Viral and Bacterial Infections Induce Expression of Multiple NK Cell Receptors in Responding CD8+ T Cells

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NK cells express several families of receptors that play central roles in target cell recognition. These NK cell receptors are also expressed by certain memory phenotype CD8$^+$ T cells, and in some cases are up-regulated in T cells responding to viral infection. To determine how the profile of NK receptor expression changes in murine CD8$^+$ T cells as they respond to intracellular pathogens, we used class I tetramer reagents to directly examine Ag-specific T cells during lymphocytic choriomeningitis virus and Listeria monocytogenes infections. We found that the majority of pathogen-specific CD8$^+$ T cells initiated expression of the inhibitory CD94/NKG2A heterodimer, the KLRG1 receptor, and a novel murine NK cell marker (10D7); conversely, very few Ag-specific T cells expressed Ly49 family members. The up-regulation of these receptors was independent of IL-15 and persisted long after clearance of the pathogen. The expression of CD94/NKG2A was rapidly initiated in naive CD8$^+$ T cells responding to peptide Ags in vitro and on many of the naive T cells that proliferate when transferred into lymphopenic (Rag-1$^{-/-}$) hosts. Thus, CD94/NKG2A expression is a common consequence of CD8$^+$ T cell activation. Binding of the CD94/NKG2A receptor by its ligand (Qa-1b) did not significantly inhibit CD8$^+$ T cell effector functions. However, expression of CD94 and NKG2A transgenes partially inhibited early events of T cell activation. These subtle effects suggest that CD94/NKG2A-mediated inhibition of T cells may be limited to particular circumstances or may synergize with other receptors that are similarly up-regulated. The Journal of Immunology, 2002, 169: 1444–1452.
Materials and Methods

Mice

C57BL/6j (B6, H-2b), BALB/c (BALB/c, H-2d), and B6-Rag-/- mice were purchased from The Jackson Laboratory (Bar Harbor, ME). B6-Ly5.1 congenic mice (catalog name, B6-Ly5.2/Cr) were purchased from Charles River Laboratories (Frederick, MD). CD94 transgenic mice (16), OT-1 TCR-transgenic mice (17), Dd/-/- mice (18), and II-15/-/- mice (19) were all maintained on the B6 background.

NGK2A transgenic mice were described in greater detail elsewhere (A. M. Jamieson and D. H. Raulet, unpublished data). In brief, NGK2A
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CD42 cDNA was cloned into the first class I promoter/IGm enhancer expression cassette (20). Transgenic founders were backcrossed to B6 at least four times, and mice were crossed to CD94 transgenic mice to yield CD94/NGK2A double-transgenic mice. Double-transgenic mice expressed the CD94/NGK2A heterodimer on the surface of all NK cells and T cells (see Fig. 8 and data not shown); these cell populations appeared to develop normally, although peripheral CD8+ T cell numbers were reduced to 60% of normal.

Viral and bacterial infections

LCMV Armstrong CA1371 strain and clone 13 variant stocks were grown in BHK-21 cells and quantitated by plaque assay on Vero cell monolayers as previously described (21). For acute infections mice were infected i.p. with 2 x 10^5 PFU of LCMV Armstrong. Chronic LCMV infections were established by injecting mice i.v. with 2 x 10^5 PFU of LCMV clone 13. In some cases, mice were rechallenged i.v. with 3 x 10^7 PFU LCMV Armstrong at least 2 mo after clearance of the primary infection.

For primary Listeria infections, BALB/c mice were infected i.v. with 3 x 10^6 CFU of Listeria monocytogenes 10403S (kindly provided by D. Portnoy, University of California, Berkeley, CA). In some cases, mice were rechallenged i.v. with 10^7 CFU at least 1 mo after clearance of the primary infection.

Generation of 10D7 mAb and MHC class I tetramers

10D7 mAb (Syrian hamster IgG) was generated using protocols described previously (22). Briefly, hamsters were immunized five times in the footpad with activated NK (day 10 A-LAK) cells from B6 mice. Popliteal lymph node cells were fused with the P3 x 63-Ag.653 murine myeloma (TIB-9; American Type Culture Collection, Manassas, VA), and hypoxan-thine/aminopterin/thymidine-resistant hybridomas were screened for Abs specific for NK cell surface proteins. 10D7 mAb was purified from supernatants by 45% ammonium sulfate precipitation following by size exclusion chromatography. The following tetrameric MHC class I-peptide complexes were produced as described previously in Esherichia coli in association with human β2-microglobulin (23–25; D5/gp33–41 (LCMV), D7/nucleoprotein (NP)96–104 (LCMV), and K/β2-microglobulin 

Genetic experiments of NK cell receptors by Ag-specific CD8+ T cells

Where applicable, anti-Ly5.2 mAb (B6 specific) or anti-Ly5.1 mAb (B6-Ly5.1 specific) was used to distinguish donor from host cells.

CTL lines and effector function assays

Derivation of the cloned CTL line 30NX/B10-1 has been described (29). A CTL line specific for OVA peptide OVA(257-264) was used to measure CTL activity of the -1 T cell line, using an E/T ratio of 4:1 and the indicated concentrations of OVA, N6 (SIFNKL), or G4 (SIIGIEFK).

Direct ex vivo CTL activity was measured using splenocytes from B6 mice infected with LCMV (Armstrong) 8 days previously. Peptide-loaded EL-4 cells and EL-4/Qa-1 transductants were used as target cells at an E/T ratio of 12:1. Standard 4-h 3Cr release assays were performed in the presence of the indicated concentrations of LCMV gp33 peptide (KAVYNFATM).

EL-4/Qa-1 stimulators were generated by subcloning Qa-1β cDNA into a MHC-IRES-GFP retroviral vector, transducing the EL-4 lymphoma (H-2b, TIB-39; American Type Culture Collection) and using a cell sorter to isolate clones with high surface expression of Qa-1β, using methods described previously (13). To measure CTL cytokine secretion, stimulator cells were preincubated for 3 h in 96-well plates (5 x 10^5 cells/well) with the indicated concentration of gp276 peptide, followed by three washes to remove free peptide. In some cases, stimulator cells were preincubated with 50 μM Qdm peptide for 12 h before addition of gp276 peptide to maximize Qa-1β expression. A total of 2 x 10^5 gp276-specific CTL were added to each well of Ag-pulsed stimulators. Cells were cultured for 5 h in the presence of brefeldin A, followed by cell staining for CD8 and intracellular TNFα using the Cytotox/Cytoperm Plus kit (BD PharMingen).

T cell activation assay

Stimulator splenocytes were irradiated (2500 Gy) and incubated in 96-well plates (10^5/well) with the indicated concentrations of staphylococcal enterotoxin B (SEB; Toxin Technology, Sarasota, FL) for 3 h at 37°C, followed by three washes to remove unbound SEB. Enriched T cells (nonlymphoid nonadherent spleen and lymph node cells) from CD94/NGK2A double-transgenic mice or nontransgenic littermates were labeled with CFSE (as above) and 3 x 10^5 responders were added to each well of stimulator cells. After culturing for 12 h, CD69 surface expression on responder CD8+ T cells was measured by flow cytometry.

Results

Viral and bacterial infections induce long-term surface expression of NK cell receptors by Ag-specific CD8+ T cells

Acute infection with LCMV (Armstrong strain) triggers a massive expansion of CD8+ T cells specific for viral epitopes, and these cells are crucial for clearance of the virus (30). Using class I tetramers folded with LCMV-derived peptides (24), we directly visualized LCMV-specific CD8+ T cells from B6 mice and assessed the expression of various NK cell receptors during and after viral infection.

Strikingly, LCMV infection induced the expression of the inhibitory CD94/NGK2A receptor in essentially all CD8+ T cells specific for an immunodominant viral epitope (Fig. 1). Most CD8+ T cells not specific for this epitope also up-regulated CD94/NGK2A. It is likely that these cells are also responding specifically to the virus, as it has been shown that the height of the immune response >50% of peripheral CD8+ T cells are LCMV specific.

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The proliferation did not trigger up-regulation of any of the NK cell receptors gradually decreased on long-term memory cells (Fig. 2). Similarly, we observed that KLRG1 was expressed at high levels on ~86% of gp33-specific CD8+ T cells on day 9 postinfection (Fig. 1). In addition, the percentage of KLRG1-bearing memory cells gradually decreased over time (Fig. 1 and data not shown), indicating a down-regulation of surface expression or a selective loss of KLRG1-bearing memory cells. Interestingly, KLRG1 expression on gp33-specific CD8+ T cells was bimodal, with a distinct population of cells remaining KLRG1 low or negative. This differed from the expression of CD94/NKG2A (Fig. 1) and 10D7 (described below in this section), which appeared to be homogeneously expressed by almost all Ag-specific cells.

The expression of Ly49 receptors was also examined. Each of the inhibitory Ly49 family members is expressed on 4–25% of memory phenotype CD8+ T cells in uninfected B6 mice (3). During LCMV infection, a small fraction of virus-specific CD8+ T cells was found to express the inhibitory receptors Ly49G2 (15), -A, -C, -F, or -I (Fig. 1 and data not shown). For each Ly49 molecule, this fraction is similar to (or lower than) the fraction of memory CD8+ T cells found in uninfected mice expressing the same receptor. Thus, only a small percentage of LCMV-specific effector cells express Ly49 receptors, and this percentage does not change significantly in the memory population (Fig. 2A).

We have generated a novel mAb, 10D7, that recognizes a cell surface molecule expressed by essentially all NK cells (Fig. 3A) and memory CD8+ T cells (Fig. 3B). Low or negligible levels of 10D7 were found on the cell surface of naive CD8+ T cells, CD4+ T cells, and B cells (Fig. 3B). We found that LCMV infection induced a striking up-regulation of the 10D7 receptor on all gp33-specific CD8+ T cells (Fig. 1). 10D7 surface expression peaked slightly earlier than that observed for the CD94/NKG2A receptor (day 7 vs day 9) and began to gradually decrease immediately thereafter (Fig. 2A). The identity of the protein recognized by the 10D7 mAb is as yet unknown; however, we have excluded many molecules 

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**TABLE 1.** Expression of NK cell receptors by LCMV-specific CD8+ T cells. B6 mice were infected with LCMV Armstrong. At the indicated times after primary infection or rechallenge, splenocytes were stained with DOPgp33–41 tetramer and mAbs specific for CD8+ and the indicated NK cell receptors or isotype control mAb. “Ly49” denotes a mixture of anti-Ly49 mAbs (14B11, 4D11, and JR9-318). Gated CD8+ T cells are shown. Data are representative of at least three mice analyzed at each time point.

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**FIGURE 1.** Expression of NK cell receptors in CTL.

Kinetic analysis revealed that expression of CD94/NKG2A was initiated early and persisted on the surface of gp33-specific memory CD8+ T cells long after acute LCMV infection. CD94/NKG2A up-regulation was observed on day 5, the first day that LCMV-specific cells could be detected in appreciable numbers (Fig. 1). Surface expression peaked on day 9 postinfection, which corresponds to the height of the CTL response, and then remained at this level for at least a month (Fig. 2A). This level of surface expression was similar to, but slightly lower than, that observed on the surface of CD94/NKG2A+ NK cells (data not shown). LCMV-specific memory T cells continued to express the CD94/NKG2A receptor 6 mo after infection; however, the surface expression levels gradually decreased on long-term memory cells (Fig. 2A). Maximal surface expression on memory CD8+ T cells was restored upon secondary LCMV infection (Fig. 2A).

A recent report demonstrated that many CD8+ T cells during acute viral infections express the KLRG1 NK cell receptor (6). Similarly, we observed that KLRG1 was expressed at high levels on ~86% of gp33-specific CD8+ T cells on day 9 postinfection (Fig. 1). In addition, the percentage of KLRG1-bearing memory cells gradually decreased over time (Fig. 1 and data not shown), indicating a down-regulation of surface expression or a selective loss of KLRG1+ memory cells. Interestingly, KLRG1 expression on gp33-specific CD8+ T cells was bimodal, with a distinct population of cells remaining KLRG1 low or negative. This differed from the expression of CD94/NKG2A (Fig. 1) and 10D7 (described below in this section), which appeared to be homogeneously expressed by almost all Ag-specific cells.

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NK cell receptors and markers of T cell activation (see Discussion).

To address whether up-regulation of these receptors occurs in other CD8+ T cell responses, we also examined epitope-specific CD8+ T cells from B6 mice infected with LCMV clone 13 and BALB/c mice infected with the intracellular bacterium L. monocytogenes. In contrast to LCMV Armstrong, the macrophage-tropic LCMV isolate clone 13 results in a persistent infection (21). As with acute LCMV infection, clone 13 infection induced the expression of CD94/NKG2A, KLRG1, and 10D7 receptors, but not Ly49 receptors, on responding CD8+ T cells (Fig. 2B). Notably, cell surface expression of these receptors appeared to remain high on Ag-specific T cells 101 days after LCMV clone 13 infection. The same NK cell receptors were up-regulated in CD8+ T cells responding to Listeria infection (Fig. 2C), with kinetics similar to what was observed during LCMV Armstrong infection. The surface expression levels of each of these receptors during Listeria infection were significantly lower than during LCMV Armstrong infection; however, high-level surface expression did occur following secondary infection (Fig. 2C). Thus, both viral and bacterial infections induce up-regulation of these NK cell receptors on Ag-specific CD8+ T cells.

**Naive CD8+ T cells initiate CD94/NKG2A expression in vitro**

We investigated whether stimulation of naive CD8+ T cells with cognate Ag was sufficient to initiate NK cell receptor expression. CD8+ T cells specific for an OVA peptide (OVAp) in the context of Kb were isolated from B6-OT-1 TCR-transgenic mice (17) and were cultured with OVAp-pulsed stimulator cells. Before culturing, the OT-1 CD8+ T cells were labeled with the intracellular dye CFSE to allow the visualization of cell divisions. As seen in Fig. 4A, expression of the CD94/NKG2A receptor was initiated early, at approximately day 2 poststimulation. Cell surface levels of CD94/NKG2A gradually increased over time, and they increased further following restimulation. Uniform, high levels of receptor expression were observed after a third in vitro stimulation (Fig. 4B, left panel). A similar up-regulation of the 10D7 receptor was observed (data not shown). Conversely, it has been documented that KLRG1 is not up-regulated in T cells stimulated in vitro (6, 32).

Interestingly, all OT-1 T cells initiated CD94/NKG2A expression to a similar degree, independent of the number of cell divisions (Fig. 4A, see day 2 or 3 postinfection). This was true even for...
The 30-fold decrease in Ly49 for an H13a-derived peptide in the context of Db. Cells were
regulation of NK cell receptors occurs on most or all murine CD8 receptors following antigenic stimulation. It is very likely that up-
receptors on murine CD8
4-fold decreases in CD44 high CD8
for the normal development or maintenance of these cell popula-
tions. In addition, human CD8
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those Vα2+ cells on day 2 or 3 with the highest level of intracellular CFSE, which were cells that had not yet divided (determined by equating to nondividing CFSE Vα2+ cells in the same cultures). Therefore, the onset of CD94/NKG2A expression preceded cell division. Taken together, these data demonstrate that naive CD8 T cells initiate de novo expression of the CD94/NKG2A receptor following antigenic stimulation. It is very likely that up-regulation of NK cell receptors occurs on most or all murine CD8 T cells restimulated in vitro, as we have observed analogous expression of CD94/NKG2A and 1D7 on 11 independent CD8 T cell lines and clones (Fig. 4B and data not shown).

**NK cell receptor up-regulation occurs in the absence of IL-15**

Mice lacking IL-15 or the high-affinity IL-15R have reduced numbers of NK cells, CD1-restricted NKT cells, and memory phenotype CD8 T cells (19, 33), demonstrating the importance of IL-15 for the normal development or maintenance of these cell populations. In addition, human CD8 T cells up-regulate the CD94/NKG2A receptor upon antigenic stimulation in vitro, but only upon the addition of exogenous IL-15 or TGF-β (34, 35). Therefore, we addressed the role of IL-15 in the expression of NK cell receptors on murine CD8 T cells.

Examination of uninfected IL-15-deficient mice revealed an almost complete absence of memory phenotype CD8 T cells that express Ly49 family members (Fig. 5A); this population normally makes up over one-quarter of CD44highCD8+ T cells in B6 mice (3). The 30-fold decrease in Ly49 CD8 T cells far exceeds the ~4-fold decreases in CD44highCD8+ T cells (Fig. 5A) and CD1-restricted NKT cells (19). We next infected IL-15-deficient mice with LCMV Armstrong and examined the expression of NK cell receptors on LCMV-specific CD8 T cells on day 9 postinfection.

Although the overall CD8+ T cell expansion in IL-15-deficient mice was reduced (13.6 ± 7% of total splenocytes compared with 37.8 ± 7% in wild type), the fractions of CD8+ T cells specific for LCMV epitopes gp33 and NP396 were very similar between IL-15-deficient and wild-type mice. Unexpectedly, the up-regulation of CD94/NKG2A, KLRG1, and 1D7 on virus-specific CD8+ T cells was not significantly affected by the absence of IL-15 (Fig. 5B). These results demonstrate that IL-15 plays a critical role in the appearance of Ly49+CD8+ T cells but is unnecessary for the expression of other NK cell receptors in CD8+ T cells responding to viral infection.

**Up-regulation of CD94/NKG2A and KLRG1 receptors on CD8+ T cells can occur during homeostasis-driven proliferation**

It has been demonstrated that naive CD8+ T cells transferred to hosts deficient in lymphocytes, such as sublethally irradiated mice or mice lacking lymphocyte compartments due to depletion or genetic manipulation, will proliferate in response to low-affinity self peptides (36–39). This T cell activation and expansion in the absence of cognate peptide is hypothesized to be a means of maintaining homeostasis of the T cell compartment. We investigated whether homeostatic proliferation of CD8+ T cells was sufficient to induce the expression of NK cell receptors.

Naive phenotype CD8+ T cells were transferred to B6-Rag-1−/− hosts, which are lymphopenic due to a complete absence of B and T lymphocytes. To serve as a negative control in which T cells do not proliferate or become activated, cells were also transferred to normal unirradiated mice. At an early time point (day 6 postransfer), many donor-derived CD8+ T cells isolated from lymphopenic recipients had acquired a CD44high phenotype (37, 38, 40), but NK cell receptor expression on these cells was not observed (data not shown). However, by day 21 postransfer a sizable fraction of donor CD8+ T cells transferred to B6-Rag-1−/− hosts expressed high levels of NK cell receptors CD94/NKG2A.

**FIGURE 4.** Ag-specific initiation of CD94/NKG2A expression on CD8+ T cells in vitro. A, Nylon wool nonadherent splenocytes from OT-1 TCR-transgenic mice were labeled with CFSE fluorescent dye and were stimulated in vitro with irradiated B6 splenocytes pulsed with OVA323 peptide. At the indicated time points poststimulation, viable cells were isolated by Histopaque 1119 gradient and stained with anti-Vα2 (specific for the OT-1 TCR) and anti-NKG2A mAbs. Gated Vα2+ cells are shown. Results are representative of three separate experiments. B, NKG2A surface expression was determined on CTL lines OT-1 (Kb/OVA peptide specific) and LCMV gp276 (Dp/gp276 specific), and on the CTL clone 30NX/B10-1 (specific for an H13a-derived peptide in the context of Dq). Cells were stained with anti-CD8 and an isotype control mAb (solid lines) or anti-NKG2A mAb (filled histograms). Gated CD8+ cells are shown.

**FIGURE 5.** NK cell receptor expression by CD8+ T cells in the absence of IL-15. A, Nylon wool nonadherent splenocytes from B6 and IL-15-deficient mice were stained with anti-CD8, anti-CD44, and a mixture of anti-Ly49 mAbs (see Fig. 1); gated CD8+ cells are shown. B, B6 and IL-15-deficient mice were infected with LCMV Armstrong, and splenocytes were stained (day 8 postinfection) with class I tetramers and mAbs specific for CD8 and the indicated NK cell receptors. Data are represented as the mean increase (over control mAb) in log MFI (± SD, n = 4 mice per group) of gated CD8+Dp/gp33–41 tetramer+ cells (see Fig. 2).
(an average of 49%) and KLRG1 (an average of 40%) (Fig. 6). It is unlikely that these cells expanded from preexisting CD94/NKG2A− or KLRG1− T cells, as CD44high and CD49/NKG2+ cells were selectively removed from the donor cell population before transfer. 10D7 and Ly49 receptors did not appear to be significantly up-regulated in B6-Rag-1−/− hosts, as the modest shifts in fluorescence seen in these cases (compared with cells from unirradiated hosts) were similar to the shift seen using an isotype control mAb (Fig. 6). Thus, homeostasis-driven proliferation can induce CD94/NKG2A and KLRG1 expression on CD8+ T cells, even without high-affinity TCR binding.

**CD94/NKG2A-mediated inhibition of CTL effector functions cannot be detected**

Binding of Qa-1 ligand by CD94/NKG2A mediates potent inhibition of NK cell killing (41). Therefore, we tested whether this interaction could inhibit peptide-specific CTL cytokine secretion and target lysis. After repeated in vitro stimulations, OVAp-specific and gp276-specific CTL lines express uniformly high levels of CD94/NKG2A (see Fig. 4B). Target cells derived from Dβ-deficient mice (Dβ−/−) lack the Qdm peptide presented by Qa-1 that is required for CD94/NKG2A recognition and are therefore expected to not inhibit CD94/NKG2A+ effector cells (42). However, CTL specific for OVAp in the context of Kβ lyzed OVAp-coated B6 and Dβ-deficient lymphoblasts equivalently (Fig. 7, left panel). Furthermore, the presence of Qa-1/Qdm ligand did not inhibit lysis of targets presenting OVAp variants N6 and G4, which are weak agonists of OVAp-specific CTL (43).

To address whether higher surface expression of Qa-1 is necessary to trigger inhibition of CD8+ T cells, EL-4 cells (H-2b) were transduced with a Qa-1β expression construct. The EL-4/Qa-1 transductants express high levels of Qa-1 on the cell surface, compared with the low levels expressed by normal EL-4 cells (Fig. 7B, right panel). Nonetheless, EL-4/Qa-1 transductants presenting gp276 peptide did not inhibit TNF-α production by a gp276-specific CTL line (Fig. 7B, left panel). In addition, EL-4 cells and EL-4/Qa-1 transductants were lysed equivalently by CTL isolated directly from LCMV-infected mice (Fig. 7C), and similar results were obtained in cytokine secretion (TNF-α, IFN-γ) and cytolyis assays using the OVAp-specific CTL line (data not shown). In all assays, preincubating the Qa-1-bearing cells (B6 lymphoblast targets or Qa-1-transduced stimulators) overnight with Qdm peptide, which enhances functional inhibition of NK cells (42), did not result in detectable CTL inhibition (Fig. 7, B and C, and data not shown).

**CD94/NKG2A heterodimers expressed as transgenes can inhibit T cell activation**

A transgenic mouse line expressing a NKG2A cDNA construct was generated and crossed to CD94 transgenic mice (16), resulting in double-transgenic mice with high-level surface expression of the CD94/NKG2A heterodimer on all peripheral NK cells and T cells (Fig. 8A and data not shown). We tested whether the expression of the CD94/NKG2A transgenes in naive T cells could inhibit
The induction of the early activation marker CD69 following TCR-mediated stimulation. T cells from CD94/NKG2A double-transgenic mice or nontransgenic littermates were cultured with stimulator cells coated with SEB. SEB, when presented by class II+ APCs, polyclonally stimulates CD4+ T cells that express reactive TCR Vβ elements (~25% of peripheral CD4+ T cells in B6 mice). However, we found significantly decreased CD69 induction in double-transgenic T cells responding to B6 APC that express the Qa-1/Qdm ligand (Fig. 8B). This inhibition of activation could be attributed to Qa-1 ligand binding by the CD94/NKG2A receptor, as double-transgenic T cells up-regulated CD69 as well as nontransgenic littermates (■ and □) were labeled with CFSE and added. After culturing for 12h, CD69 up-regulation on CFSE-CD4+ T cells was measured by flow cytometry. Data are representative of four separate experiments.

Discussion

CD8+ T cells are key mediators in controlling infections by viruses and intracellular bacteria, and for conferring immunity to these pathogens. Accumulating evidence suggests that inhibitory and stimulatory NK cell receptors can modulate T cell functions in vitro. We show in this report that most Ag-specific CD8+ T cells responding to viral or bacterial infection initiate the expression of several NK cell receptors, consistent with the notion that these receptors are involved in controlling CD8+ T cell activities during immune responses in vivo. Up-regulation of the CD94/NKG2A heterodimer is of particular interest, because this receptor is known to strongly inhibit NK cell function and the receptor ligand is known. We demonstrate in this work that significant up-regulation of CD94/NKG2A requires antigenic stimulation. In line with our data, CD94 transcripts were undetectable by Northern blotting in unstimulated spleen or lymph node populations (44). However, naive CD8+ T cells may express very low levels of CD94/NKG2A receptors, as suggested by RT-PCR experiments (45).

It is interesting that up-regulation of CD94/NKG2A and 10D7 differs in several respects from that of the KLRG1 receptor. First, whereas KLRL1 is not induced in vitro (32) and more than 10 cell divisions are required before KLRL1 surface expression is seen in vivo (6). This demonstrates that unique conditions are required for KLRL1 up-regulation; conversely, CD94/NKG2A and 10D7 expression appear to be general features of CD8+ T cell activation. Second, KLRL1 is not uniformly up-regulated on Ag-specific CD8+ T cells during infection, as ~20% of activated cells remained KLRL1 negative or low (see Fig. 1). A recent report demonstrated that sorted KLRL1+ memory T cells proliferated poorly compared with KLRL1-negative memory cells, thus correlating KLRL1 expression with senescence (6). Because the expression patterns of CD94/NKG2A and 10D7 are not bimodal, these receptors do not likewise segregate functional T cell subsets during LCMV infection.

Surprisingly, we found that the up-regulation of CD94/NKG2A and KLRL1 receptors can occur on some CD8+ T cells in the absence of stimulation by cognate Ag, during homeostasis-driven proliferation in B6-Rag-1−/− hosts. These data suggest the possibility that NK cell receptors play a role in T cell homeostasis, perhaps by helping to inhibit proliferation once homeostasis is achieved. This hypothesis is currently difficult to test experimentally. It is important to note that we did not observe expression of CD94/NKG2A or KLRL1 receptors on T cells transferred to sublethally irradiated hosts (data not shown). The discrepant results from the two types of lymphopenic mice may be related to the observation that T cells transferred to B6-Rag-1−/− hosts, unlike those transferred to irradiated hosts, continue to proliferate indefinitely (40). Thus, in lymphopenic model systems expression of NK cell receptors may only occur in T cells that have undergone prolonged proliferation or are undergoing rapid proliferation—situations that may not occur in irradiated lymphopenic mice, at least at the time points examined.

We found that the surface expression levels of CD94/NKG2A, KLRL1, and 10D7 were lower during primary Listeria infection, as compared with acute LCMV infection. This probably does not reflect a difference between viral and bacterial infections (or B6 vs BALB/c strain differences), as Listeria rechallenge resulted in high surface levels of all three receptors. Rather, we favor the idea that receptor up-regulation is governed by the potency of the CD8+ T cell response. At the height of the immune response to LCMV Armstrong, the spleen contains ~3×10^7 LCMV-specific CD8+ T cells (24). In comparison, spleens from Listeria-infected mice contain ~5×10^6 Ag-specific CD8+ T cells but significantly higher numbers (~8×10^7) after rechallenge (25). Thus, the cell surface levels of induced NK cell receptors may be subject to the extent and/or speed of T cell proliferation. The gradual increase of CD94/NKG2A expression in T cells stimulated and restimulated in vitro (Fig. 4) is consistent with this model. Along these lines, it is perhaps significant that receptor surface expression remains relatively high long after infection with the persistent LCMV variant clone 13 (Fig. 2B); this suggests that chronic stimulation may prevent the gradual receptor down-regulation observed following acute viral and bacterial infections.

The cell surface receptor recognized by the 10D7 mAb is expressed on essentially all NK cells in all mouse strains tested (B6, B10, BALB, SJL, and 129) and is also expressed at slightly lower
levels on all activated and memory (CD44^high^) CD8^+^ T cells (Fig. 3). Tests suggest that the 10D7 epitope is not any of the NK cell-specific Ags previously characterized. The variegated expression of many NK cell receptors eliminates them as candidates, such as KLRG1, CD94/NKG2 heterodimers, and individual Ly49 family members. Cells transfected with other receptors (NK2G2D, NKRP1a, -b, -c and -d) failed to stain with 10D7, eliminating them as 10D7 epitopes (data not shown). In addition, counterstaining NK cells with 10D7 vs individual mAbs to other candidate receptors (DX5, CD44, IL-2R, FcγR, Ly6C, 2B4, etc.) did not result in the staining pattern observed when a pair of labeled Abs bind to the same cell surface molecule, i.e., a double-positive population that clusters along the x = y diagonal of a two-color flow cytometry plot (Fig. 3A and data not shown). Thus, the 10D7 epitope may represent a novel NK cell receptor, and the 10D7^high^ phenotype is a useful marker for all NK cells and activated CD8^+^ T cells in many mouse strains.

We found that IL-15 was dispensable for LCMV-induced up-regulation of CD94/NKG2A, 10D7, and KLRG1 receptors in T cells (Fig. 5B). This result was somewhat unexpected, because IL-15 is thought to be important for NKG2A expression during NK cell development (46). In addition, it was shown that in vitro stimulation of human T cells induced CD94/NKG2A expression, but only when exogenous IL-15 or TGF-β was added to the culture (34, 35). These studies suggest that there are cytokine requirements for CD94/NKG2A up-regulation, but that there may be some redundancy with respect to the particular cytokine(s) required. In contrast, we found that in vitro CD94/NKG2A induction on murine T cells did not require the addition of any exogenous cytokines (Fig. 4). Although this implies that murine and human T cells require different conditions for CD94/NKG2A induction, it is also possible that sufficient levels of the required cytokine(s) were generated in our cultures to mediate receptor up-regulation.

It is unknown what circumstances are necessary for the appearance of the population of Ly49^CD8^+^ T cells found in normal mice. We found that the large majority of memory CTLs specific for different viral and bacterial Ags did not express Ly49 molecules (Figs. 1 and 2). The fact that we did always observe a small subpopulation of Ag-specific Ly49^+^ T cells suggests that Ly49 expression is not directly linked to TCR specificity, unless the Ly49^+^ cells cross-react with undefined Ags. It is noteworthy that Ly49 receptors were also not significantly up-regulated in CD8^+^ T cells during LCMV clone 13 infection (Fig. 2B). This result argues against the hypothesis that Ly49^CD8^+^ T cells may arise as a result of chronic infections (9). It still may be the case that Ly49 induction depends upon the nature of the infectious agent, such as a requirement for a type of Ag presentation that occurs only with certain routes of infection or infected cell types.

Interestingly, Ly49^CD8^+^ T cells are completely absent in IL-15-deficient mice. It is possible that IL-15 plays a direct role in Ly49 receptor up-regulation, but this is difficult to test without knowing the conditions that induce Ly49 receptor expression in T cells in vivo. Notably, IL-15 is not sufficient to trigger Ly49 expression in NK cells during in vitro development, but it may be required (47, 48). Alternatively, Ly49^CD8^+^ T cells may represent a discrete lymphocyte subpopulation that requires IL-15 for development or survival. Finally, it is plausible that the absence of Ly49^CD8^+^ T cells is due to the apparent defect in memory cell maintenance in IL-15-deficient mice. However, we feel this is unlikely, as IL-15-deficient mice have a reduced but significant number of memory phenotype CD8^+^ T cells (19), and some LCMV-specific memory cells persist in IL-15-deficient mice following infection (data not shown).

The human CD94/NKG2A receptor can inhibit T cell clones (49, 50). However, how the CD94/NKG2A receptor affects murine T cell responses in vivo remains more of an open question. We were unable to inhibit CTL effector functions using presenting cells expressing normal (or above-normal) levels of the Qa-1 ligand, even at low doses of antigenic peptide (Fig. 7). We hypothesized that inhibition might only occur in T cells responding to low-affinity peptides, thus focusing the T cell response upon high-affinity Ags. However, CD94/NKG2A ligation did not inhibit the cytolytic response to weak agonists (Fig. 7A). The only situation where we reproducibly observed CD94/NKG2A-mediated inhibition was in the initial activation of naive T cells overexpressing CD94 and NKG2A transgenes (Fig. 8).

However, two recent reports have documented CD94/NKG2A-mediated inhibition of killing by murine CTLs. In one case, non-cytotoxic NKG2^+^CD8^+^ T cells isolated from mice infected with polyoma virus could be induced to lyse target cells by first blocking and internalizing the Qa-1 ligand (11). This discrepancy from our results cannot be explained by differences in CD94/NKG2A levels, as our CTL lines and CTLs isolated from LCMV-infected mice all expressed uniformly high levels of CD94/NKG2A on the cell surface (Figs. 1 and 4B). An alternative possibility is suggested by the finding that polyoma virus-specific CTLs become cytolytic following in vitro restimulation (51), which may indicate that the in vivo environment is necessary for maintaining nonresponsiveness. However, we found that CTLs isolated from LCMV-infected mice were not inhibited by CD94/NKG2A (Fig. 7C). Therefore, reconciling the differing results requires arguing that there is something unique about the CD8^+^ T cells in polyoma virus-infected mice that renders them sensitive to CD94/NKG2A signaling. A second report found that human target cells expressing very high levels of Qa-1 and very low levels of class I were killed poorly by an allospecific CTL line (45). Thus, in this case it appears that significant inhibition requires a combination of conditions, including low levels of Ag, high levels of inhibitory receptor ligand, and perhaps a target cell that does not express murine ligands for costimulatory receptors (e.g., human cells).

There have been similar difficulties in demonstrating the inhibition of murine CTL functions by other endogenously expressed NK cell receptors, such as KLRG1 (6) and Ly49 molecules (3, 15). However, Ly49A expressed as a transgene by all T cells is a potent inhibitor of CD8^+^ T cell killing (52, 53). Similarly, a modest inhibition of CTL killing was observed upon cross-linking of transgenically expressed KLRG1 (7). Thus, these receptors clearly have the potential to dampen TCR-mediated responses, but the results taken together suggest that significant inhibition in vivo may occur only under favorable circumstances, such as high receptor and ligand levels combined with weak antigenic stimulus. Indeed, perhaps simultaneous signaling from multiple inhibitory receptors is necessary to overcome TCR-mediated activation. Because we have found that CD94/NKG2A induction is a general consequence of CD8^+^ T cell activation, it seems likely that the state of CTL nonresponsiveness found in polyoma virus-infected mice is maintained by a variety of mechanisms acting in concert, of which CD94/NKG2A-mediated inhibition may be only one. It also is possible that CD94/NKG2A signaling may play a more significant role in modifying non-effector functions such as CD8^+^ T cell activation, proliferation, or survival. Infectious model systems will prove useful in better describing how NK cell receptors may influence the waxing and waning of the CD8^+^ T cell immune response.

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References


