

# Human NK Cells Inhibit Cytomegalovirus Replication through a Noncytolytic Mechanism Involving Lymphotoxin-Dependent Induction of IFN- $\beta$ <sup>1</sup>

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NK cells play a key role in host defense against the  $\beta$ -herpesvirus CMV through perforin-dependent cytotoxicity. In this study, we show that human NK cells can also control human CMV (HCMV) infection by a noncytolytic mechanism involving induction of IFN- $\beta$  in the virus-infected cell. Both IL-2-activated primary NK cells and an IL-2-dependent NK cell line (NK-92) exhibited potent, noncytolytic anti-HCMV activity at very low E:T cell ratios (<0.1:1). Activated NK cells expressed lymphotoxin (LT) $\alpha\beta$  on their cell surface, and secreted LT $\alpha$  and TNF, all of which contributed to the NF- $\kappa$ B-dependent release of IFN- $\beta$  from infected fibroblasts. IFN- $\beta$  produced by fibroblasts and NK cell-produced IFN- $\gamma$  combined to inhibit HCMV replication after immediate early gene expression. These results highlight an efficient mechanism used by NK cells to activate IFN- $\beta$  expression in the infected target cell that contributes to the arrest of virion production and virus spread without cellular elimination. *The Journal of Immunology*, 2005, 175: 7568–7574.

Human CMV (HCMV)<sup>4</sup> infection, a  $\beta$ -herpesvirus, causes little to no pathogenicity in immunocompetent individuals, but establishes lifelong persistence with latency. However, in situations in which the immune system is compromised or immature (e.g., organ transplantation and AIDS or congenital infection), HCMV can cause serious pathology in multiple organ systems (1). Immune control of CMV in both humans (2, 3) and mice (4) requires innate immune defenses mediated by IFNs and NK cells, as well as adaptive immune responses. NK cells can function as effector cells by using both cytolytic and noncytolytic mechanisms. Cytolytic pathways include the secretion of granules containing perforin and granzymes, and inducible expression of TRAIL (5) and Fas ligand (6, 7) “death-inducing” ligands of the TNF superfamily. Noncytolytic pathways involve cytokines such as IFN- $\gamma$ . NK cells are the main source of IFN- $\gamma$  in mice infected with CMV (8). Importantly, recent evidence has indicated that both cytolytic and IFN- $\gamma$ -dependent pathways are required by NK cells to control mouse CMV replication in the liver and spleen (9).

The vast majority of studies examining the effector mechanisms used by human NK cells directed against HCMV-infected cells have focused on target cell recognition and subsequent cytolytic

pathways. The complex molecular mechanisms required for NK cell recognition and killing of target cells is mediated by the activating and inhibitory NK cell receptors and MHC-related proteins expressed on the cell surface of potential target cells (10). HCMV actively down-regulates the expression of HLA class I from the surface of infected cells, potentially sensitizing infected cells to lysis mediated by NK cells due to the resulting lack of interaction between HLA molecules and NK cell inhibitory receptors (11). However, HCMV has evolved several effective strategies to circumvent NK cell killing. HCMV glycoprotein UL40 can induce expression of HLA-E in fibroblasts, inhibiting NK cell cytotoxicity through the subsequent interaction of HLA-E with the NK cell inhibitory receptor complex CD94-NKG2A (12, 13). Additionally, HCMV UL16 inhibits the induction of “infection-induced” cellular ligands that bind the NKG2D-activating receptor (UL16-binding proteins 1 and 2 and MHC class I chain-related B), thereby suppressing this potential mechanism of NK cell activation (14, 15). Recently, UL141 in HCMV has also been shown to block surface expression of CD155, the ligand for the NK cell-activating receptors CD226 and CD96 (16). It seems likely that reported differences in the ability of HCMV strains/isolates to inhibit NK cell killing are due to the mutation or loss of one or more of these gene products after repeated passage of the virus in tissue culture (16–18).

TNF-related cytokines can initiate apoptotic cell death in virus-infected cells (19), yet herpesviruses have countered with a variety of strategies that evade proapoptotic signaling pathways (20). HCMV-infected cells can resist cell death induced by Fas ligand, TRAIL, and TNF (21, 22). However, several members of the TNF family, including lymphotoxin (LT) $\alpha\beta$  and LIGHT (lymphotoxin-like inducible protein that competes with glycoprotein D for binding herpesvirus entry mediator (HVEM) on T cells) signaling through the LT $\beta$ R or LT $\alpha$  and TNF signaling via TNFR1, are potent inhibitors of HCMV replication. We reported that activation of LT $\beta$ R or TNFR1 inhibited viral cytopathic effects and replication of HCMV in human fibroblasts by a reversible, nonapoptotic process requiring the NF- $\kappa$ B-dependent induction of IFN- $\beta$  (22). Importantly, the efficient transcriptional induction of IFN- $\beta$  required concurrent cytokine receptor signaling and viral infection.

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<sup>4</sup> Abbreviations used in this paper: HCMV, human CMV; LT, lymphotoxin; HVEM, herpesvirus entry mediator; IE1, immediate early protein 1; NHDF, normal human dermal fibroblast; MOI, multiplicity of infection.

HCMV alone was insufficient at inducing inhibitory levels of IFN- $\beta$ , and LT $\beta$ R/TNFR1 showed no ability to activate IFN- $\beta$  transcription in the absence of infection. IFN- $\beta$  produced by the infected cell acted in an autocrine fashion in the fibroblast culture to curtail HCMV replication without elimination of the infected cell, blocking virus production at a step before late gene expression.

Several cell lineages have the potential to express LT $\alpha\beta$  including B, T, NK, and embryonic lymphoid tissue inducer cells based on mouse and human studies (23). In human blood, LT $\alpha\beta$  expression is not detectable on unactivated cells, but is induced after Ag receptor is triggered in T and B lymphocytes or induced in NK cells stimulated with IL-2 (24, 25). In this study we examined whether antiviral effector cells in human blood use LT $\beta$ R and TNFR1 pathways to arrest the replication and spread of HCMV. The results reveal IL-2-activated human NK cells induce IFN- $\beta$  in virus-infected cells and, in combination with NK cell-produced IFN- $\gamma$ , inhibit the spread of HCMV in primary fibroblasts by a nonlytic, reversible mechanism. This inhibition of virus replication and spread is apparent at very low E:T ratios (<0.1:1) suggesting physiologic relevance.

## Materials and Methods

### Cytokines, Abs, and reagents

Recombinant human IL-2 and Abs against LT $\alpha$  (NC2 mAb) and LT $\beta$  (B9 mAb) (26) were a gift from Jeff Browning (Biogen, Cambridge, MA). Fusion proteins LT $\beta$ R-Fc, HVEM-Fc, and Fas-Fc were purified as described (27, 28). LIGHT was detected by anti-human LIGHT Omnicon (29), a bacterially expressed combinatorial Ab containing V<sub>H</sub> and V<sub>L</sub> chains generated from a BALB/c mouse immunized with recombinant soluble human LIGHT166 (30). Mouse anti-methamphetamine Omnicon was used for an isotype control. For FACS staining, mouse IgG1 from Sigma-Aldrich served as isotype control. Anti-TNF mAb (104c mAb) was from Covance and human  $\beta$ -actin mAb (MAB1501) from Chemicon International. Neutralizing Abs against IFN- $\alpha$  (PB31100), IFN- $\beta$  (PB31400), and IFN- $\gamma$  (PB2150) were from Research Diagnostics. Goat anti-mouse IgG (H+L chain specific) conjugated with R-PE (Southern Biotechnology Associates) was used for FACS staining, and flow cytometry was performed on a FACScalibur (BD Biosciences). Anti-HCMV mAbs against major immediate early protein 1 (IE1) and late tegument protein pp28 for Western blot detection of HCMV protein expression were prepared as described (31) and were a kind gift from W. Britt (University of Alabama, Birmingham, AL).

### Cells and virus

Neonatal normal human dermal fibroblasts (NHDF; Clonetics) were cultured in DMEM supplemented with 10 mM HEPES, 5  $\mu$ g/ml insulin, 1 ng/ml basic fibroblast growth factor (Sigma-Aldrich), and 10% FBS (HyClone Laboratories). NHDF-I $\kappa$ B $\alpha$ M cells stably express a dominant negative mutant (M) of I $\kappa$ B $\alpha$  with point mutations in serine residues 32 and 36 to alanine (also sometimes referred to as the I $\kappa$ B $\alpha$  "super repressor"). NHDF-I $\kappa$ B $\alpha$ M cells and control LXS cells were generated by retroviral vector transduction as described (22). The IL-2-dependent NK cell line NK-92 from the American Type Culture Collection (ATCC) was maintained in RPMI 1640 supplemented with 10% human A+ serum (Scripps Green Hospital) and 20 ng/ml IL-2. HCMV strains Toledo (used between p12-14 and a gift from S. Starr (Children's Hospital of Philadelphia, Philadelphia, PA) and AD169 (ATCC) stocks were prepared and quantified by limiting dilution plaque formation assay on NHDF.

### Separation of CD56<sup>+</sup> NK cells from human PBMC

NK cells were isolated from peripheral blood of healthy donors (type A+) at the General Clinical Research Center, Scripps Green Hospital (San Diego, CA) using MACS Beads magnetic separation system (Miltenyi Biotec) (32). Ficoll-Hypaque separated lymphocytes ( $10^7$  cells) were resuspended in 80  $\mu$ l of PBS with 0.5% human A+ serum, 2 mM EDTA, and 20  $\mu$ l of CD56 Microbeads and incubated on ice for 15 min and magnetically extracted. All CD56<sup>+</sup> NK cell populations used for these experiments contained <2% contaminating B cells (CD19<sup>+</sup>) or monocytes (CD14<sup>+</sup>), and the amount of CD3<sup>+</sup>CD56<sup>+</sup> NKT cells in the populations ranged between 20 and 28%. NK cells were cultured in RPMI 1640 sup-

plemented with 10% human A+ serum and used for experiments within 5 days, but no sooner than 24 h, following separation. For cell activation, 20 ng/ml IL-2 was added to  $2 \times 10^6$  NK cells per ml at 24 h following separation from PBMC, and the cells were cultured for 3-4 days before analysis. All experiments with human blood were conducted following the guidelines of the La Jolla Institute for Allergy and Immunology for human subjects.

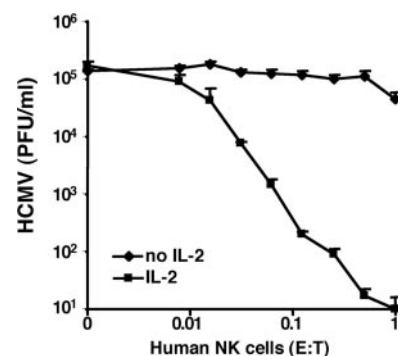
### Virus infection

Dermal fibroblasts were seeded into 96-well plates at  $1 \times 10^4$  cells per well or at  $1.5 \times 10^5$  cells per well in 12-well plates and infected the following day with HCMV at low multiplicity of infection (MOI) as described (22). Virus was allowed to adsorb for 2 h at 37°C before the cells were washed twice with medium and cultured in fresh medium for an additional 2 h. NK cells at E:T ratios  $\leq 1$  and/or specified reagents were added and cultured for a 7-day period. An E:T ratio of 1 represents the addition of  $2.5 \times 10^5$  NK cells to a confluent well of NHDF in a 12-well plate. When used, the IL-2-dependent cell line NK-92 and IL-2-activated human NK cells were washed and added to the virus infection assay in fresh medium without IL-2. For analysis of virus protein expression, 12-well cultures were harvested by dislodging the nonadherent NK cells from the adherent NHDF. Cell lysates from both cell types were prepared separately and analyzed for HCMV protein expression by Western blot as described (22). HCMV protein expression was detected as major IE1/pp28 protein and the late tegument protein pp28, and the blots were stripped and reprobed for  $\beta$ -actin to control for protein loading. The production of infectious virions in the supernatant was determined by plaque forming assay in NHDF seeded in 96-well dishes.

## Results

### Activated human NK cells inhibit HCMV spread

Purified NK cells from human blood were tested for their capacity to inhibit HCMV replication and spread in cultured monolayers of NHDF, a permissive cell type for HCMV replication. Virus was incubated with NDHF (MOI = 0.02) for 4 h to allow for attachment and entry of HCMV. NK cells were added to virus-infected NHDF for 7 days at various E:T ratios, time enough for at least two rounds of viral replication and spread to adjacent cells. Cytopathic effects (rounding of fibroblasts with plaques in the monolayer) were observed by day 7, and virion production was maximal (typically  $10^5$ - $10^6$  PFU/ml). The addition of fresh, nonactivated NK cells at E:T ratios <1 had no significant effect on HCMV-induced viral production (Fig. 1) or cytopathicity (data not shown) when incubated with the infected fibroblasts. At higher E:T target ratios (>8:1), unactivated NK cells exhibited marked cytolytic activity

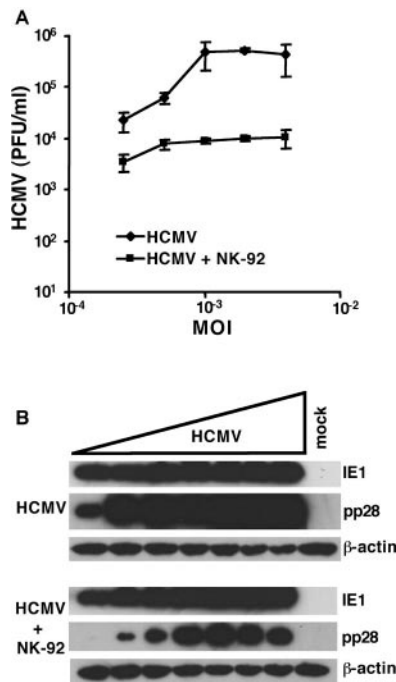


**FIGURE 1.** Human NK cell-mediated inhibition of HCMV production. NK cells were isolated from PBMC and incubated for 4 days with or without IL-2 (20 ng/ml). NHDF were infected with HCMV (Toledo strain) at an MOI of 0.02, and 2-fold dilutions of unstimulated or IL-2-activated NK cells were added 4 h later. After coculture for 7 days, the supernatants were collected and analyzed for viral PFU. Data shown are the mean  $\pm$  SD of PFU/ml from quadruplicate wells. Similar results were obtained in two other experiments, and no differences were seen between HCMV strains Toledo and AD169.

on the fibroblasts (data not shown). In contrast, NK cells preactivated for 4 days with IL-2 dramatically suppressed viral production at E:T ratios as low as 0.02 (Fig. 1) and reduced cytopathicity (data not shown). At E:T ratios  $\leq 1$ , frank cytotoxicity of the fibroblasts was not apparent suggesting that IL-2-activated NK cells mediated their anti-HCMV activity by a noncytolytic mechanism.

Inhibition of HCMV replication was observed with NK cells from all blood donors, although considerable interdonor variation was seen. To address mechanisms underlying this antiviral effect, a human NK-like cell line, NK-92 was tested for its capacity to block HCMV replication. The NK-92 cell line was established from the blood of a 50-year-old male with non-Hodgkin's lymphoma and displays a surface marker phenotype similar to primary IL-2-activated human NK cells (33). Similar to that observed for primary NK cells, NK-92 cells (E:T = 0.24) dramatically inhibited the release of infectious HCMV virions from NHDF infected over a broad MOI (Fig. 2A).

The level of HCMV IE1 gene product in NK-92-treated fibroblasts was similar to the level detected without NK-92 cells, although at the lowest MOI some reduction in IE1 expression was apparent (Fig. 2B). By contrast, expression of the late tegument protein (pp28) (34) was significantly reduced in the presence of NK-92 cells at all MOI, indicating preferential inhibition of viral gene expression downstream of IE1 (Fig. 2B). HCMV protein expression was not detected by Western blot when virus was incubated with NK-92 cells, indicating that HCMV does not replicate in these cells (data not shown). At high E:T ratios ( $>2$ ), NK-92 cells mediated frank cytolysis of the fibroblasts with a concurrent loss of IE1 detectable by Western blot (data not shown). These



**FIGURE 2.** NK-92 cell-mediated inhibition of cytopathicity and HCMV protein expression in infected NHDF. NHDF were infected with the HCMV strain Toledo at various MOI (0.001–0.02) for 4 h before addition of NK-92 cells (E:T = 0.24) or medium. *A*, Infectious virions in the supernatants after 7 days were detected by plaque forming assay (quadruplicate wells  $\pm$  SD). Similar results were obtained in four independent experiments, and similar inhibition was seen for HCMV strain AD169. *B*, NHDF were harvested after 7 days, extracted and analyzed for expression of the HCMV major IE1 protein or late tegument protein (pp28) by Western blot.

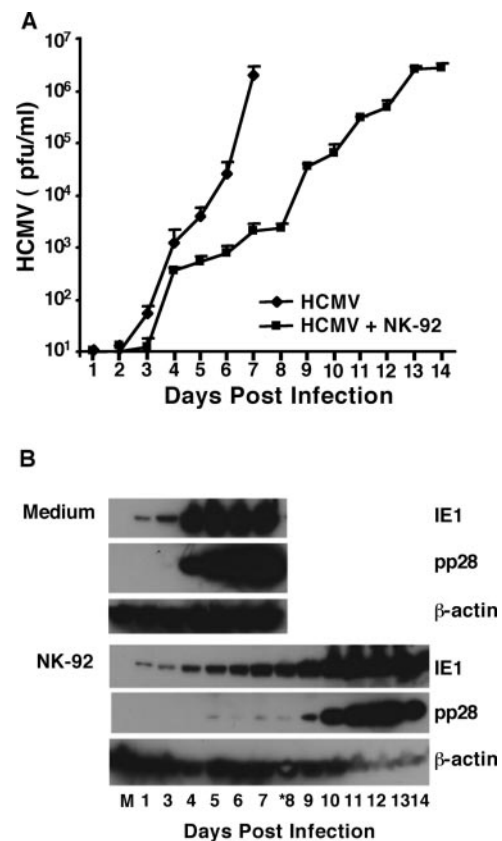
results indicate that NK-92 cells can inhibit HCMV replication and viral gene expression by both noncytolytic and lytic processes, similar to what was observed with primary activated NK cells.

#### The antiviral activity of NK-92 cells is reversible

The apparent preferential inhibition of late HCMV protein expression (pp28) by NK-92 cells suggested that a nonlytic mechanism was responsible for inhibiting virus production. To test whether this inhibition was reversible, HCMV-infected fibroblasts were incubated with NK-92 cells for 7 days, at which time NK cells were removed and fresh growth medium was added for an additional 7 days. HCMV production and protein expression was analyzed daily over this time course (Fig. 3). The addition of NK-92 cells greatly inhibited virion production ( $\sim 99.9\%$ ) and selectively inhibited pp28 expression for the initial 7 days as expected. However, upon removal of the NK cells, and continued incubation, a rapid reemergence of HCMV protein expression and virion production was observed (Fig. 3). This result supports a model in which NK-92 cells inhibit HCMV replication via a reversible, nonlytic mechanism.

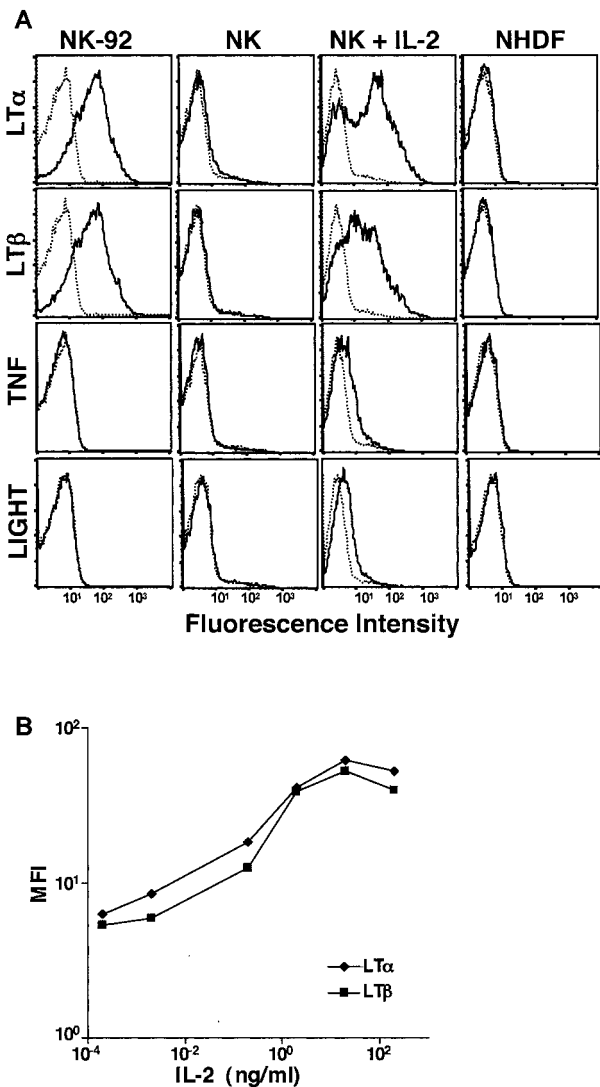
#### Expression of LTβR and TNFR ligands by NK cells

The anti-HCMV activity of activated NK cells is remarkably similar to the effects of recombinant LTαβ, LIGHT, and LTα. Flow



**FIGURE 3.** NK-92 cell-mediated anti-HCMV activity is reversible. NHDF were infected with HCMV Toledo at an MOI of 0.002 before addition of NK-92 cells (E:T = 0.24) 4 h later. After 7 days of coculture, the medium was replaced with fresh medium lacking NK-92 cells and cultured for an additional 7 days. *A*, HCMV virions present in the supernatant were determined daily ( $\pm$  SD from quadruplicate wells). *B*, NHDF were harvested daily for analysis of HCMV protein expression (IE1 and pp28) by Western blot. Similar results were obtained in three independent experiments. M, Mock-infected cells. At day 8 (\*), NK-92 cells were removed and replaced by growth media alone.

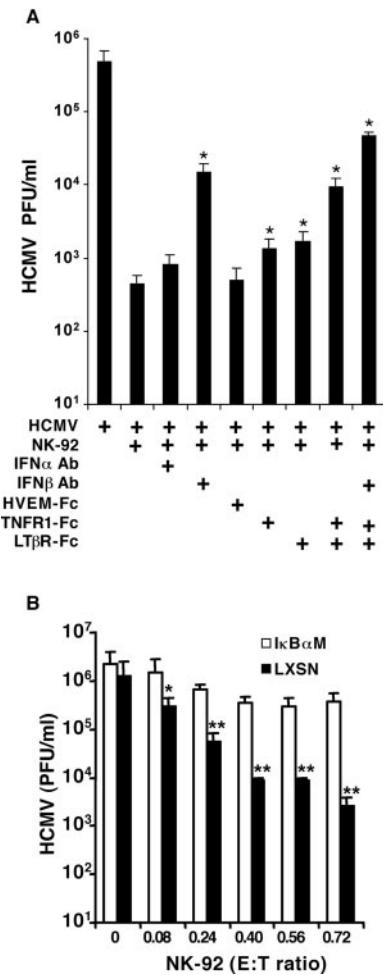
cytometry was used to analyze whether these ligands were expressed in freshly isolated and IL-2-treated primary NK cells, NK-92 cells, and NHDF. NK-92 cells expressed both cell surface LT $\alpha$  and LT $\beta$ , but not TNF or LIGHT (Fig. 4A). Primary, unactivated NK cells from human PBMC showed no detectable expression of any LT-TNF-related ligands, but all four ligands were expressed on the cell surface of primary NK cells after 3 days of treatment with IL-2. The expression of LT $\alpha$  and LT $\beta$  on the surface of NK-92 cells was coordinately enhanced in response to IL-2 (Fig. 4B). As expected, NHDF did not express TNF-related ligands on their cell surface but do express LT $\beta$ R and TNFR1. Taken together, these data indicate that IL-2-activated NK cells can produce significant levels of the LT $\alpha\beta$  complex suggesting that the nonlytic anti-HCMV activity mediated by IL-2-activated NK cells may involve LT(s), TNF, and/or LIGHT.



**FIGURE 4.** Expression of LT $\alpha$ , LT $\beta$ , TNF, and LIGHT by NK cells. *A*, NK-92 cells (cultured in IL-2, 20 ng/ml), primary NK cells isolated from PBMC and cultured for 3 days with or without IL-2 (20 ng/ml) and NHDF were analyzed by flow cytometry for cell surface expression of LT $\alpha$ , LT $\beta$ , TNF, or LIGHT. Each staining was repeated at least twice, and isotype control Ab staining is shown as dotted histograms. *B*, NK-92 cells incubated overnight in culture medium containing various concentrations of IL-2 were analyzed for cell surface expression of LT $\alpha$  or LT $\beta$  by flow cytometry. Data are presented as mean fluorescence intensity (MFI) of cells incubated with anti-LT Abs after subtracting mean fluorescence intensity of cells incubated with isotype control Ab.

*NK-92 cells inhibit HCMV by a TNFR1-LT $\beta$ R-dependent mechanism requiring the induction of IFN- $\beta$*

The similarities observed in the antiviral action of NK cells and LT $\alpha\beta$ -TNF-related cytokines suggested a potential role for these cytokines in the observed NK cell-mediated antiviral activity. To test this possibility, IL-2-activated NK-92 cells were preincubated for 2 h with cytokine-blocking reagents before the addition of cells and inhibitors to HCMV-infected fibroblasts. The ability of NK-92 cells to arrest HCMV spread was significantly reduced when LT $\beta$ R decoy protein (LT $\beta$ R-Fc) and TNFR1-Fc were added to the cultures, indicating LT $\alpha$ , LT $\alpha$ 1 $\beta$ 2, LIGHT, and/or TNF contributed to the inhibition observed (Fig. 5A). The addition of



**FIGURE 5.** NK cells induce IFN- $\beta$  production from HCMV-infected fibroblasts. *A*, NK-92 cells were preincubated for 2 h with HVEM-Fc, LT $\beta$ R-Fc, TNFR1-Fc (all 20  $\mu$ g/ml), or blocking Abs against IFN- $\alpha$  or IFN- $\beta$  (500 neutralizing U/ml) in various combinations as indicated. These NK-92 cells (E:T = 0.24) were then added to NHDF infected with HCMV Toledo (MOI = 0.002) still in the presence of blocking reagents. After 7 days of coculture, the supernatants were collected and analyzed for HCMV production (PFU shown  $\pm$  SD from quadruplicate wells). Similar results were obtained from two experiments with strain Toledo and two with AD169. Statistical significance (\*,  $p < 0.01$ ) using Student's  $t$  test for two-tailed test. *B*, NHDF transduced with retroviral vectors expressing either I $\kappa$ B $\alpha$ M or empty vector (LXSN) were infected with Toledo (MOI = 0.002), and various numbers of NK-92 cells were added 4 h postinfection. After 7 days of coculture, the supernatants were collected and analyzed for HCMV production (PFU shown  $\pm$  SD from quadruplicate wells). Similar results were obtained for three independent experiments. Statistical significance (\*,  $p < 0.05$  and \*\*,  $p < 0.01$ ) from Student's  $t$  test for two-tailed test.

HVEM-Fc however had no effect on NK-92 cell-mediated inhibition, thereby ruling out a contribution of LIGHT (Fig. 5A).

The antiviral mechanism of LT $\beta$ R and TNFR1 signaling that arrests HCMV spread in NHDF cultures requires the induction of IFN- $\beta$  in infected cells, which in turn functions in an autocrine fashion to inhibit viral replication (22). Efficient induction of IFN- $\beta$  in NHDF required signaling by LT $\alpha\beta$ -TNF ligands and virus infection, as neither alone was sufficient. To assess whether IFN- $\beta$  contributed to NK-92 cell inhibition of HCMV replication, a neutralizing anti-IFN- $\beta$  Ab was added to the coculture. Strikingly, neutralization of IFN- $\beta$  had a similar inhibitory effect on HCMV to that seen when both LT $\beta$ R-Fc and TNFR1-Fc were added in combination (Fig. 5A).

Signaling by LT $\beta$ R and TNFR1 to induce IFN- $\beta$  was dependent upon activation of the transcription factor NF- $\kappa$ B (22, 35) as revealed by I $\kappa$ B $\alpha$ M (36). NF- $\kappa$ B (p65/p50) complex, AP-1, and IFN regulatory factor 3 are necessary transcription factors for IFN- $\beta$  induction (35). NK-92 cell control of HCMV virion production was significantly reduced in NHDF expressing the I $\kappa$ B $\alpha$ M when compared with that seen in fibroblasts expressing control vector (Fig. 5B). Taken together, these results indicate that a significant component of the antiviral activity of NK cells is mediated by LT $\beta$ R/TNFR-dependent induction of IFN- $\beta$  from the infected fibroblasts.

However, some antiviral activity remained in cocultures treated with anti-IFN- $\beta$  or with a mixture of LT $\beta$ R-Fc and TNFR1-Fc ( $\sim$ 10-fold reduction in virion production, from  $4.9 \times 10^5$  to  $4.6 \times 10^4$  PFU with the mixture), suggesting an additional mechanism controlling virus replication was contributing in this model. Interestingly, this "residual" anti-HCMV activity was quantitatively similar to that mediated by NK-92 cells in NHDF-I $\kappa$ B $\alpha$ M, which are unable to induce IFN- $\beta$  (22). Activated NK cells can produce IFN- $\gamma$  whereas fibroblasts do not, suggesting the possibility that IFN- $\gamma$  may provide a component of the antiviral activity derived from NK-92 cells. Consistent with this hypothesis, the addition of a neutralizing anti-IFN- $\gamma$  Ab to the cocultures reduced virion production by  $\sim$ 6-fold (Fig. 6). Importantly, blocking both IFN- $\beta$  and

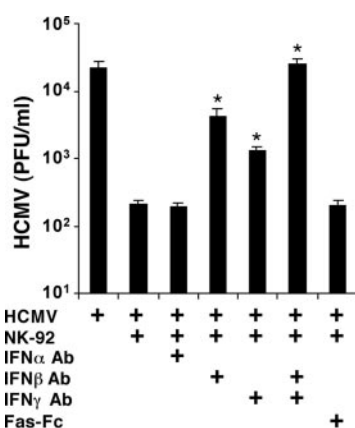
IFN- $\gamma$  in the cocultures with specific neutralizing Abs completely abolished NK-92 cell inhibition of HCMV replication. Consistent with this result, neutralization of IFN- $\alpha$  and Fas ligand had no effect on NK-92 cell inhibition, and provided further support for a noncytolytic mechanism mediated by the NK-92 cell line.

## Discussion

In this study we have shown that human NK cells can exert control over human cytomegalovirus by promoting the production of IFN- $\beta$  from the infected target cell. This mechanism functions in addition to cytolytic mechanisms used by human NK cells to destroy HCMV-infected fibroblasts (17, 18, 37, 38), and is also separate from the antiviral activity mediated by NK cell-produced IFN- $\gamma$ . The NK cell inhibition of HCMV replication in fibroblasts was reversible, and its induction was dependent upon the expression of TNF-related cytokines by the NK cells. Activated NK cells express cell surface LT $\alpha\beta$  and secrete LT $\alpha$  and TNF, which in turn signal through their cognate receptors (LT $\beta$ R and TNFR1) expressed in fibroblasts. Receptor signaling activates the canonical (p65/p50-dependent) NF- $\kappa$ B pathway, a critical component of the "enhancesome" required for IFN- $\beta$  mRNA induction (35). The induction of IFN- $\beta$  from the infected fibroblast, and its subsequent autocrine and paracrine antiviral actions, inhibits HCMV spread through the monolayer. Importantly, these data highlight the importance of the target cell in antiviral control through cross-talk with innate effector cells of the immune system. This LT-IFN- $\beta$  "axis" for control of HCMV spread by NK cells is similar to a mechanism we previously reported with purified cytokines (LT $\alpha$ -LT $\alpha\beta$ -LIGHT) and agonistic Abs that bind to the LT $\beta$ R or TNFR1 (22), and adds an important physiologic context to these earlier studies.

The finding that activated human NK cells express surface LT $\alpha\beta$  and that their antiviral activity is reduced by neutralizing with LT $\beta$ R-Fc indicates that NK cells are a source of LT $\alpha\beta$  that can deliver gene inductive signals to virus-infected target cells. The inability of HVEM-Fc (a cognate receptor for LIGHT but not LT $\alpha\beta$ ) to suppress NK-92 cell inhibition suggested that the LIGHT-LT $\beta$ R system did not contribute to the anti-HCMV activity observed. Unlike TNF, LT $\alpha$ , and LIGHT, the surface LT $\alpha_1\beta_2$  complex cannot be shed, thus cell-to-cell contact is likely a necessary step to deliver this signal to LT $\beta$ R-expressing NHDF cells. Interestingly, support for cell-to-cell contact contributing to NK cell-mediated anti-HCMV effects comes from studies in the mouse showing that NK cells are in direct contact with murine CMV infected cells in vivo (39, 40). Because secreted LT $\alpha$  and TNF can both bind and signal through TNFR1, neutralization experiments using TNFR1-Fc and NK-92 cells cannot discriminate between the relative role of these two cytokines. However, the fact that NK-92 cells have been reported to express high levels of LT $\alpha$  (41) and low to undetectable levels of TNF (42) (consistent with our inability to detect surface or intracellular TNF expression by these cells) suggests the majority of TNFR1-dependent anti-HCMV activity in NK-92 cells can be attributed to secreted LT $\alpha$ .

Differences in the potency of NK cell-mediated cytotoxicity have been described when comparing the sensitivity of various HCMV strains/isolates (17, 18), and are likely due to the absence or mutation of viral genes that dampen NK cell recognition and killing mechanisms. In these studies the Toledo strain of HCMV was primarily used, which contains several potential immunomodulatory genes located in the UL/b' region of the viral genome that are absent in the commonly used "laboratory" strains AD169 and Towne (43). However, no apparent differences were observed in NK-92 cell or primary NK cell inhibition of AD169 (ATCC



**FIGURE 6.** IFN- $\beta$  and IFN- $\gamma$  are the effector cytokines mediating anti-HCMV activity in the NK-92 coculture system. NK-92 cells were preincubated for 2 h with blocking Abs against IFN- $\alpha$  (500 neutralizing U/ml), IFN- $\beta$  (500 neutralizing U/ml), or IFN- $\gamma$  (5  $\mu$ g/ml) alone or in combination as indicated. NHDF were infected with HCMV AD169 at an MOI of 0.001, and NK-92 cells were added (E:T = 0.08) with or without blocking fusion proteins or Abs 4 h later. After 7 days of coculture, the supernatants were collected and analyzed for HCMV production (PFU shown  $\pm$  SD from quadruplicate wells). This experiment was repeated twice with both AD169 and Toledo, and similar results were seen. Statistical significance (\*,  $p < 0.01$ ) using Student's  $t$  test for two-tailed test.

strain) spread/virion production when compared with Toledo, indicating that the additional UL/b' region genes in Toledo do not confer added resistance to the noncytolytic inhibition by NK cells in this system.

As discussed, NK cell-dependent production of IFN- $\beta$  and IFN- $\gamma$  function to directly inhibit spread of HCMV in NK cell-NHDF cocultures. Our observation that this inhibition occurs at a late stage of HCMV gene expression, based upon reduced expression of the viral tegument protein pp28, is consistent with previous reports regarding noncytolytic control of both mouse (44) and human (22) CMV replication by type I IFN in fibroblasts. IL-1-mediated inhibition of HCMV replication in fibroblasts and bone marrow stromal cells has also been reported to be IFN- $\beta$ -dependent (45). Previous work by Torigoe et al. (46) has shown that control of HCMV replication by total human PBMC occurs downstream of immediate early gene expression, and is largely dependent upon IFN- $\beta$  and IFN- $\gamma$  and TNF. Interestingly, a contribution for NK cells in this multicellular coculture system was not observed when Ab depletion of this cell subset was performed. However, PBMC were not preactivated before addition to HCMV infected monolayers, and the activation status of NK cells was not analyzed. Our results indicate that activation of NK cells is necessary for LT expression and anti-HCMV activity, which may explain why NK cells were not found to contribute to HCMV inhibition in these studies.

The noncytolytic, IFN-dependent NK cell control of HCMV replication in fibroblasts suppresses viral production by several orders of magnitude. In this mixed cell model, NK cells were added to infected fibroblast cultures 4 h post HCMV infection, and the block to virus production was not as complete as we have seen previously using recombinant LT $\alpha$  (22). Interestingly, the levels of IE1 expression and virion production observed at day 4 postinfection with or without NK cells was similar (Fig. 3) suggesting that viral gene expression and replication in the initially infected fibroblasts was largely unaffected. The major impact of NK cells on inhibiting virus production was observed during the period when cell-to-cell spread occurs (at day 4–7). This difference could be explained whether the effective concentration of NK cell-expressed TNF ligands and IFNs ( $\beta$  and  $\gamma$ ) were limiting in the cocultures, consistent with the dose-dependence observed for NK cell inhibitory activity. Additionally, HCMV uses mechanisms to suppress IFN- $\alpha\beta$  and IFN- $\gamma$  signaling by inhibiting Jak/Stat-dependent signaling pathways, and this block is mediated by an immediate-early or early viral gene product (47, 48). It is possible that IFN-dependent antiviral activity is suppressed to some degree in the initial pool of infected cells, and these cytokines exhibit more potent effects in newly infected or uninfected cells, limiting spread of virus. Importantly, both type I and type II IFNs clearly regulate host defense through multiple mechanisms, including induction of inflammatory cytokines/chemokines, activation/maturation of dendritic cells, and the modulation of Ag presentation pathways (49, 50). Therefore the ultimate role that IFN produced from LT-dependent and -independent pathways plays in host defense will assuredly not be limited to direct inhibition of viral replication in the infected cells themselves.

CMV disseminates in its host replicating in multiple vital organs during acute infection (spleen, liver, kidney and intestines), suggesting the host responses may have evolved the need to use noncytolytic means of immune defense to preserve tissue integrity. Importantly, this study indicates that human NK cells can express and/or promote the release of IFN in infected cells that may aid in the control of HCMV replication via a noncytolytic mechanism. This immunological control mechanism involving the LT-IFN pathway, likely functioning in combination with cytolytic control

mechanisms used by NK cells, may contribute to the ability of CMV to ultimately establish coexistence with its host.

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## Disclosures

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