Persistent expression of CD94/NKG2 receptors by virus-specific CD8 T cells is initiated by TCR-mediated signals

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Abstract

Subsets of CD8 T cells express receptors that are critical in regulating the activity of NK cells. To characterize the expression of these receptors on CD8 T cells we made use of transgenic mice that express a H-2Kb restricted TCR specific for the immunodominant epitope located within the HSV-1 glycoprotein B (gB). Few naive gB-specific T cells express Ly49 or CD94/NKG2 receptors. Following acute infection of C57BL/6 mice with either HSV-1 or a recombinant influenza virus that encodes the gB determinant, gB-specific T cells showed a dramatic upregulation of CD94/NKG2 receptors. Moreover, gB-specific CD8 T cells that expressed CD94/NKG2 receptors were also found to express another NK receptor, KLRG1. We established that while Ag-stimulated gB-specific CD8 T cells primarily express inhibitory isoforms of CD94/NKG2 receptors, these cells remain capable of producing IFN upon peptide stimulation. While peak CD94/NKG2 expression on gB-specific cells was reached 2–3 days following infection, it remained elevated beyond 60 days post-infection with either HSV-1 or a gB-expressing recombinant influenza virus. The data imply that the prolonged expression was not due to persistence of replicating virus and suggest that while recognition of the cognate Ag is necessary to trigger expression of CD94/NKG2 receptors, it is not required for their continued expression on memory T cells.

Introduction

Activation of T cells is dependent on the recognition of specific MHC/peptide complexes by the TCR (1). However, the outcome of this interaction is modulated by a vast array of additional receptor/ligand interactions such as those involved in the adhesion of T cells to the APC and co-stimulation of the T cell (2). These additional interactions may impact on the amount of antigen required to trigger the T cell (3,4), the amount and types of cytokine that are produced following antigenic stimulation (5,6) and whether or not the T cell undergoes activation-induced cell death (7). A number of recent studies have shown that subpopulations of CD8 T cells express receptors that are perhaps more typically expressed by NK cells. These receptors include members of the KIR family (8,9), the CD85/ILT family (10,11), NKG2D (12) and the CD94/NKG2 (13) receptors in humans and members of the Ly49 family (14), CD94/NKG2A (15–17) together with NKG2D (18) and KLRG1 (16,19–21) in mice.

There is evidence that the expression of these receptors can significantly affect the functional activity of both tumour-specific and virus-specific T cells (22–24). For example NKG2D has been shown to be expressed on CMV-specific T cell clones where interaction with ligand can augment both cytotoxicity and cytokine production (25). In contrast, the expression of inhibitory KIR and the CD94/NKG2A receptors has been correlated with an impaired ability to kill various target cells, suggesting that these receptors transduce inhibitory signals that are capable of blocking T cell activation (23,26,27).

Despite the increasing number of reports of such receptors being expressed by CD8 T cells, the stimuli that initiate and maintain expression of these receptors are not clearly defined. We have examined the induction of NK receptor expression on CD8 T cells following localized infection with HSV-1. This response is highly focused on a single determinant derived from glycoprotein B (gB) in complex with H-2Kb (28). Consistent with recent findings we report that following infection with HSV-1 or a recombinant influenza virus that encodes the gB determinant (WSN/NA/gB), gB-specific T cells initiate CD94/NKG2 receptor expression that is ultimately...
NK receptor expression on Ag-specific CD8 T cells

maintained through the progression of these cells into the memory pool.

Methods

Mice

C57BL/6, gBT-I and gBT-I×B6.SJL-Ptprc°Pep3°/BoyJ [gBT-1(Ly5.1)] mice were obtained from the Department of Microbiology and Immunology, University of Melbourne and maintained in specific pathogen free conditions. gBT-I mice express a Vα2+ Vβ8.1+ T cell receptor that recognizes the gB (SSIEFARL) peptide complexed to H-2Kb and that has been described previously (29).

Viruses and infections

The KOS strain of HSV-1 was propagated and titered on VERO cells grown in MEM plus 10% FCS (CSL, Parkville, Australia). Mice were infected subcutaneously in the hind feet with 4.5 × 105 PFU of HSV-KOS diluted to a total volume of 25 µl in sterile PBS. The WSN/NA/gB and WSN/NA/OVA recombinant influenza viruses expressing the gB498–505 or OVA257–264 epitopes, respectively, in the neuraminidase (NA) stalk have been described previously (30,31) and were the kind gifts of S. Tevethia and P. Doherty. Mice were infected intranasally with 500 PFU of WSN/NA/gB or WSN/NA/OVA viruses diluted in 50 µl of sterile PBS.

Adoptive transfers

Single cell suspensions from splenocytes of naive gBT-I or gBT-1(Ly5.1) mice were prepared and 3.0 × 10^6 cells resuspended in 200 µl of sterile PBS transferred by intravenous tail vein injections into sex-matched C57BL/6 mice. In some experiments gBT-I lymph node cells were first labelled with the fluorescent intracellular dye CFSE (Molecular Probes, Eugene, USA) by culturing the cells for 10 min at 37°C in 0.1% BSA in PBS containing 0.1 µM CFSE.

Flow cytometry and reagents

All antibodies were purchased from Pharmingen (San Jose, USA). H-2Kd/gB tetramers were prepared as described by Kalergis et al. (32). Cells were incubated with either directly labelled mAb or biotinylated mAb for 30 min on ice followed by either allophycocyanin- or Alexa 488-conjugated streptavidin (Molecular Probes). Cells were stained with Kd/gB tetramers for 20 min at 37°C. Propidium iodide (Sigma Chemicals Co., St Louis, MO) was added to samples immediately prior to acquisition and used to exclude non-viable cells from further analysis. Flow cytometry was performed on Becton Dickinson FACSort and analysed using FlowJo software (Treestar, San Carlos, USA).

Intracellular γIFN staining

Splenocytes were incubated in the presence or absence of 1.0 µM gB498–505 peptide for 2 h at 37°C, followed by a 4 h incubation in the presence of Brefeldin A (10 µg/ml). Cells were stained for cell surface antigens, fixed in 1% formaldehyde and subsequently stained for intracellular γIFN in 0.2% saponin/PBS and analysed by flow cytometry.

Molecular biology

Splenocytes from C57BL/6 mice infected 7 days prior with HSV-1 were stained with Kd/gB tetramer, together with mAbs for CD8 and NKG2A/C/E and sorted for triple positive cells. The purity of the sorted population was verified by flow cytometry (>95%) and RNA was prepared using TRIzol reagent (Invitrogen, Rockville, USA) according to the manufacturer’s instructions. NKG2 cDNA was amplified in a one step RT–PCR reaction using the primers: NKG2C3'-TCAGATGGGGA-ATTTCACCTACAAAGATATGG and NKG25'-GAAAATCT- TGGAATGACAGTTTGG (Geneworks, Adelaide, Australia). Restriction enzyme analysis was performed using PvuII, Sau3AI and StuI enzymes (Promega, Madison, USA) according to the manufacturer’s instructions.

Results

gB-specific CD8 T cells express CD94/NKG2 receptors following infection with HSV-1

Subsets of CD8 T cells express receptors such as members of the Ly49 and CD94/NKG2 receptor families that regulate natural killer cell function (14–17). We wished to examine the expression of these receptors both in naïve mice and following infection with HSV-1. Consistent with previous findings (14), a small proportion of naive splenic CD8 T cells were stained with a panel of mAb specific for Ly49A, C/I or F or the NKG2A/C/E receptor subunit. Splenocytes from mice infected with HSV-1 7 days previously were also stained and the expression of Ly49 and NKG2A/C/E receptors on CD8 T cells assessed. In each case there were changes in the proportion of CD8 T cells that expressed either Ly49 receptors or NKG2A/C/E, an increased proportion of cells expressing NKG2A/C/E being particularly prominent.

To better understand the role of antigenic stimulation in the induction of NK receptor expression by CD8 T cells, we made use of gBT-I transgenic mice (29) that express an H-2Kd-restricted TCR specific for the immunodominant determinant derived from the HSV-1 gB and tetramer MHC complexes that facilitate the identification of gB-specific T cells (33). These reagents allowed us to assess the expression of NK receptors both on naïve virus-specific T cells and on activated cells immediately following their encounter with Ag. Consequently gBT-I splenocytes were adoptively transferred into normal C57BL/6 mice that were subsequently infected with HSV-1 24 h later. Mice were sacrificed 7 days post-infection, the peak of the gB-specific CD8 T cell response in the spleen (34) and stained with gB tetramer and CD8-specific mAb together with antibodies specific for NK receptors. In control mice that received gBT-I cells, only a small proportion of naïve tetramer positive CD8 T cells were stained by mAb specific for Ly49 receptors or CD94/NKG2 receptors (Fig. 2).

However, following infection with HSV-1, a dramatic increase in both the number and proportion of gB-specific CD8 T cells found in the spleen was observed. Despite the increase in the numbers of gB-specific T cells, there was little change in the proportion that stained with mAb specific for Ly49A, Ly49C/I or Ly49F. In contrast to this, a marked increase was observed in the numbers of tetramer positive cells that stained with mAb specific for either CD94 or NKG2A/C/E following infection with...
HSV-1. The coordinate increase in staining using both CD94 and NKG2A/C/E-specific mAb suggests that following HSV-1 infection, gB-specific T cells induced expression of heterodimeric CD94/NKG2 receptors (35).

To establish which isoform of NKG2 was upregulated on CD8 T cells following HSV-1 infection, a method described by Vance et al. was employed (36). RNA was extracted from gB tetramer+, CD8+, NKG2A/C/E+ cells isolated from mice 7 days post-infection with HSV-1 (Fig. 3). Primers that anneal to sequences that are conserved between NKG2A, -C and -E mRNA were used to amplify a 298 bp fragment of NKG2 cDNA and the identity of the amplified product was then determined by restriction enzyme analysis. Digestion of the amplified product with Sau3A (that is predicted to cut NKG2A but not NKG2C or -E) produced two fragments (194 bp and 104 bp) suggesting that the major species of NKG2 mRNA present in these cells was derived from the nkg2a gene. This interpretation was further strengthened by the observation that incubation of the amplified product with either StuI or PvuII (that will digest products amplified from NKG2C or NKG2E mRNA, respectively) did not digest the amplified cDNA. Nevertheless, these enzymes were capable of digesting control plasmid DNA. In summary, these data indicate that the predominant NKG2 mRNA species is that of NKG2A.

gB-specific T cells that express CD94/NKG2 receptors are capable of responding to Ag

Given that the NKG2A receptor contains an ITIM motif and has been shown to be capable of inhibiting both NK and CD8 T cells...
cell activation, we examined whether both CD94/NKG2 positive and negative gB-specific T cells were equally capable of responding to antigen. Mice were adoptively transferred with naive splenocytes from gBT-I(Ly5.1) mice that express the Ly5.1 allele of CD45, and 7 days post-infection with HSV-1 mice were sacrificed. The ability of transgenic gB-specific cells to respond to Ag following short term in vitro stimulation was then assessed by intracellular staining for IFN. Consistent with previous data, few transgenic Ly5.1+CD8+ cells obtained from naive mice expressed CD94/NKG2 receptors (Fig. 4). Short term stimulation of these cells with synthetic peptide corresponding to the immunodominant determinant of gB stimulated ~6% of transgenic cells to produce IFN. In contrast, following infection with HSV-1, there was a marked increase in both the number of transgenic CD8 T cells observed in the spleen (data not shown), and the proportion that expressed CD94/NKG2A/C/E receptors. There was also a dramatic increase in the proportion of transgenic CD8 T cells that were able to produce IFN following short term in vitro stimulation with the gB498-505 peptide. Moreover, >90% of the IFN producing gB-specific cells expressed CD94/NKG2A/C/E receptors. These data demonstrate that CD94/NKG2 expressing T cells remain capable of responding to antigen and furthermore suggest that cells with effector function are predominantly found within this subset.

**CD94/NKG2 expression by gB-specific CD8 T cells is initiated in the lymph nodes draining the site of infection**

In order to examine the kinetics of CD94/NKG2 receptor expression, lymph node cells from gBT-I transgenic mice were labelled with CFSE and adoptively transferred into naive recipients 24 h prior to infection with HSV-1. Cells were then recovered from the draining popliteal lymph node 24, 48 and 60 h post-infection and the expression of CD94/NKG2 receptors assessed by flow cytometry. Consistent with published data indicating that cell division can be first observed at 30 h (37), CFSE-labelled gBT-I cells remained undivided 24 h post-infection (Fig. 5). At this point in time a marginal increase in NKG2A/C/E expression was observed. However, by 48 h post-infection there was a significant increase in the proportion of CFSE-labelled cells that expressed NKG2A/C/E, with maximal expression levels being reached after the cells had undergone 2–3 divisions. Similarly, at 60 h post-infection, NKG2A/C/E expression was still evident on cells that had undergone division. gBT-I cells transferred into naive recipients did not express significant levels of NKG2A/C/E.

**Co-expression of CD94/NKG2 and KLRG1 receptors by gB-specific T cells**

Recent studies have shown that KLRG1, another receptor initially thought to regulate NK cell activity, is also expressed on T cells following stimulation with antigen (16,19–21). Consequently, we wished to determine whether KLRG1 was also expressed by gB-specific T cells following infection with HSV-1. Again, naive transgenic gBT-I cells were adoptively transferred into C57BL/6 recipients. Staining of splenocytes from these animals with gB-tetramer together with antibodies specific for CD8, NKG2A/C/E and KLRG1 revealed that KLRG1 is not expressed on naive gB-specific CD8 T cells (Fig. 6). However, analysis of splenocytes from mice that had been infected with HSV-1 7 days earlier revealed a marked induction in the expression of KLRG1 by gB-specific T cells. Notably, all cells that expressed NKG2A/C/E also
Interestingly, there appears to be two distinct populations of gB-specific CD8 T cells that express NKG2A/C/E receptors that can be identified largely on differences in the expression level of KLRG1.

Recombinant influenza induces CD94/NKG2 expression on Ag-specific T cells

In order to determine whether the expression of CD94/NKG2 receptors by Ag-specific T cells might be a general feature of the response of CD8 T cells to viral infection, we made use of a recombinant influenza virus that encoded the gB determinant from HSV-1 (WSN/NA/gB) (30). A similar approach to that used for HSV-1 was employed, in that gBT-I splenocytes were transferred into naive recipients 24 h prior to intranasal infection. Seven days post-infection, cells from the mediastinal lymph nodes were stained with gB tetramer, CD8 and NKG2A/C/E-specific mAb and then analysed by flow cytometry. As expected, CD8 T cells obtained from naive mice that had received gBT-I cells expressed little NKG2A/C/E (Fig. 7). In contrast, following infection with WSN/NA/gB, NKG2A/C/E was expressed on >90% of tetramer positive CD8 T cells. Furthermore, an additional cohort of mice that had received gBT-I cells were infected with a recombinant influenza virus that did not express the gB determinant (WSN/NA/OVA) (31). Unlike mice infected with WSN/NA/gB, gB-tetramer positive cells from mice infected with WSN/NA/OVA did not express appreciable levels of NKG2A/C/E.

Fig. 4. gB-specific T cells expressing NKG2A/C/E produce γIFN. gBT-I(Ly5.1) splenocytes were transferred into C57BL/6 mice and 24 h later these mice were infected with HSV-1 or left uninfected. Splenocytes were harvested 7 days later and incubated in the presence or absence of 2.0 μM gB498–505 peptide. After 6 h cells were stained for NKG2A/C/E, CD8, CD45.1 and for intracellular γIFN and analysed by flow cytometry. γIFN production is shown on control mice that had only received transferred cells (AT) and mice that were subsequently infected with HSV-1 (AT+HSV). Dotplots are gated on CD45.1+ve CD8+ve T cells and are representative of at least three independent experiments.

Fig. 5. CD94/NKG2 receptor expression is initiated in the lymph nodes draining the site of infection. 3.0 × 10⁶ CFSE-labelled gBT-I lymph node cells were transferred into C57BL/6 mice that either remained uninfected (Naive) or were infected with HSV-1 24 h later. Cells from the draining popliteal lymph node were recovered from either naive mice or 24 h, 48 h or 60 h post-infection with HSV-1. Cells were stained with mAb specific for CD8 and NKG2A/C/E and analysed by flow cytometry. Dotplots shown are gated on CD8+ve, CFSE+ve cells and are representative of at least three independent experiments.
suggesting that Ag-recognition rather than bystander exposure to an antiviral inflammatory immune response is required for upregulation of CD94/NKG2 receptor expression.

**Persistent expression of CD94/NKG2 receptors on virus-specific T cells**

While the expression of CD94/NKG2 receptors was evident shortly after viral infection and was dependent on TCR-mediated signals, we wished to determine whether CD94/NKG2 receptor expression could be observed on virus-specific memory cells. Consequently, using the adoptive transfer model, mice that had received gBT-I cells were infected with HSV-1 and 60 days later splenocytes stained with tetramer, CD8 and NKG2A/C/E specific mAb. Following adoptive transfer into naive recipients, few gB-specific CD8 T cells expressed appreciable levels of CD94/NKG2 receptors (Fig. 8). In contrast, 60 days post-infection with HSV-1, CD94/NKG2 receptor expression was evident on the vast majority of gB-specific T cells. Given that HSV-1 can form a persistent infection (38), we also assessed the level of CD94/NKG2 receptor expression on gB-specific T cells up to 60 days following infection with WSN/NA/gB, a virus that is cleared from mice (39,40). Again, following intranasal infection with this recombinant virus, CD94/NKG2 expression remains...
evident on the majority of gB-specific T cells. These data demonstrate that antigenic stimulation of virus-specific T cells during viral infections leads to expression of CD94/NKG2 receptors that can persist well beyond the acute phase of the infection.

Discussion

Recent evidence has demonstrated that subsets of CD8 T cells express a number of receptors that are critical in regulating the activity of NK cells (15–17,19,24). In this study we have made use of a localized model of HSV-1 infection in which many of the early events critical in the development of an anti-viral T cell response have been well characterized (28,34,37). In the lymph node draining the site of infection, antigen presentation can be detected as early as 6 h post-infection and following the adoptive transfer of HSV-specific T cells, cytotoxic activity and proliferation can be detected by 30 h (37). It is in this context that we wanted to assess NK receptor expression on virus-specific T cells, and whether this expression was limited to the site of infection, was Ag-driven, or a by-product of the anti-viral inflammatory response.

Consistent with recent studies, we observed induction of CD94/NKG2 receptors on gB-specific T cells following HSV-1 infection (15,16,24). The use of CFSE-labelled transgenic T cells from gBT-I mice enabled us to examine the timing and kinetics of the induction of these receptors within the draining lymph nodes in more detail. While there were marginal changes in the expression of CD94/NKG2 receptors on transgenic T cells as early as 24 h post-infection in the popliteal lymph nodes following subcutaneous infection with HSV-1, it is in the ensuing 24 h that the most dramatic changes in NKG2A/C/E expression occurred. In particular, NKG2A/C/E expression was markedly elevated on cells that had divided with peak expression levels being reached after 2–3 cell divisions. Interestingly, the induction of CD94/NKG2 receptor expression by gB-specific T cells appears to parallel functional responses such as proliferation and the capacity to lyse Ag-bearing target cells (37).

Recent studies have reported the upregulation of another NK receptor, KLRG1, on CD8 T cells following infection with various pathogens (16,19–21). In contrast to the rapid induction of CD94/NKG2 receptors, KLRG1 expression is evident only following extensive proliferation of CD8 T cells (19). Co-staining of gB-specific T cells with KLRG1 and NKG2A/C/E-specific mAb revealed that >90% of gB-specific KLRG1+ cells obtained from the spleen 7 days post-infection also expressed NKG2A/C/E. These data contrast somewhat from experiments described by Robbins et al., in which there is a significant KLRG1 negative, NKG2A/C/E positive population of CD8 T cells in the spleen following MCMV infection (21). It should be noted that the subcutaneous model of HSV-1 infection leads to a relatively localized response with little virus being detected even in the draining lymph node after 5 days (33). Consequently, the gB-specific T cells are obtained from an anatomical site in which infectious virus cannot be detected and have undergone proliferation prior to their arrival in the spleen.

The other issue we sought to clarify was the role that TCR-mediated signals played in the induction of CD94/NKG2 receptor expression by CD8 T cells. The use of transgenic T cells allowed us to formally compare the expression of NK receptors on both naive and memory cells of the same specificity. Following infection with either HSV-1 or WSN/NA/gB, there was a striking induction in the expression of CD94/ NKG2 receptors. Moreover, this altered surface phenotype of gB-specific cells was maintained in excess of 60 days following infection, suggesting that expression of CD94/NKG2 receptors might be a hallmark of T cells that had been stimulated by Ag-bearing APC. Alternatively, CD94/NKG2 receptor expression may have been induced on bystander CD8 cells that had been exposed to the inflammatory milieu associated with a localized anti-viral immune response. This latter explanation appears unlikely given that infection with WSN/NA/OVA fails to induce CD94/NKG2 receptor expression on transgenic gB-specific T cells.

While these data implicate TCR-mediated signals in the induction of CD94/NKG2 receptor expression, the role of TCR/
MHC interactions in maintaining expression remains unclear. Following HSV-1 infection, CD94/NKG2 receptor expression was stably maintained in excess of 60 days. However, HSV-1 infection in mice can generate a persistent infection resulting in continued presentation of the gB determinant (38). In contrast to HSV-1, influenza virus is cleared from mice following intranasal infection (39,40). This in turn should result in the cessation of presentation of the gB determinant. As observed following HSV-1 infection, CD94/NKG2 receptor expression persists beyond 60 days following infection with WSN/NA/gB. These data suggest that presentation of the cognate ligand for gBT-I CD8 T cells is not required to maintain CD94/NKG2 receptor expression. It is also possible that following Ag-dependent induction of CD94/NKG2 receptor expression, relatively low affinity interactions between the TCR and various self-peptides may be sufficient to maintain this expression.

The functional significance of CD94/NKG2 receptor expression by CD8 T cells remains unclear. Studies examining the T cell response to polyoma virus in C3H/HeN mice have shown that the CD94/NKG2-expressing T cells have an impaired ability to kill peptide pulsed target cells (24). In contrast, two recent studies focusing largely on LCMV-specific CD8 T cells found little evidence that the functional activity of CD94/NKG2 positive cells was greatly impaired (15,16). Consistent with the latter, we have previously demonstrated the in vivo expansion and acquisition of effector functions by gB-specific CD8 T cells following HSV-1 infection over the time periods described in the current experiments (28,34,37). In the current study we have shown that CD94/NKG2 receptor expression was clearly evident 48 h and perhaps as early as 24 h post-infection. Ag-stimulated cells continued to proliferate well beyond these time points, suggesting that the early induction of CD94/NKG2 receptor expression does not block the initial antigen-driven expansion of virus-specific CD8 T cells in vivo.

We have also formally demonstrated that gB-specific T cells that express CD94/NKG2 receptors are capable of producing γIFN following short term ex vivo stimulation with antigen. Moreover, through the adoptive transfer of gBT-I cells we were able to assess the response of CD94/NKG2 negative CD8 T cells of identical specificity. Over 90% of transgenic CD8 T cells that produced γIFN in response to short term ex vivo stimulation with Ag expressed CD94/NKG2 receptors. The data indicate that the gB-specific T cells that have the ability to respond rapidly to Ag reside almost entirely within the CD94/ NKG2 positive population.

It is possible that the functional differences attributed to CD94/NKG2 expression observed in some systems may be dependent on as yet undefined genetic differences between mouse strains. These could include differences in the level of expression of CD94/NKG2 receptors or their ligands. In this respect, T cells that expressed transgenic CD94/NKG2A receptors at high levels on a C57BL/6 background have been shown to have an impaired ability to upregulate CD69 following stimulation with SEB (16). Similarly, while there is little data that demonstrate that CD94/NKG2 receptors inhibit effector functions such as cytotoxicity and the production of γIFN in normal C57BL/6 mice, it is possible that they serve some other critical role. The observation that CD94/NKG2 receptors are expressed by nearly all memory gB-specific T cells raises the possibility that expression of this receptor is important in the generation or maintenance of an effective CD8 memory T cell response, perhaps serving to prevent activation-induced cell death or unwanted activation that might result from weak cross-reactivity with self-Ag.

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Abbreviations
Ag antigen
CFSE carboxyfluorescein diacetate succinimidyl ester
CMV cytomegalovirus
gB glycoprotein B
HSV-1 herpes simplex virus 1
LCMV lymphocytic choriomeningitis virus

References
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