Viral and Bacterial Infections Induce Expression of Multiple NK Cell Receptors in Responding CD8$^+$ T Cells[1]

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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-1^b) 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.

Natural killer cells use a variety of cell surface receptors to recognize and attack target cells. Many of the best-described NK cell receptors are inhibitory, such as the CD94/NKG2A heterodimer and most members of the murine Ly49 receptor family. These receptors bind to MHC class I ligands on potential target cells and inhibit NK cells from attacking cells that express normal levels of class I (1). Other NK cell receptors are stimulatory and contribute to the activation of NK cells upon binding constitutive or induced ligands on potential targets (2).

Although termed "NK cell receptors," the expression of many of these molecules is not restricted to NK cells. We and others (3, 4) found that a significant fraction of murine CD8$^+$ T cells in the periphery express inhibitory members of the Ly49 receptor family. By several criteria these cells appear to be memory CD8$^+$ T cells (3), but it remains unclear under what circumstances Ly49 receptor expression is initiated. A subset of these cells also shows variable expression of other NK cell receptors, such as the CD94/NKG2A receptor (5), KLRG1 (6, 7), CD49b (recognized by mAb DX5), and NKR-P1 family members (3, 8). Similarly, NK cell receptors are expressed by some human CD8$^+$ T cells, and evidence indicates that signaling by these receptors can modulate Ag-specific T cell responses (9, 10).

It is commonly hypothesized that CD8$^+$ T cells initiate the expression of NK cell receptors during an immune response to fine-tune the TCR-mediated response. Indeed, several NK cell receptors have been recently shown to be up-regulated on murine T cells during viral infections. Looking specifically at the populations of CD8$^+$ T cells responding to different viruses, the majority of these cells expressed high levels of KLRG1 (6) and CD94/NKG2 (11), and 12–40% of these cells expressed modest levels of NKR-P1 and CD49b (8, 12). The stimulatory NKG2D receptor is also induced in all Ag-specific murine CD8$^+$ T cells following in vitro activation (13) or viral infection (14). Whether infection leads to up-regulation of Ly49 family members is not as clear-cut. Only ~2% of lymphocytic choriomeningitis virus (LCMV)[3]-specific CD8$^+$ T cells in infected mice express Ly49G2 (15), which is similar to the percentage of Ly49G2$^+$CD8$^+$ T cells in uninfected mice. However, a significantly larger population of Ly49$^+$CD8$^+$ T cells was reported in mice infected with influenza virus (12).

In this report, we document the expression of several murine NK cell receptors in Ag-specific CD8$^+$ T cells responding to in vivo infections, and we explore the conditions under which receptor expression is initiated. We found that expression of the CD94/NKG2A receptor could be induced in CD8$^+$ T cells under many circumstances, such as during viral and bacterial infections, during in vitro stimulation, in the absence of IL-15, and even during T cell proliferation in the absence of cognate Ag. Additional experiments addressed the effects of CD94/NKG2A receptor ligation on Ag-driven T cell activation and effector functions.

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Received for publication February 28, 2002. Accepted for publication June 3, 2002.

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[1] This work was supported by National Institutes of Health Grants RO1 AI35021 (to D.H.R.), RO1 AI49360 (to A.J.Z.), and R37 AI30048 (to R.A.), and a fellowship from the Cancer Research Institute/Chase Manhattan Bank (to C.W.M.).

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[3] Abbreviations used in this paper: LCMV, lymphocytic choriomeningitis virus; SEB, staphylococcal enterotoxin B; MFI, mean fluorescence intensity; NP, nucleoprotein.

0022-1767/02/$02.00

Materials and Methods

Mice

C57BL/6J (B6, H-2^b), BALB/cJ (BALB/c, H-2^d), and B6-Rag-1$^{-/-}$ 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), D$^{b-/-}$ mice (18), and IL-15$^{-/-}$ mice (19) were all maintained on the B6 background.

NKG2A transgenic mice will be described in greater detail elsewhere (A. M. Jamieson and D. H. Raulet, unpublished data). In brief, NKG2A^{B6} cDNA was cloned into the class I promoter/Igμ 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/NKG2A double-transgenic mice. Double-transgenic mice expressed the CD94/NKG2A heterodimer on the surface of all NK cells and T cells (see Fig. 8*A* 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 injected i.p. with 2×10^5 PFU of LCMV Armstrong. Chronic LCMV infections were established by injecting mice i.v. with 2×10^6 PFU of LCMV clone 13. In some cases, mice were rechallenged i.v. with 3×10^6 PFU LCMV Armstrong at least 2 mo after clearance of the primary infection.

For primary *Listeria* infections, BALB/c mice were injected i.v. with 3×10^3 CFU of *Listeria monocytogenes* 10403S (kindly provided by D. Portnoy, University of California, Berkeley, CA). In some cases, mice were rechallenged i.v. with 10^5 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 × 63-Ag.8.653 murine myeloma (TIB-9; American Type Culture Collection, Manassas, VA), and hypoxanthine/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 followed by size exclusion chromatography. The following tetrameric MHC class I-peptide complexes were produced as described previously in *Escherichia coli* in association with human β_2-microglobulin (23–25): D^b/gp33–41 (LCMV), D^b/nucleoprotein (NP)$_{396-404}$ (LCMV), and K^d/listeriolysin O$_{91-99}$ (*Listeria*). Monomeric complexes were tetramerized by the addition of PE-streptavidin (Molecular Probes, Eugene, OR).

Ab staining

Labeled mAbs specific for the following molecules were purchased: Ly5.1, Ly5.2, NKR-P1c (NK1.1), CD49b (DX5), CD19, Qa-1^b, CD4, TCR Vα2, CD3ϵ, TCR$\alpha\beta$, and TNF-α (BD PharMingen, San Diego, CA); and CD8α and CD44 (Caltag Laboratories, Burlingame, CA). Additional anti-NK receptor mAbs were prepared in our laboratory: NKG2A/C/E (20d5) (26), NKG2A^{B6} (16a11) (16), KLRG1 (2F1) (22), Ly49C/F/H/I (14B11) (27), Ly49A (JR9-318) (28), and Ly49G2 (4D11) (HB-240; American Type Culture Collection). These mAbs were used as direct conjugates to FITC, except for the 20d5-biotin conjugate used in Fig. 4*A*. Before staining with labeled mAbs, cells were preincubated for 20 min with 2.4G2 (HB-197; American Type Culture Collection) hybridoma supernatant to block FcγII/III receptors. Flow cytometry was performed on EPICS XL-MCL machines (Coulter, Hialeah, FL) and data were analyzed on FlowJo software (Tree Star, San Carlos, CA).

Adoptive cell transfers

Donor cells (B6 splenocytes) were enriched for naive CD8$^+$ T cells by passage over nylon wool followed by mAb staining and removal of CD44^{high} and CD94/NKG2$^+$ cells using an autoMACS magnetic cell sorter (Miltenyi Biotec, Auburn, CA). Postsort CD8$^+$ T cells (~40% of total) were >95% CD44^{low} and >99% CD94/NKG2$^-$. A total of 5×10^6 donor cells were injected i.v. into lymphopenic hosts (B6-Rag-1$^{-/-}$ mice) or into nonlymphopenic hosts (unirradiated B6-Ly5.1 mice). Splenocytes from at least three mice of each group were analyzed by flow cytometry on day 21. 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 OVAp (SIINFEKL) in the context of K^b was established by culturing splenocytes from OT-1 TCR-transgenic mice with irradiated B6 splenocytes pulsed with 1 μM OVAp peptide. In some experiments, nylon wool nonadherent OT-1 splenocytes were first labeled with the fluorescent intracellular dye CFSE (Molecular Probes) by culturing the cells (5×10^6/ml) for 10 min at 37°C in PBS containing 0.1 μM CFSE. CTL were restimulated weekly, supplementing the culture with 5% T-STIM supernatant (BD Biosciences, Franklin Lakes, NJ) during the third stimulation and thereafter. A CTL line specific for LCMV gp276 peptide (SGVENPGGYCL) in the context of D^b was established in a similar manner from B6 mice infected 8 days before with LCMV Armstrong. CTL lines expressed uniformly high surface levels of CD94/NKG2A and were used as effectors 5–8 days after restimulation.

Con A blast targets were made by culturing B6 or B6-D$^{b-/-}$ splenocytes for 24 h with 2 μg/ml Con A, followed by Con A neutralization with methyl α-mannopyranoside. Targets were radiolabeled with ^{51}Cr for 1 h and were isolated with Histopaque 1119 (Sigma-Aldrich, St. Louis, MO). Standard 4-h ^{51}Cr release assays were performed to measure CTL activity of the OT-1 T cell line, using an E:T ratio of 4:1 and the indicated concentrations of peptides OVAp, N6 (SIINFNKL), or G4 (SIIGFEKL).

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 ^{51}Cr 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-1^b cDNA into a MSCV-IRES-GFP retroviral vector, transducing the EL-4 lymphoma (H-2^b, TIB-39; American Type Culture Collection) and using a cell sorter to isolate clones with high surface expression of Qa-1^b, using methods described previously (13). To measure CTL cytokine secretion, stimulator cells were preincubated for 3 h in 96-well plates (5×10^5 cells/well) with the indicated concentrations 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-1/Qdm presentation. A total of 2×10^5 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 TNF-α using the Cytofix/Cytoperm Plus kit (BD PharMingen).

T cell activation assay

Stimulator splenocytes were irradiated (2500 cGy) and incubated in 96-well plates (10^6/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 (nylon wool nonadherent spleen and lymph node cells) from CD94/NKG2A double-transgenic mice or nontransgenic littermates were labeled with CFSE (as above) and 3×10^5 responders were added to each well of stimulator cells. After culturing for 12 h, CD69 surface expression on responder CD4$^+$ 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/NKG2A 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/NKG2A. It is likely that these cells are also responding specifically to the virus, as it has been shown that at the height of the immune response >50% of peripheral CD8$^+$ T cells are LCMV specific

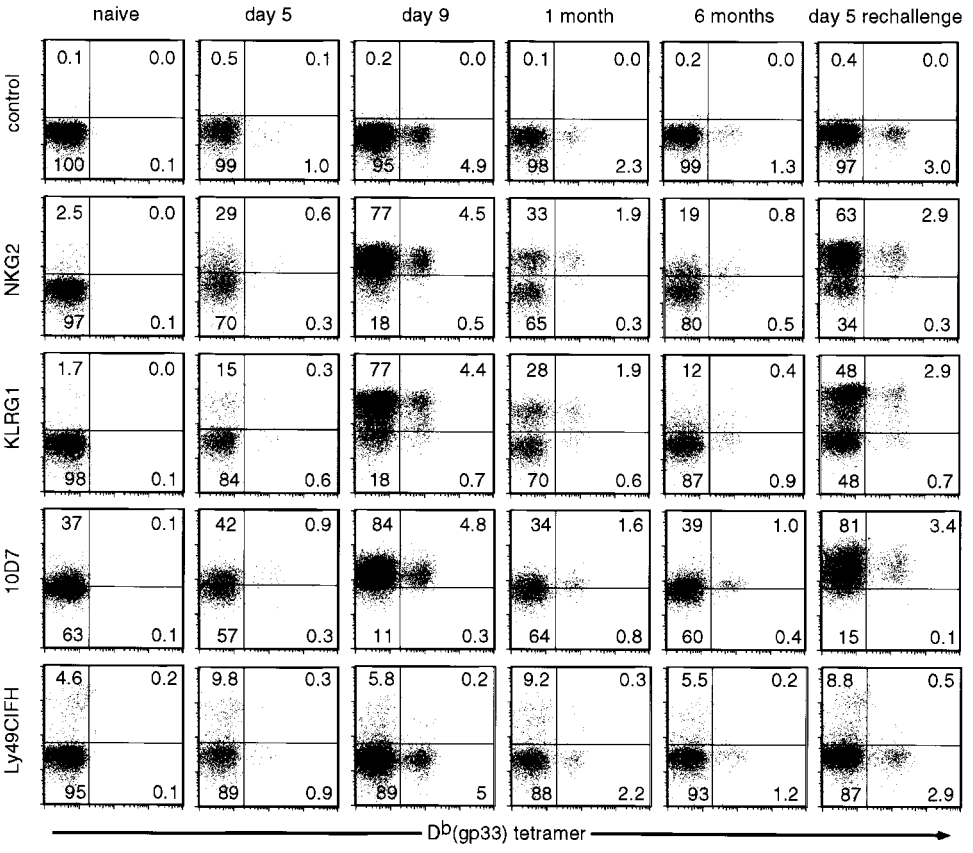

FIGURE 1. Expression of NK cell receptors by LCMV-specific $CD8^+$ T cells. B6 mice were infected with LCMV Armstrong. At the indicated times after primary infection or rechallenge, splenocytes were stained with D^b/gp33–41 tetramer and mAbs specific for CD8 and the indicated NK cell receptors or isotype control mAb. "Ly49" denotes a mixture of anti-Ly49 mAbs (14B11, 4D11, and JR9-318). Gated $CD8^+$ cells are shown. Data are representative of at least three mice analyzed at each time point. Similar results were obtained with $D^b/NP_{396-404}$ tetramer.

(24). In addition, although cytokines induced by infectious agents trigger the Ag-independent "bystander proliferation" of memory $CD8^+$ T cells (31), we found that poly(I:C)-induced bystander proliferation did not trigger up-regulation of any of the NK cell receptors studied (data not shown).

It should be noted that the data shown use the NKG2-specific mAb 20d5, which recognizes NKG2A, -C, and -E (26). However, the same results were obtained with the NKG2A-specific mAb 16a11 (see Fig. 2, *A* and *B*) and the CD94-specific mAb 18d3 (data not shown). These results confirm that the inhibitory CD94/NKG2A heterodimer is up-regulated by virus-specific CTL but do not eliminate the possibility that stimulatory NKG2C or -E receptors may also be expressed. To address this issue, a quantitative assay taking advantage of gene-specific restriction enzymes (26) revealed that NKG2A message represents >90% of the total NKG2 mRNA in virus-specific CTL (data not shown).

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. 2*A*). 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. 2*A*). Maximal surface expression on memory $CD8^+$ T cells was restored upon secondary LCMV infection (Fig. 2*A*).

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.

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. 2*A*).

We have generated a novel mAb, 10D7, that recognizes a cell surface molecule expressed by essentially all NK cells (Fig. 3*A*) and memory $CD8^+$ T cells (Fig. 3*B*). Low or negligible levels of 10D7 were found on the cell surface of naive $CD8^+$ T cells, $CD4^+$ T cells, and B cells (Fig. 3*B*). 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. 2*A*). The identity of the protein recognized by the 10D7 mAb is as yet unknown; however, we have excluded many

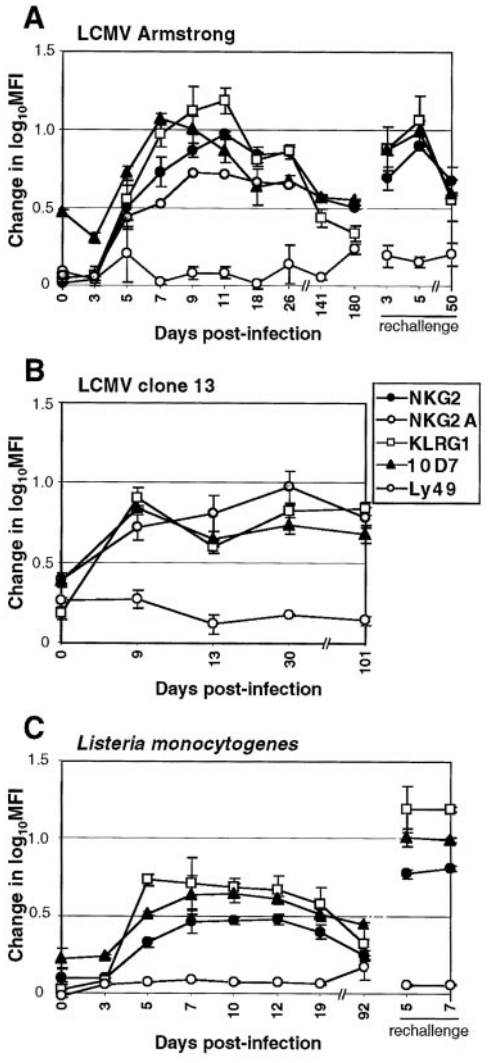

FIGURE 2. NK cell receptor expression by $CD8^+$ T cells responding to viral and bacterial infections. B6 mice were infected with LCMV Armstrong (*A*) or LCMV clone 13 (*B*), and BALB/c mice (*C*) were infected with *L. monocytogenes*. At the indicated time points, splenocytes were stained as in Fig. 1. NKG2 denotes staining with 20d5 mAb, while NKG2A denotes staining with 16a11 mAb. The mean fluorescence intensities (MFIs) for NK receptor mAbs and isotype control mAbs were measured on gated $CD8^+D^b$/gp33–41 $tetramer^+$ cells (*A* and *B*) or $CD8^+$ K^d/listeriolysin $O_{91–99}$ $tetramer^+$ cells (*C*). Gated $CD8^+$ cells were examined on days 0 and 3 due to a lack of detectable $tetramer^+$ cells. Data are displayed as $[\log(MFI^{NKR\ mAb}) - \log(MFI^{control\ mAb})]$, so that a value of "1" represents a one-log shift above the negative control. Values are the mean ± SD from at least three mice at each time point, with the exception of NKG2A (16a11) staining in *A*, which are from single mice at each time point. Note that the time line (*x*-axis) is not to scale.

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. 2*B*). Notably, cell surface expression of these receptors appeared to remain

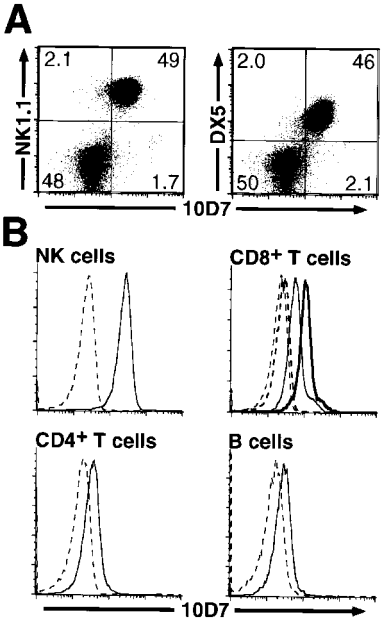

FIGURE 3. Expression of the 10D7 epitope on the surface of lymphocytes. Nylon wool nonadherent splenocytes from 3-mo-old B6 mice were analyzed by flow cytometry. *A*, Counterstaining of 10D7 against NK cell markers NK1.1 (*left panel*) and DX5 (CD49b, *right panel*). Gated CD3-negative cells are shown. *B*, Staining of lymphocyte subsets with 10D7 mAb (solid lines) or control mAb (dashed lines): NK cells ($NK1.1^+CD3^-$); B cells ($CD19^+CD3^-$); $CD4^+$ T cells ($CD4^+CD3^+$); naive $CD8^+$ T cells (thin line, $CD8^+CD44^{low}$); memory $CD8^+$ T cells (thick line, $CD8^+CD44^{high}$).

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. 2*C*), 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. 2*C*). 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 K^b 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. 4*A*, 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. 4*B*, *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. 4*A*, see day 2 or 3 postinfection). This was true even for

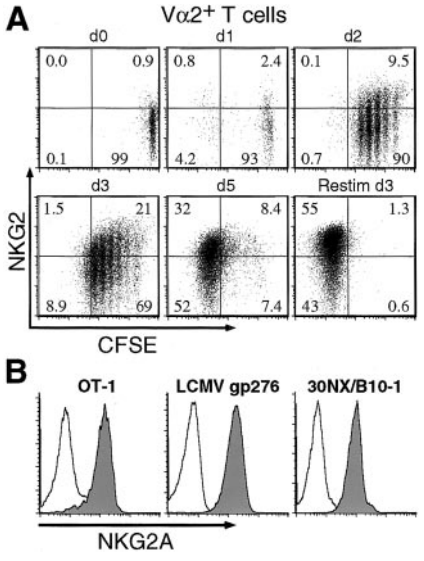

FIGURE 4. Ag-specific initiation of CD94/NKG2A expression on $CD8^+$ T cells in vitro. *A*, Nylon wool nonadherent splenocytes from OT-1 TCR-transgenic mice were labeled with CFSE fluorescent dye and were stimulated in vitro with irradiated B6 splenocytes pulsed with OVAp peptide. At the indicated time points poststimulation, viable cells were isolated by Histopaque 1119 gradient and stained with anti-Vα2 (specific for the OT-1 TCR) and anti-NKG2 mAbs. Gated $V\alpha2^+$ cells are shown. Results are representative of three separate experiments. *B*, NKG2A surface expression was determined on CTL lines OT-1 (K^b/OVAp specific) and LCMV gp276 (D^b/gp276 specific), and on the CTL clone 30NX/B10-1 (specific for an $H13^a$-derived peptide in the context of D^b). Cells were stained with anti-CD8 and an isotype control mAb (solid lines) or anti-NKG2A mAb (filled histograms). Gated $CD8^+$ cells are shown.

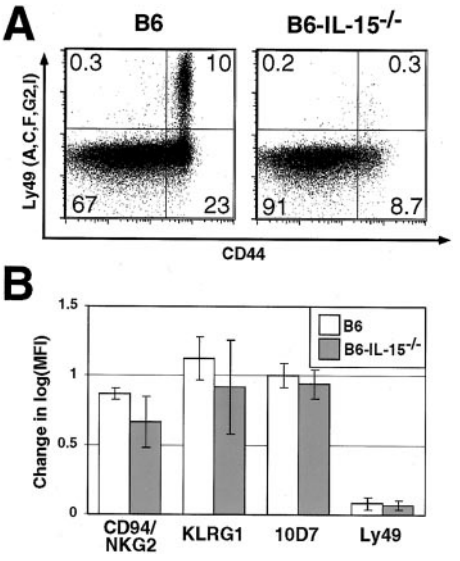

FIGURE 5. NK cell receptor expression by $CD8^+$ T cells in the absence of IL-15. *A*, Nylon wool nonadherent splenocytes from B6 and IL-15-deficient mice were stained with anti-CD8, anti-CD44, and a mixture of anti-Ly49 mAbs (see Fig. 1); gated $CD8^+$ cells are shown. *B*, B6 and IL-15-deficient mice 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 ($\pm$ SD, $n = 4$ mice per group) of gated $CD8^+D^b$/gp33–41 tetramer^+ cells (see Fig. 2).

those $V\alpha2^+$ 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 $CFSE^+V\alpha2^-$ 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 10D7 on 11 independent $CD8^+$ T cell lines and clones (Fig. 4*B* 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. 5*A*); this population normally makes up over one-quarter of $CD44^{high}CD8^+$ T cells in B6 mice (3). The 30-fold decrease in $Ly49^+CD8^+$ T cells far exceeds the ~4-fold decreases in $CD44^{high}CD8^+$ T cells (Fig. 5*A*) 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 $\pm$ 7% of total splenocytes compared with 37.8 $\pm$ 7% in wild type), the fractions of $CD8^+$ cells specific for LCMV epitopes gp33 and NP_{396} were very similar between IL-15-deficient and wild-type mice. Unexpectedly, the up-regulation of CD94/NKG2A, KLRG1, and 10D7 on virus-specific $CD8^+$ T cells was not significantly affected by the absence of IL-15 (Fig. 5*B*). 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 posttransfer), many donor-derived $CD8^+$ T cells isolated from lymphopenic recipients had acquired a $CD44^{high}$ phenotype (37, 38, 40), but NK cell receptor expression on these cells was not observed (data not shown). However, by day 21 posttransfer a sizable fraction of donor $CD8^+$ T cells transferred to B6-Rag-$1^{-/-}$ hosts expressed high levels of NK cell receptors CD94/NKG2A

(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 CD44^{high} and CD94/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. 4*B*). Target cells derived from D^b-deficient mice (D$^{b-/-}$) 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 K^b lysed OVAp-coated B6 and D^b-deficient lymphoblasts equivalently (Fig. 7*A*, *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-2^b) were transduced with a Qa-1^b 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. 7*B*, *right panel*). Nonetheless, EL-4/Qa-1 transductants presenting gp276 peptide did not inhibit TNF-α production by a gp276-specific CTL line (Fig. 7*B*, *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. 7*C*), and similar results were

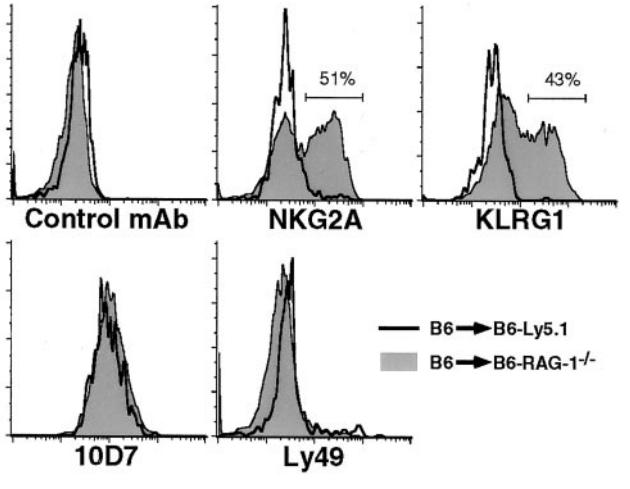

FIGURE 6. Expression of NK cell receptors by T cells transferred to lymphopenic hosts. Enriched naive CD8$^+$ T cells from B6 mice were transferred to normal hosts (unirradiated B6-Ly5.1 mice) or to lymphocyte-deficient hosts (B6-Rag-1$^{-/-}$ mice). On day 21 posttransfer, NK cell receptor expression on splenocytes was measured, using the following gates to identify donor-derived CD8$^+$ T cells: CD8$^+$Ly5.1$^-$ cells (from unirradiated B6-Ly5.1 hosts, solid lines) or CD8$^+$CD3$^+$ cells (from B6-Rag-1$^{-/-}$ hosts, filled histograms). A mixture of anti-Ly49 mAbs was used (see Fig. 1).

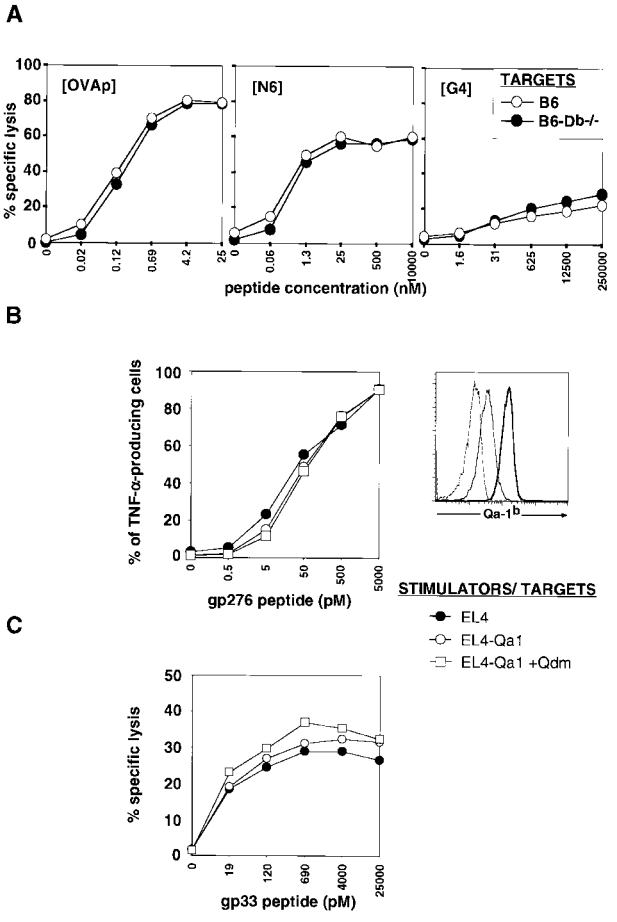

FIGURE 7. Effector functions of CD94/NKG2A$^+$CD8$^+$ T cells. *A*, An OVAp-specific CTL line was tested for the ability to kill Con A blast targets from B6 mice (Qa-1/Qdm$^+$, ○) or D$^{b-/-}$ mice (Qa-1/Qdm$^-$, ●). Targets were coated with the indicated concentrations of agonist peptide (OVAp) or partial agonist OVAp variants (N6, G4). Specific lysis was measured as nonspontaneous ^{51}Cr release from labeled targets. *B*, TNF-α production by a LCMV gp276-specific CTL line was measured following a 5-h stimulation with gp276 peptide-pulsed EL-4 cells (Qa-1^{low}, ●), EL-4/Qa-1 transductants (Qa-1^{high}, ○), or EL-4/Qa-1 transductants preincubated with Qdm peptide (Qa-1^{high}, □). The percentage of CD8$^+$ cells producing TNF-α was determined by intracellular staining. The histogram (*right panel*) shows Qa-1^b expression on EL-4 cells (thin line) and EL-4/Qa-1 transductants (thick line) compared with isotype control mAb staining (dashed line). *C*, Direct ex vivo activity of CTL from LCMV-infected mice was measured in a standard ^{51}Cr release assay. EL-4 cells or EL-4/Qa-1 transductants (as in *B*) served as target cells, coated with the indicated doses of LCMV gp33 peptide.

obtained in cytokine secretion (TNF-α, IFN-γ) and cytolysis 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. 8*A* and data not shown). We tested whether the expression of the CD94/NKG2A transgenes in naive T cells could inhibit

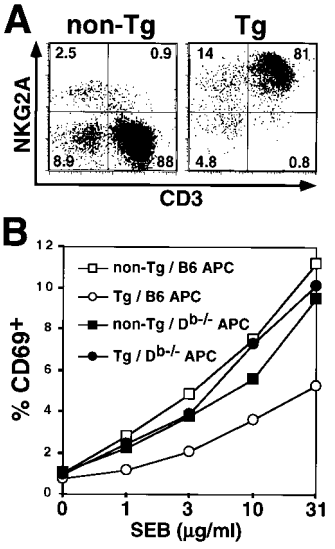

FIGURE 8. Activation of T cells expressing CD94 and NKG2A transgenes. *A*, Nylon wool nonadherent splenocytes from a CD94/NKG2A double-transgenic mouse (Tg) and a nontransgenic littermate (non-Tg) were stained with anti-CD3ε and anti-NKG2 mAbs. *B*, Irradiated stimulator cells from B6 mice (Qa-1/Qdm$^+$, □ and ○) or D$^{b-/-}$ mice (Qa-1/Qdm$^-$, ■ and ●) were preincubated with the indicated concentrations of SEB. Responder T cells from CD94/NKG2A double-transgenic mice (● and ○) or nontransgenic littermates (■ and □) were labeled with CFSE and added. After culturing for 12 h, CD69 up-regulation on CFSE$^+$CD4$^+$ T cells was measured by flow cytometry. Data are representative of four separate experiments.

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. 8*B*). 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 T cells in response to APCs that lack Qa-1/Qdm (D$^{b-/-}$). Using similar assay conditions, the CD94/NKG2A transgenes also partially inhibited T cell proliferation, but this effect was not consistently reproducible (data not shown). It is notable that the inhibition of T cell activation and proliferation were relatively modest, especially when considering that the surface expression of transgenic CD94/NKG2A on T cells is even higher than that of the endogenous receptor on NK cells (Fig. 8*A*).

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, CD94/NKG2A and 10D7 are rapidly induced on naive CD8$^+$ T cells upon in vitro stimulation (Fig. 4 and data not shown), whereas KLRG1 is not induced in vitro (32) and more than 10 cell divisions are required before KLRG1 surface expression is seen in vivo (6). This demonstrates that unique conditions are required for KLRG1 up-regulation; conversely, CD94/NKG2A and 10D7 expression appear to be general features of CD8$^+$ T cell activation. Second, KLRG1 is not uniformly up-regulated on Ag-specific CD8$^+$ T cells during infection, as ~20% of activated cells remained KLRG1 negative or low (see Fig. 1). A recent report demonstrated that sorted KLRG1$^+$ memory T cells proliferated poorly compared with KLRG1-negative memory cells, thus correlating KLRG1 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 KLRG1 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 KLRG1 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, KLRG1, 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 ~5×10^5 Ag-specific CD8$^+$ T cells but significantly higher numbers (~8×10^6) 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. 2*B*); 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 (NKG2D, NKR-P1a, -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-2Rβ, 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. 3*A* 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. 5*B*). 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. 2*B*). 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. 7*A*). 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 4*B*). 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. 7*C*). 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.

Acknowledgments

We thank Parakash Pratibu for assistance with LCMV clone 13 infections and FACS analysis, Laurel Lenz for assistance with *Listeria* infections, and Eric

Pamer and Dirk Busch for providing class I expression vectors and helpful advice on tetramer preparation. We also thank Francois Lemonnier for providing us with $D^{b-/-}$ mice, James Allison for OT-1 mice, Lisa Mendoza for the 30NX/B10-1 CTL clone, and Stephen Jameson for N6 and G4 peptides.

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