T cell–dependent production of IFN-γ by NK cells in response to influenza A virus

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The role of human NK cells in viral infections is poorly understood. We used a cytokine flow-cytometry assay to simultaneously investigate the IFN-γ response of NK and T lymphocytes to influenza A virus (fluA). When PBMCs from fluA-immune adult donors were incubated with fluA, IFN-γ was produced by both CD56dim and CD56bright subsets of NK cells, as well as by fluA-specific T cells. Purified NK cells did not produce IFN-γ in response to fluA, while depletion of T lymphocytes reduced to background levels the fluA-induced IFN-γ production by NK cells, which indicates that T cells are required for the IFN-γ response of NK cells. The fluA-induced IFN-γ production of NK cells was suppressed by anti–IL-2 Ab, while recombinant IL-2 replaced the helper function of T cells for IFN-γ production by NK cells. This indicates that IL-2 produced by fluA-specific T cells is involved in the T cell–dependent IFN-γ response of NK cells to fluA. Taken together, these results suggest that at an early stage of recurrent viral infection, NK-mediated innate immunity to the virus is enhanced by preexisting virus-specific T cells.

Introduction
Influenza A virus (fluA) is the major pathogen of humans and several animal species causing annual winter epidemics in the United States and has the potential to cause worldwide pandemics (1). Studies in humans and mice have implicated adaptive immune responses including Ab responses and T cell responses in protective immunity against fluA infection (2–6). Previous studies in the mouse model suggested that NK cells were also involved in the control of fluA infection (7, 8). The role of the innate immune response in protective immunity against fluA is poorly understood, however, especially in humans.

NK cells are important effector cells in the innate immune response against infections and tumors. Two mechanisms are involved in the protective effects of NK cells against viral infections: cytokine production and cytotoxic activity (9–12). Human NK cells are characterized phenotypically by the presence of CD56 and the lack of CD3 expression (10). Two subsets of human peripheral blood NK cells have been identified and characterized. The majority subset (approximately 90%) expresses low levels of CD56 (CD56dim), whereas the minority subset (approximately 10%) expresses high levels of CD56 (CD56bright) (13). These 2 NK subsets are thought to have unique functional attributes and, therefore, distinct roles in the human immune response (13, 14). The CD56dim NK subset is more naturally cytotoxic and may serve as the major cytotoxic effectors. By contrast, the CD56bright subset has the capacity to produce abundant cytokines and may serve as immunoregulators (15, 16). One of the cytokines produced by CD56bright NK cells is IFN-γ, which has immune regulatory activity (17–21) as well as direct antiviral activity (22–25). The role of these 2 subsets of human NK cells in the context of a viral infection has not been extensively investigated.

In this study, we analyzed the production of cytokines by human NK cells and T cells during ex vivo incubation of PBMCs with fluA and explored the relationship between the cytokine response of NK cells and T cells to the virus. We demonstrate that both CD56bright and CD56dim NK cells produce IFN-γ in response to fluA and that IL-2 produced by virus-specific T cells influences the IFN-γ production of NK cells. These results indicate a role of adaptive immune lymphocytes in regulating the function of innate immune cells.

Results
The fluA virus induces production of IFN-γ in the CD56bright and CD56dim subsets of NK cells as well as in T cells. In our previous work, we developed a cytokine flow-cytometry assay for the detection and characterization of fluA-specific memory CD8+ T cells. PBMCs were incubated with fluA ex vivo, followed by intracellular staining for IFN-γ (26). In the current study, we modified the assay to simultaneously investigate the IFN-γ response of NK cells and T cells to fluA. When PBMCs from adult donors were incubated with purified fluA for 17 hours, IFN-γ–producing (IFN-γ+) cells were detected in CD3– and CD3+ lymphocyte subsets (Figure 1A). The majority of IFN-γ+ cells in the CD3– T cell population did not express CD3 (Figure 1B). Most of the CD3– IFN-γ+ cells expressed CD56 (Figure 1C), indicating that they were NK cells. Incubating PBMCs with heat-inactivated (56°C/35 min) purified fluA induced similar IFN-γ response in CD3+ T cells and CD3-CD56– NK cells (data not shown).

To examine the IFN-γ production by the CD56bright and CD56dim subsets of NK cells, the fluA-stimulated PBMCs were costained for cell surface CD56 and intracellular perforin in addition to CD3 and IFN-γ. Perforin is a main effector molecule in rapid natural cytotoxicity. As previously reported (27), only the CD56dim subset of NK cells expressed perforin (Figure 1D). Thus, the CD3–CD56– NK cell population could be further resolved into 2 subsets: CD56bright perforin+ and CD56dim perforin+. Both subsets produced...
IFN-γ in response to fluA, with a higher percentage of IFN-γ+ cells in the CD56bright population than in the CD56dim population (Figure 1, E and F). When PBMCs were incubated in the absence of fluA, IFN-γ was produced neither in NK cells (Figure 1G) nor in CD3+ T cells (Figure 1H), indicating that IFN-γ production in both NK cells and T cells was induced by fluA.

The same experiments as those shown in Figure 1 were conducted with PBMCs from 25 adult donors. Perforin staining was incorporated in all experiments to improve the resolution of CD56bright and CD56dim NK populations, which, for some donors, overlapped significantly with each other when defined by CD56 signal intensity alone (data not shown). For all donors, IFN-γ production was detected for both NK subsets in PBMC samples incubated with fluA. Both the percentage of NK cells producing IFN-γ (Figure 2A) and the IFN-γ staining intensity (Figure 2B) varied among donors. The percentage of IFN-γ+ cells in the CD56bright perforin- and CD56dim perforin+ subsets was positively correlated (rS = 0.878, P < 0.001), with average percentage of IFN-γ+ cells in the CD56bright subset approximately 7-fold higher than that in the CD56dim subset (P < 0.001) (Figure 2B). Since the number of NK cells in the CD56dim subset was approximately 10-fold higher than that of the CD56bright subset (data not shown), the absolute number of IFN-γ+ cells in the 2 NK cell subsets from the same aliquot of PBMCs was approximately the same (Figure 2C).

T cells are required for the IFN-γ response of NK cells to fluA. To assess if the IFN-γ response of NK cells to fluA involves other cell types, we first determined if fluA could directly induce IFN-γ production in virus-exposed NK cells. We purified NK cells from PBMCs by depleting T cells, B cells, and monocytes prior to incubating them with fluA. Only background levels of IFN-γ were observed for the CD56bright and the CD56dim NK cell subsets in all 4 donors tested (Figure 3, A and B). IFN-γ production was induced from the purified NK cells by incubation with recombinant cytokines IL-12 and IL-2 (Figure 3B), indicating that NK cells remained functional after the purification procedure. When purified NK cells were incubated with recombinant IFN-γ in the presence of fluA, no IFN-γ+ NK cells were detected (Figure 3B), indicating that the detection of IFN-γ+ NK cells was not due to the uptake of extracellular IFN-γ by NK cells. These results demonstrate that the fluA-induced IFN-γ production of NK cells requires other leukocyte subset(s) present in PBMCs.
To assess what other cells are necessary for the IFN-γ response of NK cells to fluA, we first investigated the contribution of T cells. PBMCs were depleted of T cells by negative selection with anti-CD3 Ab. The remaining CD3-depleted PBMCs were incubated with fluA and examined for IFN-γ production by NK cells. In the absence of CD3+ cells, IFN-γ production was reduced to background levels in both NK cell subsets, a result obtained for all donors tested (Figure 4). This result indicates that CD3+ cells are required for the IFN-γ response of NK cells to fluA.

A small subset of the CD3+ population expressed the NK cell marker CD56. Some of these cells have been defined as NK T cells. Of note, NK T cells have been suggested to provide helper functions for cytokine production by NK cells (28). To determine if the CD3+CD56+ subset was required for the production of IFN-γ by NK cells in response to fluA, we depleted all CD3+ cells or just CD3+CD56+ cells from PBMCs by a strategy shown in Figure 5, A and B. When CD3-depleted PBMCs, CD3+CD56+ cell-depleted PBMCs, and unfractionated PBMCs were incubated with fluA, IFN-γ production in NK cells was observed in the unfractionated PBMCs and the CD3+CD56+ cell-depleted PBMCs but not in the CD3-depleted PBMCs (Figure 5C). In all 3 donors tested, the levels of IFN-γ production in NK cells were reduced to less than 10% of the original level when all CD3+ cells were depleted, while remaining at greater than 80% of the original level when only CD3+CD56+ cells were depleted (Figure 5C and data not shown). This result indicates that only CD56+ T cells are required for the IFN-γ production of NK cells in response to fluA.

We also sought to estimate the correlation between the levels of fluA-induced IFN-γ production in the NK and T cell populations, taking advantage of the fact that both responses were measured simultaneously in the same PBMC aliquot from each donor. For the 25 donors tested, the percentage of IFN-γ+ cells in CD56bright and CD56dim NK subsets were each positively correlated with the percentage of IFN-γ+ T cells or fluA-specific T cells (P ≤ 0.002; Figure 6). Taken together, these results suggest that the IFN-γ response of NK cells to fluA is associated with the fluA-specific T cell subset.
IFN-γ production of NK cells. Taken together, these results indicate that IL-2 produced by fluA-specific T cells is one of the regulatory factors for the IFN-γ response of NK cells to fluA.

Discussion
The host immune response to an infection involves orchestrated activities of different components of the immune system. Therefore, a comprehensive approach is necessary for understanding protective immunity to a virus, which is likely to encompass innate as well as adaptive immunity. In this set of experiments, we used a single cell-based flow-cytometry assay to detect and quantify IFN-γ+ cells in the 2 NK subsets and the T cell population from PBMCs exposed to fluA. We observed that in addition to fluA-specific T cells, CD56bright and CD56dim NK cells produced IFN-γ after a 17-hour ex vivo incubation of PBMCs with fluA. This IFN-γ response of NK cells depends on the T cell population in the PBMCs and was correlated with the level of the T cell response to fluA. IFN-γ production by NK cells responding to fluA could be suppressed by neutralizing Ab against IL-2, while addition of recombinant IL-2 could replace the effect of T cells on the IFN-γ production by NK cells, indicating that IL-2 is a cytokine primarily produced by activated antigen-specific T cells (31).
In the current study we demonstrate that after 17 hours of ex vivo incubation of PBMCs with fluA, both CD56\textsuperscript{bright} and CD56\textsuperscript{dim} NK cells produced IFN-\(\gamma\). In agreement with previously published results, the CD56\textsuperscript{bright} NK subset was the more potent IFN-\(\gamma\) producer as indicated by greater percentage of IFN-\(\gamma\)\textsuperscript{+} cells and amount of IFN-\(\gamma\) per cell in this subset (Figure 2, A and B). The number of IFN-\(\gamma\)\textsuperscript{+} cells contributed by the 2 NK subsets in PBMCs was similar (Figure 2C), however, and on average the difference between these 2 subsets in the amount of IFN-\(\gamma\) per cell was only approximately 2-fold (Figure 2B). There are 2 possible explanations for the apparently greater capability of IFN-\(\gamma\) production by the CD56\textsuperscript{dim} NK subset observed in our study than that reported previously (15, 16). This subset of NK cells may require different regulatory factors for IFN-\(\gamma\) production, which were provided by fluA but not by the monokines used in the previous studies; alternatively, the IFN-\(\gamma\) production of CD56\textsuperscript{dim} subset may be less sustained and may only be detected at the earlier stage of culturing.

IFN-\(\gamma\) is known for its immune regulatory activity as well as direct antiviral activity (17–25). Rapid production of IFN-\(\gamma\) and other inflammatory cytokines by NK cells is an important component of the innate immune response against viral infections (12), which has been shown to be mediated by IL-12 in a murine CMV-infected mouse model (32, 33). Our results suggest that both CD56\textsuperscript{bright} and CD56\textsuperscript{dim} NK subsets participate in the innate immune response against fluA by producing IFN-\(\gamma\) during the early stage of infection.

Resting CD56\textsuperscript{bright} and CD56\textsuperscript{dim} NK cells are known to express distinct panels of lymphocyte homing receptors, suggesting their different homing potential (34). CD56\textsuperscript{bright} NK cells express the chemokine receptor CCR7 (35) and high levels of the adhesion molecule CD62L (\(\alpha\)-selectin) (36); both are receptors mediating the homing of lymphocytes to secondary lymphoid organs. Consistent with their expression of lymph node homing receptors, CD56\textsuperscript{bright} NK cells have been shown to constitute the major NK population in lymph nodes (16, 37). In contrast, NK cells in the peripheral blood and the spleen are overwhelmingly CD56\textsuperscript{dim} (13, 37). Resting CD56\textsuperscript{dim} NK cells lack the expression of CCR7 and CD62L but express high levels of the chemokine receptors CXCR1, CXCR2, CXCR3, CXCR4, and CX3CR1 (34). Although the homing potential of CD56\textsuperscript{dim} NK cells is not clear, they have the ability to home to secondary lymphoid organs.

Figure 7
IFN-\(\gamma\) and IL-2 production by NK cells and T cells in response to fluA. PBMCs from adult donors were incubated with fluA or SPG (negative control) for 12 hours, with brefeldin A added during the last 5 hours (A and B) or were incubated with fluA for 4–12 hours, with brefeldin A added for the last 4 hours (C). The cells were stained for CD56, fixed and permeabilized, and then stained intracellularly for CD3, IFN-\(\gamma\), and IL-2. (A) Dot plots for a representative donor (no. 7) displaying cells gated on CD3\textsuperscript{–}CD56\textsuperscript{+} NK cell population (left panels) or CD3\textsuperscript{+} T cell population (right panels). Numbers in the dot plots refer to the percentage of cytokine-producing cells in each quadrant. (B) Summary of the levels of IFN-\(\gamma\) and IL-2 production by T cells from 4 donors (nos. 7–10). IL-2 was not detected in NK cells from any of these 4 donors. (C) Kinetics of IL-2 production by T cells and IFN-\(\gamma\) production by NK cells (donor no. 20).

Figure 8
Suppression of IFN-\(\gamma\) production of NK cells in response to fluA by IL-2–neutralizing Ab. PBMCs from 6 donors (nos. 11–16) were incubated with fluA for 17 hours in the presence of anti–IL-2 Ab or its isotype control (4 \(\mu\)g/ml), respectively, followed by intracellular staining for IFN-\(\gamma\) to determine frequencies of IFN-\(\gamma\)\textsuperscript{+} CD3\textsuperscript{–}CD56\textsuperscript{+} NK cells. Displayed in the bar graph is the percentage suppression for each donor, which is defined as \([1 – (frequency of IFN-\(\gamma\)\textsuperscript{+} NK cells in the presence of anti–IL-2)/ (frequency of IFN-\(\gamma\)\textsuperscript{+} NK cells in the presence of isotype control)] \times 100.

not been found in the lymph nodes and are likely to migrate to other sites in the body and exert their antiviral activity by killing infected cells and producing antiviral cytokines, including IFN-γ, during the early phase of fluA infection.

The infection of host cells by fluA is mediated by binding of viral HA molecules to the sialic acid residues present on cell surface receptors (38). Previous studies have shown that fluA infects different subsets of leukocyte and induces production of innate cytokines, including various IFNs and interleukins (39–41). Of particular interest, DCs are the major producer of type I IFN and IL-12, which have profound effects on other immune cell subsets (42). These innate cytokines can activate NK cells and induce production of IFN-γ (12, 15, 16). Therefore, they are likely to play a critical role in the fluA-induced IFN-γ production of NK cells observed in our current study.

The major finding of this study, however, is that the IFN-γ response of NK cells to fluA also depends on T cells. It has been reported that IL-2, a cytokine produced by activated T cells, enhances IL-12–induced IFN-γ production by CD56bright NK cells (16). In the experiments reported here, we observed that depletion of T cells always reduced to background the fluA-induced IFN-γ production by NK cells (Figure 4), which upon exposure to the virus, fluA-specific T cells produced IL-2 prior to production of IFN-γ by NK cells (Figure 7), and that the T cell–dependent IFN-γ production of NK cells can be suppressed by IL-2–neutralizing Ab for the majority of donors (Figure 8). We have also observed exogenous IL-2 to replace T cells in facilitating IFN-γ production of NK cells exposed to fluA (Figure 9). In addition, the level of IFN-γ response of NK cells appears to correlate positively with the level of fluA-specific T cells (Figure 6).

Based on these results, as well as previously reported effects of DC-derived innate cytokines on the activation and IFN-γ production of NK cells (15, 16), we propose the following model for the IFN-γ response of NK cells to fluA. The production of IFN-γ by NK cells requires regulatory signals from both DC and T cells. Incubation of PBMCs with fluA results in the infection of DCs (43, 44), which produces innate cytokines including IFN-α, IFN-β, IL-12, and other monokines with the potential to activate NK cells (12, 15, 16, 45–47). On the other hand, fluA-infected DCs process and present fluA antigens to fluA-specific T cells, which produce IL-2 and other cytokines. Under the collective actions of DC-derived monokines and T cell–derived cytokines, the NK cells respond by producing IFN-γ (16). Of note, while important proof of concept for this model was provided by the experiments of purified NK cells incubated with recombinant IL-12 plus IL-2 (ref. 16 and Figure 3B) and T cell–depleted PBMCs incubated with fluA plus IL-2 (Figure 9), other cytokines derived from DCs and T cells could be involved in the underlying mechanism for this model as well. In particular, the fact that IL-2–neutralizing Ab only partially blocked the IFN-γ production by NK cells (Figure 8) suggests that IL-2 is not the only T cell–derived regulatory factor for the IFN-γ response of NK cells.

Taken together, our results suggest a dependence of one of the innate immune functions, that is, IFN-γ production by NK cells, on the fluA-specific T cell recall reaction, which is a part of adaptive immunity. FluA infection does not persist, but occurs at multiple times throughout the life of an individual. Since only donors without recent flulike disease were used in this study, the IFN-γ+ T cells detected in PBMCs stimulated ex vivo for 17 hours or fewer with our cytokine-flow cytometric assay are likely to represent pre-existing fluA-specific memory T cells.

The innate immune response, which is rapid but not thought to be antigen specific, provides a first line of defense against viral infection and influences the subsequent adaptive T cell response (48). Recent studies have revealed a complex interaction between NK cells and DCs that may lead to NK cell activation, DC activation, or NK cell–mediated killing of DCs under different circumstances (49–53), indicating an important role of NK cells in the regulation of adaptive immunity to infections. It has been shown in a mouse model that NK cells are necessary for optimal priming of adenovirus-specific T cells (54). Of particular interest, depletion of NK cells abrogated fluA-specific CD8+ T cell responses both in vitro and in vivo (55). Conversely, the experiments reported here suggest that at the very early stage of infection, preexisting memory T cells specific for the infecting virus may also play a critical role in regulating the antiviral functions of NK cells. In addition, the correlation we observed between the levels of IFN-γ responses in NK cells and T cells to fluA suggests that at a later stage of infec-

**Figure 9**
The helper function of T cells for fluA-induced IFN-γ production in NK cells can be replaced by exogenous IL-2. PBMCs or CD3-depleted PBMCs from 3 donors (nos. 17–19) were incubated with fluA or control for 17 hours, with or without addition of recombinant IL-2 (250 U/ml). Displayed in the graphs are frequencies of IFN-γ+ cells in the CD56bright or CD56dim NK cell subsets under each condition.
Methods

Human subjects and blood samples. Twenty-three adult donors (ages 25–65) without recent flu-like symptoms were enrolled with informed consent. The study protocol was approved by the institutional review board at Stanford University. Venous blood samples were collected using Vacutainer tubes with sodium heparin (Vacutainer Systems; BD). In addition, buffy coats obtained from 3 healthy blood donors at a blood bank were also included in the study, yielding a total of 26 subjects.

Preparation and fractionation of PBMCs. PBMCs were prepared with standard Ficoll-Paque (Pharmacia Biotech Inc.) gradient centrifugation from the whole blood or buffy coats of NK C3 cell enrichment. NK and T cells were conducted using respective RosetteSep reagents (StemCell Technologies Inc.) or MACS MicroBeads (Miltenyi Biotech) following the manufacturer’s instructions.

Preparation of influenza virus. Purified fluA Panama/2007/99 strain (H3N2) was prepared as previously described (26). In brief, virus was grown in 11-day-old embryonated specific pathogen-free hen eggs (Charles River Laboratories Inc.). Allantoic fluid was harvested 48 hours after infection and assayed for virus by measuring the concentration of influenza HA. Virus-containing allantoic fluid was pooled and centrifuged to pellet fluA particles. The virus pellet was resuspended in PBS and further purified by a continuous 15–60% sucrose gradient centrifugation. The purified virus was reconstituted in PBS, stabilized with sucrose-phosphate-glutamate (SPG) (BioWhittaker Inc.), dispensed into single-use aliquots, and stored at –70°C. The virus titer was determined with Madin-Darby canine kidney cells by standard procedures (56).

Cytokine flow cytometry. Unfractionated or fractionated PBMCs or lymphocyte subsets were incubated with fluA as previously described (26). In brief, 2 × 10⁶ cells were resuspended in 0.1 ml of RPMI-1640 medium without serum. Purified fluA virus was added to the cells at a MOI of 3 and incubated at 37°C with 5% CO₂ for 1 hour. The same volume of SPG was used as the negative control. RPMI-1640 medium supplemented with 10% FCS and antibiotics was then added to a final volume of 0.7 ml, with or without the addition of 1 of the following reagents: rat anti-human IL-2–neutralizing Ab or its isotype control (BD Biosciences — Pharmingen), recombinant human IL-2 (Chiron Inc.), recombinant human IL-12 (Sigma-Aldrich) or recombinant human IFN-γ (a gift from L. Blatt, Internume Inc.). The cells were incubated for another 11 hours (for detection of IL-2 and IFN-γ) or 16 hours (for detection of IFN-γ). Brefeldin A (Sigma-Aldrich) was added to a final concentration of 10 µg/ml for the last 5 hours of incubation. Staining and flow-cytometric analysis were done as described previously (26). In brief, the cells were first stained with allopthyocyanin-labeled anti-CD56 (BD Biosciences — Pharmingen) and then treated with FACScalibur flow cytometer, with Cellquest software (BD Biosciences).

Statistical analyses. Subsets’ means were compared using paired Student’s t tests, in some cases after logarithmic transformation of the data. Correlation testing was based on Spearman’s rank-correlation statistic, a robust, distribution-free measure of correlation (57). Individual test’s P-value thresholds for declaring statistical significance were set at or below 0.05 to control the total type I error rate collectively across all statistical tests at 0.05 (58).

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