indicate percentage of MT389-specific CD8 T cells that are BrdU^+ and values in parentheses indicate percentage of BrdU^+ cells within CD94/NKG2A^+ MT389-specific or CD94/ NKG2A^- MT389-specific CD8 T cells. The frequency of BrdU^+ MT389-specific CD8 T cells at day 4 after infection by VV-WR was equivalent to unchallenged persistently infected mice (data not shown). Data are representative of five experiments, with two to three mice per time point. B and C, Numbers of BrdU^+ MT389-specific CD8 T cells and total MT389-specific CD8 T cells, respectively, at the indicated time point (Figure legend continues)
to Ag-induced apoptosis or proliferative capacity. Experiments using plate-bound anti-CD3 or plate-bound MT389-specific monomers failed to reveal differential staining by annexin V or activated caspase 3 on CD94/NKG2A⁺ or CD94/NKG2A⁻ MT389-specific CD8 T cells during the effector (day 7 p.i.), effector-memory transition (day 10–21 p.i.), or memory (past day 40 p.i.) phases of the anti-PyV immune response (data not shown). Likewise, intracellular staining for Bcl-2 at these p.i. time points did not uncover differences in expression of this antiapoptotic molecule between CD94/NKG2A⁺ and CD94/NKG2A⁻ MT389-specific CD8 T cells (data not shown).

To monitor the proliferation of PyV-specific memory CD8 T cells in vivo, we examined incorporation of the thymidine analog BrdU by MT389-specific CD8 T cells during the persistence phase of PyV infection. Mice were sacrificed 5–8 h after receiving 1 mg of BrdU i.p. to capture a snapshot of cell proliferation by anti-PyV CD8 T cells expressing a particular level of CD94/NKG2A. Although few BrdU⁺ cells were detected during this short-term pulse, MT389-specific CD8 T cells expressing CD94/NKG2A during the persistent phase of infection clearly showed preferential proliferation compared with those lacking expression of this iNKR (Fig. 2A).

To increase numbers of proliferating MT389-specific CD8 T cells and improve resolution of the in vivo BrdU snapshot of CD94/NKG2A-expressing cells, we examined BrdU incorporation during recall responses. Because virus-neutralizing Ab precludes rechallenge by PyV, persistently infected C3H/HeN mice were infected by a recombinant VV carrying a minigene encoding aa 389–397 of the viral MT protein (VV-MT389) (27). Use of the VV-MT389 Ag rechallenge approach may also be seen as serving to amplify infrequent repetitive recall responses that take place during persistent PyV infection, thereby facilitating visualization of MT389-specific CD8 T cell responses in persistently infected mice. After VV-MT389 infection of PyV-immune C3H/HeN mice, the percentage of MT389-specific CD8 T cells that incorporated BrdU increased ~8-fold, with peak incorporation by MT389-specific CD8 T cells occurring 4 days after VV-MT389 challenge (Fig. 2, A and B). Infection by control VV-WR did not drive expansion of MT389-specific CD8 T cells at any time point postchallenge, indicating that expansion of these cells after VV-MT389 challenge is Ag-specific and not a bystander response to vaccinia infection (Fig. 2, B and C).

As seen in basal memory, MT389-specific CD8 T cells expressing CD94/NKG2A showed markedly greater numbers of BrdU⁺ cells after VV-MT389 infection (Fig. 2, A, D, and E). Although the CD94/NKG2A⁺ MT389-specific CD8 T cells did not increase relative to the CD94/NKG2A⁻ MT389-specific CD8 T cells after the peak of proliferation (Fig. 2F), this discrepancy is unlikely to be due to preferential death of the CD94/NKG2A⁺ population after proliferation. Ex vivo analysis did not reveal statistically significant differences in the percentage of annexin V⁺ cells between CD94/NKG2A⁺ and CD94/NKG2A⁻ MT389-specific CD8 T cells after the peak of proliferation (day 4 postchallenge; annexin V⁺ NKG2A⁺ = 28.73 ± 2.91 (SEM), annexin V⁺ NKG2A⁻ = 27.95 ± 3.92, n = 6, p = 0.94; day 5 postchallenge: annexin V⁺ NKG2A⁺ = 31.42 ± 5.33, annexin V⁺ NKG2A⁻ = 33.68 ± 5.92, n = 6, p = 0.82). A decrease in the CD94/NKG2A⁺ population relative to the largely nonproliferating CD94/NKG2A⁻ population, concomitant with preferential proliferation of the CD94/NKG2A⁻ population, indicates that MT389-specific memory CD8 T cells lose expression of this iNKR after Ag-driven proliferation (Fig. 2G).

Preferential proliferation of CD94/NKG2A⁺ MT389-specific CD8 T cells after recall is Ag dependent

To more clearly discern proliferation differences between CD94/NKG2A⁺ and CD94/NKG2A⁻ MT389-specific CD8 T cells, CD8⁺ spleen cells from persistently infected C3H/HeN mice were sorted based on their expression of CD94/NKG2A. We deliberately avoided sorting using D8 MT389 tetramers to prevent phenotypic and functional changes due to TCR engagement, most notably induction of CD94/NKG2A (35, 36). FACS-sorted CD94/NKG2A⁺ and CD94/NKG2A⁻ CD8 T cells were CFSE-labeled and equal numbers of MT389-specific CD8 T cells (determined by postsort D8 MT389 tetramer staining) from each group were cocultured with MT389 peptide-pulsed syngeneic spleen cells for 5 days. A portion of the CD94/NKG2A⁺ MT389-specific CD8 T cell population underwent several rounds of division while the CD94/NKG2A⁻ population failed to divide (Fig. 3). The rare dividing cells in the MT389 peptide-stimulated CD94/NKG2A⁻ population (Fig. 3, top right) most likely represent contaminating CD94/NKG2A⁺ cells, which are also visualized in the absence of peptide (Fig. 3, bottom right). Addition of IL-7, IL-15, or TGF-β alone or with peptide did not influence cell division by either group (data not shown).

In vivo proliferation of CD94/NKG2A⁺ and CD94/NKG2A⁻ MT389-specific CD8 T cells was monitored by adoptively transferring equivalent numbers of FACS-sorted, CFSE-labeled CD94/NKG2A⁺ and CD94/NKG2A⁻ MT389-specific memory CD8 T cells into mice persistently infected with A2.M516, a PyV carrying point mutations in MT that eliminate the MT389 epitope (37, 38). We opted to use A2.M516 virus-immune mice rather than naive mice as recipients to 1) use PyV-specific humoral immunity to neutralize potential carryover wild-type virus and 2) recapitulate the persistently infected environment of the donor T cells. One day after transfer, recipient mice were inoculated with VV-MT389 or VV-WR virus. As shown in Fig. 4, upper panel, by 4 days after VV-MT389 rechallenge, a portion of the donor CD94/NKG2A⁺ MT389-specific CD8 T cells underwent two to three cell divisions while the CD94/NKG2A⁻ MT389-specific CD8 T cells did not substantially divide. Neither transferred population divided in mice infected by VV-WR virus (Fig. 4, middle panel). To more clearly visualize this difference in proliferation between CD94/NKG2A⁺ and CD94/NKG2A⁻ MT389-specific CD8 T cells, we repeated
CD94/NKG2A+ T CELLS PREFERENTIALLY PROLIFERATE

CD94/NKG2A+ PyV-specific CD8 T cells preferentially proliferate in vitro. Spleen cells from persistent PyV-infected (>day 50 p.i.) C3H/HeN mice were FACS sorted based on CD94/NKG2 and CD8α expression and labeled with 1 μM CFSE. Equal numbers of CD94/NKG2A+ or CD94/NKG2A− MT389-specific CD8 T cells were cultured with MT389 peptide-pulsed (1 μM) or unpulsed syngeneic spleen cells. After 5 days, cells were stained with D11 MT389 tetramers and mAbs to CD8α, NKG2A/C/E, and CFSE dilution was analyzed by flow cytometry. Plots are gated on MT389-specific CD8 T cells and numbers indicate the percentage of cells that have undergone at least one division. Data are representative of duplicate wells from three experiments.

FIGURE 3. CD94/NKG2A+ PyV-specific CD8 T cells preferentially proliferate in vitro. Spleen cells from persistent PyV-infected (>day 50 p.i.) C3H/HeN mice were FACS sorted based on CD94/NKG2 and CD8α expression and labeled with 1 μM CFSE. Equal numbers of CD94/NKG2A+ or CD94/NKG2A− MT389-specific CD8 T cells were cultured with MT389 peptide-pulsed (1 μM) or unpulsed syngeneic spleen cells. After 5 days, cells were stained with D11 MT389 tetramers and mAbs to CD8α, NKG2A/C/E, and CFSE, and CFSE dilution was analyzed by flow cytometry. Plots are gated on MT389-specific CD8 T cells and numbers indicate the percentage of cells that have undergone at least one division. Data are representative of duplicate wells from three experiments.

CD94/NKG2A+ MT389-specific CD8 T cells selectively express IL-2

The γ chain cytokines IL-7 and IL-15 have been implicated in the identification of memory precursors during the effector stage and homeostatic memory maintenance, respectively, of Ag-specific CD8 T cells (39–43). Although we found that these cytokines did not modulate CD94/NKG2A expression on MT389-specific CD8 T cells in vitro, we asked whether receptors for these cytokines were differentially expressed by MT389-specific CD8 T cells expressing CD94/NKG2A+. We examined expression of CD25 (IL-2Rα), CD122 (IL-2Rβ/IL-15Rβ), and CD127 (IL-7Rα) on either CD94/NKG2A+ or CD94/NKG2A− MT389-specific CD8 T cells in persistently infected mice pre- and postchallenge by VV-MT389. As shown in Fig. 5A, more CD94/NKG2A+ MT389-specific CD8 T cells coexpressed CD25, CD122, and CD127 during persistent PyV infection and after VV-MT389 challenge infection than did CD94/NKG2A− MT389-specific CD8 T cells. Statistical analysis determined that only differences in CD25 expression were significant during secondary proliferation. Thus, increased Ag-driven proliferation of the CD94/NKG2A+ subset may be linked to its enhanced capacity to respond to IL-2 due to elevated expression of high-affinity IL-2R.

IL-2 is a major survival and mitogenic cytokine for CD8 T cells during viral infection (44). To determine whether autocrine IL-2 production might contribute to the preferential proliferation of CD94/NKG2A+ MT389-specific CD8 T cells, we examined the capacity of CD94/NKG2A+ and CD94/NKG2A− MT389-specific CD8 T cells to make IL-2 upon ex vivo MT389 peptide stimulation. Using intracellular IFN-γ production to identify MT389-specific CD8 T cells, we found that ~80% of IL-2 production originated from the CD94/NKG2A+ expressing CD8 T cells (Fig. 5B), suggesting that autocrine synthesis of IL-2 combined with increased expression of IL-2 receptors may contribute to the increased proliferative capacity of the CD94/NKG2A+ MT389-specific CD8 T cells.
Distinct populations of Ag-specific memory T cells that differ according to trafficking properties, effector capabilities, and proliferative potential have been identified in a variety of microbial infections (4). The most commonly used classification scheme demarcates memory T cells into subpopulations based on cell surface expression of the CD62L-selectin lymph node homing molecule and the CCR7 chemokine receptor. Central memory T cells are CD62L<sup>high</sup> CCR7<sup>high</sup>, lack immediate effector activity and proliferate well whereas effector memory T cells are CD62L<sup>low</sup> CCR7<sup>low</sup>, exhibit immediate effector activity, but proliferate poorly (7). Despite their widespread usage, strict correlation between these phenotypically defined memory T cell subsets with effector functionality and proliferative potential does not uniformly apply to memory T cells generated in response to different infections (14). Additionally, the lineage relationship between central and effector memory T cell populations is unclear (1, 5, 8, 45). Another complication is that memory T cells in persistent viral infections are phenotypically distinct from those generated in response to infections that are completely resolved (10–12, 46–52). Thus, classification criteria for memory T cells in the context of persistent infection depart from those used to categorize memory T cells from acutely cleared infections. The strong association between expression of the CD94/NKG2A iNKR on PyV-specific CD8 T cells during persistent infection and their proliferative potential suggest that iNKR expression may be used to define functionally distinct subsets of memory CD8 T cells in persistently infected hosts.

The proportion of CD8 T cells expressing CD94/NKG2A has been shown to increase over time in hosts infected by a variety of pathogens (16, 17). Unlike KIR and Ly49 molecules, commitment to expression of CD94/NKG2A has been shown to be a clonal attribute (53). The similarities in fine antigenic specificity profiles between MT389-specific CD8 T cells differing in CD94/NKG2A expression (Fig. 1C) is consistent with this finding. CD94/NKG2A expression is maintained long-term on virus-specific CD8 T cells in persistent infections (26, 27), but expression declines slowly on memory CD8 T cells elicited by acutely resolved infections (25, 26); this has led to speculation that repeated TCR stimulation is required to maintain expression of this iNKR. In persistent PyV-infected C3H/HeN mice, there is little heterogeneity in CD62L and CCR7 surface expression, with >90% of MT389-specific CD8 T cells from spleen, liver, and lung having CD62L<sup>low</sup> CCR7<sup>low</sup> expression profiles (37). The long-term persistence of PyV DNA in multiple organs, together with evidence that priming of naive virus-specific CD8 T cells occurs during persistent infection (54, 55), indicates that PyV-specific CD8 T cells repetitively encounter viral Ag during the persistent phase of PyV infection.

Enhanced proliferative capacity of CD94/NKG2A<sup>+</sup> MT389-specific CD8 T cells, combined with continuous priming and expansion of naive precursors, provide an explanation for the preferential maintenance of CD94/NKG2A<sup>+</sup> MT389-specific CD8 T cells during the persistent phase of PyV infection. CD94/NKG2A<sup>+</sup> MT389-specific CD8 T cells do not appear to contribute to preferential accumulation of the CD94/NKG2A<sup>+</sup> population, as cognate Ag rechallenge of persistence phase CD94/NKG2A<sup>+</sup> MT389-specific CD8 T cells does not induce surface expression of CD94/NKG2A (Figs. 1, 3, and 4). Molecular evidence that phenotypically similar effector and memory CD8 T cells can express markedly different transcriptional profiles (56) is consistent with our findings that CD94/NKG2A<sup>+</sup> MT389-specific CD8 T cells during persistent and acute infection, although indistinguishable by surface phenotype, differ in sensitivity to Ag-mediated up-regulation of this iNKR. In this connection, the capacity for CD94/NKG2A induction on human CD8 T cells has been shown to be associated with T cell differentiation status (57).

The accumulation of CD94/NKG2A<sup>+</sup> MT389-specific CD8 T cells during persistent PyV infection may derive from negative
signaling by this iNKR. A frequently proposed scenario is that CD94/NKG2A receptor engagement dampens TCR-mediated activation signals to an extent that confers protection against activation-induced cell death resulting from repetitive Ag encounter during persistent infection, while providing sufficient signal strength to permit proliferation. This concept fits the negative feedback model proposed by Jabri et al. (53) that proposes that TCR engagement up-regulates CD94/NKG2A expression, which in turn, negatively regulates TCR activation, such that CD94/NKG2A expression falls, T cell proliferation wanes, and the T cell returns to a state of heightened readiness. This model fits the observation that surface CD94/NKG2A expression by antiviral CD8 T cells eventually drops after resolution of acute infection (25, 26).

Persistent exposure to Ag, as in PyV infection, would maintain long-term expression of CD94/NKG2A; this expression would be predicted to modify TCR-mediated activation signals on CD94/NKG2A+ CD8 T cells to prevent clonal exhaustion or immunopathological consequences of excessive antiviral effector activity. We found that re-exposure to cognate Ag increased the mean fluorescence intensity of CD94/NKG2A expression on CD94/NKG2A+ MT389-specific CD8 T cells both in vitro (Fig. 3, top left) and in vivo (data not shown); this elevated per cell expression of CD94/NKG2A may be necessary to further attenuate TCR-mediated activation signals on previously activated PyV-specific CD8 T cells.

Based on our data, we propose that expression of CD94/NKG2A delineates antiviral CD8 T cells into subpopulations based on proliferative potential during persistent infection. CD94/NKG2A expression, via its ability to dampen TCR-mediated activation signals, maintains a population of intermediately differentiated, self-renewing PyV-specific CD8 T cells that do not express CD94/NKG2A proliferate poorly and have greatly diminished IL-2 production; these traits are associated with an increasingly differentiated, or “exhausted” population incapable of effective reactivation by loss of CD94/NKG2A expression. Because persistent PyV infection triggers a developmental program in naive cells.

Recent studies suggest that robust proliferative capacity is associated with protective immunity (14, 58). Linking the proliferative potential of memory T cells with the extent of their differentiation instead of defining memory T cell subsets by effector functionality (e.g., cytotoxicity and cytokine production) or anatomic localization may clarify relationships between memory T cell subsets, especially in the case of persistent infections. The ability to distinguish the proliferative capabilities of memory CD8 T cells based on expression of CD94/NKG2A receptors could aid in designing strategies to combat persistent viral infections.

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Disclosures

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References


