The Journal of Immunology

NK Cells Regulate CD8$^+$ T Cell Effector Function in Response to an Intracellular Pathogen

Ramakrishna Vankayalapati, Peter Klucar, Benjamin Wizel, Stephen E. Weis, Buka Samten, Hassan Safi, Homayoun Shams and Peter F. Barnes

J Immunol 2004; 172:130-137; ;
doi: 10.4049/jimmunol.172.1.130
http://www.jimmunol.org/content/172/1/130

The Journal of Immunology is published twice each month by
The American Association of Immunologists, Inc.,
1451 Rockville Pike, Suite 650, Rockville, MD 20852

Print ISSN: 0022-1767 Online ISSN: 1550-6606.

NK Cells Regulate CD8$^+$ T Cell Effector Function in Response to an Intracellular Pathogen[1]

Ramakrishna Vankayalapati,*† Peter Klucar,*† Benjamin Wizel,*† Stephen E. Weis,§ Buka Samten,*† Hassan Safi,* Homayoun Shams,*† and Peter F. Barnes[2]*†‡

We studied the role of NK cells in regulating human CD8$^+$ T cell effector function against mononuclear phagocytes infected with the intracellular pathogen *Mycobacterium tuberculosis*. Depletion of NK cells from PBMC of healthy tuberculin reactors reduced the frequency of *M. tuberculosis*-responsive CD8$^+$IFN-γ^+ cells and decreased their capacity to lyse *M. tuberculosis*-infected monocytes. The frequency of CD8$^+$IFN-γ^+cells was restored by soluble factors produced by activated NK cells and was dependent on IFN-γ, IL-15, and IL-18. *M. tuberculosis*-activated NK cells produced IFN-γ, activated NK cells stimulated infected monocytes to produce IL-15 and IL-18, and production of IL-15 and IL-18 were inhibited by anti-IFN-γ. These findings suggest that NK cells maintain the frequency of *M. tuberculosis*-responsive CD8$^+$IFN-γ^+ T cells by producing IFN-γ, which elicits secretion of IL-15 and IL-18 by monocytes. These monokines in turn favor expansion of Tc1 CD8$^+$ T cells. The capacity of NK cells to prime CD8$^+$ T cells to lyse *M. tuberculosis*-infected target cells required cell-cell contact between NK cells and infected monocytes and depended on interactions between the CD40 ligand on NK cells and CD40 on infected monocytes. NK cells link the innate and the adaptive immune responses by optimizing the capacity of CD8$^+$ T cells to produce IFN-γ and to lyse infected cells, functions that are critical for protective immunity against *M. tuberculosis* and other intracellular pathogens. *The Journal of Immunology,* 2004, 172: 130–137.

Natural killer cells are large granular lymphocytes that can kill infected cells without prior sensitization and play an important role in innate immunity to microbial pathogens. NK cells mediate protection against viruses, bacteria, and parasites through destruction of infected cells and by secretion of cytokines that shape the adaptive immune response (1–5). Increased NK cell cytolytic activity has been observed soon after primary viral infections, and there is a striking correlation between poor NK cell function and susceptibility to viral and other microbial infections (6, 7). NK cells are an important early source of IFN-γ, which is critical for activation of macrophages (1–3), and NK cells may favor clonal expansion of Th1 cells, processes that are important for elimination of many intracellular pathogens.

The immune response to *Mycobacterium tuberculosis* is mediated by CD4$^+$ and CD8$^+$ T cells. The latter contribute to protection by lysing infected cells and by producing cytokines such as IFN-γ (8, 9). CD16$^+$ NK cells are essential for the generation of human CD8$^+$ allogeneic CTL responses, and this effect is mediated in part through CD56 (10). NK cells also contribute to the capacity of CD8$^+$ T cells to reject tumors through interactions of CD27 on NK cells and CD70 on tumor cells, and this activity depends on IFN-γ production (11).

Limited information is available on the effects of NK cells on the CD8$^+$ CTL response to infection. Depletion of NK cells by Abs to asialo-GM1 or to NK1.1 suppressed influenza virus-specific CTL responses (7). Abs to asialo-GM1 also abrogated the capacity of DNA immunization to elicit protection through induction of CD8$^+$ CTL against *Plasmodium yoelii*, suggesting that NK cells are essential to optimize the CTL response (4). However, because anti-asialo-GM1 and anti-NK1.1 can bind to T cells (12), it is uncertain whether these effects were mediated entirely by depletion of NK cells. The contribution of NK cells to development of CD8$^+$ CTL activity against intracellular pathogens in humans is largely unexplored. In the present study, we investigated the mechanisms by which human NK cells regulate CD8$^+$ T cell effector function against cells infected with *M. tuberculosis*.

Materials and Methods

Patient population

Blood was obtained from 17 healthy tuberculin reactors, under protocols approved by the institutional Review Boards of the University of Texas Health Center at Tyler and the University of North Texas Health Science Center at Fort Worth. Five subjects had been vaccinated with *Mycobacterium bovis* bacillus Calmette-Guérin during childhood.

Cytofluorometric analysis

To measure the percentages of CD8$^+$, CD3$^+$, and CD56$^+$ cells in various cell populations, we used FITC anti-CD8, FITC anti-CD3, and PE-conjugated anti-CD56 (all from BD PharMingen, San Diego, CA), and immunostaining was performed by standard techniques (13). Based on forward scatter and side scatter characteristics, we gated on lymphocytes and the percentages of single- and double-stained cells were determined with an EPICS flow cytometer (Coulter, Hialeah, FL). To measure CD40 ligand (CD40L)[3] expression on purified NK cells, we used PE-conjugated anti-CD40L (BD PharMingen).

*Center for Pulmonary and Infectious Disease Control and Departments of †Microbiology and Immunology and ‡Medicine, University of Texas Health Center, Tyler, TX 75708; and §Department of Internal Medicine, University of North Texas Health Sciences Center, Fort Worth, TX 76107

Received for publication June 23, 2003. Accepted for publication October 24, 2003.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] This work was supported by grants from the National Institutes of Health (AI44935), the Potts Memorial Foundation, the Cain Foundation for Infectious Disease Research, and the Center for Pulmonary and Infectious Disease Control. P.F.B. holds the Margaret E. Byers Cain Chair for Tuberculosis Research.

[2] Address correspondence and reprint requests to Dr. Peter F. Barnes, Center for Pulmonary and Infectious Disease Center, University of Texas Health Center, 11937 US Highway 271, Tyler, TX 75708-3154. E-mail address: peter.barnes@uthct.edu

[3] Abbreviations used in this paper: CD40L, CD40 ligand; MOI, multiplicity of infection.

0022-1767/04/$02.00

Preparation of target cells for cytotoxicity assays

PBMC were isolated by differential centrifugation over Ficoll-Paque (Amersham Pharmacia Biotech, Piscataway, NJ). Monocytes were isolated with magnetic beads conjugated to anti-CD14 (Miltenyi Biotec, Auburn, CA), and positively selected cells were >95% $CD14^+$, as measured by flow cytometry. Monocytes (10^6/well) were plated in 12-well plates (BD Labware, Franklin Lakes, NJ) in 1 ml of RPMI 1640 (Life Technologies, Grand Island, NY) containing 10% heat-inactivated human serum (PelFreez Biologicals, Rogers, AR). Some monocytes were uninfected and others were infected with single-cell suspensions of *M. tuberculosis* H37Ra at a multiplicity of infection (MOI) of 5:1, as previously described (14). Cells were incubated for 4 h at 37°C in a humidified 5% CO_2 atmosphere, washed to remove extracellular bacilli, and cultured in RPMI 1640 containing 10% heat-inactivated human serum. Approximately 25–40% of the monocytes were infected. After 48 h, cells were collected using a cell scraper and used as targets in cytotoxicity experiments. These cells were >90% monocytes, as assessed by staining with nonspecific esterase. Cell viability was >90%, as judged by trypan blue exclusion.

Isolation of cell subpopulations

Most experiments involved evaluation of PBMC and CD56-depleted PBMC, obtained by incubation with magnetic beads conjugated to anti-CD56 (Miltenyi Biotec), using standard methods (15). PBMC and CD56-depleted PBMC were cultured with *M. tuberculosis* H37Ra at a MOI of 0.5:1. Because ~10% of the PBMC were monocytes, the ratio of *M. tuberculosis* to monocytes was roughly 5:1. After varying periods of incubation, $CD8^+$ cells were isolated by positive selection with magnetic beads conjugated to anti-CD8 (Miltenyi Biotec) and used in assays to measure cytokine production or CTL activity. These cells were 92–99% $CD8^+$, as assessed by flow cytometry.

In some experiments, PBMC were cultured with *M. tuberculosis* H37Ra at a MOI of 0.5:1 for 24–48 h, $CD56^+$ cells were isolated by positive immunomagnetic selection, and $CD3^+$ cells were then immunomagnetically depleted (Dynal Biotech, Lake Success, NY) to yield $CD56^+CD3^-$ cells, which we considered to be activated NK cells. These cells were >95% $CD56^+CD3^-$, as measured by flow cytometry.

Culture of monocytes and NK cells

In some experiments, we cultured infected and uninfected monocytes in 12-well plates at 10^6 cells/well in 1 ml of RPMI 1640 containing penicillin (Life Technologies) and 10% heat-inactivated human serum in the absence or presence of 10^5 NK cells/well. In other experiments, 10^5 *M. tuberculosis*-activated autologous NK cells, prepared as outlined above, were cultured in Transwell inserts (Costar, Milpitas, CA) in the 12-well plates. The insert contains 0.4-μm diameter pores that allow diffusion of soluble factors but not cell-cell contact. In some cases, neutralizing Abs to IFN-γ (BD PharMingen), IL-12, IL-15 (both from R&D Systems, Minneapolis, MN), and IL-18 (MBL International, Nagoya, Japan) were added to the Transwells and to the large wells.

Cytotoxicity assay

$CD8^+$ T cell-mediated cytotoxicity against infected and uninfected monocytes was assayed in a ^{51}Cr release assay using standard methods (16). Briefly, target cells were labeled overnight with 100 μCi of sodium chromate. Target cells were washed three times, and triplicate wells of 10^4 cells/well were mixed with effector cells at an E:T ratio of 40:1 in 200 μl of RPMI 1640 with 10% heat-inactivated human serum. Six hours after incubation, 100 μl of supernatant was removed from each well and radioactivity was measured in a gamma counter. The percentage of lysis was calculated as 100 × ((experimental release − spontaneous release/maximum release − spontaneous release)).

Measurement of cytokine concentrations

For measurement of IL-18, IL-12, and IL-15, supernatants from cultured cells were collected after 10 h and for IFN-γ, supernatants were collected after 1–5 days and stored at −70°C until cytokine concentrations were measured by ELISA. Paired Abs were used to detect IFN-γ (BD PharMingen). ELISA kits were used to measure IL-15, IL-12 p70 (both from R&D Systems), and IL-18 (MBL International). The lower limits of detection were 0.6 pg/ml for IL-12, 5 pg/ml for IFN-γ, 3 pg/ml for IL-15, and 13 pg/ml for IL-18.

Frequency of IFN-γ-producing cells

To measure the percentage of $CD8^+$ cells that produce IFN-γ, we used the ELISPOT assay (17). Briefly, $CD8^+$ cells were isolated by positive immunomagnetic selection from PBMC or CD56-depleted PBMC, and triplicate wells of 10^5 cells were cultured in 96-well nitrocellulose-backed plates for 18 h using the anti-human IFN-γ mAbs 1-DIK and 7-B6–1 (Mabtech, Nacka, Sweden) as coating and detection Abs, respectively. The wells were developed according to the manufacturer's instructions, and the spots in the air-dried plates were counted with a stereomicroscope.

To measure the frequency of *M. tuberculosis*-activated NK cells that produced IFN-γ, $CD56^+CD3^-$ cells were isolated from PBMC cultured with H37Ra for 24 h, and the ELISPOT assay was used to detect IFN-γ^+ NK cells as outlined above for $CD8^+$ cells.

Statistical analysis

Results are shown as the mean ± SE. For data that were normally distributed, comparisons between groups were performed by a paired or unpaired *t* test, as appropriate. For data that were not normally distributed, the Wilcoxon rank sum test was used. Values of $p < 0.05$ were considered statistically significant.

Results

$CD56^+$ cells contribute to IFN-γ production by PBMC

One of the important mechanisms by which $CD8^+$ T cells contribute to immunity against tuberculosis is by producing IFN-γ (18, 19). To evaluate the contribution of NK cells to *M. tuberculosis*-induced IFN-γ production, PBMC and CD56-depleted PBMC from four healthy tuberculin reactors were cultured with live *M. tuberculosis* for 24–120 h, and IFN-γ levels were measured by ELISA. Depletion of $CD56^+$ cells markedly reduced IFN-γ levels after 24 h of culture (63 ± 39 pg/ml vs 858 ± 273 pg/ml, $p = 0.06$, Fig. 1), when NK cells are expected to be the major source of IFN-γ. However, even after 5 days, when T cells are the predominant source of IFN-γ (19), IFN-γ production was greatly reduced in CD56-depleted PBMC (924 ± 232 pg/ml vs 3749 ± 251 pg/ml, $p = 0.02$). This suggests that $CD56^+$ cells, which include NK cells and NKT cells, affect the capacity of T cells to produce IFN-γ in response to *M. tuberculosis*. Previous studies showed that $CD56^+CD3^-$ NK cells exposed to *M. tuberculosis*-infected monocytes are potent sources of IFN-γ (15). However, $CD56^+$ cells alone cultured with *M. tuberculosis* did not secrete IFN-γ (Fig. 1), indicating that exposure of NK cells to infected monocytes is required for IFN-γ production.

$CD56^+$ cells maintain the frequency of M. tuberculosis*-responsive $CD8^+IFN\text{-}\gamma^+$ T cells*

To determine whether $CD56^+$ cells affect the capacity of $CD8^+$ cells to produce IFN-γ in response to *M. tuberculosis*, we cultured

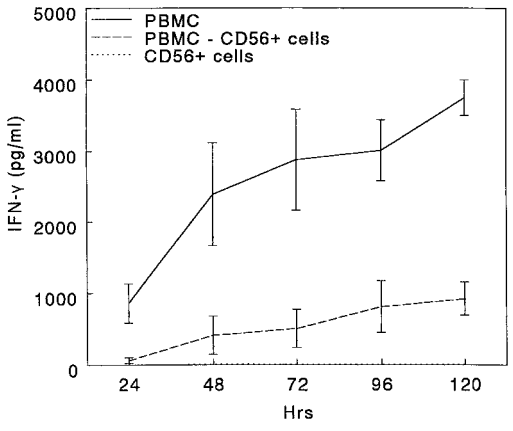

FIGURE 1. Effect of depletion of $CD56^+$ cells on IFN-γ production by PBMC. PBMC were obtained from healthy tuberculin reactors and $CD56^+$ cells were depleted from some PBMC. PBMC, CD56-depleted PBMC, and $CD56^+$ cells alone were cultured with live *M. tuberculosis* for 1–5 days, and IFN-γ levels were measured by ELISA. Mean values and SEs for experiments in four donors are shown.

PBMC or CD56-depleted PBMC from 15 healthy tuberculin reactors with live *M. tuberculosis* for 48 h. CD8$^+$ cells were then isolated from PBMC and from CD56-depleted PBMC and incubated overnight on an ELISPOT plate to detect IFN-γ-producing cells. Depletion of CD56$^+$ cells reduced the frequency of IFN-γ-producing CD8$^+$ cells by two-thirds (929 ± 179 per 10^5 cells vs 319 ± 93 per 10^5 cells, $p = 0.001$, Fig. 2*A*).

CD56$^+$ cells enhance CD8$^+$ cell-mediated cytotoxicity

To determine whether CD56$^+$ cells affect the capacity of CD8$^+$ T cells to lyse *M. tuberculosis*-infected mononuclear phagocytes, we cultured PBMC and CD56-depleted PBMC from five healthy tuberculin reactors with live *M. tuberculosis* for 6 days. CD8$^+$ cells were then isolated and used as effectors against autologous *M. tuberculosis*-infected monocytes as outlined in *Materials and Methods*. Depletion of CD56$^+$ cells markedly reduced net specific lysis by CD8$^+$ effectors from 24 ± 7% to 3 ± 2% ($p = 0.01$, Fig. 2*B*). This reduction was not due to depletion of APCs by CD56 depletion, as the percentage of CD14$^+$ cells in CD56-depleted PBMC was similar to that in PBMC (13% vs 14%).

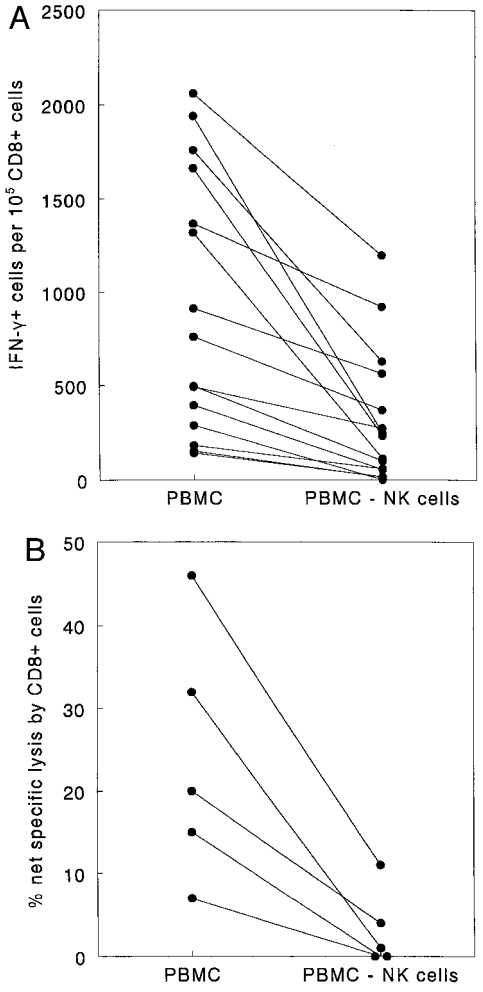

FIGURE 2. Effect of depletion of CD56$^+$ cells on the frequency of *M. tuberculosis*-responsive CD8$^+$IFN-γ$^+$ cells (*A*) and CD8$^+$ CTL activity (*B*). *A,* PBMC and CD56-depleted PBMC from 15 healthy tuberculin reactors were cultured with live *M. tuberculosis* at a MOI of 0.5:1 for 48 h. CD8$^+$ cells were isolated from PBMC and from CD56-depleted PBMC and incubated overnight in triplicate wells on an ELISPOT plate to detect IFN-γ-producing cells. Each spot shows mean values from one subject. *B,* PBMC and CD56-depleted PBMC from five healthy tuberculin reactors were cultured with live *M. tuberculosis* at a MOI of 0.5:1 for 5 days. CD8$^+$ cells were then isolated and cultured in triplicate wells with uninfected and *M. tuberculosis*-infected autologous monocytes at an E:T ratio of 40:1. Each spot shows the percent net specific lysis for one subject, calculated by subtracting percent lysis of uninfected monocytes from that of infected monocytes.

Effect of soluble factors produced by activated NK cells on IFN-γ production and CTL activity of CD8$^+$ cells

Depletion of CD56$^+$ cells removes both CD56$^+$CD3$^+$ NKT cells and CD56$^+$CD3$^-$ NK cells. To focus on the mechanisms by which NK cells regulate CD8$^+$ cell function, we first asked whether soluble factors produced by *M. tuberculosis*-activated NK cells upregulate IFN-γ production and cytotoxicity by CD8$^+$ cells. At day 0, PBMC from healthy tuberculin reactors were cultured with live *M. tuberculosis* for 24 h, and CD56$^+$CD3$^-$ cells were isolated by immunomagnetic selection and designated as activated NK cells. At day 1, more blood was obtained from the same donor, and PBMC, as well as CD56-depleted PBMC, were prepared by negative immunomagnetic depletion. These cells were cultured in separate wells of 12-well plates with live *M. tuberculosis*. The activated NK cells were cultured in Transwell inserts in some wells containing CD56-depleted PBMC. For measurement of IFN-γ$^+$CD8$^+$ cells, CD8$^+$ cells were isolated after 48 h from PBMC, CD56-depleted PBMC, and CD56-depleted PBMC exposed to activated NK cells in the Transwell. The CD8$^+$ cells were incubated overnight on an ELISPOT plate to detect IFN-γ-producing cells. For measurement of CTL activity, CD8$^+$ cells were isolated after 5 days from PBMC, CD56-depleted PBMC, and CD56-depleted PBMC exposed to activated NK cells in the Transwell. CD8$^+$ cells were used as effectors against autologous *M. tuberculosis*-infected monocytes.

Similar to results in Fig. 2, depletion of CD56$^+$ cells from PBMC lowered the frequency of IFN-γ-producing CD8$^+$cells (Fig. 3*A*). Coculture of CD56-depleted PBMC with activated NK cells restored the number of IFN-γ-producing CD8$^+$ cells to baseline levels (973 ± 281 per 10^5 cells vs 511 ± 176 per 10^5 cells, $p = 0.02$, Fig. 3*A*). Addition of activated NK cells also restored IFN-γ concentrations to baseline levels (Fig. 3*B*). In contrast, CTL activity of CD8$^+$ cells was not restored by addition of activated NK cells in a Transwell (Fig. 3*C*).

M. tuberculosis*-activated NK cells produce IFN-γ*

NK cells exposed to *M. tuberculosis*-infected monocytes are potent sources of IFN-γ (15). Therefore, we hypothesized that IFN-γ produced by activated NK cells elicits production of cytokines by mononuclear phagocytes that in turn increase IFN-γ production by CD8$^+$ cells. To test this hypothesis, we first cultured PBMC of five donors with or without live *M. tuberculosis* for 24 h, then isolated CD56$^+$CD3$^-$ NK cells and placed them in triplicate wells on an ELISPOT plate coated with anti-IFN-γ Abs. The frequency of IFN-γ$^+$ cells in *M. tuberculosis*-activated NK cells was extremely high (2198 ± 448 cells per 10^5 cells) compared with that in unactivated NK cells (2 ± 2 cells per 10^5 cells, $p < 0.001$).

Activated NK cells enhance production of IL-15 and IL-18

We hypothesized that IFN-γ produced by activated NK cells elicited production of IL-12, IL-15, and/or IL-18 by *M. tuberculosis*-infected monocytes, which in turn increased IFN-γ production by CD8$^+$ T cells. As a first step to test this hypothesis, we cultured infected monocytes with activated NK cells in Transwells in the presence or absence of anti-IFN-γ. IL-12 and IL-15 were not detected in cell culture supernatants. However, activated NK cells in Transwells enhanced IL-18 production by infected monocytes, and this effect was reduced by anti-IFN-γ (147 ± 8 pg/ml vs 89 ± 5

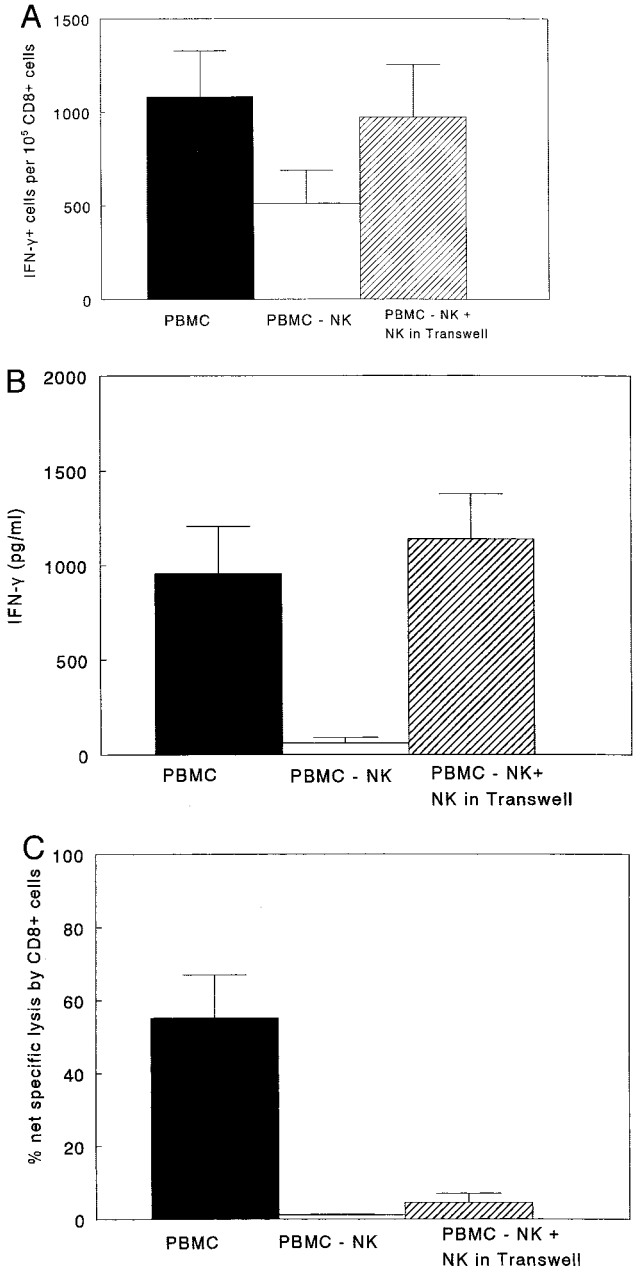

FIGURE 3. Effect of soluble factors produced by activated NK cells on the frequency of *M. tuberculosis*-responsive $CD8^+IFN\text{-}\gamma^+$ cells (*A*), the concentration of IFN-γ (*B*), and $CD8^+$ cell-mediated cytotoxicity against *M. tuberculosis*-infected monocytes (*C*). PBMC and CD56-depleted PBMC from healthy tuberculin reactors were cultured with live *M. tuberculosis* at a MOI of 0.5:1. To some wells of CD56-depleted PBMC, $CD56^+CD3^-$ NK cells preactivated by culture with live *M. tuberculosis* for 24 h were added in Transwell inserts. *A,* After 48 h, $CD8^+$ cells were positively selected from PBMC, CD56-depleted PBMC, and CD56-depleted PBMC exposed to activated NK cells. The $CD8^+$ cells were incubated in triplicate wells overnight on an ELISPOT plate to detect IFN-γ-producing cells. Mean values and SEs for experiments in seven donors are shown. *B,* After 24 h, IFN-γ concentrations were measured by ELISA in supernatants from wells containing PBMC, CD56-depleted PBMC, and CD56-depleted PBMC to which activated NK cells were added in Transwell inserts. Mean values and SEs for experiments in four donors are shown. *C,* After 5 days, $CD8^+$ cells were positively selected from the PBMC, CD56-depleted PBMC, and CD56-depleted PBMC exposed to activated NK cells. The $CD8^+$ cells were cultured in triplicate wells with uninfected or *M. tuberculosis*-infected autologous monocytes at an E:T ratio of 40:1. Mean values and SEs for percent net specific lysis of $CD8^+$ cells from five donors are shown, calculated by subtracting percent lysis of uninfected monocytes from that of infected monocytes.

pg/ml, $p = 0.004$, Fig. 4). Isotype control Abs did not affect IFN-γ production (data not shown).

Separation of infected monocytes and NK cells by Transwells does not permit cell-cell contact and may reduce monokine production. To evaluate production of IL-12, IL-15, and IL-18 under more physiologic conditions, activated NK cells were added directly to wells containing *M. tuberculosis*-infected monocytes. Addition of activated NK cells increased production of IL-15 and IL-18 ($p < 0.05$ for both monokines, Fig. *5*). IL-12 levels were <5 pg/ml in the presence or absence of NK cells. Anti-IFN-γ reduced production of IL-15 and IL-18 by infected monocytes cultured with activated NK cells (Fig. 5, $p = 0.002$ for IL-15 and $p = 0.004$ for IL-18). In contrast, isotype control Abs had no effect (data not shown).

IFN-γ and monokines are required for NK cells to restore the frequency of $CD8^+$ IFN-γ cells

The experiments above suggest that IFN-γ produced by activated NK cells elicits production of IL-15 and IL-18 by *M. tuberculosis*-infected monocytes, which may in turn increase IFN-γ production by $CD8^+$ cells. To investigate this hypothesis, we added anti-IFN-γ to CD56-depleted PBMC cultured with activated NK cells in Transwells. We also used Abs to IL-15 and IL-18, alone or in combination, to determine the contribution of these monokines to IFN-γ production by $CD8^+$ cells. Anti-IFN-γ abrogated the capacity of NK cells to restore the frequency of $CD8^+IFN\text{-}\gamma^+$ cells in CD56-depleted PBMC to baseline levels (488 ± 167 per 10^5 cells vs 1247 ± 426 per 10^5 cells, $p = 0.04$, Fig. 6). Isotype control Abs did not affect IFN-γ concentrations (data not shown). We considered the possibility that incubation of cells with anti-IFN-γ artifactually reduced the number of $IFN\text{-}\gamma^+CD8^+$ cells visualized by ELISPOT. Therefore, we cultured PBMC with PMA, with or without anti-IFN-γ Abs, for 48 h, then positively selected $CD8^+$ cells and placed them on an ELISPOT plate for 18 h. Anti-IFN-γ did not reduce the number of $IFN\text{-}\gamma^+CD8^+$ cells (data not shown).

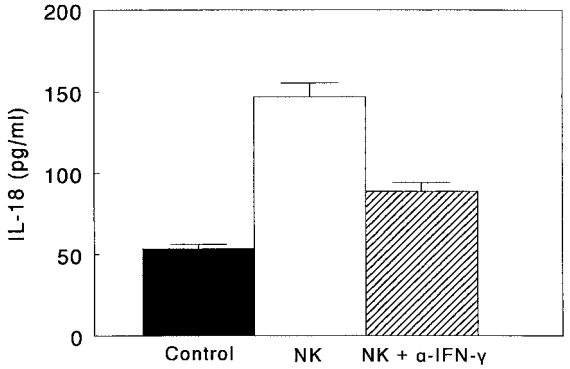

FIGURE 4. Effect of NK cells and anti-IFN-γ on IL-18 production by *M. tuberculosis*-infected monocytes. Monocytes from healthy tuberculin reactors were infected with *M. tuberculosis* at a MOI of 5:1 for 24 h. To some wells, autologous $CD56^+CD3^-$ NK cells, preactivated by culture with live *M. tuberculosis* for 24 h, were added in Transwell inserts at a ratio of 1 NK cell to 5 monocytes. Anti-IFN-γ (10 μg/ml) was added to some wells containing NK cells in Transwells. After an additional 48 h, supernatants were collected and IL-18 levels were measured by ELISA. Mean values and SEs for experiments in four donors are shown. ■, Infected monocytes alone; □, infected monocytes and NK cells in Transwell; ▨, infected monocytes and NK cells in Transwell and anti-IFN-γ.

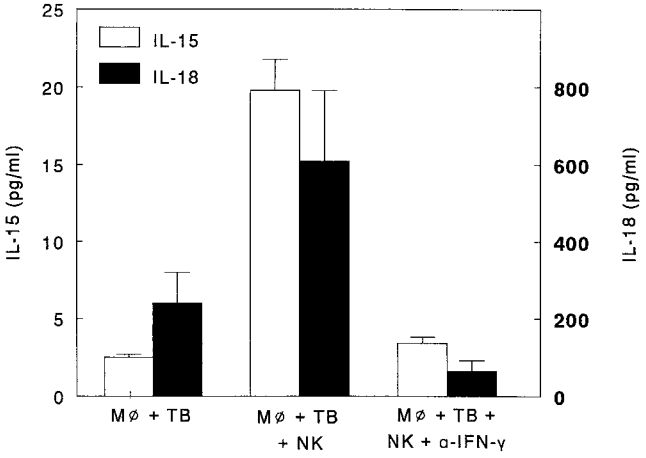

FIGURE 5. Effect of activated NK cells and anti-IFN-γ on monokine production. Monocytes from four healthy tuberculin reactors were infected with *M. tuberculosis* (TB) at a MOI of 5:1. After 24 h, autologous CD56$^+$CD3$^-$ NK cells, preactivated by culture with live *M. tuberculosis* for 24 h, were added directly to some wells at a ratio of 1 NK cell to 10 monocytes. To other wells, preactivated NK cells and anti-IFN-γ (10 μg/ml) were added. After 48 h, supernatants were collected and IL-15 and IL-18 levels were measured by ELISA. Mean values and SEs are shown.

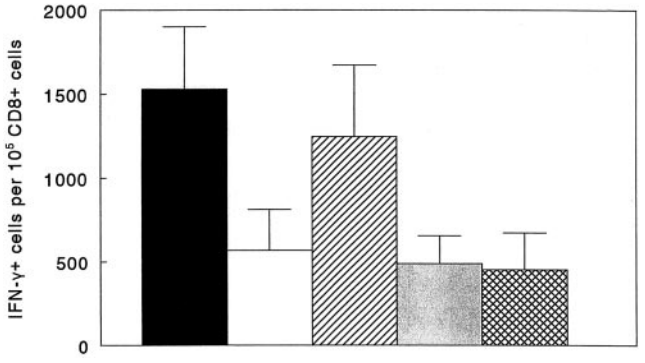

FIGURE 6. Effect of anti-cytokine Abs on the frequency of CD8$^+$IFN-γ^+ cells. PBMC and CD56-depleted PBMC from healthy tuberculin reactors were cultured with live *M. tuberculosis* at a MOI of 0.5:1. To some wells of CD56-depleted PBMC, CD56$^+$CD3$^-$ NK cells, preactivated by culture with live *M. tuberculosis* for 24 h, were added in Transwell inserts. To some wells containing CD56-depleted PBMC and activated NK cells, anti-IFN-γ or the combination of anti-IL-15 and anti-IL-18 were also added (all at 10 μg/ml). After 48 h, CD8$^+$ cells were isolated from PBMC, CD56-depleted PBMC, and CD56-depleted PBMC exposed to activated NK cells, with or without anti-cytokine Abs, and incubated overnight on an ELISPOT plate to detect IFN-γ-producing cells. Mean values and SEs for experiments in five donors are shown. ■, PBMC; □, CD56-depleted PBMC; ▨, CD56-depleted PBMC and NK cells in Transwell; ▩, CD56-depleted PBMC, NK cells in Transwell, and anti-IFN-γ; ▩, CD56-depleted PBMC, NK cells in Transwell, and anti-IL-15 and anti-IL-18.

The combination of anti-IL-15 and anti-IL-18 prevented NK cells from increasing the frequency of CD8$^+$IFN-γ^+ cells in CD56-depleted PBMC (453 $\pm$ 219 per 10^5 cells vs 1247 $\pm$ 426 per 10^5 cells, $p = 0.02$, Fig. 6). Addition of anti-IL-15 and anti-IL-18 individually had effects similar to that of adding both Abs (data not shown). We also added 25–2500 pg/ml rIL-15 and rIL-18 individually to CD56-depleted PBMC. At concentrations of 100 pg/ml, both recombinant cytokines restored the number of CD8$^+$IFN-γ^+ cells to normal, but lower concentrations had variable effects in different individuals (data not shown). We were therefore unable to determine the relative contribution of IL-15 and IL-18 to enhancing IFN-γ production by CD8$^+$ cells.

CD40L contributes to the capacity of NK cells to optimize CTL activity of CD8$^+$ T cells

The results in Figs. 2*B* and 3*C* suggest that NK cells enhance lysis of *M. tuberculosis*-infected monocytes by CD8$^+$ T cells and that this effect requires cell-cell contact between NK cells and either infected monocytes or CD8$^+$ cells. We hypothesized that *M. tuberculosis*-activated NK cells express CD40L, which binds to CD40 on infected monocytes, up-regulating CD8$^+$ CTL activity in a manner similar to that mediated by CD4$^+$ cells (20, 21). To evaluate this possibility, we first isolated CD56$^+$CD3$^-$ cells from PBMC that had been cultured with or without live *M. tuberculosis* and measured the percentage of NK cells that expressed CD40L. In seven donors, the mean fluorescence intensity of CD40L on unstimulated CD56$^+$CD3$^-$ NK cells was 24 $\pm$ 1, and this increased to 41 $\pm$ 2 on *M. tuberculosis*-activated NK cells ($p < 0.001$).

CD40L on CD4$^+$ T cells can provide costimulation for CD8$^+$ T cells by binding either to CD40 on APCs (20, 21) or by binding to CD40 on CD8$^+$ cells (22). Because we hypothesized that the former was more likely, we devised an experimental system in which NK cells had contact with infected monocytes but not with CD8$^+$ cells. We first isolated CD14$^+$ monocytes from four healthy tuberculin reactors and infected them with *M. tuberculosis*. Monocytes were then cultured under four conditions: 1) alone, 2) with autologous NK cells and anti-CD40 mAb, 3) with autologous NK cells and anti-CD40L mAb, and 4) with autologous CD56$^+$CD3$^-$ NK cells and isotype control Abs. After 2 days of culture, monocytes from each well were cultured with autologous CD8$^+$ cells. After 6 additional days, CD8$^+$ cells were isolated and tested for CTL activity against *M. tuberculosis*-infected monocytes. CD8$^+$ T cells cultured with infected monocytes alone showed minimal CTL activity. In contrast, infected monocytes that had been exposed to NK cells and isotype control Abs primed CD8$^+$ T cells to lyse 80–90% of infected target cells (Fig. 7). Anti-CD40 and anti-CD40L abrogated the capacity of NK cell-exposed infected monocytes to prime CTL activity of CD8$^+$ cells. In three additional donors, anti-CD40 had similar effects (data not shown). These findings indicate that interactions between CD40L on NK cells and CD40 on monocytes prime CTL activity of CD8$^+$ T cells.

Blocking CD40L-CD40 interactions alters IL-15 and IL-18 production by infected monocytes

Because IL-15 and IL-18 can favor development of CD8$^+$ CTL, we determined whether interactions between CD40L on NK cells and CD40 on monocytes contributes to IL-15 and IL-18 production by infected monocytes. We isolated NK cells from three healthy donors and cultured them with autologous *M. tuberculosis*-infected monocytes in the absence and presence of Abs to CD40 and CD40L. After 48 h, culture supernatants were collected and IL-18 and IL-15 levels were measured by ELISA. Baseline IL-18 concentrations of 87 $\pm$ 15 pg/ml were reduced to 38 $\pm$ 8 pg/ml by anti-CD40 and to 42 $\pm$ 10 by anti-CD40L Abs. Similarly, baseline IL-15 concentrations of 5.1 $\pm$ 0.5 pg/ml were reduced to 1.9 $\pm$ 0.6 pg/ml by anti-CD40 and to 0.9 $\pm$ 0.2 pg/ml by anti-CD40L.

Discussion

NK cells are a central component of the innate immune response to intracellular pathogens. It is widely believed that NK cells shape

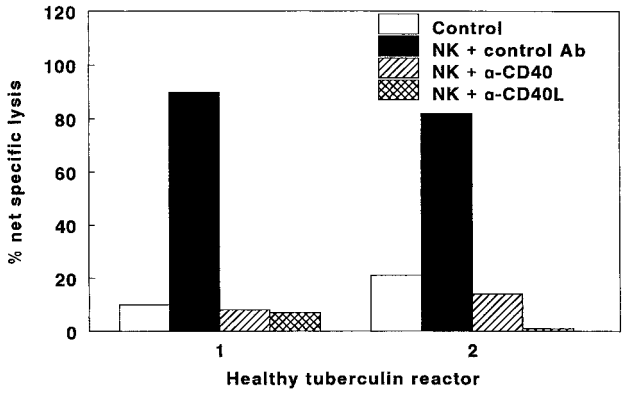

FIGURE 7. Effect of CD40 and CD40L interactions on the capacity of infected monocytes to prime CTL activity of $CD8^+$ T cells. $CD14^+$ monocytes from healthy tuberculin reactors were infected with *M. tuberculosis* at a MOI of 5:1. These were cultured: 1) alone (control), 2) with autologous $CD56^+CD3^-$ NK cells at a ratio of 1 NK cell to 5 monocytes and isotype control Abs (1gG1, 5 μg/ml; BD PharMingen), 3) with autologous NK cells and neutralizing anti-CD40 mAb (M2, 5 μg/ml; Amgen, Seattle, WA), and 4) with autologous NK cells and neutralizing anti-CD40L mAb (M91, 5 μg/ml; Amgen). After 2 days, $CD14^+$ cells from each well were positively selected and cultured with autologous $CD8^+$ cells at a ratio of 25 $CD8^+$ cells to 1 $CD14^+$ cell. After 6 additional days, $CD8^+$ cells were isolated and tested for CTL activity against *M. tuberculosis*-infected and uninfected monocyte targets. Percent net specific lysis was calculated by subtracting the percent lysis of uninfected monocytes from the percent lysis of infected monocytes. Each value shows the mean of triplicate values for a single subject. The SE was $<10\%$ of the mean in all cases. Experiments were performed with cells from four subjects, and two representative results are shown.

the adaptive immune response through production of cytokines and other mechanisms. However, we are unaware of published evidence in humans demonstrating that NK cells contribute to the T cell response to an infectious agent. We studied the relationship between NK cells and $CD8^+$ T cell in persons infected with *M. tuberculosis*, a facultative intracellular bacterium that infects an estimated two billion persons worldwide (23).

We found that depletion of $CD56^+$ cells from PBMC of healthy tuberculin reactors reduced the frequency of *M. tuberculosis*-responsive $CD8^+$ IFN-γ producing cells and decreased the capacity of $CD8^+$ T cells to lyse *M. tuberculosis*-infected mononuclear phagocytes. The frequency of $CD8^+$IFN-γ^+ T cells was restored by soluble factors secreted by activated $CD56^+CD3^-$ NK cells and was dependent on the presence of IFN-γ, IL-15, and IL-18. *M. tuberculosis*-activated NK cells produced IFN-γ, addition of activated NK cells to infected monocytes enhanced secretion of IL-15 and IL-18, and production of IL-15 and IL-18 was abrogated by anti-IFN-γ. The capacity of NK cells to prime CTL activity of $CD8^+$ T cells depended on cell-cell contact between NK cells and infected monocytes and was inhibited by anti-CD40 and anti-CD40L. These findings demonstrate that NK cells enhance IFN-γ production by $CD8^+$ cells through a cascade of cytokines and optimize $CD8^+$ CTL activity through the CD40L/CD40 pathway. Therefore, NK cells utilize at least two distinct mechanisms to enhance $CD8^+$ T cell effector functions that are likely to contribute to protective immunity against *M. tuberculosis*.

IFN-γ increases production of IL-15 and IL-18 by mononuclear phagocytes exposed to mycobacteria (24, 25). Furthermore, IL-15 enhances expansion of virus- and mycobacterium-specific $CD8^+$ T cells (26, 27), and IL-18 favors development of Tc1 $CD8^+$ T cells that produce IFN-γ (28). In the current report, NK cells were potent sources of IFN-γ, addition of activated NK cells to infected monocytes significantly increased production of IL-15 and IL-18 (Fig. 5), and IL-15 and IL-18 concentrations were reduced by anti-IFN-γ (Figs. 4 and 5). In our experimental system, IL-15 concentrations were extremely low and others have found that IL-15 is barely detectable in biological fluids despite abundant evidence for its myriad immunologic effects (29, 30). This paradox has recently been resolved by the demonstration that activated monocytes express complexes of IL-15 bound to the IL-15α receptor on the cell membrane, and these complexes have the potential to stimulate proliferation by $CD8^+$ T cells (31). We speculate that monocyte IL-15 may act predominantly as membrane-bound rather than soluble cytokines that affect $CD8^+$ T cells in our experimental system. Our current findings suggest that NK cells secrete IFN-γ, which stimulates monocytes to produce IL-15 and IL-18, which in turn facilitates expansion of $CD8^+$ T cells that produce IFN-γ in response to *M. tuberculosis*-infected monocytes. Because NK cells produce IFN-γ early in the course of infection with *M. tuberculosis* (32), this pathway is likely to be important in facilitating expansion of $CD8^+$ effector cells during the immune response to *M. tuberculosis* in vivo.

$CD8^+$ effector cells have classically been considered to both produce IFN-γ and exhibit CTL activity. However, a growing body of evidence indicates that these functional characteristics can be dissociated. For example, HIV-specific and polyoma virus-induced $CD8^+$ T cells produce IFN-γ in response to viral peptides, but showed reduced CTL activity (33, 34). In addition, a novel assay that permits identification of individual cytokine-producing and cytolytic cells demonstrated independent regulation of these effector functions (35). In the current report, soluble factors secreted by $CD56^+CD3^-$ NK cells increased the number of IFN-γ^+CD8^+ T cells, whereas interactions between CD40 on APCs and CD40 ligand on NK cells enhanced $CD8^+$ CTL activity against *M. tuberculosis*-infected monocytes. This suggests that NK cells may utilize distinct mechanisms to regulate IFN-γ production and CTL activity by $CD8^+$ cells in the course of the immune response to infection with intracellular pathogens. In NK cells, IFN-γ production and cytolytic activity are mediated through different cell surface receptors and signal transduction pathways. Stimulation through the receptors CD137 or KIR2DL4 elicits IFN-γ production but not cytotoxicity (36, 37). In addition, signaling through mitogen-activated protein kinases and Vav1 control cytotoxicity, whereas the p38 signaling pathway mediates IFN-γ production (38, 39). We speculate that IFN-γ production and CTL activity by $CD8^+$ cells are also controlled by different intracellular signaling pathways.

$CD4^+$ T cells provide help for development of $CD8^+$ CTL in response to several intracellular pathogens through interactions between the CD40L on $CD4^+$ T cells and CD40 on APCs (40, 41), and the CD40L trimer increased human $CD8^+$ CTL activity against *M. tuberculosis*-infected monocytes (42). In the current report, we found that NK cells enhanced lysis of *M. tuberculosis*-infected monocytes by $CD8^+$ T cells through mechanisms that require direct contact between NK cells and infected monocytes. CD40L expression was up-regulated on activated NK cells exposed to infected monocytes. In addition, both anti-CD40 and anti-CD40L reduced production of IL-15 and IL-18 and abrogated the capacity of NK cell-exposed monocytes to prime CTL function by $CD8^+$ T cells. These data suggest that interactions between CD40L on activated NK cells and CD40 on infected monocytes were necessary to optimize $CD8^+$ CTL activity. CD40 was originally believed to be expressed only on APCs, but recent data indicate that CD40 is also present on $CD8^+$ T cells (22). CD40L

on NK cells did not bind to CD40 on CD8$^+$ T cells in our experimental system, because NK cells did not have contact with CD8$^+$ T cells (Fig. 7).

We found that depletion of CD56$^+$ cells from PBMC markedly reduced CD8$^+$ T cell effector function. Approximately 20% of CD8$^+$ T cells in PBMC were CD56$^+$, and some of the reduced cytotoxicity by CD56-depleted cells may have been due to removal of CD8$^+$CD56$^+$ cells. CD56$^+$ cells include CD56$^+$CD3$^-$ NK cells and CD56$^+$CD3$^+$ NKT cells. One recent study showed that NK cells and NKT cells are both important for dendritic cell-dependent priming of CD8$^+$ T cells that recognize a hepatitis B viral peptide (43). Nevertheless, we found that CD56$^+$CD3$^-$ cells restored the capacity of CD8$^+$ T cells to produce IFN-γ and exhibit CTL activity through secretion of soluble factors and cell-cell contact with infected monocytes, respectively. These findings clearly demonstrate a role for NK cells in maintaining CD8$^+$ T cell function. Additional studies are needed to determine the relative contributions of NK cells and NKT cells to the CD8$^+$ T cell response to *M. tuberculosis*.

One shortcoming of our studies is that we evaluated NK cells obtained from peripheral blood rather than from the lungs and lymph nodes of persons with primary *M. tuberculosis* infection. Such experiments would be extremely informative but unfortunately, ethical and logistical constraints make it difficult to perform these studies in humans. Additional work in animals is needed to better delineate the contribution of NK cells to development of the CD8$^+$ T cell response at the site of disease in vivo.

CD8$^+$ T cells are a critical component of the adaptive immune response that contributes to protective immunity against *M. tuberculosis* through lysis of infected cells, production of IFN-γ, and secretion of the mycobactericidal peptide granulysin (8, 18, 44). It is therefore important to delineate the innate immune mechanisms that favor expansion of CD8$^+$ T cells that recognize and lyse pathogen-infected cells. Most studies have emphasized the role of APCs. For example, dendritic cells can shape cytokine production and CTL activity of CD8$^+$ T cells through production of IL-12, TNF-α, and other cytokines (45, 46). Our results demonstrate that NK cells constitute another innate mechanism that can modulate the human CD8$^+$ T cell response to intracellular pathogens.

References

1. Orange, J. S., B. Wang, C. Terhorst, and C. A. Biron. 1995. Requirement for natural killer cell-produced interferon gamma in defense against murine cytomegalovirus infection and enhancement of this defense pathway by interleukin 12 administration. *J. Exp. Med. 182:1045*.
2. Tay, C. H., E. Szomolanyi, and R. M. Welsh. 1998. Control of infections by NK cells. *Curr. Top. Microbiol. Immunol. 230:193*.
3. Gregory, S. H., X. Jiang, and E. J. Wing. 1996. Lymphokine-activated killer cells lyse *Listeria*-infected hepatocytes and produce elevated quantities of interferon-γ. *J. Infect. Dis. 174:1073*.
4. Doolan, D. L., and S. L. Hoffman. 1999. IL-12 and NK cells are required for antigen-specific adaptive immunity against malaria initiated by CD8$^+$ T cells in the *Plasmodium yoelii* model. *J. Immunol. 163:884*.
5. Garcia-Penarrubia, P., F. T. Koster, R. O. Kelley, T. D. McDowell, and A. D. Bankhurst. 1989. Antibacterial activity of human natural killer cells. *J. Exp. Med. 169:99*.
6. Biron, C. A., K. S. Byron, and J. L. Sullivan. 1989. Severe herpesvirus infections in an adolescent without natural killer cells. *N. Engl. J. Med. 320:1731*.
7. Kos, F. J., and E. G. Engleman. 1996. Immune regulation: a critical link between NK cells and CTLs. *Immunol. Today 17:174*.
8. Serbina, N. V., C. C. Liu, C. A. Scanga, and J. L. Flynn. 2000. CD8$^+$ CTL from lungs of *Mycobacterium tuberculosis*-infected mice express perforin in vivo and lyse infected macrophages. *J. Immunol. 165:353*.
9. Tascon, R. E., E. Stavropoulos, K. V. Lukacs, and M. J. Colston. 1998. Protection against *Mycobacterium tuberculosis* infection by CD8 T cells requires production of gamma interferon. *Infect. Immun. 66:830*.
10. Kos, F. J., and E. G. Engleman. 1995. Requirement for natural killer cells in the induction of cytotoxic T cells. *J. Immunol. 155:578*.
11. Kelly, J. A., P. K. Darcy, J. L. Markby, D. I. Godfrey, K. Takeda, H. Yagita, and M. J. Smyth. 2002. Induction of tumor-specific T cell memory by NK cell-mediated tumor rejection. *Nat. Immunol. 3:83*.
12. Kos, F. J. 1998. Regulation of adaptive immunity by natural killer cells. *Immunol. Res. 17:303*.
13. Vankayalapati, R., B. Wizel, B. Samten, D. E. Griffith, H. Shams, M. R. Galland, C. F. von Reyn, W. M. Girard, R. J. Wallace, Jr., and P. F. Barnes. 2001. Cytokine profiles in immunocompetent persons infected with *Mycobacterium avium* complex. *J. Infect. Dis. 183:478*.
14. Zhang, M., J. Gong, Y. Lin, P. F. Barnes. 1998. Growth of virulent and avirulent *Mycobacterium tuberculosis* in human macrophages. *Infect. Immun. 66:794*.
15. Vankayalapati, R., B. Wizel, S. E. Weis, H. Safi, D. L. Lakey, O. Mandelboim, B. Samten, A. Porgador, and P. F. Barnes. 2002. The NKp46 receptor contributes to NK cell lysis of mononuclear phagocytes infected with an intracellular bacterium. *J. Immunol. 168:3451*.
16. Wizel, B., M. Palmieri, C. Mendoza, B. Arana, J. Sidney, A. Sette, and R. Tarleton. 1998. Human infection with *Trypanosoma cruzi* induces parasite antigen-specific cytotoxic T lymphocyte responses. *J. Clin. Invest. 102:1062*.
17. Shams, H., B. Wizel, S. E. Weis, B. Samten, and P. F. Barnes. 2001. Contribution of CD8$^+$ T cells to γ interferon production in human tuberculosis. *Infect. Immun. 69:3497*.
18. Cho, S., V. Mehra, S. Thoma-Uszynski, S. Stenger, N. V. Serbina, R. J Mazzaccaro, J. L. Flynn, P. F. Barnes, S. Southwood, E. Celis, et al. 2000. Antimicrobial activity of MHC class I-restricted CD8$^+$T cells in human tuberculosis. *Proc. Natl. Acad. Sci. USA 97:12210*.
19. Kemp, K., L. Hviid, A. Kharazmi, and M. Kemp. 1997. Interferon-γ production by human T cells and natural killer cells in vitro in response to antigens from the two intracellular pathogens *Mycobacterium tuberculosis* and *Leishmania major*. *Scand. J. Immunol. 46:495*.
20. Bennett, S. R., F. R. Carbone, F. Karamalis, R. A. Flavell, J. F. Miller, and W. R. Heath. 1998. Help for cytotoxic-T-cell responses is mediated by CD40 signalling. *Nature 393:478*.
21. Ridge, J. P., F. DiRosa, and P. Matzinger. 1998. A conditioned dendritic cell can be a temporal bridge between a CD4$^+$ T-helper and a T-killer cell. *Nature 393:474*.
22. Bourgeois, C., B. Rocha, and C. Tanchot. 2002. A role for CD40 expression on CD8$^+$ T-cells in the generation of CD8$^+$ T cell memory. *Science 297:2060*.
23. Dye, C., S. Scheele, P. Dolin, V. Pathania, and M. C. Raviglione. 1999. Global burden of tuberculosis: estimated incidence, prevalence, and mortality by country. *JAMA 282:677*.
24. Vankayalapati, R., B. Wizel, D. L. Lakey, Y. Zhang, K. A. Coffee, D. E. Griffith, and P. F Barnes. 2001. T cells enhance production of IL-18 by monocytes in response to an intracellular pathogen. *J. Immunol. 166:6749*.
25. Doherty, T. M., R. A. Seder, and A. Sher. 1996. Induction and regulation of IL-15 expression in murine macrophages. *J. Immunol. 156:735*.
26. Umemura, M., H. Nishimura, K. Hirose, T. Matsuguchi, and Y. Yoshikai. 2002. Overexpression of IL-15 in vivo enhances protection against *Mycobacterium bovis* bacillus Calmette-Guérin infection via augmentation of NK and T cytotoxic 1 responses. *J. Immunol. 167:946*.
27. Schluns, K. S., K. Williams, A. Ma, X. X. Zheng, and L. Lefrancois. 2002. Requirement for IL-15 in the generation of primary and memory antigen-specific CD8 T cells. *J. Immunol. 168:4827*.
28. Okamoto, I., K. Kohno, T. Tanimoto, H. Ikegami, and M. Kurimoto. 1999. Development of CD8$^+$ effector T cells is differentially regulated by IL-18 and IL-12. *J. Immunol. 162:3202*.
29. Musso, T., L. Calosso, M. Zucca, M. Millesimo, D. Ravarino, M. Giovarelli, F. Malavasi, A. N. Ponzi, R. Paus, and S. Bulfone-Paus. 1999. Human monocytes constitutively express membrane-bound, biologically active, and interferon-γ-upregulated interleukin-15. *Blood 93:3531*.
30. Neely, G. G., S. M Robbins, E. K Amankwah, S. Epelman, H. Wong, J. C Spurrell, K. K. Jandu, W. Zhu, D. K. Fogg, C. B. Brown, and C. H Mody. 2001. Lipopolysaccharide-stimulated or granulocyte-macrophage colony-stimulating factor-stimulated monocytes rapidly express biologically active IL-15 on their cell surface independent of new protein synthesis. *J. Immunol. 167:5011*.
31. Dubois, S., J. Mariner, T. A. Waldmann, and Y. Tagaya. 2002. IL-15Rα recycles and presents IL-15 in *trans* to neighboring cells. *Immunity 17:537*.
32. Junqueira-Kipnis, A. P., A. Kipnis, M. G. Juarrero, J. Turner, A. Diefenbach, D. H. Raulet, and I. M. Orme. 2003. Lung NK cells participate in the acute phase of *Mycobacterium tuberculosis* mouse infection. In *Abstracts of the Keystone Symposium Tuberculosis: Integrating Host and Pathogen Biology.* Abstract 325, Keystone Symposia, Silverthorne, CO, p. 91.
33. Appay, V., D. F. Nixon, S. M. Donahoe, G. M. A. Gillespie, T. Dong, A. King, G. S. Ogg, H. M. L. Spiegel, C. Conlon, C. A. Spina, et al. 2000. HIV-specific CD8$^+$ T cells produce antiviral cytokines but are impaired for cytolytic function. *J. Exp. Med. 192:63*.
34. Moser, J. M., J. D. Altman, and A. E. Lukacher. 2001. Antiviral CD8$^+$ T cell responses in neonatal mice: susceptibility to polyoma virus-induced tumors is associated with lack of cytotoxic function by viral antigen-specific T cells. *J. Exp. Med. 193:595*.
35. Snyder, J. E., W. J. Bowers, A. M. Livingstone, F. E.-H. Lee, H. J. Federoff, and T. R. Mosmann. 2003. Measuring the frequency of mouse and human cytotoxic T cells by the Lysispot assay: independent regulation of cytokine secretion and short-term killing. *Nat. Med. 9:231*.

36. Rajagopalan, S., J. Fu, and E. O. Long. 2001. Cutting edge: induction of IFN-γ production but not cytotoxicity by the killer cell Ig-like receptor KIR2DL4 (CD158d) in resting NK cells. *J. Immunol. 167:1877*.
37. Wilcox, R. A., K. Tamadda, S. E. Strome, and L. Chan. 2002. Signaling through NK cell-associated CD137 promotes both helper function for $CD8^+$ cytolytic T cells and responsiveness to IL-2 but not cytotoxic activity. *J. Immunol. 169:4230*.
38. Chuang, S. S., P. R. Kumaresan, and P. A. Matthew. 2001. 2B4 (CD244)-mediated activation of cytotoxicity and IFN-γ release in human NK cells involves distinct pathways. *J. Immunol. 167:6210*.
39. Colucci, F., E. Rosmaraki, S. Bregenholt, S. I. Samson, V. Di Bartolo, M. Turner, L. Vanes, V. Tybulewicz, and J. P. Di Santo. 2001. Functional dichotomy in natural killer cell signaling: Vav1-dependent and –independent mechanisms. *J. Exp. Med. 193:1413*.
40. Yang, Y., and J. M. Wilson. 1996. CD40 ligand-dependent T cell activation: requirement of B7-CD28 signaling through CD40. *Science 273:1862*.
41. Gurunathan, S., K. R. Irvine, C. Y. Wu, J. I. Cohen, E. Thomas, C. Prussin, N. P. Restifo, and R. A. Seder. 1998. CD40 ligand/trimer DNA enhances both humoral and cellular immune responses and induces protective immunity to infectious and tumor challenge. *J. Immunol. 161:4563*.
42. Samten, B., B. Wizel, H. Shams, S. E. Weis, P. Klucar, S. Wu, R. Vankayalapati, E. K. Thomas, S. Okada, A. M. Krensky, et al. 2003. CD40 ligand trimer enhances the response of $CD8^+$ T cells to *Mycobacterium tuberculosis*. *J. Immunol. 170:3180*.
43. Stober, D., I. Jomantaite, R. Schirmbeck, and J. Reimann. 2003. NKT cells provide help for dendritic cell-dependent priming of MHC class I-restricted $CD8^+$ T cells in vivo. *J. Immunol. 170:2540*.
44. Stenger, S., D. A. Hanson, R. Teitelbaum, P. Dewan, K. R. Niazi, C. J. Froelich, T. Ganz, S. Thoma-Uszynski, A. Melian, C. Bogdan, et al. 1998. An antimicrobial activity of cytolytic T cells mediated by granulysin. *Science 282:121*.
45. Zarling, A. L., J. G. Johnson, R. W. Hoffman, and D. R. Lee. 1999. Induction of primary human $CD8^+$ T lymphocyte responses in vitro using dendritic cells. *J. Immunol. 162:5197*.
46. Voisine, C., F. X. Hubert, B. Trinite, M. Heslan, and R. Josien. 2002. Two phenotypically distinct subsets of spleen dendritic cells in rats exhibit different cytokine production and T cell stimulatory activity. *J. Immunol. 169:2284*.