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Preferential Survival of CD8 T and NK Cells Expressing High Levels of CD94

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The Qa-1\textsuperscript{b}/Qdm tetramer binds to CD94/NKG2 receptors expressed at high levels on \textasciitilde50\% of murine NK cells. Although very few CD8 T cells from naive mice express CD94/NKG2 receptors, \textasciitilde50\% of CD8 T cells taken from mice undergoing a secondary response against \textit{Listeria monocytogenes} (LM) are CD94\textsuperscript{high} and bind the tetramer. Although CD94\textsuperscript{int} NK cells do not bind the tetramer, CD94\textsuperscript{int} CD8 T cells do, and this binding is dependent on the CD8 coreceptor. We found that the extent of apoptosis in CD8 T and NK cells was inversely related to the expression of CD94, with lower levels of apoptosis seen in CD94\textsuperscript{high} cells after 1–3 days of culture. The difference in CD8 T cell survival was evident as early as 6 h after culture and persisted until nearly all the CD94\textsuperscript{neg/int} cells were apoptotic by 48 h. In contrast, expression of inhibitory Ly-49A,G2,C/I molecules was associated with higher levels of apoptosis. Cross-linking CD94/NKG2 receptors on CD8 T cells from a mouse undergoing an LM infection further reduced the percentage of apoptotic cells on the CD94-expressing populations, while cross-linking Ly-49I had no effect on CD8 T cells expressing Ly-49I. Cross-linking CD3 on CD8 T cells from a mouse undergoing a secondary LM infection increases the extent of apoptosis, but this is prevented by cross-linking CD94/NKG2 receptors at the same time. Similar results were observed with NK cells in that the CD94\textsuperscript{high} population displayed less apoptosis than CD94\textsuperscript{int} cells after 1–3 days in culture. Therefore, the expression of apoptosis, but this is prevented by cross-linking CD94/NKG2 receptors at the same time. Similar results were observed with NK cells in that the CD94\textsuperscript{high} population displayed less apoptosis than CD94\textsuperscript{int} cells after 1–3 days in culture. Therefore, the expression of CD94/NKG2 is correlated with a lower level of apoptosis and may play an important role in the maintenance of CD8 T and NK cells. The \textit{Journal of Immunology}, 2003, 170: 1737–1745.

Human and murine NK cells express families of receptors that bind to class I molecules. In humans, killer-inhibitory receptors (KIRs)\textsuperscript{1} are members of the Ig superfamily, while CD94/NKG2 heterodimers belong to a group of C-type lectins. Although mice express CD94/NKG2 receptors, they lack KIR molecules and instead express Ly-49 homodimers, which also belong to the group of C-type lectins (1). In mice, CD94/NKG2 receptors are expressed on \textasciitilde50\% of adult splenic NK cells and a small subset of CD8 T cells (2, 3). These receptors bind to the nonclassical class I molecule, Qa1, in mice and its homologue, HLA-E, in humans (2, 4). Both HLA-E and Qa1 present peptides that are derived from the leader sequences of other class I molecules (5).

Depending on the NKG2 molecule that CD94 dimerizes with, either an inhibitory or an activating receptor is formed. NKG2A contains immunoreceptor tyrosine-based inhibitory motifs (ITIMs) in its cytoplasmic domain, which results in the activation of SHP-1 and SHP-2 phosphatases, resulting in the inhibition of cytotoxicity (6). The expression of this receptor, which inhibits antiseif NK activity, supports the missing-self hypothesis first postulated by Karre (7). In contrast, some KIR, Ly-49, and CD94/NKG2 receptors have cytoplasmic domains that lack ITIMs. NKG2C, for example, interacts with the adaptor molecule DAP12, which contains immunoreceptor tyrosine-based activation motifs (8). As a result, this leads to cell activation and cytotoxic function.

Recently, it has been reported that both KIR and Ly-49 molecules play other roles in immune cell function. Ugolini et al. (9) reported that a KIR molecule functions in promoting CD8 T cell survival. KIRs have been shown to contribute to the downstream activation of an antiapoptotic protein kinase (10). Ly-49A ligation has been shown to inhibit apoptosis of a T cell hybridoma (11). CD94 ligation has been shown to play a role in costimulation or promote apoptosis of NK cells (12).

In addition to CD94/NKG2 expression on NK cells, this receptor is also expressed on a small percentage of CD8 T cells (3). We and others have noted that its expression on CD8 T cells is significantly increased upon infection with \textit{Listeria monocytogenes} (LM) as well as other infectious agents, and therefore could be a common marker for CD8 T cell activation (13–15) (data not shown). The role this receptor plays on CD8 T cells is controversial. Several reports suggest that expression of CD94/NKG2 down-regulates the cytotoxic activity of CD8 T cells specific for various Ags (6, 13, 16–19). However, recent studies using lymphocytic choriomeningitis virus and LM showed contradictory results indicating that CD94/NKG2 expression does not inhibit CD8 T cell effector function (14, 15). Therefore, we wished to examine whether this receptor may have additional functions. In this study, we examine the role of CD94/NKG2 expression on both NK and CD8 T cell survival in vitro and compare that with expression of Ly-49 receptors.

Materials and Methods

\textbf{Mice}

C57BL/6 (B6) mice, purchased from The Jackson Laboratory (Bar Harbor, ME), were housed in a traditional specific pathogen-free colony. Ly-49I transgenic mice were a generous gift from M. Bennett (University of Texas Southwestern Medical Center, Dallas, TX 75390).

\textsuperscript{1}Abbreviations used in this paper: KIR, killer-inhibitory receptor; 7-AAD, 7-amino actinomycin D; AICD, activation-induced cell death; int, intermediate; ITIM, immunoreceptor tyrosine-based inhibitory motif; LM, \textit{Listeria monocytogenes}; neg, negative.
Southwestern Medical Center, Dallas, TX) (20). Adult mice were used at 6–12 wk of age. Aged mice were used at 13–15 mo of age.

**Bacteria**

LM 10403 serotype 1 was originally provided by H. G. A. Bouwer (Veterans Affairs Medical Center, Portland, OR). Bacteria were grown on brain-heart infusion agar plates (Difco, Detroit, MI), and virulent stocks were maintained by repeated passage through B6 mice. The LD₅₀ for B6 mice is ~2 × 10³ bacteria. For infection of mice, log phase cultures of LM grown in brain-heart infusion broth were washed twice and diluted in PBS before i.v. injection. For a primary LM infection, B6 mice were injected with 2 × 10⁶ bacteria. For a secondary LM infection, B6 mice were injected with 2 × 10⁷ bacteria, rested for ~3 wk, and injected a second time with 2 × 10⁶ bacteria. Spleens were then harvested for analysis between 6 and 9 days after infection.

**Cell culture**

Single cell suspensions were prepared from freshly isolated spleens of B6 mice. After lysing RBCs with 1 mM Tris ammonium chloride, splenocytes were enriched for T and NK cells by passing them through a nylon wool column, as described previously (21). For T cell cultures, RPMI 1640 medium (Life Technologies, Grand Island, NY), supplemented with 10% FCS, 25 mM HEPES, 2 mM glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin, 1 mM sodium pyruvate, 0.1 mM nonessential amino acids, 20 µM 2-ME, and 1.3 × 10⁻² µM human rIL-2, was used. For NK cell cultures, DMEM supplemented with 10% FCS, 25 mM HEPES, 2 mM glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin, 100 µg/ml 2-ME, and 5 × 10⁻² µM human rIL-2, was used. Cells were incubated for 3 days at 37°C in humidified air containing 7% CO₂.

**Abs and cell staining**

For flow cytometric analysis, anti-CD3 FITC (145-2C11), anti-CD8 FITC (53-6.7), anti-CD4 APC (IM7), anti-CD44 bio (18d3), anti-NKG2A/C/E FITC (20d5), anti-NK1.1 PE (PK136), anti-Ly-49C/I FITC (18d3), anti-NKG2A/C/E FITC (20d5), anti-CD8 PE (53-6.7), anti-CD44 APC (IM7), and anti-CD94 bio (18d3), purchased from BD Pharmingen (San Diego, CA).

For Tetramer Facility and National Institutes of Health AIDS Research and Reference Reagent Program (Rockville, MD). Cells were washed with PBS before proceeding to the staining protocol with mAbs.

**Detection of apoptosis**

The percentage of cells undergoing apoptosis was determined by staining 0.5 × 10⁶ cells with Annexin V-FITC plus 7-amino actinomycin D (7-AAD), according to the manufacturer’s instructions (BD Pharmingen), and performing FACS analysis. CasPASE FITC-VAD-FMK, purchased from Promega (Madison, WI), was also used to determine the level of apoptosis. A total of 0.5 × 10⁶ cells were stained with CasPASE FITC-VAD-FMK, according to the manufacturer’s instructions, and FACS analysis was performed.

**Calculation of percentage of viable input cells**

A total of 3 × 10⁶ nylon wool-purified splenocytes isolated from a B6 mouse undergoing a secondary LM-specific response were cultured in IL-2–enriched T cell medium. The total number of cells at a given time point was determined using a Coulter Counter (Miami, FL). Viable CD94⁺⁹⁶ low and CD94⁺⁹⁶ high populations were identified by FACS staining as CD8⁺ CD94⁺⁹⁶ low or CD8⁺ CD94⁺⁹⁶ high cells that were CasPASE FITC-VAD-FMK negative. The absolute numbers of viable CD94⁺⁹⁶ high and CD94⁺⁹⁶ high CD8 T cells for a given time point were calculated by multiplying the total number of cells and the percentage of the individual viable population. The percentage of viable input cells was then calculated by dividing the absolute number of viable cells at a given time point by the absolute number of viable cells at 0 h and multiplying by 100.

**Results**

Expression of CD94/NKG2 receptors on NK and CD8 T cells

It has previously been reported that ~50% of adult NK cells express CD94/NKG2 or bind Qa-1b/Qdm tetramers (2, 22). Our results confirm this finding by showing the same proportion of tetramer staining in B6 NK cells (Fig. 1A). The tetramer-binding cells coexpress CD94, as expected (Fig. 1A). All CD94⁺⁹⁶ high cells also coexpress NKG2 (Fig. 1B). Most CD8 T cells from naive mice do not express CD94/NKG2 receptors (Fig. 1E) nor bind the Qa-1b/Qdm tetramer (data not shown). However, when we challenged B6 mice with a primary LM infection and examined their spleens 6 days later, we noted that ~25% of the CD8 T cells were CD94⁺⁹⁶ high (Fig. 1E). By 21 days after the primary LM infection, this percentage drops to ~10% (Fig. 1E). When we rechallenged LM-immune mice with LM and examined their spleen cells 6 days after treatment with LM, we observed a significant increase in the percentage of CD94⁺⁹⁶ high CD8 T cells. Additionally, we observed a decrease in the percentage of Qa-1b/Qdm tetramer-binding cells (Fig. 1D). These results indicate that CD94 expression is associated with increased longevity of CD8 T cells in LM-infected mice.

**FIGURE 1.** CD94 expression correlates with NKG2 expression and Qa-1b/Qdm tetramer binding. Freshly isolated splenocytes from either naive B6 mice or B6 mice undergoing a 2nd LM-specific response were analyzed by FACS for Qa-1b/Qdm tetramer binding and the expression of CD94/NKG2 receptors on NK1.1⁺ and CD8⁺ cells, respectively. Qa-1b/Qdm tetramer binding (A) and anti-NKG2A/C/E Ab staining (B) of CD94⁺⁹⁶ high cells that were Qa-1b/Qdm tetramer conjugated to PE for 20 min at 37°C. This tetramer was kindly provided by National Institute of Allergy and Infectious Diseases Tetramer Facility and National Institutes of Health AIDS Research and Reference Reagent Program (Rockville, MD). Cells were washed with PBS before proceeding to the staining protocol with mAbs.

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later, we noted that >50% of CD8 T cells were CD94\textsuperscript{high} (Fig. 1E), while the rest could be divided into CD94\textsuperscript{neg} and CD94\textsuperscript{int} cells. Meanwhile, CD44, a commonly used memory T cell marker, is expressed at high levels after primary LM infection and persists at that level after secondary challenge (Fig. 1E). Thus, to study the role of CD94 on CD8 T cells, we used splenocytes from a B6 mouse infected with a secondary LM infection 6–9 days prior. The CD94\textsuperscript{high} population in such a mouse shows ~98% Qa-1\textsuperscript{b}/Qdm tetramer staining (Fig. 1C). This binding is CD8 independent because it is not blocked by anti-CD8 Ab (Fig. 1D). In contrast, 69% of the CD94\textsuperscript{int} cells stain Qa-1\textsuperscript{b}/Qdm tetramer in the absence of the anti-CD8-blocking Ab, but this percentage is reduced to only 16% in the presence of the blocking Ab, suggesting a dependence on the CD8 coreceptor for Qa-1\textsuperscript{b}/Qdm tetramer binding (Fig. 1C). Therefore, we wished to examine the expression of CD94 receptors on both NK and CD8 T cells in culture and correlate this with cellular apoptosis and proliferation. NK populations were generated by culturing nylon wool-purified splenocytes from naive B6 mice in IL-2-enriched NK culture medium. CD8 T cells were generated by culturing nylon wool-purified splenocytes from B6 mice undergoing a secondary LM-specific response in culture. Both populations were cultured in vitro for 1–3 days and stained with anti-NK1.1 and anti-CD8 Abs, respectively, to identify distinct populations. As expected, ~50% of the NK cells are CD94\textsuperscript{high} (Fig. 2A). When cells were stained with CFSE to monitor cell division, no differences were noted between the CD94\textsuperscript{high} vs CD94\textsuperscript{int} populations (data not shown). Apoptosis was also monitored in these cultures by Annexin V/7-AAD staining. The percentage of NK cells from the B6 naive mouse that stains with both Annexin V/7-AAD, which represents the late apoptotic and dead cells, is slightly lower in the CD94\textsuperscript{high} than the CD94\textsuperscript{int} population on days 0 and 1 (Fig. 2, B–E). On day 2 in culture, only 19% of the NK cell population that expresses high levels of CD94 show apoptosis, compared with 34% of cells in the CD94\textsuperscript{int} population (Fig. 2, F and G). Similarly, on day 3, only 4% cell death is observed in the CD94\textsuperscript{high} population, compared with 27% in the CD94\textsuperscript{int} population (Fig. 2, H and I).

When CD8 T cells from a secondary LM-immune mouse were examined, ~43% expressed high levels of CD94 in this experiment (Fig. 2J). Similar to NK cells, CD8 T cells showed a significant difference in the level of apoptosis between the CD94\textsuperscript{high} and CD94\textsuperscript{int} populations. Only a slight difference was noted on day 0 (Fig. 2, K and L). However, after 1 day in culture, 2% of CD94\textsuperscript{high} cells stain for annexin V/7-AAD, while 21% of the CD94\textsuperscript{int} population stain for these markers (Fig. 2, M and N). By day 2, 10% of CD94\textsuperscript{high} CD8 T cells are dead, while 62% cell death is seen in the CD94\textsuperscript{int} CD8 T cells (Fig. 2, O and P). On day 3, the CD94\textsuperscript{high} population contains mostly viable cells, while the CD94\textsuperscript{int} population shows 33% cell death (Fig. 2, Q and R). Similar results were seen when CD8 T cells were cultured in medium that contained IL-15 or both IL-2 and IL-15 (data not shown). However, culturing these cells in medium without cytokines resulted in very high levels of death, preventing any useful analysis (data not shown).

In addition to annexin V/7-AAD staining, CaspACE VAD-FMK, an inhibitor of activated caspases, was used to detect apoptosis in the T cell cultures. CD8 T cells taken from nylon wool-purified splenocytes of a B6 mouse undergoing a secondary LM-specific response were cultured for 2 days in vitro. The percentage of apoptotic cells was determined by CaspACE VAD-FMK staining. Results were similar to the Annexin V/7-AAD staining.

Expression of CD94 is associated with an inhibition of apoptosis. Nylon wool-purified splenocytes from naive B6 mice or B6 mice undergoing a secondary LM-specific response were cultured in vitro. NK cells from the naive B6 mice were analyzed for expression of CD94 and monitored for the level of apoptosis by annexin V/7-AAD staining. CD94 staining of NK cells is shown for day 0 (Fig. 2A). As expected, ~50% of the NK cells are CD94\textsuperscript{high} (Fig. 2A). When cells were stained with

FIGURE 2. Expression of CD94 is associated with an inhibition of apoptosis. Nylon wool-purified splenocytes from naive B6 mice or B6 mice undergoing a secondary LM-specific response were cultured in vitro. NK cells from the naive B6 mice were analyzed for expression of CD94 and monitored for the level of apoptosis by annexin V/7-AAD staining. CD94 staining of NK cells is shown for day 0 (A). Values shown are the percentages of NK cells that express high or intermediate levels of CD94. Annexin V/7-AAD staining of NK1.1\textsuperscript{+} CD94\textsuperscript{high} cells (B, D, F, and H) and of NK1.1\textsuperscript{+} CD94\textsuperscript{int} cells (C, E, G, and I) is shown from days 0 to 3. Similarly, CD94 staining of CD8 T cells from B6 mice undergoing a secondary LM-specific response is shown for day 0 (J). Values shown are the percentages of CD8 T cells that express high or intermediate levels of CD94. Annexin V/7-AAD of CD8\textsuperscript{+} CD94\textsuperscript{high} T cells (K, M, O, and Q) and CD8\textsuperscript{+} CD94\textsuperscript{int} cells (L, N, P, and R) is shown from days 0 to 3. Values shown are the percentages of annexin V/7-AAD\textsuperscript{+} cells. Results shown are representative of four independent experiments.
staining (data not shown). Furthermore, the percentage of viable input cells was calculated at 3, 6, 12, 24, and 48 h in culture. The difference in viability between the CD94^high and CD94^neg/int cells is apparent as early as after 6 h in culture (Fig. 3A). After 12 h in culture, the percentage of viable input cells in the CD94^high population remains high at greater than 80%, while the percentage of viable input cells in the CD94^neg/int population drops dramatically to ~50%. The rapid decline of viable CD94^neg/int cells continues into the latter part of the culture period. After 48 h in culture, ~95% of CD94^neg/int cells are dead. Meanwhile, ~50% of the CD94^high cells are still viable. In contrast, both CD8^+CD44^high and CD8^-CD44^neg/int cells show a similar rate of decline in cell viability during the 48-h culture (Fig. 3B). It is unlikely that the CD44^high cells represent naive T cells that acquired CD44 expression due to culture conditions because ~50% of the CD8 T cells are CD44^high on day 0, similar to the percentage of CD94^high cells.

**Association of Ly-49 expression with levels of apoptosis on NK and CD8 T cells during in vitro culture**

Because the expression of CD94 is associated with less apoptosis in both cultured NK and CD8 T cells in vitro, we analyzed the same populations for Ly-49 expression with a cocktail of Abs specific for the inhibitory Ly-49 molecules, Ly-49A, Ly-49G2, and Ly-49C/I. When the NK cells were stained with both anti-Ly-49 and anti-CD94 Abs after 2 days in culture, four distinct populations were seen (Fig. 4A). The CD94^high cells were mostly viable, as assessed by lack of 7-AAD staining, while the CD94^int population showed a slightly higher extent of apoptosis (15 vs 30%) (Fig. 4, B and C). In contrast, the opposite result was observed in the extent of apoptosis in the Ly-49^- vs Ly-49^+ cells. In this study, the Ly-49^- cells showed a higher extent of cell death (28 vs 8%) (Fig. 4, D and E). However, CD94^high cells within these populations may be contributing to the observed levels of death. Thus, cells were examined for the expression of both CD94 and Ly-49. We noted that the CD94^highLy-49^- and CD94^intLy-49^- NK populations had low levels of cell death, 12 and 17%, respectively (Fig. 4, F and G), while the CD94^intLy-49^+ and CD94^highLy-49^+ NK populations had slightly higher levels, 29 and 25%, respectively (Fig. 4, H and I).

When CD8 T cells were costained with CD94 and Ly-49 Abs after 2 days in culture, interestingly, almost all of the CD94^int cells were Ly-49^- (Fig. 5A). Approximately 50% of CD94^int cells express Ly-49 receptors, while a much smaller percentage of the CD94^high cells express these molecules (22%). As noted with the NK cells, the extent of apoptosis decreases as CD94 expression increases (Fig. 5, B, C, and D). In contrast, the expression of Ly-49 is associated with an increase in the level of apoptosis. The CD94^highLy-49^- population has a low percentage of cell death.

**FIGURE 3.** Lack of CD94 expression is correlated with a decline in viable cells in culture. Nylon wool-purified cells from B6 mice undergoing a 2"LM-specific response were cultured for 2 days in vitro. Viable CD94^high and CD94^neg/int populations were identified by FACS staining as CaspACE FITC-VAD-FMK-negative, CD8^+, CD94^high, or CD94^neg/int cells, respectively. Similarly, viable CD44^high and CD44^neg/int populations were identified as CaspACE FITC-VAD-FMK-negative, CD8^-, CD44^high, or CD44^neg/int cells, respectively. The absolute numbers of viable cells were calculated at each time point. The data are plotted as the percentage of viable input cells in the CD94^high and CD94^neg/int populations over time in culture (A). The percentage of viable input cells in the CD44^high and CD44^neg/int populations is also shown (B). Data shown are an average of values from triplicate wells with SE bars. Results shown are representative of three independent experiments.

**FIGURE 4.** Expression of Ly-49 molecules is not associated with inhibition of apoptosis in NK cells. Nylon wool-purified cells from naive B6 mice were cultured in vitro for 2 days. NK1.1^+ cells were gated on and analyzed for their expression of CD94 and Ly-49 molecules with a cocktail of Abs against Ly-49A, Ly-49G2, and Ly-49 C/I (A). The 7-AAD staining was used to detect cell death. The 7-AAD staining in the CD94^int and CD94^high NK cells (B, C), as well as in the Ly-49^- and Ly-49^+ NK cells (D, E) from naive B6 mice is shown. The percentage of dead cells in the CD94^intLy-49^- (F), CD94^highLy-49^- (G), CD94^intLy-49^- (H), and CD94^highLy-49^- (I) populations was also determined by 7-AAD staining. Results shown are representative of two independent experiments.
(5%), while the CD94$^{neg}$/Ly-49$^{-}$ population has a higher amount (45%) (Fig. 5, G and H). However, this latter population represents only 7% of the total CD8 T cells. Similarly, the CD94$^{neg}$/Ly-49$^{-}$ cells show only 9% cell death, while CD94$^{neg}$/Ly-49$^{-}$ show 81%. The latter population represents only 10% of the total CD8 T cell population. The CD94$^{neg}$/Ly-49$^{-}$ cells display 59% cell death, while CD94$^{neg}$/Ly-49$^{-}$ cells have the highest percentage of apoptotic cells, 97% (Fig. 5, I–L).

To determine whether the low extent of apoptosis in CD8 T cells was directly related to expression of CD94 or simply a result of memory cells preferentially surviving in culture, we compared the level of apoptosis in CD8$^{-}$ CD44$^{high}$ memory vs CD8$^{-}$ CD44$^{neg/int}$ naive cells taken from the spleen of a B6 mouse undergoing a secondary LM-specific response and determined its relation to CD94 expression. Our results show that most of the CD8 T cells are CD44$^{high}$ and could be further separated into CD44$^{high}$/CD94$^{neg/int}$ populations. All CD94$^{high}$ T cells express high levels of CD44 (Fig. 6A). As expected, the CD94$^{neg/int}$ population shows a higher level of cell death than the CD44$^{high}$ population (Fig. 6, D and E). There is a slightly higher amount of cell death in the CD44$^{neg/int}$ population (36%), compared with the CD44$^{high}$ population (27%) (Fig. 6, B and C). When the CD44$^{high}$ population was analyzed on the basis of CD44 expression, we noted that the extent of apoptosis is low in the CD44$^{hi}$/CD44$^{hi}$ population (14% 7-ADD staining), while the CD44$^{neg/int}$/CD44$^{hi}$ population shows a higher level of cell death (54% 7-ADD staining) (Fig. 6, F and G).

**Effect of cross-linking CD94/NKG2 receptors on cell survival in culture**

To better understand the role CD94/NKG2 plays in the survival of CD8 T cells, plate-bound mAbs were used to cross-link these receptors. Isotype control, anti-CD94, and anti-NKG2A/C/E Abs were coated onto wells in microtiter plates overnight. The secondary LM-immune splenocytes were monitored for CD94 expression on their CD8$^{-}$ population (Fig. 7, A, D, and G) and apoptosis was detected by annexin V/7-AAD staining. The extent of annexin V/7-AAD staining is much lower in the CD94$^{high/int}$ vs CD94$^{neg}$ populations cultured in the presence of the isotype control Ab, consistent with our previous results (Fig. 7, B and C). Culture of these cells with plate-bound anti-CD94 Ab reduced the extent of annexin V/7-AAD staining in the CD94$^{high/int}$ population early as 12 h after culture (data not shown). The most significant reduction was seen after 2 days, in which culture with plate-bound anti-CD94 Ab decreased the level of apoptosis in the CD94$^{high/int}$ population from 38 to 18%, while culture with plate-bound anti-NKG2A/C/E Ab reduced it to 8% (Fig. 7, B, E, and H). No difference was noted in CD94$^{neg}$ cells cultured with these Abs (Fig. 7, C, F, and I). When sorted CD94$^{high}$ CD8 T cells from a B6 mouse undergoing a 2° LM-specific response were cultured in vitro. After 2 days, CD94 and CD44 expression was assessed on CD8$^{-}$ cells (A). Percentage of apoptotic cells in the CD94$^{neg/int}$ (B) and CD44$^{high}$ populations (C) as well as CD94$^{neg/int}$ (D) and CD94$^{high}$ populations (E) was measured using 7-AAD staining. The percentage of dead cells in the CD94$^{neg}$/CD44$^{neg}$ (F) and CD94$^{neg}$/CD44$^{high}$ (G) populations was also determined by 7-AAD staining. Results shown are representative of three independent experiments.

**FIGURE 5.** Expression of Ly-49 molecules is not associated with an inhibition of apoptosis in CD8 T cells. Nylon wool-purified splenocytes from B6 mice undergoing a 2° LM-specific response were cultured in vitro for 2 days. CD8$^{-}$ cells were gated on and analyzed for their expression of CD94 and Ly-49 molecules with a cocktail of Abs against Ly-49A, Ly-49G2, and Ly-49C/I (Fig. 5, I–L). The 7-AAD staining in CD94$^{neg}$/Ly-49$^{+}$ (A), CD94$^{int}$/Ly-49$^{+}$ (B), CD94$^{hi}$/Ly-49$^{+}$ (C), CD94$^{neg}$/Ly-49$^{-}$ (D), and CD94$^{neg}$/Ly-49$^{-}$ (E) populations was also determined by 7-AAD staining. Results shown are representative of two independent experiments.

**FIGURE 6.** Inhibition of apoptosis by CD94 is independent of CD44 expression. Nylon wool-purified splenocytes from B6 mice undergoing a 2° LM-specific response were cultured in vitro. After 2 days, CD94 and CD44 expression were assessed on CD8$^{-}$ cells (A). Percentage of apoptotic cells in the CD94$^{neg/int}$ (B) and CD44$^{high}$ populations (C) as well as CD94$^{neg/int}$ (D) and CD94$^{high}$ populations (E) was measured using 7-AAD staining. The percentage of dead cells in the CD94$^{neg}$/CD44$^{neg}$ (F) and CD94$^{neg}$/CD44$^{high}$ (G) populations was also determined by 7-AAD staining. Results shown are representative of three independent experiments.
FIGURE 7. Cross-linking of CD94/NKG2 provides protection from apoptosis. Nylon wool-purified splenocytes from B6 mice undergoing a 2° LM-specific response were cultured with plate-bound Abs for 2 days. CD94 expression was detected in CD8 T cells cultured with isotype control rat IgG2a (A), anti-CD94 (D), and anti-NKG2A/C/E Abs (G). Values shown represent the percentage of CD8 T cells that are CD44^hi or CD94^neg. Annexin V/7-AAD staining of CD8^CD44^hi (B) and CD94^neg (F) cells cross-linked with isotype control (H, anti-CD94 (E), and anti-NKG2A/C/E Abs (I) is shown. Annexin V/7-AAD staining of CD8^CD44^hi cells cross-linked with isotype control (C), anti-CD94 (F), and anti-NKG2A/C/E Abs (J) are also shown. Values shown represent percentages of annexin V^+ cells. Results shown are representative of two independent experiments.

FIGURE 8. Cross-linking of Ly-49I does not provide protection from apoptosis. Nylon wool-purified splenocytes from B6 and Ly-49I transgenic mice undergoing primary LM-specific responses were cultured with plate-bound Abs for 2 days. Annexin V staining of CD8^CD44^hi (C) and CD94^neg (D) cells cross-linked with isotype control (A) and anti-CD94 (B) Abs is shown. Annexin V staining of CD8^CD44^hi /Ly-49I^+ cells cross-linked with isotype control (C) and anti-Ly-49I (D) Abs is also shown. Values shown represent percentages of annexin V^+ cells.

mouse undergoing a secondary LM-specific response were cultured with plate-bound anti-CD94 Ab, their expression of CD94 was down-regulated to intermediate levels (data not shown). Thus, to include these cells in our analysis, we compared the level of apoptosis in the CD94^hi/int cells with the CD94^neg cells.

To investigate whether this apparent protection from apoptosis upon receptor engagement was specific for CD94, we compared the effect of cross-linking CD94 with the cross-linking of Ly-49I, an inhibitory Ly-49 molecule. Due to the low expression of Ly-49I on CD8 T cells taken from the spleen of B6 LM-infected mice, Ly-49I transgenic mice were used as a source for Ly-49^+ CD8 T cells. Greater than 90% of CD8 T cells in these mice express the Ly-49I receptor (20). Nylon wool-purified splenocytes from B6 and Ly-49I transgenic mice undergoing a primary LM-specific response were cultured for 2 days in the absence or presence of plate-bound anti-CD94 or anti-Ly-49I Abs, respectively. Apoptosis in the CD44^hi CD8 T cells, which represent cells involved in the immune response, was detected by annexin V staining. Culture with anti-CD94 Ab decreased the level of apoptosis in CD44^hi CD8 T cells from 42 to 28% (Fig. 8, A and B), while culture with anti-Ly-49I Ab had no effect on the survival of CD44^hi CD8 T cells (Fig. 8, C and D).

We next examined what effect cross-linking the CD94/NKG2 receptor would have on activation-induced cell death (AICD) using anti-CD3 Ab. In the previous experiments, there were negligible numbers of cells that were Annexin V^+ and 7-AAD^− during culture, suggesting that apoptosis occurred rapidly, similar to other reports (24). However, when cells were exposed to anti-CD3 Ab, we noted a higher percentage of Annexin V^+ 7-AAD^− cells, and thus included them in our analysis. The percentage of apoptotic CD8 T cells detected by Annexin V/7-AAD staining after 2 days in culture is 62% in the presence of the isotype control Ab (Fig. 9A). Most apoptotic cells are in the CD44^neg population (Fig. 9D). This baseline level of apoptosis increases to 85% when cross-linked with the anti-CD3 Ab (Fig. 9E). Nevertheless, the extent of apoptosis in the CD44^hi/int population is less than that seen in the CD44^neg population, 61 vs 86% (Fig. 9, G and H). To determine whether cross-linking CD94 could protect cells from AICD induced by anti-CD3, we cross-linked both receptors at the same time. Cross-linking with anti-CD94 Ab down-regulated the expression of CD94 (Fig. 9, compare J and N with F), and this was more marked than seen in experiments in which only CD94 or NKG2 was cross-linked without concomitant cross-linking of CD3 (compare Fig. 7, D, G, and A with Fig. 9, J, N, and F). Therefore, we only examined the CD8 T cells that retained expression of CD94. It is clear that the CD94^hi/int cells show only 19–22% annexin V/7-AAD staining (Fig. 9, K and O), compared with 73–77% in the CD44^neg population (Fig. 9, L and P). The extent of apoptosis in the entire CD8 T cell population cross-linked with either anti-CD3 and anti-CD94 or anti-CD3 and anti-NKG2A/C/E Abs is also shown (Fig. 9, I and M).

Discussion

Inhibitory receptors bearing ITIM motifs including CD94/NKG2A, KIR in humans, and Ly-49 molecules in mice have been well characterized for their ability to inhibit the cytotoxic activity of both NK and CD8 T cells (6, 16, 25–27). Although these receptors appear to be constitutively expressed on NK cells, they are induced on CD8 T cells as a result of their activation or from cytokine stimulation (19, 28–31). Thus, inhibitory Ly-49 receptors
and adaptors such as FcR
SHP-1 and SHP-2 to inhibit signaling through activating receptors ITIMs of human NKG2A interact with the tyrosine phosphatases provides negative signaling because murine NKG2A has an ITIM genes against a variety of Ags (16). The expression of these receptors on both NK and CD8 T cells was shown to be attractive candidates for molecules that play a role in T cell survival or persistence in addition to its known function of inhibiting cytotoxicity. Accordingly, we decided to test whether the expression of these receptors on both NK and CD8 T cells was correlated with their level of apoptosis.

Although most LM-specific TCR transgenic cells transferred into normal hosts respond to a secondary LM infection and acquire CD94/NKG2 receptors 6 days after infection, we also noted that ~50% of host CD8 T cells express CD94 upon secondary infection (data not shown). This percentage most likely represents Ag-specific cells. Consistent with this, Pamer and colleagues (36) have shown that nearly 20% of CD8 T cells in a mouse undergoing a secondary LM infection are specific for a single known LM epitope. However, although LM-specific CD8 T cells up-regulate their expression of CD94 upon activation, cytokines such as IL-15, TGF-β, IL-10, and IL-12 could also be involved (19, 28, 30, 31). We took advantage of LM infection to analyze CD94 expression of CD8 T cells and their level of apoptosis in culture. CD94 expression divides CD8 T cells into three populations, CD94high, CD94med, and CD94low. Interestingly, in the presence of anti-CD8 Abs, the Qa-1β/Qdm tetramer binds the CD94highCD8 T cells, but is inhibited from binding the CD94low cells, suggesting that there is

FIGURE 9. Cross-linking CD94/NKG2 inhibits apoptosis induced by anti-CD3 Ab. Nylon wool-purified splenocytes from B6 mice undergoing a 2nd LM-specific response were cultured with plate-bound Abs for 2 days. The level of apoptosis in total CD8 cells cross-linked with isotype control rat IgG2a (A), anti-CD3 (E), anti-CD3 and anti-CD94 (I), and anti-CD3 and anti-NKG2A/C/E Abs (M) is shown. The values shown are percentages of annexin V+/7-AAD− plus annexin V+/7-AAD+ cells. CD94 expression was detected in CD8 T cells cultured with isotype control (B), anti-CD3 (F), anti-CD3 and anti-CD94 (J), and anti-CD3 and anti-NKG2A/C/E Abs (N). Values shown represent the percentage of CD8 T cells that are CD94high/int or CD94 neg . Apoptosis was measured by annexin V+/7-AAD staining in the CD8 cell population mostly dimerizes with NKG2A, and therefore could account for the reduced levels of apoptosis in culture (data not shown). The pairing of CD94 with NKG2A provides negative signaling because murine NKG2A has an ITIM motif in its cytoplasmic domain (34). Both N- and C-terminal ITIMs of human NKG2A interact with the tyrosine phosphatases SHP-1 and SHP-2 to inhibit signaling through activating receptors and adaptors such as FcRy or DAP12 (6, 35). CD94/NKG2A expression has been documented on both human and murine CD8 T cells (2) and has been shown to inhibit effector function of CTL against specific target cells (16). The expression of these receptors on CD8 T cells is associated with inhibition of lysis of polyoma virus-infected cells and was suggested to allow for the maintenance of CD8 memory T cells specific for this pathogen (13). However, recently, other studies showed that CD94 expression on CD8 T cells specific for lymphocytic choriomeningitis virus and LM does not inhibit CD8 T cell effector function (14). Other sequelae of triggering through inhibitory receptors have been described. For example, Roger et al. (11) showed that simultaneous ligation of Ly-49A and the TCR in a T cell hybridoma inhibited IL-2 secretion and apoptosis. Expression of transgenic KIR2DL3 on CD8 T cells promoted their accumulation in vivo, and interaction of this receptor with its ligand inhibited AICD (9). Although KIR and Ly-49 receptors are generally allele specific and therefore not necessarily reactive with self class I molecules, CD94/NKG2A is specific for Qa-1 in mice and HLA-E in humans (2, 4). These molecules show little polymorphism, and thus interact with their receptors from most members of their species. The interaction of this receptor is due to its binding of ubiquitous self class Ia leader sequences from other class I molecules (5). The appearance of these receptors on Ag-activated CD8 T cells could be attractive candidates for molecules that play a role in T cell survival or persistence in addition to its known function of inhibiting cytotoxicity. Accordingly, we decided to test whether the expression of these receptors on both NK and CD8 T cells was correlated with their level of apoptosis.

Although most LM-specific TCR transgenic cells transferred into normal hosts respond to a secondary LM infection and acquire CD94/NKG2 receptors 6 days after infection, we also noted that ~50% of host CD8 T cells express CD94 upon secondary infection (data not shown). This percentage most likely represents Ag-specific cells. Consistent with this, Pamer and colleagues (36) have shown that nearly 20% of CD8 T cells in a mouse undergoing a secondary LM infection are specific for a single known LM epitope. However, although LM-specific CD8 T cells up-regulate their expression of CD94 upon activation, cytokines such as IL-15, TGF-β, IL-10, and IL-12 could also be involved (19, 28, 30, 31).

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both CD8 dependent and independent binding of Qa-1\textsuperscript{b}/Qdm by T cells. Culture of CD8 T cells indicated that the level of apoptosis was correlated with expression of CD94. Apoptosis was lowest in CD94\textsuperscript{high} cells, intermediate in CD94\textsuperscript{int} cells, and highest in CD94\textsuperscript{neg} cells. In contrast, Ly-49\textsuperscript{+} expression was not associated with a decrease in apoptosis. In fact, it was higher in this population of CD8 T cells. Using a T cell hybridoma, Roger et al. (11) showed that cross-linking the inhibitory receptor Ly-49A resulted in a lower level of apoptosis. In our present study, however, CD8 T cells were isolated from mice undergoing infection and cultured in vitro. Furthermore, our data show higher levels of apoptosis in Ly-49\textsuperscript{+} cells in the absence of cross-linking. Cross-linking of Ly-49I, however, does not confer protection from apoptosis. In contrast, both the expression of CD94 and the cross-linking of the CD94/NKG2 receptors result in a decrease of apoptosis.

During LM infection, our data show that a large population of CD8 T cells becomes activated, as assessed by expression of CD94 and CD44 molecules. Interestingly, only a subset of the CD44\textsuperscript{high} CD8 T cells expresses high levels of CD94. The CD44\textsuperscript{neg} CD44\textsuperscript{int} cells could be bystander T cells, while the CD44\textsuperscript{high} CD44\textsuperscript{int} cells are the true memory T cells. Moreover, our data suggest that the expression of CD94, not CD44, denotes protection from apoptosis. The expression of CD94 may allow for the survival of these cells by interacting with Qa-1\textsuperscript{b}/Qdm or perhaps other ligands in the environment. Furthermore, the presence of CD94\textsuperscript{neg/int} CD8 T cells remains relatively high (data not shown).

Clearly, the expression of CD94 is correlated with a reduced level of apoptosis in CD8 T cell cultures. The percentage of apoptotic cells in the CD94\textsuperscript{high} population, as detected by both annexin V/7-AAD and CaspACE VAD-FMK staining, was lower than in the CD94\textsuperscript{neg/int} population. When the kinetics of this phenomenon were observed closely, the percentage of viable input cells appeared lower than the percentages extrapolated from the direct Annexin V/7-AAD staining in other experiments. An explanation for this apparent difference is that the direct Annexin V/7-AAD staining simply shows results from a particular time in culture, while the percentage of viable input cells incorporates the loss of absolute cell numbers during time in culture into its value. The difference in viability between CD94\textsuperscript{neg/int} and CD94\textsuperscript{high} cells is evident as early as after 6 h in culture. The rapid decline of viable CD94\textsuperscript{neg/int} cells eventually resulted in the near disappearance of this population by 48 h in culture. These results imply that during T cell activation, the cells expressing high levels of CD94 have an advantage of survival when compared with the CD94\textsuperscript{neg/int} cells. Whether this is due to signaling through the CD94 receptor or CD94 is a marker coincident with T cell survival is yet to be determined.

Cross-linking of CD94/NKG2 receptors reduced the level of spontaneous apoptosis in CD8 T cells taken from a B6 mouse undergoing a secondary LM-specific response. Thus, while the experiments described above indicate a correlation between CD94/NKG2 expression and lack of apoptosis, these data indicate that engagement of these receptors further protects cells from cell death. We also noted that AICD induced by cross-linking the TCR can also be prevented by cocross-linking CD94/NKG2A receptors on the CD8 T cells taken from a B6 mouse undergoing a secondary LM-specific response. Inhibition of AICD by other NK receptors has also been demonstrated (9, 11). This may be attributed to preventing downstream signaling events that lead to AICD in Ag-activated cells. We also noted that cross-linking these receptors on CD8 T cells leads to their down-regulation, suggesting that recycling of these receptors is rapid and may allow for NK and CD8 T cells to rapidly survey their environment.

Our data for CD94 expression and apoptosis on NK cells are similar to that observed with CD8 T cells. Approximately 50% of NK cells express high levels of CD94 and bind the Qa-1\textsuperscript{b}/Qdm tetramer. Unlike CD8 T cells, the CD94\textsuperscript{high}-expressing NK cells do not bind tetramer probably due to the fact that they cannot use the CD8 molecule as a coreceptor for binding. CD94\textsuperscript{high} NK cells showed less apoptosis in culture than CD94\textsuperscript{neg} NK cells. Cross-linking this receptor enhanced the protection. These data are in contrast to the report by Ida et al. (23), who noted an increase in apoptosis. In their report, only 25% of the human NK cells showed an increase in apoptosis, which could be due to the differential expression of the inhibitory and activating CD94/NKG2 molecules between mice and humans.

In this study, we examined the role of CD94/NKG2 receptors in the survival of NK and CD8 T cells in culture. Our studies clearly show that CD94 expression as well as cross-linking of CD94/NKG2 receptors result in a decrease of apoptosis in NK and CD8 T cells, and therefore attribute a novel function to these receptors. Our data suggest that the expression of CD94/NKG2 may play an important role in the maintenance of NK and CD8 T cells by protecting them from apoptosis.

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