

Distinct KIR/HLA compound genotypes affect the kinetics of human antiviral natural killer cell responses

Golo Ahlenstiel,¹ Maureen P. Martin,² Xiaojiang Gao,² Mary Carrington,² and Barbara Rehermann¹

¹Immunology Section, Liver Diseases Branch, National Institute of Diabetes and Digestive and Kidney Diseases (NIDDK), NIH, Bethesda, Maryland, USA.

²Laboratory of Genomic Diversity, Science Applications International Corporation–Frederick, Inc., National Cancer Institute–Frederick, Frederick, Maryland, USA.

Genetic studies suggest a role for killer cell immunoglobulin-like receptor/HLA (KIR/HLA) compound genotypes in the outcome of viral infections, but functional data to explain these epidemiological observations have not been reported. Using an in vitro model of infection with influenza A virus (IAV), we attribute functional differences in human NK cell activity to distinct KIR/HLA genotypes. Multicolor flow cytometry revealed that the HLA-C–inhibited NK cell subset in HLA-C1 homozygous subjects was larger and responded more rapidly in IFN- γ secretion and CD107a degranulation assays than its counterpart in HLA-C2 homozygous subjects. The differential IFN- γ response was also observed at the level of bulk NK cells and was independent of KIR3DL1/HLA-Bw4 interactions. Moreover, the differential response was not caused by differences in NK cell maturation status and phenotype, nor by differences in the type I IFN response of IAV-infected accessory cells between HLA-C1 and HLA-C2 homozygous subjects. These results provide functional evidence for differential NK cell responsiveness depending on KIR/HLA genotype and may provide useful insights into differential innate immune responsiveness to viral infections such as IAV.

Introduction

As part of the innate immune system, NK cells present a first line of defense against viral infections and tumors (1). NK cell effector functions, such as cytotoxicity and cytokine release, are controlled by integrated signals from a large panel of both activating and inhibitory receptors (2–4). Killer cell immunoglobulin-like receptors (KIRs) on NK cells and their ligands, HLA class I molecules, play an essential part in this tight regulation. Both the *KIR* gene cluster and the *HLA* class I loci are extraordinarily diverse, which led to the hypothesis that NK cell immune responses are genetically predetermined to some extent (4). This is supported by recent epidemiological observations that *KIR/HLA* compound genotypes with a supposedly activating profile (i.e., presence of activating *KIR* or lack of inhibitory *KIRs* or their respective ligands) are associated with resistance to HCV (5) and HIV infection (6), slower HIV disease progression (7), and better reproductive fitness (8). On the other hand, activating profiles enhance the risk for autoimmune disease such as psoriasis/psoriatic arthritis (9–13), type I diabetes (14), and scleroderma (15).

Several of these studies take into account *HLA-C*, the gene locus encoding ligands for KIR2DL receptor, where a functional dimorphism determines KIR specificity. *HLA-C* group 1 (*HLA-C1*) alleles, encoding Ser77/Asp80 of the HLA-Cw α 1 domain, bind to the inhibitory receptors KIR2DL2 and KIR2DL3 and probably also to the activating KIR2DS2 (16, 17). In contrast, the *HLA-C* group 2 (*HLA-C2*) alleles, encoding Asp77/Lys80, bind to KIR2DL1 and possibly to KIR2DS1 (16, 17). Homozygosity for *HLA-C1* alleles and *KIR2DL3* is associated with resolution of HCV infection as compared with homozygosity or heterozygosity for *HLA-C2* (5).

In contrast, *HLA-C2* alleles are associated with protection against cervical neoplasia (18) and, to some extent, nasopharyngeal carcinoma (19). Thus, *KIR/HLA-C* compound genotypes contribute to susceptibility or resistance to a variety of infectious diseases and cancer (20). In particular, homozygosity for *HLA-C1* and *KIR2DL3* may be advantageous in viral infections, but detrimental in chronic inflammatory conditions that play a role in carcinogenesis (21).

To date, the functional mechanisms responsible for these epidemiological associations are poorly defined. It has been proposed that improved resistance to virus infections among *KIR2DL3/HLA-C1*–positive individuals may be the result of weaker NK cell inhibition through KIR2DL3 compared with NK cell inhibition through KIR2DL1 in *KIR2DL1/HLA-C2*–positive individuals (5, 8, 22). Consistent with this notion, Winter et al. showed weaker binding between a KIR2DL3-Fc fusion protein and *HLA-C1* transfectants than between KIR2DL1-Fc fusion protein and *HLA-C2* transfectants (23). However, direct measurements by surface plasmon resonance revealed almost similar affinity between KIR2DL3 and *HLA-C1* and KIR2DL1 and *HLA-C2*, respectively (24–26).

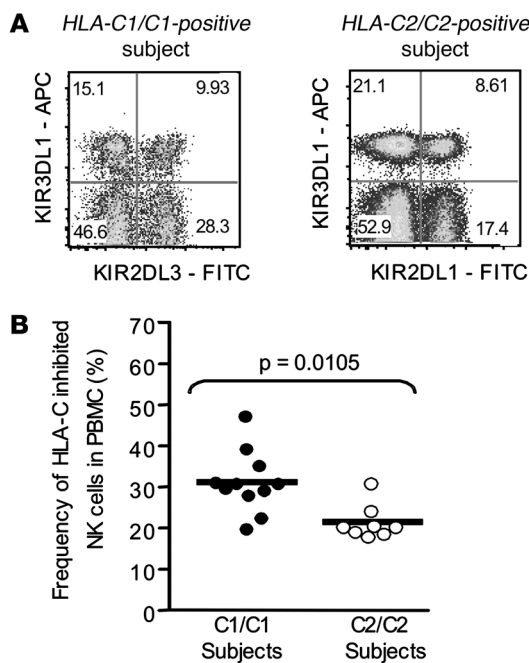
Apart from their role in inhibiting NK cell function, KIR and *HLA-C* molecules also play an essential role during the development of NK cells, because functional maturation of NK cells requires specific interaction with MHC class I molecules. For example, NK cells that express MHC class I–specific inhibitory receptors have been found to be functionally reactive, but NK cells that lack MHC class I–specific inhibitory receptors are hyporeactive to the same stimuli and do not exhibit cytotoxicity to MHC class I–deficient target cells (27, 28). Likewise, inhibitory receptor–expressing NK cells of MHC class I–deficient patients (29–31) and NK cells of $\beta_2m^{-/-}$ (32–34), *TAP^{-/-}* (31, 35), or MHC-deficient (34, 36) mice do not kill MHC class I–deficient normal cells.

In order to characterize NK cell responsiveness in the context of different *HLA* alleles, we studied the kinetics of NK cell

Nonstandard abbreviations used: EIA, enzyme immunoassay; EMA, ethidium monoazide; IAV, influenza A virus; KIR, killer cell immunoglobulin-like receptor.

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cells was analyzed in *HLA-C1/C1* and *HLA-C2/2* subjects, respectively. KIR2DL3⁺KIR2DL1⁺ double-positive NK cells were included in the KIR2DL3⁺ NK cell population in *HLA-C1* homozygous subjects and in the KIR2DL1⁺ NK cell population in *HLA-C2* homozygous subjects. This was justified because KIR2DL3⁺KIR2DL1⁺ NK cells were inhibited by HLA-C1 in *HLA-C1* homozygous subjects and by HLA-C2 in *HLA-C2* homozygous subjects, as *HLA-C1/C2* heterozygous subjects were excluded from our study.

As shown in Figure 1A for a single subject from each group and in Figure 1B for all subjects, the frequency of HLA-C-inhibited NK cells was significantly higher in *HLA-C1* homozygous than in *HLA-C2* homozygous subjects, that is, KIR2DL3 was expressed on a greater percentage of NK cells in *HLA-C1* homozygous subjects than was KIR2DL1 in *HLA-C2* homozygous subjects ($P = 0.0105$; Figure 1B). Of note, there were no differences in the absolute number of NK cells between groups (*HLA-C1* group, mean 253.3 NK cells/ μ l blood; *HLA-C2* group, mean 290.9 NK cells/ μ l blood; $P = \text{NS}$) or their frequency in total PBMCs (mean, 15.5% vs. 12.9%; $P = \text{NS}$).

Multicolor flow cytometry demonstrated that a substantial percentage of NK cells was negative for CD25, CD117, and Nkp44 and positive for CD122, CD16, NKG2A, and NKG2D (Figure 2A), consistent with a mature phenotype (46). This phenotype extended to NK cells that were inhibited by HLA-C (Figure 2B) as well as to those NK cells that were not inhibited by HLA-C (Figure 2C) and did not differ between the *HLA-C1* and the *HLA-C2* homozygous groups (Figure 2, A–C).

To examine whether NK cells from *HLA-C1* homozygous subjects displayed intrinsically different cytotoxic responses relative to *HLA-C2* homozygous subjects, the level of NK cell cytotoxicity against HLA-negative K562 target cells was determined in a standard cytotoxicity assay. As shown in Supplemental Figure 1 (available online with this article; doi:10.1172/JCI32400DS1), NK cell cytotoxicity of *HLA-C1* homozygous subjects did not differ from those of *HLA-C2* homozygous subjects at any of the effector/target ratios or assay time points. Furthermore, IFN- γ release was studied after 5, 7, and 9 h of NK cell incubation with K562 cells and

Figure 1

Identification and quantitation of HLA-C-inhibited NK cells by flow cytometry. (A) Gating strategy used to identify HLA-C-inhibited NK cells by flow cytometry. After gating on single cells (forward scatter–height versus forward scatter–area) and lymphocytes (forward scatter versus side scatter), NK cells were identified by gating on CD56⁺ cells and by excluding CD3⁺ T cells, CD14⁺ monocytes, CD19⁺ B cells, and EMA⁺ dead cells. Samples from *HLA-C1/C1* subjects were stained with antibodies to KIR2DL3, and samples from *HLA-C2/C2* subjects were stained with antibodies to KIR2DL1. All samples were stained with antibodies to KIR3DL1 to allow further subset analysis as shown in Figure 5. Numbers indicate the percentage of events in each quadrant. (B) Frequency of HLA-C-inhibited NK cells in PBMCs. The HLA-C-inhibited NK cell subset consists of KIR2DL3⁺ NK cells in *HLA-C1* homozygous subjects and KIR2DL1⁺ NK cells in *HLA-C2* homozygous subjects. Horizontal lines indicate the mean.

did not differ between subject groups (data not shown). Therefore, bulk NK cells of both groups did not differ in their ability to kill HLA-C-negative K562 cells (Supplemental Figure 1).

NK cells of *HLA-C1* and *HLA-C2* homozygous subjects display differential degranulation and cytokine responses to IAV infection. To examine NK cell responses in the context of each subject's HLA haplotype, T cell-depleted PBMCs of *HLA-C1* or *HLA-C2* homozygous subjects were infected with IAV. This model had the advantage of physiological expression levels of (a) KIR on NK cells and (b) HLA molecules on autologous, IAV-infected target cells, namely monocytes. T cells were depleted from PBMCs prior to IAV infection, because T cell-derived IL-2 enhances NK cell responses (47) and because IL-2 secretion by IAV-specific memory T cells may vary among individuals based on their IAV infection history.

Because IAV-infected monocytes label poorly with ⁵¹Cr and are therefore not optimal for a traditional cytotoxicity assay, NK cells were analyzed for CD107a expression. An increase in the expression of CD107a on the cell membrane is a marker for degranulation of perforin- and granzyme-containing vesicles, corollaries for NK cell cytotoxicity (48, 49). Bulk NK cells of *HLA-C1* homozygous subjects showed higher CD107a mean fluorescence intensity (MFI) than did bulk NK cells of *HLA-C2* homozygous subjects during the first 3, 5, 7, 9, and 17 h of the IAV assay (Figure 3A). Differential CD107a expression was most pronounced specifically for HLA-C-inhibited NK cell populations of *HLA-C1* and *HLA-C2* homozygous groups (Figure 3B). In contrast, the CD107a expression level of NK cells not inhibited by HLA-C did not differ between *HLA-C1* and *HLA-C2* homozygous subjects (Figure 3C).

Next, IFN- γ production was studied as additional readout of NK cell function. To better understand the differential response kinetics of HLA-C-inhibited NK cells in the 2 subject groups, the percentage of IFN- γ -secreting HLA-C-inhibited NK cells was determined 3, 5, 7, 9, and 17 h after IAV infection using T cell-depleted PBMCs from 5 donors in each group. Using an IFN- γ secretion assay, we calculated how many HLA-C-inhibited NK cells were secreting IFN- γ at each time point after IAV infection relative to the frequency of NK cells at the end of the assay (17-h time point). The results were displayed as a percentage of the 17-h time point rather than as absolute values in order to assess the early kinetics of the NK cell response and to eliminate an effect of differences in the absolute response strength between individuals. Consistent with the results of the CD107a assay (Figure 3B), HLA-C-inhibited (i.e., KIR2DL3⁺) NK cells from *HLA-C1* homozygous subjects displayed a faster response than did

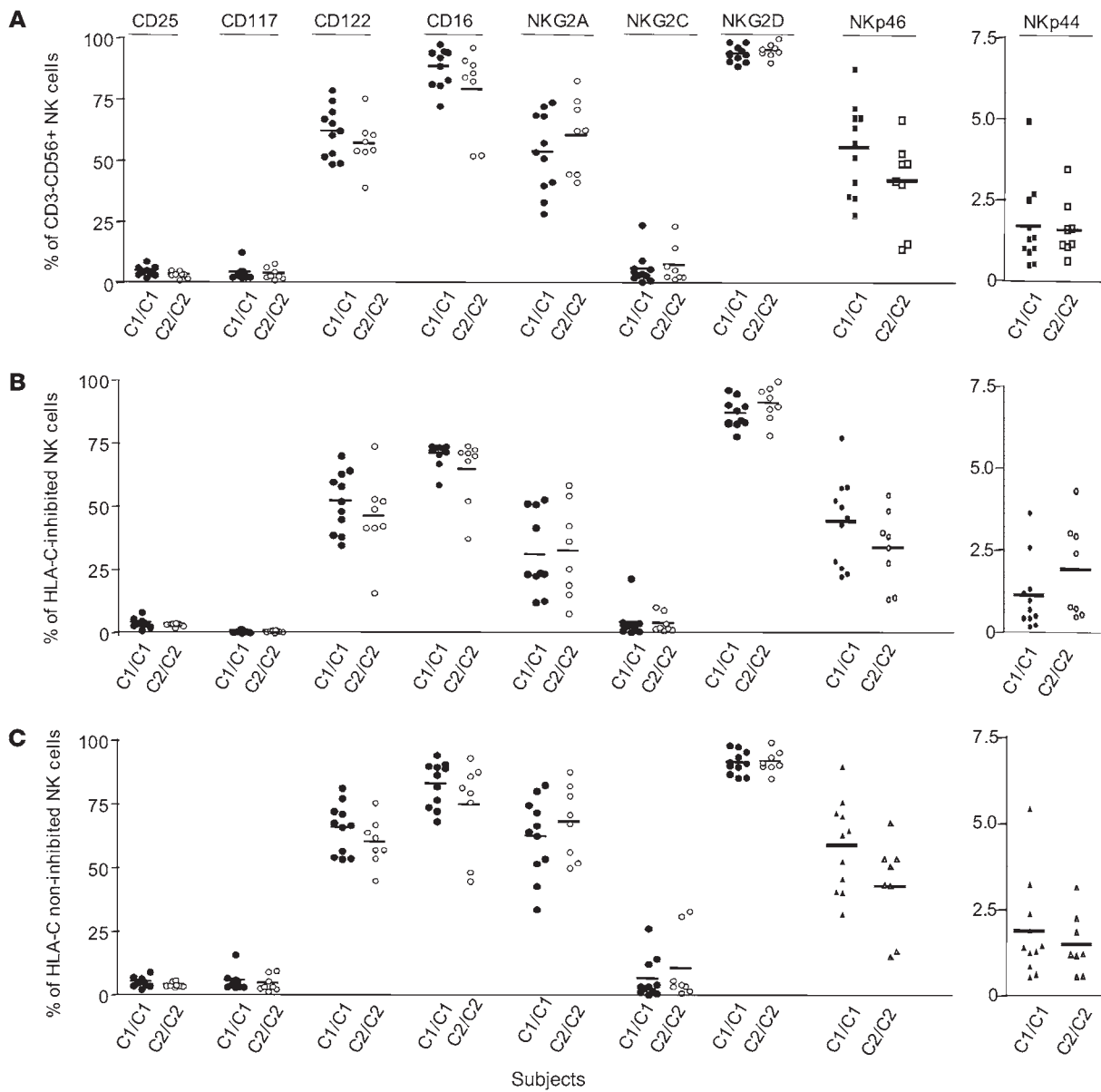


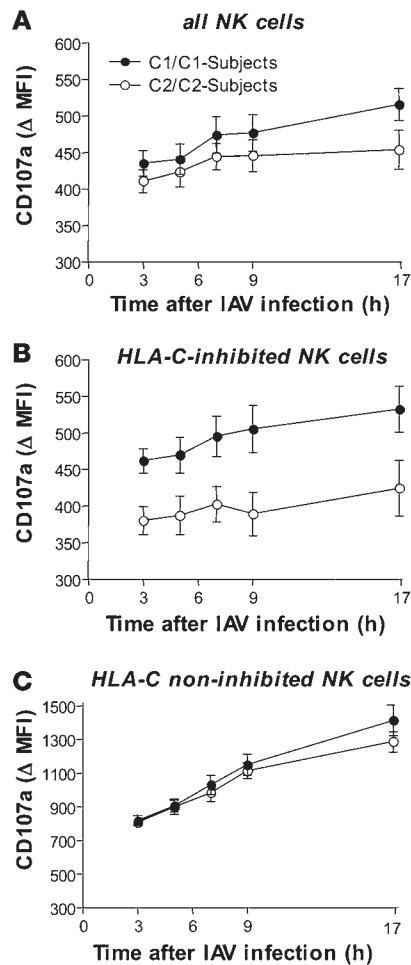
Figure 2 Phenotype of HLA-C–inhibited NK cells in *HLA-C1* and *HLA-C2* homozygous subjects. The total NK cell population (A) and the HLA-C–inhibited (B) and HLA-C–noninhibited (C) subpopulations displayed a mature phenotype and did not differ between *HLA-C1* and *HLA-C2* homozygous subjects. Horizontal lines indicate the mean.

HLA-C–inhibited (i.e., KIR2DL1⁺) NK cells from *HLA-C2* homozygous subjects, as evidenced by a greater slope of the response curve during the first 5 h of IAV infection (Figure 4A).

These results were confirmed by assessing the percentage of IFN- γ -secreting HLA-C–inhibited NK cells at the 9-h and 17-h time points for a larger number of subjects in the *HLA-C1* and *HLA-C2* homozygous groups. As shown in Figure 4B, the 9-h/17-h ratio of IFN- γ -secreting HLA-C–inhibited NK cells was significantly higher for the *HLA-C1* homozygous group than for the *HLA-C2* homozygous group ($P = 0.021$). There was no significant difference in the percentage of IFN- γ -secreting HLA-C–inhibited NK cells in the *HLA-C1* and *HLA-C2* homozygous groups at the final, 17-h assay time point (data not shown). These results indicated that there may be

functional differences between *HLA-C1* and *HLA-C2* homozygous subjects due to faster response kinetics of KIR2DL3⁺ NK cells from *HLA-C1* homozygous subjects compared with KIR2DL1⁺ NK cells from *HLA-C2* homozygous subjects.

To investigate whether the differential response of the HLA-C–inhibited NK cell subsets was associated with a differential overall bulk NK cell response, IFN- γ release into the supernatant was measured by enzyme immunoassay (EIA) at 3, 5, 7, 9, 12, 15, and 17 h after IAV infection. Figure 4C shows the amount of IFN- γ released into the supernatant at each time point relative to the total amount released during the entire 17 h of the assay (set as 100%). Consistent with the previous results, bulk NK cells of the *HLA-C1* homozygous group released IFN- γ faster than did bulk NK cells of the *HLA-C2*

**Figure 3**

HLA-C-inhibited NK cells of *HLA-C1* homozygous subjects show a stronger increase in CD107a expression after IAV infection than do HLA-C-inhibited NK cells of *HLA-C2* homozygous subjects. Upregulation of CD107a, a surrogate marker of degranulation and cytotoxicity, is shown at the indicated times after IAV infection of CD3-depleted PBMCs for the total NK cell population (A) and the HLA-C-inhibited (B) and HLA-C-noninhibited (C) NK cell subpopulations. Values are mean \pm SEM. $n = 7$ per group.

these data show a differential response of HLA-C1- and HLA-C2-inhibited NK cells in *HLA-C1* and *HLA-C2* homozygous subjects. Thus, *HLA-C* genotypes may influence the strength of the early NK cell response when HLA-C allotypes are present on target cells.

KIR3DL1 and *HLA-Bw4* do not mediate any confounding effect. To analyze potential confounding effects, we examined a possible influence of *KIR3DL1*, a locus that displays significant polymorphism and is associated with HLA-Bw4-mediated inhibition of NK cells (50–52). This was important because a substantial proportion of *KIR2DL1*⁺ and *KIR2DL3*⁺ cells coexpressed *KIR3DL1* (Figure 5A), because coexpression of multiple inhibitory KIRs affects the functional capacity of NK cells (53), and because the *KIR3DL1* ligand, HLA-Bw4, was observed less frequently in the *HLA-C1* homozygous group (6 of 12 subjects, 50.0%) than in the *HLA-C2* homozygous group (8 of 10 subjects, 80.0%). Antibodies against *KIR3DL1* were therefore included in the flow cytometry staining panel, and *KIR3DL1*⁺ cells were excluded from the analysis. As shown in Figure 5, B and C, the differences in the time kinetics described above for the total HLA-C-inhibited NK cell population of *HLA-C1* and *HLA-C2* homozygous subjects were completely reproducible after exclusion of *KIR3DL1*⁺ cells from the assays. Consistent with our above findings, the 9-h/17-h ratio of cytokine-secreting NK cells after IAV infection was significantly higher for *HLA-C1* homozygous subjects than for *HLA-C2* homozygous subjects ($P = 0.008$; Figure 5B). Moreover, detailed kinetic analysis confirmed that the response pattern of HLA-C-inhibited NK cells of both groups did not change after exclusion of *KIR3DL1*⁺ cells (Figure 5C). Thus, the differential response of HLA-C-inhibited NK cells of *HLA-C1* and *HLA-C2* homozygous subjects was independent of *KIR3DL1*.

homozygous group. The time interval from the start of the assay to the time point at which 50% of the total IFN- γ release was achieved was significantly shorter for the *HLA-C1* homozygous group than for the *HLA-C2* homozygous group ($P = 0.024$; Figure 4C).

This differential response kinetics of the bulk NK cell population was confirmed by studying samples from a larger number of subjects in the *HLA-C1* and *HLA-C2* homozygous groups at the 9- and 17-h time points. As shown in Figure 4D, bulk NK cells of *HLA-C1* homozygous subjects released a greater percentage of the total IFN- γ amount within the first 9 h of the 17-h infection assay than did bulk NK cells of *HLA-C2* homozygous subjects ($P = 0.006$). As mentioned above, the total amount of IFN- γ released during the full 17 h of the assay did not significantly differ between *HLA-C1* and *HLA-C2* homozygous subjects (mean IFN- γ , *HLA-C1* homozygous 1,423 pg/ml; *HLA-C2* homozygous 832 pg/ml; $P = \text{NS}$).

To evaluate whether NK cell-independent factors might contribute to this differential response, we asked whether macrophages and dendritic cells from *HLA-C1* and *HLA-C2* homozygous subjects responded differentially to IAV infection. Supernatants from the same IAV assays used to study NK cell functions were used to assess production of IFN- α and - β . Those cytokines were chosen because they are produced by dendritic cells and by virus-infected cells (in our IAV assay mainly by monocytes) and influence NK cell function. However, IFN- α and - β release did not differ between *HLA-C1* and *HLA-C2* homozygous groups (data not shown). Collectively,

Discussion

A growing number of epidemiological studies suggest an influence of the *KIR/HLA* compound genotypes on the outcome of a variety of diseases (5–9, 11, 12). However, to our knowledge, functional data to explain these genetic data have not been reported to date. Here we describe a model system to investigate human NK cell responses to IAV infection. In this model, NK cell responses were studied in the context of physiological expression levels of KIR on NK cells and HLA molecules on autologous, IAV-infected monocytes. Multicolor flow cytometry analysis of NK cell subsets allowed us to attribute differences in frequency and antiviral function of HLA-C-inhibited NK cell activity to distinct *KIR/HLA-C* compound genotypes.

For this study, we chose a unique cohort of well-characterized subjects who were all homozygous for the *KIR* haplotype A, the most common *KIR* haplotype worldwide. Homozygosity for this haplotype is found in 30% of white individuals and 56% of Japanese individuals (45). Moreover, nearly all *KIR* haplotypes worldwide contain *KIR2DL1* along with either *KIR2DL3* or *KIR2DL2*. HLA-C allotypes, the ligands for *KIR2DL* molecules, therefore almost

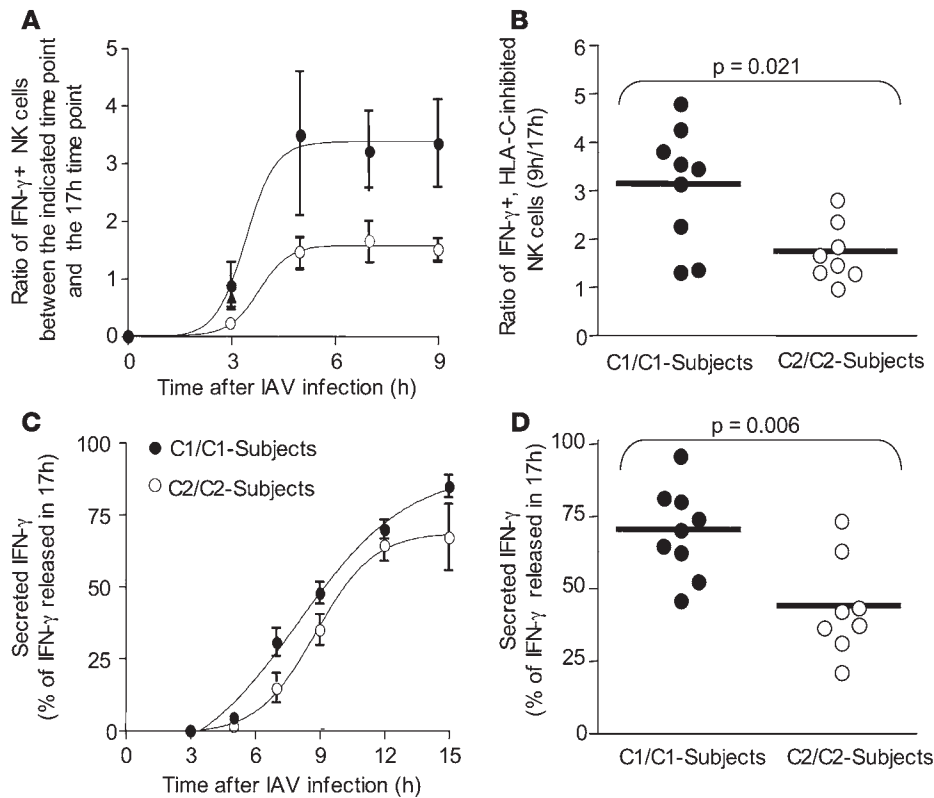


Figure 4 HLA-C–inhibited NK cells of *HLA-C1* homozygous subjects secrete IFN- γ more rapidly after IAV infection than do HLA-C–inhibited NK cells of *HLA-C2* homozygous subjects. (A and C) Kinetic analysis of the IFN- γ response of (A) HLA-C–inhibited NK cells and (C) the total NK cell population for a subgroup of *HLA-C1* and *HLA-C2* homozygous subjects. The respective NK cell populations were identified in IAV-infected, CD3-depleted PBMCs in a cytokine-secretion assay by flow cytometry. Mean and SEM at each time point of the infection assay are shown relative to the study end point at 17 h (expressed as ratio). $n = 5$ per group. (B) Frequency of IFN- γ ⁺, HLA-C–inhibited NK cells at the 9-h time point of the IAV assay relative to the 17-h time point (expressed as 9-h/17-h ratio). (D) IFN- γ released by the total NK cell population during the first 9h of the IAV assay relative to IFN- γ released during the total 17h of the IAV assay (set as 100%). Experiments in B and D were performed as in A, but included a larger number of *HLA-C1* ($n = 9$) and *HLA-C2* homozygous subjects ($n = 8$). Horizontal lines indicate the mean.

always inhibit a subset of each individual’s NK cell population. In contrast, inhibition by HLA-A and -B allotypes is less common, because it has only been shown for HLA-A3/-A11 (54, 55) and HLA-Bw4 (56, 57), which bind to KIR3DL2 and KIR3DL1, respectively.

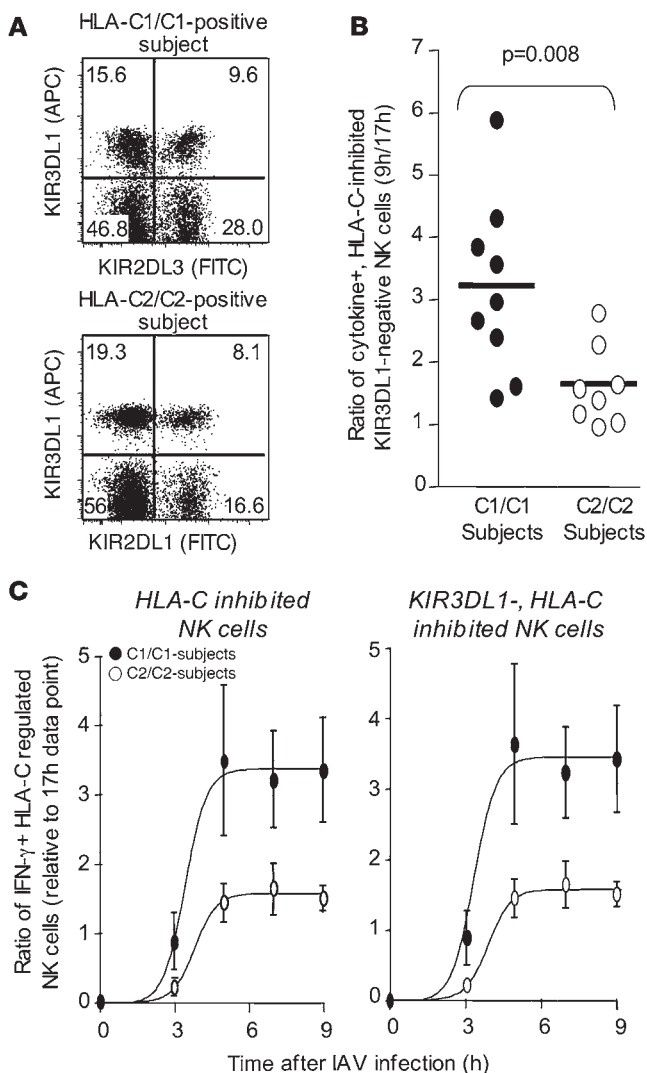
A distinction between the effect of the compound genotypes *HLA-C2/KIR2DL1* and *HLA-C1/KIR2DL3* on the outcome of various diseases has been previously reported and attributed to differential activation of NK cells (5, 18, 19, 21, 45). To address the functional basis for this finding, we studied the frequency, phenotype, and function of NK cells from healthy subjects who had both KIR2DL3 and KIR2DL1 (and not KIR2DL2), but were homozygous for either HLA group C1 or group C2 alleles. We found that *HLA-C1* homozygous subjects displayed a significantly higher frequency of HLA-C–inhibited NK cells than did *HLA-C2* homozygous subjects (26.3% vs. 16.3%; $P = 0.019$). This observation is consistent with recent in vitro studies showing that NK cells acquire the HLA-C1–specific KIR2DL3 at earlier time points during development from hematopoietic cells and with higher frequency than the HLA-C2–specific KIR2DL1 (58).

Functional differences in the properties of the *KIR2DL3* and *KIR2DL1* promoters (59) may account for the higher frequency of NK cells expressing KIR2DL3 compared with KIR2DL1.

We also demonstrated that HLA-C–inhibited KIR2DL3⁺ NK cells from *HLA-C1* homozygous subjects secreted more IFN- γ at earlier time points after infection and displayed greater degranulation than did HLA-C–inhibited KIR2DL1⁺ NK cells from *HLA-C2* homozygous subjects. Because all subjects were KIR2DL3⁺ and KIR2DL1⁺, these results suggest that NK cell inhibition through interaction between KIR2DL3 and HLA-C1 is weaker than the inhibition conferred through KIR2DL1/HLA-C2 interaction. Given that the affinities of KIR2DL1 and KIR2DL3 for their respective ligands are very similar (24, 25, 26), the basis for weaker inhibition by KIR2DL3 could be differences in negative signaling controlled by the cytoplasmic tails (26). Thus, our data provide a functional correlate for previous epidemiological observations (5, 18, 19, 21, 45).

Our study extends the results of previous publications that used similar in vitro infection systems (38, 47). First, Draghi et al. reported that NK cells are stimulated by IAV-infected dendritic cells (38), and He et al. observed that the strength of NK cell responses in IAV-infected PBMCs differed among subjects (47). This may be explained, at least in part, by differences in IAV-specific memory T cell responses, because T cells were not depleted in the latter study and T cell–derived IL-2 was clearly shown to enhance NK cell responses. However, as we show now, KIR/HLA interaction also affects NK cell responsiveness, especially in the early phase of this viral infection. Second, Artavanis-Tsakonas et al. studied IFN- γ responses of human NK cells to *Plasmodium falciparum*–infected erythrocytes in vitro (60). The presence of the *KIR3DL2*002* allele was associated with stronger NK cell responses in this parasite infection model. However, due to the lack of HLA expression on erythrocytes, the underlying mechanisms of KIR/ligand interactions could not be evaluated (60).

Because of the complexity of factors that regulate NK cell activation, we also considered potential confounders. Although the *HLA-C1* homozygous and *HLA-C2* homozygous groups were identical with regard to the genes present on their *KIR* haplotypes, subjects were diverse with regard to presence of Bw4, the ligand for KIR3DL1. Nevertheless, after exclusion of KIR3DL1⁺ cells, the difference between the responses of HLA-C–inhibited NK cells in *HLA-C1* homozygous versus *HLA-C2* homozygous groups was preserved (Figure 5, B and C). Thus, the observed difference between



HLA-C1 homozygous and *HLA-C2* homozygous groups appeared to be independent of Bw4/KIR3DL1 interactions. Although it is not possible to control for all genetic differences in a functional study, the fact that we still observed differences in the overall IFN- γ responses between NK cells from the *HLA-C1* homozygous and *HLA-C2* homozygous groups strengthens our conclusions.

An additional factor for further investigation is the possibility that HLA-C-bound viral or host peptides contribute to the differential inhibitory effect of HLA-C1 and HLA-C2 molecules. Such peptides may be produced in response to infection and introduce allosteric changes in HLA-C molecules or enhance the binding of HLA-C molecules by their cognate KIR receptor. Indeed, KIR preference for certain peptides sequences has been previously demonstrated in studies examining NK cell recognition of HLA-B27-expressing target cells (61, 62) and in KIR2DL recognition of HLA-C molecules (63, 64). However, we consider it unlikely that differential peptide recognition explains the better NK cell response conferred by the KIR2DL3/HLA-C1 compound genotype compared with the KIR2DL1/HLA-C2 compound genotype for the following reasons. First, each HLA-C group is composed of multiple HLA-C alleles, which do not contain a common peptide

Figure 5

The differential response of HLA-C-inhibited NK cell subsets in *HLA-C1* and *HLA-C2* homozygous subjects is not influenced by KIR3DL1 and Bw4 expression. (A) Examples of KIR2DL3+KIR3DL1+ cells in an *HLA-C1* homozygous subject and KIR2DL1+KIR3DL1+ NK cells in an *HLA-C2* homozygous subject. Both plots are gated on CD56+CD3- NK cells. Numbers indicate the percentage of events in each quadrant. (B) Frequency of IFN- γ -secreting NK cells in the HLA-C-inhibited, KIR3DL1- population 9 h after IAV infection relative to 17 h after IAV infection (expressed as 9-h/17-h ratio). Horizontal lines indicate the mean. (C) Frequencies of IFN- γ -producing cells in the HLA-C-inhibited cell population (reproduced from Figure 4A for reference) and the KIR3DL1- and HLA-Bw4-independent, HLA-C-inhibited NK cell population at various time points of the IAV infection assay. The frequency of the respective IFN- γ -secreting NK cell population in each subject group is shown as a ratio relative to the maximum frequency at the 17-h time point. Mean and SEM are indicated. $n = 5$ per group.

binding motif (65). Therefore, they all present different peptides, even though they are recognized by the same KIR. Second, the HLA molecules of a given cell display a large number of different peptides, so that only a small percentage (<1%) of all HLA molecules is occupied by the same self or viral peptide.

We also considered that NK cells from *HLA-C1* homozygous and *HLA-C2* homozygous groups may have intrinsically different responsiveness. This is an interesting topic in light of recent studies implicating a role of inhibitory receptors for MHC class I in the education and eventual functional capacity of NK cells (27, 28, 66–69). According to these prior studies, NK cells that can be inhibited by MHC class I are functionally reactive because they express the corresponding inhibitory receptors. In contrast, NK cells that cannot be inhibited by MHC class I are hyporeactive because they lack the corresponding MHC class I-specific inhibitory receptors. However, the *HLA-C1* and *HLA-C2* homozygous patients in our study did not differ in their cytotoxic response to HLA-negative K562 cells. Functional differences only became evident in a more physiologic model of IAV infection in the context of HLA-C-expressing autologous cells.

While the previous studies compared NK cells that expressed MHC-specific inhibitory receptors with those that did not (27, 28, 66–69), our study focused on NK cell subsets that all expressed MHC-specific inhibitory receptors, but were inhibited by different groups of HLA-C molecules. The observation that HLA-C1- and HLA-C2-inhibited NK cells displayed differential functional responsiveness to IAV infection is an important extension of a previous observation Johansson et al. made in transgenic mice (70). By generating mice expressing different single MHC class I molecules, the authors show that individual MHC class I molecules have a differential impact on NK cell function (70). Thus, differential inhibition of NK cell function by recognition of distinct class I allotypes does not only occur in transgenic mice, but also in humans with different HLA-C ligands.

To our knowledge, there is presently no in vivo infection model to demonstrate that differential response kinetics in the first 9 h of NK cell activation result in earlier or better viral control. However, it should be noted that NK cells are abundant in inflamed and noninflamed human lymph nodes (71, 72) and thus may exert a rapid antiviral effect. Fast-replicating viruses such as IAV reach significant viral titers within 24 h of infection (73); human NK cells respond within 24 h to IAV (47); and IFN- γ levels in nasopharyngeal lavage fluid correlate significantly to IAV titers, clinical symptoms, oral temperature, and nasal discharge (74). In addition to its anti-



viral effect, NK cell responses promote early CD8 T cell responses against viruses (75), and depletion of NK cells from mice has been shown to result in inefficient priming of virus-specific T cells (76).

In summary, this study presents an *in vitro* model to study the functional impact of KIR/HLA interactions in an autologous system of HLA-C-expressing, IAV-infected monocytes and KIR-expressing NK cells. The data provide what we believe to be the first functional evidence in support of previous genetic studies that describe associations between distinct *KIR/HLA* compound genotypes and susceptibility to infectious diseases (5).

Methods

Study cohort. Blood samples were drawn in acid citrate/dextrose tubes from 22 healthy individuals who were selected based on their KIR/HLA compound genotype: individuals who were homozygous for *KIR* haplotype A (*KIR3DL3-KIR2DL3-KIR2DL1-KIR2DL4-KIR3DL1-KIR2DS4-KIR3DL2*) were studied (Table 1). Subjects were defined as HLA-C1 positive ($n = 12$) if they expressed *HLA-Cw*01*, **03*, **07*, **12*, or **1601*, which all encode serine and asparagine at positions 77 and 80, respectively, in the $\alpha 1$ domain of the HLA-Cw molecule (16, 17). Subjects were defined as HLA-C2 positive ($n = 10$) if they expressed *HLA-Cw*02*, **04*, **05*, **06*, or **15*, which all encode asparagine and lysine at these positions (16, 17). This strategy excluded the possible confounding effect of *KIR2DL2*, which also recognizes *HLA-C1* alleles, and all activating KIRs other than *KIR2DL4* and *KIR2DS4*. All subjects gave written informed consent for research testing under protocols approved by the Institutional Review Board of the National Cancer Institute (CR OH99-C-NO46).

KIR and HLA typing. The presence or absence of 10 KIR genes (*KIR2DL1*, *KIR2DL2*, *KIR2DL3*, *KIR3DL1*, *KIR2DS1*, *KIR2DS2*, *KIR2DS3*, *KIR2DS4*, *KIR2DS5*, and *KIR3DS1*) was determined by PCR with sequence-specific primers as previously described (7). HLA class I typing was performed by amplification of genomic DNA with locus-specific primers that flanked exons 2 and 3 of the HLA gene on chromosome 6, and PCR products were blotted onto nitrocellulose membranes and hybridized with sequence-specific oligonucleotide probes according to the protocol recommended by the 13th International Histocompatibility Workshop (<http://www.ihwg.org/components/ssopr.htm>). Alleles were assigned according to the reaction patterns of the sequence-specific oligonucleotide probes, and ambiguities were resolved by sequence analysis.

NK cell frequency and phenotype. Complete blood count, white blood cell differential, and immune status analyses were performed at the Department of Laboratory Medicine at NIH Clinical Center. As part of this analysis, the absolute number and percentage of NK cells (defined as CD3⁻ and either CD56⁺ or CD16⁺) in PBMCs of *HLA-C1* and *HLA-C2* homozygous individuals were determined.

The phenotype and frequency of NK cell subpopulations was determined in our research laboratory as follows. PBMCs were stained with antibodies to either KIR2DL2/2DL3/2DS2 (CD158b-FITC; BD Biosciences) or KIR2DL1/2DS1 (CD158a-FITC; R&D Systems). All samples were simultaneously stained with KIR3DL1-APC (R&D Systems) and CD56-PeCy7 (BD Biosciences) antibodies as well as CD3-AlexaFluor700 (BD Biosciences), CD19-PeCy5 (BD Biosciences), and CD14-PeCy5 antibodies (Serotec) and EMA to exclude T cells, B cells, monocytes, and dead cells. To study maturation status and phenotype, cells were stained with CD16-PacificBlue, CD25-APC-Cy7, CD117-APC (BD Biosciences), CD122-FITC, NKG2C-PE (R&D Systems), NKp44-PE, NKp46-PE, NKG2A-PE, and NKG2D-PE (Beckman Coulter) antibodies. Cells were analyzed on an LSRII system using FACS Diva Version 4.1 (BD Biosciences) and FlowJo Version 8.5.2 (Tree Star) software.

IAV infection assay. PBMCs were separated on Ficoll-Histopaque (Mediatech) density gradients, washed 3 times with PBS (Mediatech), and cryopre-

served. After thawing of the cryopreserved PBMCs, T cells were depleted with anti-CD3-coated magnetic beads (77) and the autoMACS (77) according to the manufacturer's instructions (Miltenyi Biotec). Depletion efficiency was assessed by flow cytometry using anti-CD14-FITC, anti-CD19-PE, anti-CD56-PeCy7, and anti-CD3-APC antibodies (all from BD Biosciences) and EMA (Invitrogen) staining. In the live cell gate, the remaining T cell contamination was below 5% for all subjects, with the exception of 2 samples with 7% and 11% T cells. T cell-depleted PBMCs were not cultured overnight and not preactivated with any cytokines prior to IAV infection. For IAV infection, 3.5×10^6 T cell-depleted PBMCs (17.5×10^6 /ml) were incubated with or without $10^{7.2}$ copies purified influenza A/PR/8/34 (H1N1) virus (kind gift of J. Yewdell and J. Bennink, National Institute of Allergy and Infectious Diseases [NIAID], NIH, Bethesda, Maryland, USA) in the absence of any exogenously added cytokines at ambient temperature in 0.2 ml cell RPMI 1640 in 5-ml tubes. After 1 h, 0.5 ml cell culture medium (RPMI 1640 supplemented with 2 mM L-glutamine, 100 μ g/ml streptomycin, 100 U/ml penicillin, and 10% FCS; U.S. Bio-Technologies) was added, and the incubation was continued for another 2–16 h (as indicated in Figures 2–5) at 37°C, 5% CO₂, so that the maximum assay time was 17 h. Monocytes are the primary IAV-infected cell population in this assay (78, 79). As previously reported, the level of MHC class I expression does not change on the surface of IAV-infected cells (80).

Cytokine EIAs, secretion assays, and bead arrays. Cytokine secretion by NK cells was determined by EIA and by cytokine secretion assay. For the IFN- γ EIA, 50 μ l cell culture supernatant was collected after 3, 5, 7, 9, 12, 15, and 17 h in the IAV infection assay and after 5, 7, and 9 h in the K562 assay and frozen until they were subjected to an IFN- γ EIA (Quantikine Human IFN- γ ; R&D Systems). To study type I IFN release by IAV-infected and accessory cells and chemokine release, culture supernatant was collected at 7, 9, and 17 h, and EIAs for IFN- α (PBL Biomedical Laboratories) and IFN- β (FUJIREBIO Inc.) as well as bead arrays for IP-10, MIG, MIP-1 α , and MIP-1 β (BD Biosciences) were performed according to the manufacturers' instructions.

For IFN- γ secretion assays (77), replicate cultures of T cell-depleted PBMCs were infected with IAV so that cytokine staining could be performed at multiple assay time points. At each assay time point, cells were washed in cold PBS (Mediatech) supplemented with 0.5% albumin bovine fraction V (MP Biomedicals Inc.) and 2 mM EDTA (Quality Biological Inc.). Cells were then incubated in cell culture medium with 5% FCS (US Bio-Technologies) for 45 min at 37°C in the presence of IFN- γ -specific Catch Reagent (a conjugate of antibodies against the cytokine and the universal leukocyte antigen CD45; Miltenyi) to allow attachment of the secreted cytokine to the cell surface of CD45-expressing cells. Cells were subsequently labeled with a secondary PE-conjugated detection antibody (77). In the absence of IAV infection, release of cytokines did not significantly increase throughout the duration of the assay.

To identify HLA-C-inhibited NK cells in *HLA-C1* or *HLA-C2* homozygous subjects, cells were stained with antibodies to KIR2DL2/2DL3/2DS2 (CD158b-FITC; BD Biosciences) or KIR2DL1/2DS1 (CD158a-FITC; R&D Systems). All samples were simultaneously stained with CD56-PeCy7 (BD Biosciences) and KIR3DL1-APC (R&D Systems) antibodies and with CD3-PeCy5 (BD Biosciences), CD19-PeCy5 (BD Biosciences), CD14-PeCy5 antibodies (Serotec) and EMA to exclude T and B cells, monocytes and dead cells. Cells were analyzed on an LSRII using FACS Diva Version 4.1 (BD Biosciences) and FlowJo Version 8.5.2 (Tree Star) software.

Degranulation assay. NK cell degranulation in response to influenza infection was studied using CD107a-PE as a marker for degranulation (48, 49). IAV infection of CD3-depleted PBMCs was performed as described above, with the exception that CD107-PE (5 μ l/ml; BD Biosciences) was added prior to infection. Uninfected controls were included for each individual time point. To identify HLA-C-inhibited NK in *HLA-C1* or *HLA-C2* homo-



zygous subjects, cells were stained with antibodies to KIR2DL2/2DL3/2DS2 (CD158b-FITC; BD Biosciences) or KIR2DL1/2DS1 (CD158a-FITC; R&D Systems). All samples were simultaneously stained with CD56-PeCy7 (BD Biosciences) antibodies and CD3-AlexaFluor700 (BD Biosciences), CD19-PeCy5 (BD Biosciences), and CD14-PeCy5 antibodies (Serotec) as well as with EMA to exclude T and B cells, monocytes and dead cells. Cells were analyzed on an LSRII using FACS Diva Version 4.1 (BD Biosciences) and FlowJo Version 8.5.2 (Tree Star) software. MFI was calculated as MFI of CD107a⁺ cells minus that of the parent population.

NK cell isolation and ⁵¹Cr release assay. Cryopreserved PBMCs from all subjects were thawed and cultured at 4 × 10⁶ cells/ml overnight at 37°C in cell culture medium without any exogenously added cytokines. NK cells were isolated by depletion of CD3⁺, CD4⁺, CD14⁺, CD15⁺, CD19⁺, CD36⁺, CD123⁺, and/or CD235a⁺ cells using the NK Cell Isolation Kit II (77) and the autoMACS system (77) according to the manufacturer's instructions (Miltenyi Biotec). Cytotoxicity of these negatively isolated NK cells was assessed in a standard ⁵¹Cr release assay (81) using MHC-negative K562 cells labeled with ⁵¹Cr (Amersham Biosciences) as target cells. Triplicate cultures of NK cells and K562 cells were incubated at effector/target ratios of 30:1, 15:1, 7.5:1, and 3.8:1 in 96-well round-bottomed plates containing cell culture medium. Ten percent purified IL-2 (equivalent to a concentration of 50 U/ml; Hemagen) was added to the culture and did not affect cytotoxicity, as determined in additional experiments (data not shown). At 1, 3, 5, 7, and 9 h after the start of the cytotoxicity assay, 25 μl cell culture supernatant was harvested to quantitate the amount of released ⁵¹Cr in a Topcount microplate scintillation counter (Packard Biosciences). These assay time points were chosen because they cover the standard assay time that is commonly reported in the literature (27) and because the percentage of cytotoxicity reached a plateau between 7 and 9 hours (Figure 1). Percent lysis was calculated as (experimental release – spontaneous release)/(maximum release – spontaneous release), in which spontaneous release and maximum release reflected target cell lysis in the absence of effector cells and in the presence of 10% Triton-X100 (Sigma-Aldrich), respectively. Spontaneous release was less than 5% of maximum release in all assays.

Statistics. All statistical analyses were performed with GraphPad Prism Version 3.0cx software (GraphPad). Mann-Whitney *U* tests were used to analyze differences in NK cell responses between the 2 groups. Two-sided

P values less than 0.05 were considered significant. Nonlinear regression analysis was used to create time/response curves for the results of cytotoxicity and IFN-γ assays. A nonlinear mixed-effects model was used to fit the parameters of a logistic model to the data on IFN-γ production.

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Address correspondence to: Barbara Rehermann, Immunology Section, Liver Diseases Branch, NIDDK, NIH, DHHS, 10 Center Drive, Building 10, Room 9B16C, Bethesda, Maryland 20892-1800, USA. Phone: (301) 402-7144; Fax: (301) 402-0491; E-mail: rehermann@nih.gov.

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