The Inhibitory Receptor NKG2A Determines Lysis of Vaccinia Virus-Infected Autologous Targets by NK Cells

Collin R. Brooks, Tim Elliott, Peter Parham and Salim I. Khakoo

J Immunol 2006; 176:1141-1147; doi: 10.4049/jimmunol.176.2.1141
http://www.jimmunol.org/content/176/2/1141

References This article cites 51 articles, 22 of which you can access for free at: http://www.jimmunol.org/content/176/2/1141.full#ref-list-1

Subscription Information about subscribing to The Journal of Immunology is online at: http://jimmunol.org/subscription

Permissions Submit copyright permission requests at: http://www.aai.org/About/Publications/JI/copyright.html

Email Alerts Receive free email-alerts when new articles cite this article. Sign up at: http://jimmunol.org/alerts
The Inhibitory Receptor NKG2A Determines Lysis of Vaccinia Virus-Infected Autologous Targets by NK Cells

Collin R. Brooks,* Tim Elliott,* Peter Parham,† and Salim I. Khakoo2*

Signals transduced by inhibitory receptors that recognize self-MHC class I molecules prevent NK cells from being activated by autologous healthy target cells. In order for NK cells to be activated upon contact with an infected cell, the balance between the activating and inhibitory signals that regulate NK cell function must be altered in favor of activation. By studying liver-derived NK cells, we show that only a subpopulation of NK cells expressing high levels of the inhibitory receptor NKG2A are able to lyse autologous vaccinia-infected targets, and that this is due to selective down-regulation of HLA-E. These data demonstrate that release from an inhibitory receptor:ligand interaction is one mechanism that permits NK cell recognition of a virally infected target, and that the variated expression of inhibitory receptors in humans generates a repertoire of NK cells with different antiviral potentials. The Journal of Immunology, 2006, 176: 1141–1147.

Natural killer cells are lymphocytes of the innate immune system. They have a critical role in antiviral defense (1). This can be through the direct interaction with virally infected targets, or via recruitment of cells of the adaptive immune system (2, 3) and their interactions with dendritic cells (4, 5). Their functions, cytotoxicity and cytokine secretion, are controlled by activating and inhibitory cell surface receptors including several specific for MHC class I. These MHC class I receptors are especially important in the phenomenon of “missing-self” recognition (6). Such receptors on NK cells include those of the killer cell Ig-like receptor (KIR) and CD94:NKG2 families in humans, and the Ly49 and CD94:NKG2 families in rodents (7–10). These receptors recognize either classical polymorphic MHC class I molecules (KIR and Ly49), nonclassical MHC class I (KIR2DL4 and CD94:NKG2A/C/E) or MHC class I-like molecules (NKG2D) (11). Furthermore, whereas the NKG2A-E genes are present in all individuals, the KIR exhibit substantial population diversity (12–14). A second level of diversity is found at the level of individual NK cell clones. Although KIR2DL4 and NKG2D are expressed on all NK cells, the other receptors are expressed in various combinations on individual NK cells (15, 16). This combinatorial pattern of expression, also called variaged expression, generates an NK cell repertoire. Exactly how the final NK cell repertoire is shaped is not clearly understood; however, one feature is that the mature human repertoire consists predominantly of NK cells that express at least one inhibitory receptor for self MHC class I (15, 16). Population study and family analysis shows that a person’s HLA genotype and KIR genotype combine to determine the final NK cell repertoire (17, 18).

At a functional level, it is thought that there is a balance of inhibitory and activating receptors on each NK cell. Such a balance permits both self-tolerance and discrimination of infected from uninfected autologous cells. One prediction of the variated expression of receptors and the requirement for a balance of inhibitory and activating receptors is that different NK cells have different potentials to recognize infected cells. Despite the many described NK cell receptors, relatively few of them have been shown to be directly responsible for the recognition of virally infected cells (19). Activating receptors have been implicated in the immune response to HIV (20), murine CMV infection (21, 22), and also human CMV infection (23). Although conversely, no clear correlation was found between the expression of NK cell receptors and the ability to lyse CMV-infected autologous fibroblasts in vitro (24). Inhibitory NK cell receptors also play a role in generating diversity in the immune response to viral infections. However, they have been implicated predominantly in the evasion of the immune response by viruses (25–27). Recently, we performed an epidemiological study that described a protective inhibitory receptor:ligand interaction, KIR2DL3 in combination with its cognate HLA-C ligands, in hepatitis C virus infection (28). Thus, it appears that different NK cell receptors are protective in the context of different viral infections, and that this protection can be associated with both activating and inhibitory receptors.

Vaccinia virus is a member of the pox virus family, which infects a wide variety of cell types. The immune response against vaccinia virus is thought to be in part dependent on NK cells (29, 30), and NK cell proliferation in response to vaccinia infection can be detected in mice (31). Although vaccinia has many specific mechanisms for evading the host immune response, it does not appear to specifically target MHC class I and induces moderate (~50%) MHC class I down-regulation in murine cells (30, 32). Indeed, vaccinia infection is used experimentally to specifically target Ags to the MHC class I presentation pathway and sensitize targets for lysis by T cells. Furthermore, vaccinia is a clinically relevant infection because it is used for prophylactic vaccination against smallpox infection and is also being tested in trials of therapeutic vaccines against malaria and tumors (33). The aim of this study was to investigate the NK cell response to this virus in the context of autologous MHC class I, to determine whether specific receptor:ligand combinations might be associated with a protective NK cell response.
sequence analysis (34). Typing according to the method of Uhrberg et al. (12), and HLA typing by from the BLCL using the QIAamp blood kit (Qiagen) and used for KIR line from the individual was generated (34). Genomic DNA was extracted and MEM-E/08 (Abcam) were used to assess total HLA class I and HLA-E. L. Lanier (University of California, San Francisco, CA). These Abs were DX27 (anti-KIR2DL2/3), and DX31 (anti-KIR3DL2) were a gift from Dr. NKG2A) were obtained from Beckman Coulter; DX9 (anti-KIR3DL1), positive by flow cytometry. The mAbs EB6 (anti-KIR2DL1) and Z199 (anti-NKG2A) were used at a final concentration of 10 mcg/ml.

Quantitative PCR
RNA was extracted from each clone using RNAzol and cDNA synthesized as described previously (35). This was used for analysis by quantitative PCR (TaqMan; Applied Biosystems). The primers and probes used in this assay are listed in Table I. Initially, plasmids containing the KIR and NKG2 genes of interest (16) were used to setup and standardize the assay. For each reaction, 2.5 μl of cDNA was amplified in a total volume of 25 μl using 2× Universal Mastermix (Applied Biosystems), 12.5 μM of both sense and antisense primer, and 2.5 μM of labeled probe. Quantitative TaqMan PCR was performed on an Applied Biosystems 7700 Sequence Detector using SDS 1.9.1 software, under the following conditions: 50°C for 2 min, 95°C for 10 min followed by 40 – 45 cycles of 95°C for 15 s, and 60°C for 1 min. All reactions were run in duplicate or triplicate and repeated at least once. In all experiments, a standard dilution series representing between 5 × 10^7 and 50 plasmid copies per reaction was run in triplicate for direct quantification of expression of each gene of interest, as were negative control samples. Normalization of the cDNA quantity to GAPDH was performed according to the manufacturer’s instructions using a known

Materials and Methods

Cells and cell lines
NK cells were extracted, with approval of the Southampton General Hospital Institutional Review Board (Southampton, U.K.), from the surplus portion of a liver biopsy from an individual undergoing examination for the confirmation of successful resolution of hepatitis C virus infection. NK cells were isolated using CD56+ beads (Dynal Biotech). They were then cloned by limiting dilution, cultured as described previously (16), and clones used in this study were confirmed to be CD3-negative, CD56-positive by flow cytometry. The mAbs EB6 (anti-KIR2DL1) and Z199 (anti-NKG2A) were obtained from Beckman Coulter; DX9 (anti-KIR3DL1), DX27 (anti-KIR2DL2/3), and DX31 (anti-KIR3DL2) were a gift from Dr. L. Lanier (University of California, San Francisco, CA). These Abs were used to confirm clonal expression of NK cell receptors. The Abs W6/32 and MEM-E/08 (Abcam) were used to assess total HLA class I and HLA-E expression, respectively. An autologous B-lymphoblastoid cell (BLCL) line from the individual was generated (34). Genomic DNA was extracted from the BLCL using the QIAamp blood kit (Qiagen) and used for KIR typing according to the method of Uhrberg et al. (12), and HLA typing by sequence analysis (34).

Cytotoxicity assays
Cell lines were infected with vaccinia virus (WR strain) at a multiplicity of infection of 10 or “mock infected,” and infection was confirmed by Western blot using a polyclonal Ab (AMS Biotechnology). At 16 h after infection, these targets were used in51Cr-release cytotoxicity assays. Assays were performed at an E:T ratio of 6:1, unless otherwise stated, and were repeated at least once. Percentage-specific lysis was calculated using the following formula: percentage-specific lysis = (measured release-spontaneous release) × 100/(maximal release-spontaneous release). Spontaneous release was <20% of total release for all assays. Blocking Abs Z199 (anti-NKG2A) and the isotype-matched control Eric-1 (anti-CD56; Serotec) were used at a final concentration of 10 mcg/ml.

Table I. Sequences of primers and probed used in the quantitative PCR assay for the NK cell receptor genes studied

<table>
<thead>
<tr>
<th>Target</th>
<th>Sense Primer</th>
<th>Antisense Primer</th>
<th>Probe</th>
</tr>
</thead>
<tbody>
<tr>
<td>KIR2DL1</td>
<td>GTCAGATGCTCAAGTGTTGAAA</td>
<td>GTCAGTGGACGAGTGACAC</td>
<td>ACTTCTTCCATCAGTGCGATGAGCAAGACTCTTG</td>
</tr>
<tr>
<td>KIR2DL2</td>
<td>CCACTGCTGTTCTTGATGAAA</td>
<td>CAGGGAGAAATCTTGGATCAA</td>
<td>CACAGTTGAACTCAGTGTTTTCACACAGG</td>
</tr>
<tr>
<td>KIR2DL3</td>
<td>CCACTGACCTCTTCTGACATC</td>
<td>CAGGGAGAAATCTTGGATCAA</td>
<td>CACAGTTGAACTCAGTGTTTTCACACAGG</td>
</tr>
<tr>
<td>KIR2DL4</td>
<td>CCACTGACCTCTTCTGACATC</td>
<td>CAGGGAGAAATCTTGGATCAA</td>
<td>CACAGTTGAACTCAGTGTTTTCACACAGG</td>
</tr>
<tr>
<td>KIR3DL2</td>
<td>CGGATCTGCTGTTCTGACATC</td>
<td>CAGGGAGAAATCTTGGATCAA</td>
<td>CACAGTTGAACTCAGTGTTTTCACACAGG</td>
</tr>
<tr>
<td>KIR3DL3</td>
<td>TCCGCAGATGCTCTTCCGCAA</td>
<td>AAGGGCAAGCATCATGGA</td>
<td>TGGATCTTCATGACCGCTTCCACGCT</td>
</tr>
<tr>
<td>KIR3DL4</td>
<td>CGGATCTGCTGTTCTGACATC</td>
<td>CAGGGAGAAATCTTGGATCAA</td>
<td>CACAGTTGAACTCAGTGTTTTCACACAGG</td>
</tr>
<tr>
<td>KIR3DS1</td>
<td>GACCACCCCTCCATCACCAGTAAA</td>
<td>AAGGGCAAGCATCATGGA</td>
<td>TGGATCTTCATGACCGCTTCCACGCT</td>
</tr>
<tr>
<td>NKG2A</td>
<td>CAGGATCAGCAGGAAATAAACATTATGCG</td>
<td>ATGTTCATGACGAGGCAAATT</td>
<td>AGATTATCATACAGTCAGTTGAAGCTCA</td>
</tr>
<tr>
<td>NKG2C</td>
<td>TGTTGGTCCTTCTCACAACATAT</td>
<td>CAGGATCAGCCTTGAACCGAAAAT</td>
<td>CCTAGTTGACATACACTGCTGTATTAAAG</td>
</tr>
<tr>
<td>NKG2D</td>
<td>CCAGCGCTTCTGTATGACAGTCC</td>
<td>CAGGATCAGCCTTGAACCGAAAAT</td>
<td>CCTAGTTGACATACACTGCTGTATTAAAG</td>
</tr>
</tbody>
</table>

Primer sequence previously described by Uhrberg et al. (12).

FIGURE 1. Lysis of the vaccinia-infected autologous BLCL is clonally distributed and correlates with expression of NKG2A. A. A cytotoxicity assay of 12 NK cell clones tested against the 721.221 cell line (■), and the autologous BLCL either mock-infected (□) or infected with vaccinia virus (●). The assay was performed 16 h after vaccinia virus infection. The staining profile of each clone with the EB6 (KIR2DL1) and Z199 (NKG2A) Abs as determined by flow cytometry and the mean Z199 fluorescence intensity is illustrated. Clones were tested at an E:T ratio of 6:1. B. A flow cytometry histogram of the surface expression of NKG2A on two NK cell clones and a negative control T cell line as determined using the mAb Z199.
FIGURE 2. NK cell inhibition by the HLA-A*0101 allele, but not by HLA-C alleles, is overcome by vaccinia virus infection. A, A cytotoxicity assay of four NK cell clones comparing the lysis of 721.221 cells either untransfected (●) or transfected with HLA-A*0101 (●), and mock-infected (solid lines) or infected with vaccinia virus (dashed lines) at various E:T ratios. Clones 7, 10, and 12 were KIR2DL2/Eβ6%, and clone 1 was KIR2DL3/Eβ6%. B, A cytotoxicity assay of eight NK cell clones against the 721.221 cell line either untransfected or transfected with HLA-Cw*1503 or Cw*0702, and mock-infected or infected with vaccinia virus (VV). Above the chart are the results of the flow cytometry staining with the EB6, DX27, and Z199 Abs. These clones were tested at an E:T ratio of 6:1. C, A cytotoxicity assay of two NK2GA/Eβ6% NK cell clones tested against the 721.221 cell line (●), and the autologous BLCL either mock-infected (□) or infected with vaccinia virus (△). The assay was done in the presence of the anti-NKG2A Ab Z199, or an isotype-matched control (clg) as indicated. Clones were tested at an E:T ratio of 6:1.

standard concentration of RNA from the Raji cell line, which was serially diluted and used to generate a standard curve.

Due to the high degree of sequence homology between KIR genes, the specificity of each primer and probe set was tested by the following: performing each assay with 5 x 10^4 copies per reaction of a nontarget KIR; performing each assay with increasing concentrations of nontarget KIR alongside the KIR of interest; and performing the assay with mixed cDNA species derived from PBMCs. In these experiments, cross reactivity was detected only for the KIR2DL1 reaction against the KIR2DL1 cDNA clone, and this was noted only when KIR2DL3 was present at a level of 5 x 10^7 copies, which is >100-fold the level of expression found in the NK cell clones. This was determined to be insignificant because in all cases there were negligible effects, until >5 x 10^5 copies per reaction of competing KIR were introduced, and such quantities were not found during actual analysis of NK cell-derived cDNA. To further ensure the correct identity of the target gene amplified, for selected amplicons, the quantitative PCR products were run on a 3% agarose gel to determine amplicon size and directly sequenced to determine identity. All assays were also run using cDNA from the KIR-negative cell lines RPMI8866 and 721.221. The limit of detection was <50 copies per reaction for all genes apart from KIR2DS4 and KIR3DS4 (50–500 copies per reaction).

Results

Lysis of a vaccinia-infected autologous target by NK cells is clonally distributed

The NK cell donor studied in this study had the HLA type: HLA-A*0201, -A*3402, -B*0801, -B*3501, -Cw*0401; and KIR type: KIR2DL1, KIR2DL2, KIR2DL3, KIR2DL4, KIR3DL1, KIR3DL2, KIR2DS2, KIR2DS3, and KIR2DS4. We studied tissue-derived NK cell clones from a liver biopsy sample taken from this individual. Twelve NK cell clones were tested in cytotoxicity assays against the class I-negative BLCL line, 721.221, the autologous BLCL, and the autologous BLCL-infected with wild-type vaccinia virus. In 4-h 51Cr release assays, all NK cell clones killed the uninfected 721.221 cell line, and all were strongly inhibited by the mock-infected autologous BLCL (Fig. 1A). However, some NK cell clones lysed the vaccinia-infected autologous BLCL, suggesting that the strong MHC class I inhibition of NK cell lysis by the autologous BLCL can be overcome by infection with vaccinia virus, and that this phenomenon is clonally distributed.

Expression of NKG2A correlates with lysis of vaccinia-infected targets

For the NK cells of the individual studied in this study, there are two potential functional inhibitory receptor:MHC I interactions: KIR2DL1 with HLA-Cw*0401; and CD94:NKG2A with HLA-E. We found that lysis of the vaccinia-infected autologous BLCL was confined to those NK cell clones with the EB6%, Z199+ phenotype, i.e., those clones expressing NKG2A, but not KIR2DL1. Furthermore, heterogeneity was observed in the level of expression of NKG2A as determined by the mAb Z199 (Fig. 1B). The NK cell clones formed two groups: Z199 bright clones (median fluorescence intensity >200) or Z199 dim clones (median fluorescence intensity <90). Six of 7 Z199 bright clones lysed the vaccinia-infected autologous BLCL, as compared with none of the Z199 dim clones.

To determine the role of the CD94:NKG2A receptor in this interaction, we studied lysis of the MHC class I-negative 721.221 cell line either untransfected or transfected with HLA-A*0101. This HLA class I allele provides the leader peptide permissive for cell surface expression of HLA-E and thus a ligand for the CD94:NKG2A receptor. NK cell clones lysed the uninfected 721.221 cell line and the 721.221 cell line infected with vaccinia virus to a similar or slightly lesser degree (Fig. 2A). NK cell clones expressing high levels of the CD94:NKG2A receptor were inhibited from lysing the 721.221-HLA-A*0101 transfectant, and this inhibition was overcome by infection with vaccinia virus. However, no significant change in lysis was observed for the cell lines expressing low levels of NKG2A.

Conversely, NK cell clones that were inhibitable by HLA-C transfectants of the 721.221 cell line were inhibited to the same significant degree whether the transfectant was infected by vaccinia virus or mock infected. This was found to be the case for transfectants with either the C1 motif (HLA-Cw*0702) or C2 motif (HLA-Cw*1503), which did not significantly inhibit NKG2A-positive...
NK cell clones. HLA-Cw*0702 expresses a leader peptide, which can up-regulate HLA-E, but does not result in significant inhibition of NK cells by NKG2A because CD94:NKG2A exhibits peptide selectivity (36, 37), and, thus, the leader peptides of this allele is not cognate for CD94:NKG2A. However, HLA-A*0101 and HLA-Cw*1503 have similar leader peptides; therefore, the lack of inhibition of NKG2A-positive NK cell clones is most likely related to the lower expression of HLA-E on the HLA-Cw*1503 transfectant as compared with the HLA-A*0101 transfectant (data not shown). This is because of the lower expression of HLA-Cw*1503 as compared with HLA-A*0101 on these cell lines. Thus, these transfectants could be used to study the effects of vaccinia on KIR-mediated inhibition in isolation from NKG2A. Also, NK cell clones that were not inhibited by these HLA-C transfectants, but were inhibited by the 721.221-HLA-A*0101 transfectant, lysed the mock-infected and infected HLA-C transfectants to similar degrees (Fig. 2B). These data are consistent with the model that the inhibitory interaction between CD94:NKG2A and HLA-E, but not that between HLA-Cw*0401 and KIR2DL1, can be overcome by vaccinia virus infection. Lysis of the 721.221 cell line and BLCL is determined predominantly by the activating receptor Nkp46, which is expressed on all NK cells (38). In these experiments, infection of the untransfected 721.221 cells did not augment their lysis, implying that loss of inhibitory signal, and not up-regulation of an activating receptor:ligand interaction, is the primary mechanism for the clonal response to vaccinia infection. Furthermore, consistent with this model, if the inhibitory receptor NKG2A was blocked using the mAb Z199, then vaccinia infection did not augment lysis of the autologous BLCL (Fig. 2C).

Lysis of vaccinia-infected targets correlates with high levels of NKG2A and low levels of inhibitory KIR

To further study the receptor complement on the NK cells in this study, quantitative PCR (TaqMan) assays for NKG2A, NKG2C, NKG2D, KIR2DL1, KIR2DL2, KIR2DL3, KIR2DL4, KIR3DL2, KIR2DS2, KIR2DS3, and KIR2DS4 were devised. cDNA was derived from each clone as well as four others not tested in cytotoxicity assays and used to determine the copy number of each transcript relative to GAPDH expression (Fig. 3A). Selected transcripts for each gene were sequenced, and these further confirmed the fidelity of the assay. In general, transcripts measured at <1000 copies per nanogram of GAPDH did not generate interpretable sequence data and so may be either low-level transcript of the gene of interest, as observed by Chan et al. (39), or true cross-reactive transcripts.

Expression of KIR and C-type lectin-like receptors was variaged among the NK cell clones, consistent with the clonal expression of these receptors. The two exceptions to this were KIR2DL4 and NKG2D. These receptors are expressed on all NK cells and were found to be more tightly regulated with expression levels of $10^4$–$10^5$ copies/ng GAPDH and $10^3$–$10^6$ copies/ng GAPDH, respectively. NKG2A expression was inversely correlated with that of the inhibitory MHC-C-specific KIR (Fig. 3B). No correlations were observed between the expression of the following: NKG2A and the activating KIR (KIR2DS2, KIR2DS3, and KIR2DS4); NKG2A and KIR2DL4; NKG2C and the activating KIR; and NKG2D and the activating KIR (Fig. 3, C–F).

The level of expression of NKG2A also correlated well with the ability to lyse the vaccinia-infected autologous BLCL (Fig. 4). Six of 7 clones with high levels of expression of NKG2A lysed the infected autologous BLCL, whereas all those with low levels of expression did not. Clone 6, which did not lyse the vaccinia-infected BLCL and expressed high levels of NKG2A, also expressed LILRB1 (ILT2). Because this clone did lyse the vaccinia-infected 721.221-A*0101 cell line and not the mock-infected 721.221-A*0101 cell line, this is consistent with LILRB1 conferring an additional inhibitory signal on the clone, which was not affected by vaccinia infection. No further correlations were observed with the other receptors tested and the
ability to lyse the vaccinia-infected target, including the activating receptor NKG2D.

**Vaccinia virus infection induces down-regulation of HLA-E**

The levels of MHC class I expression were determined in infected or mock-infected autologous BLCL at the midtime point of the cytotoxicity assays using the autologous BLCL and the 721.221 cell line transfected with HLA-Cw*0401. At 18 h postinfection, total MHC class I was intact, but HLA-E was consistently reduced by ~40% (Fig. 5). This implies that the NKG2A ligand HLA-E, but not the KIR ligand HLA-C, is down-regulated in this system. Hsp60 has a leader sequence that binds HLA-E but is not permissive for recognition by NKG2A (40). However, we did not observe a change in the levels of hsp60 by Western blot (not shown). Therefore, we suggest that the level of class I down-regulation that we observed is adequate to permit lysis by NK cell clones inhibited predominantly by NKG2A.

**Discussion**

We have used an autologous system to study the interaction of NK cells with virally infected cells. This implies that all potential interactions between MHC class I and NK cells are present. These interactions are perturbed by vaccinia infection in a manner that is distributed clonally among NK cells and determined by expression of the inhibitory receptor NKG2A. This implies that release from inhibition, by down-regulation of HLA-E, is a mechanism that permits lysis of these cells via activating signals such as those derived from the natural cytotoxicity receptors or NKG2D. The inverse correlation of NKG2A with inhibitory KIR expression suggests that the level of inhibitory receptor expression is regulated, either as a primary mechanism or via a selective process. Thus, differential expression of inhibitory receptors can generate NK cell clones with different antiviral potentials. This model is consistent with the protective role of the inhibitory interaction KIR2DL3-HLA-C^*A0607, in hepatitis C virus infection (28). Furthermore, because NKG2A is nonpolymorphic and most individuals have populations of KIR-negative NKG2A-positive NK cells, these results should be extendable to other individuals.

The correlation between expression of NKG2A and inhibitory KIR suggests that in these tissue-derived NK cells, there is, at a functional level, either an interaction between the regulation of these two receptor families to maintain a specific level of inhibitory receptors on the cell surface, or that NK cell clones with a specific level of inhibitory receptors have been selected for during ontogeny. By contrast, there appears to be no clear relationship between NKG2C or NKG2D and the activating KIR, implying that different mechanisms coordinate the expression of inhibitory and activating receptors on NK cells. These differences may relate to the differing functions of these molecules: inhibitory receptors recognize ligands expressed on healthy cells, but activating receptors recognize stress-induced ligands and viral products (22, 41, 42). A system in which the expression of inhibitory receptors is well controlled, permits NK cells to become activated following the subtle changes in MHC class I expression that we have observed, while simultaneously preserving self-tolerance to healthy cells. Thus, although we have not exhaustively excluded a role for other receptors, our data imply that the NKG2A:HLA-E interaction is a major determinant in the lysis of these vaccinia-infected cells.

The correlation for NKG2A and KIR2DL1 was not as precise as that for NKG2A vs all KIR2DL1/KIR2DL2/KIR2DL3 because one
clone that expressed relatively low levels of both KIR2DL1 and NKG2A also expressed very high levels of KIR2DL3. Potentially, the quantitative PCR assay may not have recognized an allelic variant of KIR2DL1, although this was not confirmed by sequence analysis of complimentary DNA from the clone. Alternatively, as the clone did not lyse the autologous BLCL, a different receptor, such as CEACAM1 (43), may provide the inhibitory signal that prevents auto-reactivity. Although we cannot completely exclude the induction of NKG2A during the culture techniques (44), this is unlikely for two reasons: all NK cell clones were cultured under the same conditions, and we observed substantially different levels of NKG2A expression; and NK cells cultured under these conditions previously have been shown to have stable receptor repertoires (16).

In this system, in which MHC class I expression is high, down-regulation appears to be confined to HLA-E, with relative sparing of other HLA class I molecules. Allelic specificity of MHC class I to the effects of vaccinia virus have been observed in L929 cells, in which H-2Dk is more sensitive to vaccinia-induced down-regulation than H-2Kk (30). Such allelic specificity may explain the lower levels of class I down-regulation that we have observed in our experiments as compared with the previous study. Alternatively, these differences may be related to the cell line studied, because BLCL express relatively high levels of MHC class I. The mechanism inducing this down-regulation is, at present, unclear. However, it has recently been shown that Qa-1 and HLA-E are unstable at the cell surface in comparison to classical MHC class I. These nonclassical class Ib molecules thus require a continuous supply of cognate peptide to maintain cell surface expression and thence mediate effective NK cell inhibition (45). One possibility to explain the specificity of MHC class I down-regulation is that vaccinia virus inhibits host protein synthesis (46, 47). Thus, it could disrupt the supply of cognate peptide to HLA-E. Because HLA-E expression on the cell surface is dependent on its binding to a cognate peptide (48), a loss of supply of these peptides prevents newly synthesized HLA-E molecules from appearing on the cell surface. HLA-E complexes are more unstable than those of classical MHC class I (45). Thus, similar to its murine homologue Qa-1, it likely turns over more rapidly on the cell surface than classical MHC class I molecules. This implies that down-regulation of HLA-E could be evident before that of classical MHC class I molecules. This process could therefore represent a mechanism of NK cell recognition that is common to the many other viral infections that disrupt host protein synthesis (49). Alternatively, because we observed no classical MHC class I down-regulation in our system, vaccinia virus may encode a novel mechanism that specifically targets HLA-E, but leaves cell surface expression of other HLA class I molecules intact. Thus, the potential mechanisms for allele-specific MHC class I down-regulation require further study.

Inhibitory receptors for MHC class I provide a dominant signal on NK cells that prevents auto-reactivity. The clonal distribution of these receptors provides a key component that generates diversity in the NK cell repertoire within an individual. In both humans and mice, CD94:NKG2A sense global MHC class I expression via presentation of their leader peptides by nonclassical class Ib molecules (48, 50). This conserved relationship is in contrast to the rapid evolution of the KIR and Ly49 receptors (35, 51). We have shown that expression of this inhibitory receptor confers a clonally distributed antiviral function to NK cells.

Acknowledgments
We thank S. Cooper and R. Gish for the liver biopsy sample, and W. Rosenberg, V. Braud and A. Williams for helpful discussion.

Disclosures
The authors have no financial conflict of interest.

References
Downregulation of natural killer cell-activating ligand CD155 by human cyto-
inhibitory receptor genes in resolving hepatitis C virus infection. Science 305:
872–874.
Inhibitory receptor genes in natural killer cell function. J. Immunol. 131:
1531–1538.
to infections of cells by natural killer (NK) cytophilic cytolysis correlates with
enhanced NK cell triggering and is concomitant with a decrease in
31. Dokun, A. O., S. Kim, H. R. Smith, H. S. Kang, D. T. Chu, and
D. T. Chu, J. W. Barrett, H. Everett, C. Cameron,
natural killer cell depletion enhances virus synthesis and virus-induced hepatitis
32. Seet, B. T., J. B. Johnston, C. R. Brunetti, J. W. Barrett, H. Everett, C. Cameron,
34. Cooper, S., A. L. Erickson, E. J. Adams, A. J. Weiner, D. Y. Chien,
M. Houghton, P. Parham, and C. M. Walker. 1999. Specific and nonspecific NK cell activation during virus
35. Khakoo, S. I., R. Rajalingam, B. P. Shum, K. Weidenbach, L. Flodin, D. G. Muir,
D. Saha, and N. K. Gupta. 1997. Viral infection. I. Regulation of
M.-A. Mei-Lin, W. M. Yokoyama. 2001. Specific and nonspecific NK cell activation during virus
37. Kaiser, B. K., F. Barahmand-Pour, W. Paulsene, S. Medley, D. E. Geraghty, and
D. Llano, M., N. Lee, F. Navarro, P. Garcia, J. P. Albar, D. E. Geraghty, and
D. E. Geraghty, and N. J. Chalupny. 2004. The mechanisms con-
trolling NK cell autoreactivity in TAF2-deficient patients. Blood 103:
1770–1778.
38. Sivori, S., D. Pende, C. Bottino, E. Marcenaro, A. Pessino, R. Biaissoni,
L. Moretta, and A. Moretta. 1999. Nonclassical MHC class I molecule Qa-1 forms unstable
39. Chan, H. W., Z. B. Kurage, C. A. Stewart, M. J. Wilson, M. P. Martin,
maintains allele-specific KIR gene expression in human natural killer cells.
40. Michaelsson, C., J. Teixeira de Matos, A. Achour, L. L. Lanier, K. Karre, and
S. Jiang, H. W., Z. B. Kurage, C. A. Stewart, M. J. Wilson, M. P. Martin,
maintains allele-specific KIR gene expression in human natural killer cells.
2001. Activation of NK cells and T cells by NKG2D, a receptor for stress-
42. Cosman, D., J. Mullberg, C. L. Sutherland, W. Chin, R. Armitage, W. Fanslow,
M. Kubin, and N. J. Chalupny. 2001. ULBP, novel MHC class I-related mole-
cules, bind to CMV glycoprotein UL16 and stimulate NK cytotoxicity through the
NKG2D receptor. Immunity 14: 123–133.
43. Markel, G., H. Mussaffi, K. L. Ling, M. Salio, S. Gadola, G. Steuer, H. Blau,
trolling NK cell autoreactivity in TAF2-deficient patients. Blood 103:
1770–1778.
inhibition by HLA class I. Blood 105: 2028–2035.
45. Kamayashi, T., J. R. Kralj-Leavy, J. G. Dauner, A. D’Andrea,
46. Guerra, S., A. L. Lopez-Fernandez, A. Pascual-Montano, M. Munoz,
N. J. Chalupny. 2004. The mechanisms con-
trolling NK cell autoreactivity in TAF2-deficient patients. Blood 103:
1770–1778.
binds to natural killer cell receptors CD94/NKG2A, B and C. Nature 391:
795–799.
Ib molecule Qa-1 by putative activating receptors CD94/NKG2C and CD94/
cluster in C57BL/6 mice: a rapidly evolving multigene family in the immune