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CD56^{bright} NK Cells Are Enriched at Inflammatory Sites and Can Engage with Monocytes in a Reciprocal Program of Activation¹

Nicola Dalbeth,^{*†‡} Roger Gundle,[‡] Robert J. O. Davies,[§] Y. C. Gary Lee,[§] Andrew J. McMichael,[†] and Margaret F. C. Callan^{2*†}

Human NK cells may be divided into a CD56^{dim} subset and a CD56^{bright} subset. In peripheral blood, CD56^{dim} NK cells dominate, whereas in lymph nodes, CD56^{bright} NK cells are more common. In this study we show that CD56^{bright} NK cells accumulate within inflammatory lesions in a wide variety of clinical diseases affecting several different anatomical sites. We demonstrate that when activated by the monokines IL-12, IL-15, and IL-18, these NK cells promote TNF- α production by CD14⁺ monocytes in a manner that is dependent on cell:cell contact. Conversely, CD14⁺ monocytes synergize with monokines to promote IFN- γ production by these NK cells. Again, this interaction is dependent on cell:cell contact. The experiments show that CD56^{bright} NK cells accumulate in inflammatory lesions and, in the appropriate cytokine environment, can engage with CD14⁺ monocytes in a reciprocal activatory fashion, thereby amplifying the inflammatory response. Such a positive feedback loop is likely to be important in the pathogenesis of chronic inflammatory conditions such as rheumatoid arthritis. *The Journal of Immunology*, 2004, 173: 6418–6426.

Natural killer cells are an important component of the innate immune system (1). These cells account for 10–15% of PBLs and are defined by the expression of CD56 and the lack of expression of CD3 (2). NK cells have a number of effector functions, including recognition and lysis of virus-infected or tumor cells and production of immunoregulatory cytokines, particularly IFN- γ . Their activity is regulated by integration of both activatory and inhibitory signals from a wide range of cell surface receptors (3). These include members of the C-type lectin family, such as CD94 and NKG2A, -B, -C, and -D; members of the Ig superfamily, such as the killer Ig-like receptors (KIRs)³ and the leukocyte Ig-like receptor-1; as well as natural cytotoxicity receptors (NCRs), cytokine receptors, and a range of costimulatory molecules.

Two subsets of NK cells in peripheral blood have been recognized (4). The majority (CD56^{dim} NK cells) express moderate levels of CD56 and high levels of CD16. The CD56^{dim} NK cells usually express KIRs and are heterogeneous with respect to the expression of CD94 and NKG2A. The minor subset of NK cells (CD56^{bright} NK cells) accounts for only 10% of circulating NK cells. These cells express high levels of CD56, CD94, and NKG2A and tend to lack expression of CD16 and the KIRs. The two sub-

sets of NK cells differ also in terms of chemokine receptor and adhesion molecule expression, suggesting that they have different homing properties (5). Indeed, the CD56^{bright} cells have been found to be the dominant NK cell subset in human lymph nodes (6). Lastly, they show important functional differences; the CD56^{dim} subset has superior cytotoxic capacity, whereas the CD56^{bright} subset has greater ability to produce proinflammatory cytokines on exposure to low concentrations of monokines (4, 7). The underlying developmental relationship between the two NK cells subsets remains controversial (8, 9).

We have recently demonstrated that the CD56^{bright} subset of NK cells is greatly expanded in synovial fluid of patients with inflammatory arthritis (10). In this study we have analyzed the phenotype of NK cells within synovial tissue from patients with inflammatory arthritis; within exudative pleural fluid from patients with infectious, reactive, or malignant pulmonary disease; and within peritoneal fluid from patients with bacterial peritonitis. We then investigated the hypothesis that these NK cells are capable of interacting in a reciprocal fashion with the CD14⁺ mononuclear cell population and that they may thereby promote the development or maintenance of inflammation.

Materials and Methods

Patients and samples

Samples of peripheral blood or fluid from an inflammatory site were collected from five different groups of individuals. First, paired samples of peripheral blood and synovial fluid were obtained from 33 patients with inflammatory arthritis who presented to the out-patient clinic with knee effusions. Diagnoses included rheumatoid arthritis, psoriatic arthritis, ankylosing spondylitis, gout, and juvenile idiopathic arthritis. Second, paired samples of blood and fresh inflammatory synovial tissue were obtained from the operating room at the time of knee joint replacement or arthroscopic synovectomy from 7 patients. These were 5 patients with rheumatoid arthritis, 1 patient with ankylosing spondylitis, and 1 patient with juvenile idiopathic arthritis. Third, paired samples of peripheral blood and exudative pleural fluid were obtained from 17 patients. These included 5 patients with pleural infection (complicated parapneumonic effusion or empyema), 10 patients with histocytologically proven pleural malignancy (four with mesothelioma and six with metastatic pleural malignancy), and 2 patients with inflammatory exudative pleural effusions. Fourth, samples

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³ Abbreviations used in this paper: KIR, killer Ig-like receptor; NCR, natural cytotoxicity receptor.

of chronic ambulatory peritoneal dialysis fluid were obtained from 8 patients with end-stage renal failure and bacterial peritonitis. Last, samples of peripheral blood were obtained from healthy donors.

Fresh synovial tissue specimens were digested with 1 mg/ml collagenase type IV (Sigma-Aldrich, Poole, U.K.) in RPMI 1640 supplemented with 10% FCS, 2 mM glutamine, 100 U/ml penicillin, and 100 μ g/ml streptomycin (culture medium) for 1 h at 37°C in the presence of 5% CO₂. The tissue was then passed through a 70- μ m pore size filter (BD Biosciences, Oxford, U.K.) and washed three times in complete medium. Mononuclear cells were separated from blood and inflamed sites by Lymphoprep (Nycomed Pharma, Oslo, Norway) gradient centrifugation.

The local ethics committees at Oxford Radcliffe Trust and Hammer-smith Hospital Trust approved the study.

Staining for expression of cell surface molecules

Mononuclear cells were washed in PBS supplemented by 0.16% BSA, 0.1% sodium azide, and 1% human serum and incubated for 30 min on ice with saturating amounts of one or more of the following panel of mAb: anti-CD16 (DakoCytomation, Carpinteria, CA), anti-CD94 (BD Biosciences, San Diego, CA), anti-CD69 (BD Biosciences), anti-CD62L conjugated to FITC (BD Biosciences), anti-CCR7 (BD Biosciences), anti-NKG2A conjugated to PE (Immunotech, Marseille, France), anti-CD158a (KIR2DL1 and KIR2DS1; Immunotech), anti-CD158b (KIR2DL3, KIR2DS2, and KIR2DL2), anti-KIR3DL1 (clone DX9; gift from L. Lanier, University of California, San Francisco, CA), and anti-KIR3 DL2 (clone DX31; gift from L. Lanier). The four mAb specific for the KIRs were all added to the same sample, and the staining is referred to as staining for KIRs. Cells were washed and, where necessary, stained for 30 min on ice with an FITC-conjugated anti-mouse mAb (DakoCytomation). After this, cells were washed and stained with saturating amounts of an anti-CD56 mAb conjugated to allophycocyanin (Immunotech), and anti-CD3-PE (DakoCytomation) or anti-CD3-FITC (BD Biosciences) in the presence of 1% mouse serum. All samples were analyzed on a FACSCalibur using CellQuest software (both from BD Biosciences). Lymphocytes were identified by their forward and side scatter characteristics.

Staining for expression of intracellular perforin

After surface staining for CD3 and CD56 as described above, mononuclear cells were fixed in 4% formaldehyde, washed twice in PBS containing 0.1% saponin and 1% FCS (permeabilization buffer), and stained with 0.5 μ g of anti-perforin-FITC (Ansell, Bayport, MN), or 0.5 μ g of mouse IgG2b-FITC (DakoCytomation) as a control. Cells were washed, resuspended in PBS, and analyzed on a FACSCalibur as described above.

Detection of intracellular IFN- γ expression by NK cells

Paired samples of mononuclear cells from peripheral blood and inflammatory sites were incubated in culture medium alone or in culture medium supplemented with low concentrations of IL-12 (5–10 ng/ml) and IL-18 (10 ng/ml; Medical and Biological Laboratories, Nagoya, Japan) in 24-well plates for 18 h at 37°C in the presence of 5% CO₂. Brefeldin A (Sigma-Aldrich) was subsequently added at a final concentration of 5 μ g/ml. The cells were washed, stained with anti-CD56 and anti-CD3 mAbs as described above, fixed, permeabilized, and then stained with 0.5 μ g of anti-IFN- γ -allophycocyanin (BD Biosciences) or with 0.5 μ g of mouse IgG1-allophycocyanin (BD Biosciences) as a control. Cells were subsequently washed, resuspended in PBS, and analyzed on a FACSCalibur as described above.

Isolation of pure populations of NK cell and monocytes

Two approaches were taken to sorting populations of NK cells and monocytes. To obtain pure populations of the CD56^{dim} and CD56^{bright} NK cell subsets and monocytes from mononuclear cells separated from peripheral blood of healthy individuals, cells were stained with anti-CD56-PE (BD Biosciences), anti-CD3-allophycocyanin (BD Biosciences), and anti-CD14-FITC (DakoCytomation), and the populations of CD56^{dim} and CD56^{bright} NK cells and CD14⁺ monocytes were sorted using a FACSVantage SE flow cytometer (BD Biosciences). The purity of the sorted populations was confirmed using FACS analysis and was always >95%.

To obtain populations of NK cells and CD14⁺ monocytes from patient samples, an approach using magnetic beads was taken. NK cells were positively selected by MACS using a one-step positive selection with anti-CD56-coated magnetic microbeads (Miltenyi Biotec, Auburn, CA). CD3⁺ cells were then removed using anti-CD3 Dynabeads (DynaL Biotech, Oslo, Norway). In all experiments analyzed, NK cell purity was >90%, with <1% CD3⁺ cells. Purified CD14⁺ monocytes from patient samples were positively selected by MACS using a one-step positive selection with anti-

CD14-coated magnetic microbeads (Miltenyi Biotec). In all experiments the purity of CD14⁺ monocyte preparations was >90%.

Coculture of NK cells and monocytes

NK cells and monocytes were cocultured for 16 h at a 1:1 ratio in complete medium or in complete medium supplemented with IL-12, IL-15, and IL-18, all at a concentration of 10 ng/ml. Brefeldin A (Sigma-Aldrich) was subsequently added at a final concentration of 5 μ g/ml. After cell harvesting, NK cells were stained for surface expression of CD69 and CD56 and intracellular expression of IFN- γ . Monocytes were stained for surface expression of CD14-FITC (DakoCytomation) and intracellular expression of TNF- α using 1 μ g of anti-TNF- α -allophycocyanin (BD Biosciences). Mouse IgG1-allophycocyanin (BD Biosciences) was used as an isotype-matched control Ab. To investigate the contact dependence of the interaction, monocytes and NK cells were separated by a 0.4- μ m pore size membrane in Transwell plates (Costar, Corning NY) in some experiments. To investigate involvement of selected molecules, blocking experiments were performed by adding the following mAb: anti-IFN- γ (clone B27, IgG1; BD Biosciences), anti-HLA class I (clone W6/32, IgG2a), anti-CD94 (clone HP-3B1, IgG2a; Immunotech), and anti-NKG2D (clone 1D11, IgG1; BD Biosciences). Control experiments were performed using isotype-matched murine Abs (Sigma-Aldrich). All mAbs were used at a final concentration of 10 μ g/ml.

Statistical analysis

Data were analyzed using two-tailed Student's *t* tests. All results shown are representative of at least three separate experiments. Unless otherwise stated, values are expressed as the mean \pm SEM. To adjust for the significant variation between monocyte cytokine production at baseline (1–48%), changes in monocyte cytokine production were calculated as the fold difference, where this fold change was equal to the percentage of monocytes producing cytokine in a given condition divided by the percentage of monocytes producing cytokine at baseline.

Results

CD56^{bright} subset of NK cells is expanded at sites of inflammation

We investigated the frequency and phenotype of NK cells at sites of inflammation, including exudative pleural fluid from patients with infective, reactive, or malignant pulmonary disease; synovial fluid and synovial tissue from patients with inflammatory arthritis; and peritoneal fluid from patients with bacterial peritonitis.

Overall, we found that the frequency of NK cells at inflamed sites (15.1 \pm 1.8% of lymphocytes) was similar to that in peripheral blood (14.3 \pm 1.4% of lymphocytes; blood vs inflamed site, *p* = 0.7). The phenotypes of NK cells at the two sites, however, were strikingly different. Staining of NK cells within a paired sample of peripheral blood and pleural fluid taken from a patient with reactive pleuritis associated with Takayasu's disease is shown in Fig. 1A. The majority of NK cells within peripheral blood expressed intermediate levels of CD56 and had a phenotype consistent with previous reports of CD56^{dim} NK cells. In contrast, CD56^{bright} NK cells dominated in the pleural fluid. Very few of these cells expressed CD16 or the KIRs, whereas the majority expressed very high levels of CD94 and NKG2A. They were also more likely to express CCR7 and CD62L and much less likely to express perforin. A subset expressed CD69, suggesting activation. Extensive analysis of NK cells found in paired samples of peripheral blood vs pleural fluid from patients with other infective, reactive, or malignant types of pleural disease revealed similar results (Fig. 1B). CD56^{bright} NK cells were enriched in both malignant and nonmalignant pleuritis (malignant vs nonmalignant, *p* = 0.7). Detailed analysis confirmed that the phenotype of the CD56^{bright} NK cells in pleural fluid taken from patients with malignant disease was indistinguishable from that of the NK cells in pleural fluid from patients with infective or reactive pleural disease. Furthermore, analysis of paired samples of peripheral blood vs synovial fluid or synovial tissue from patients with inflammatory arthritis also revealed the dominance of the CD56^{bright} NK

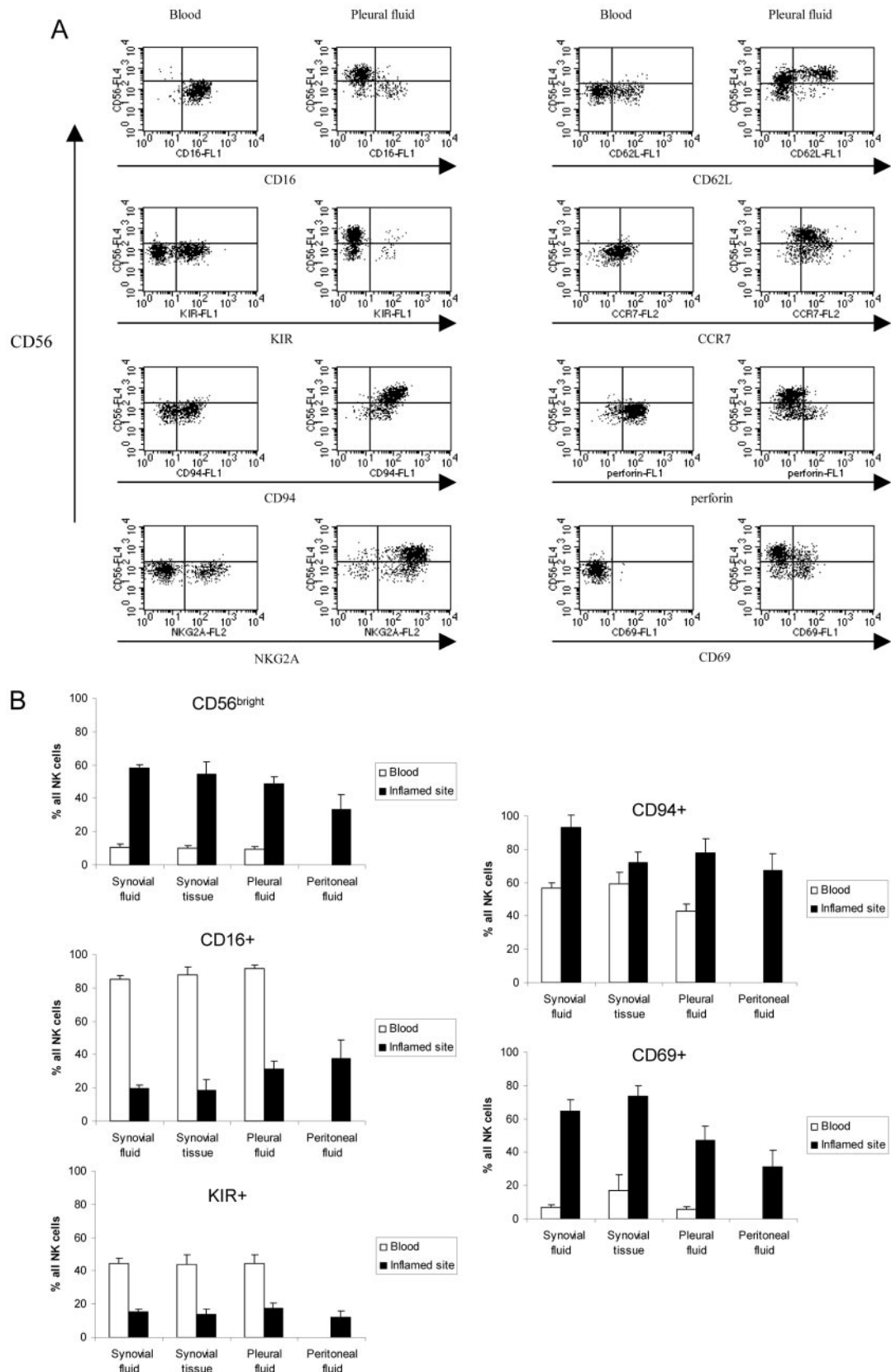


FIGURE 1. The CD56^{bright} subset of NK cells is expanded at sites of inflammation. *A*, Mononuclear cells obtained from paired blood and inflamed fluid/tissue were stained with mAb specific for selected cell surface and intracellular molecules and analyzed by flow cytometry. This is a representative example obtained from a patient with reactive pleuritis associated with Takayasu's disease. Gates have been set on the NK cell (CD56⁺, CD3⁻) population. *B*, Results of staining NK cells in all samples to detect expression of CD56, CD16, KIRs, CD94, and CD69 are summarized. Values are expressed as the mean (SEM) percentage of NK cells expressing each molecule. Synovial fluid, $n = 22$; synovial tissue, $n = 7$; pleural fluid, $n = 11$; peritoneal fluid, $n = 8$.

subset at the inflamed site (Fig. 1B). We examined the phenotype of NK cells in peritoneal fluid from patients with acute peritonitis, although we did not have access to peripheral blood from this group of patients. In four of eight samples we found that CD56^{bright} NK cells accounted for >50% of all NK cells in the peritoneal fluid (Fig. 1B). At all sites, a significant proportion of the NK cells showed evidence of activation, as assessed by the expression of CD69 (Fig. 1B).

NK cells from inflamed sites express more IFN- γ than peripheral NK cells

To further examine the properties of the NK cells found at inflamed sites, we studied the ability of these cells to produce proinflammatory cytokines, in particular IFN- γ . We cultured mononuclear cells obtained from paired samples of peripheral blood and inflamed synovial fluid, synovial tissue, or pleural fluid in medium, with or without IL-12 and IL-18, and measured intracellular IFN- γ expression. One example of an experiment performed on each of these three types of pairs is shown in Fig. 2. Very few peripheral blood NK cells produced IFN- γ , even when stimulated with low concentrations of monokines. In samples obtained from inflamed sites, there was variable expression of IFN- γ without stimulation. In one of the examples shown, almost 10% NK cells in synovial tissue expressed low levels of IFN- γ constitutively. After culture with low concentrations of monokines, a large proportion of NK cells from inflamed sites produced IFN- γ . The results shown are representative of a total of 19 experiments performed using cells from synovial fluid ($n = 7$), synovial tissue ($n = 5$), and pleural fluid ($n = 5$). Very similar results were obtained regardless of the anatomical site from which the NK cells were derived or the specific underlying disease.

NK cells, particularly the CD56^{bright} subset, stimulate monocyte TNF- α production

The observation that large numbers of activated CD56^{bright} NK cells, sensitive to monokines and with potent capacity to secrete IFN- γ , were present at inflammatory sites, stimulated us to investigate the possibility that these cells were capable of interacting with other mononuclear cells to promote the inflammatory process (11, 12). In initial experiments we sorted pure populations of monocytes and CD56^{bright} and CD56^{dim} NK cell subsets from the peripheral blood of healthy individuals. We then cultured the monocytes alone or with the different NK subsets, in either the absence or the presence of supplementary monokines (IL-12, IL-15, and IL-18). We reasoned that addition of monokines might be required in vitro to activate NK cells. The concentrations of monokines used were very low so as to more closely reflect an inflammatory reaction in vivo.

An example of the results is shown in Fig. 3A. In the absence of added monokines we did not observe a significant effect of NK cells on monocyte expression of TNF- α . Addition of the monokines to monocytes alone had no significant effect on TNF- α production. However, addition of NK cells in the presence of monokines led to a small increase in the number of monocytes that produced TNF- α . The effect of CD56^{bright} NK cells was greater than that of CD56^{dim} NK cells. Five additional experiments performed using cells from different healthy individuals gave similar results (Fig. 3B). Constitutive expression of TNF- α by monocytes was variable between donors; therefore, the results are expressed in terms of fold change in the number of monocytes expressing TNF- α to allow comparison between donors. Although the effect of NK cells on TNF- α expression by monocytes in these experiments was small, it was reproducible and statistically significant.

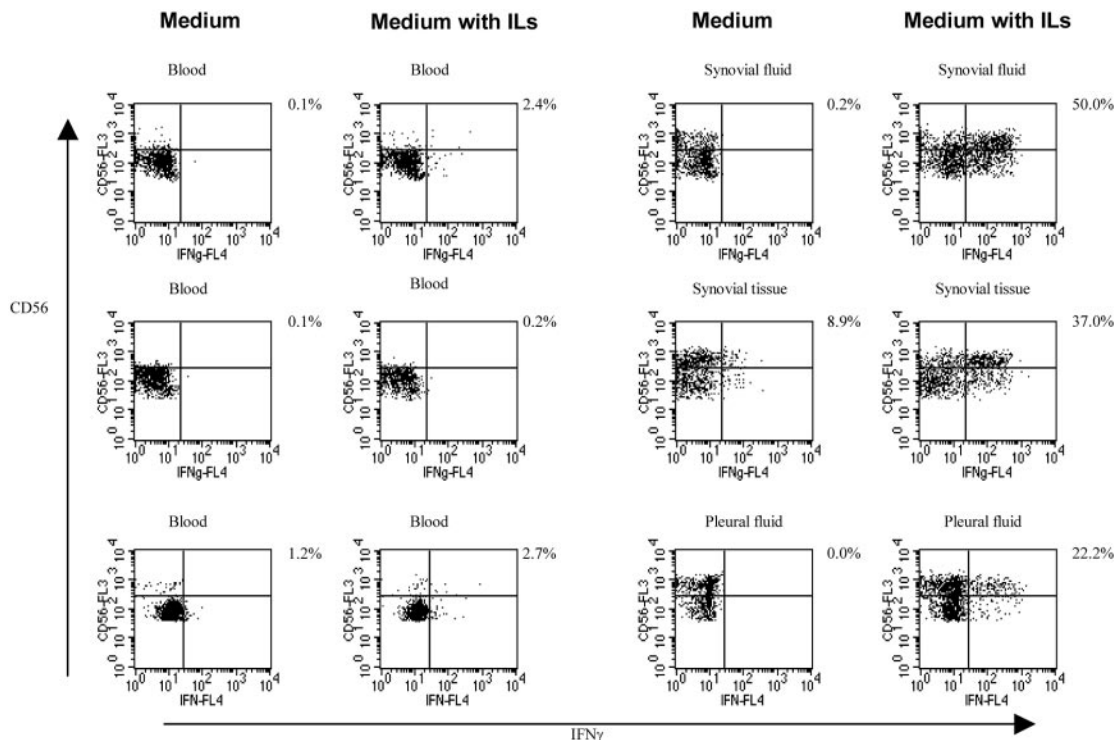


FIGURE 2. NK cells from inflamed sites express more IFN- γ than peripheral NK cells. Mononuclear cells were obtained from paired samples of blood and inflamed synovial fluid, blood and synovial tissue, or blood and pleural fluid from three different donors. These cells were cultured overnight in complete medium and brefeldin A in the absence or the presence of IL-12 and IL-18 (designated ILs). After surface staining for CD3 and CD56, cells were fixed, permeabilized, stained for intracellular IFN- γ , and analyzed by flow cytometry. Cells in the NK cell gate (CD56⁺, CD3⁻) are shown. These representative plots show that after stimulation, NK cells from sites of inflammation have a greater capacity to produce IFN- γ .

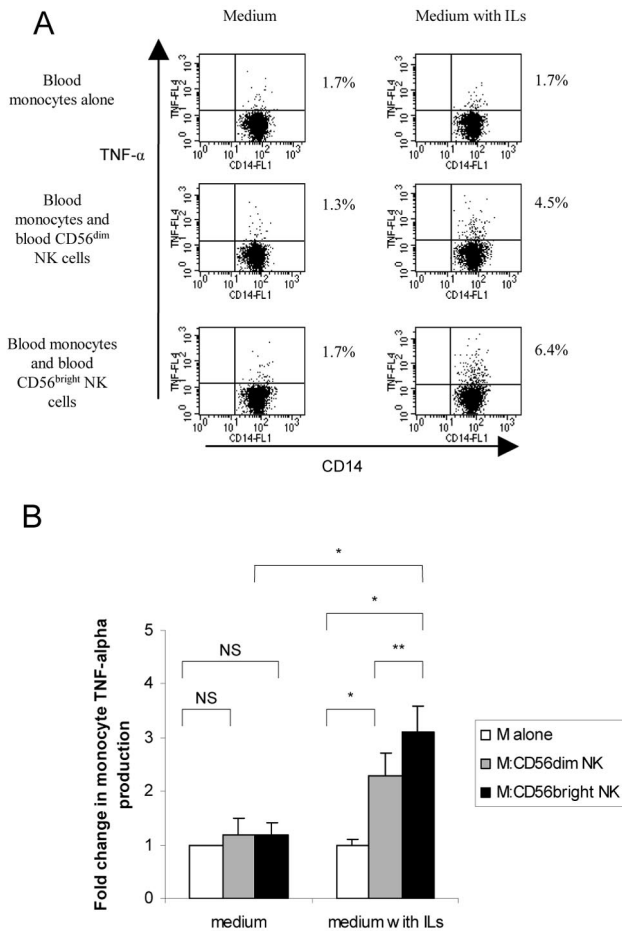


FIGURE 3. NK cells, particularly the CD56^{bright} subset, stimulate monocyte TNF- α production. CD56^{bright} and CD56^{dim} NK cell subsets and CD14⁺ monocytes (M) were isolated from healthy donor blood by FACS sorting and cocultured at a 1:1 ratio in complete medium in the absence or the presence of IL-12, IL-15, and IL-18 (designated ILs). CD14⁺ monocytes were then analyzed for the expression of intracellular TNF- α . **A**, Representative experiment showing that addition of NK cells, particularly CD56^{bright} NK cells, leads to increased monocyte TNF- α production when monokines are present. Values indicate the percentage of CD14⁺ cells producing TNF- α . **B**, Summary of six separate experiments performed in medium alone or medium supplemented with monokines. Values indicate the mean (SEM) fold change in monocyte TNF- α from baseline. *, $p < 0.05$; **, $p < 0.01$.

The effect of CD56^{bright} NK cells was greater than that of the CD56^{dim} NK cells in this respect ($p < 0.01$).

NK cells from inflamed sites stimulate monocyte TNF- α production

We then proceeded to perform similar experiments, using samples taken from our patients. To this end we sorted populations of monocytes and NK cells from paired samples of peripheral blood and inflammatory fluid and investigated the capacity of the NK populations to modulate the expression of TNF- α by the monocytes. Fig. 4A shows the results of an experiment performed using cells taken from a patient with rheumatoid arthritis. Approximately 30% of monocytes in peripheral blood expressed TNF- α constitutively, and this proportion did not increase when the cells were cultured in the presence of the monokines IL-12, IL-15, and IL-18. Addition of peripheral blood NK cells to the monocytes led to an increase in the expression of TNF- α by the monocytes, particularly where the culture medium was supplemented with monokines. Ad-

dition of synovial fluid NK cells to the monocytes led to an even greater increase in TNF- α expression. Parallel experiments, in which monocytes from synovial fluid were cultured, without or with monokines in the absence or the presence of NK cells from peripheral blood or synovial fluid revealed very similar results; the synovial fluid monocytes were as susceptible to the effect of NK cells as were peripheral blood monocytes (data not shown). These results are representative of a total of five experiments performed using three paired samples of peripheral blood and synovial fluid and two paired samples of peripheral blood and pleural fluid. A summary of all results is shown in Fig. 4B. Overall, the effect of adding peripheral NK cells to monocytes in the absence of supplementary monokines was very modest. However, the effect was consistently enhanced by monokines and by the use of NK cells taken from inflammatory fluid (effect of fluid NK cells vs blood NK cells, $p < 0.01$). To study whether IFN- γ does play a role in monocyte activation by NK cells in our system, we investigated the effect of addition of anti-IFN- γ Ab to the monocyte-NK cell cocultures in five additional experiments, all performed using cells isolated from an inflammatory site (Fig. 4C). Although this maneuver did decrease the capacity of NK cells to promote monocyte TNF- α , the effect was only partial and did not reach statistical significance. In seven additional experiments, again all performed using cells isolated from an inflammatory site, we investigated the effect of separating the fluid monocyte and NK cell populations using a Transwell (Fig. 4D). Preventing cell contact between NK cells and monocytes clearly diminished the capacity of NK cells to promote monocyte TNF- α , and the effect was statistically significant ($p < 0.05$). Thus, the experiments show that NK cells from inflamed sites are able to activate monocytes and that the full extent of activation is dependent on contact between NK cells and monocytes.

Monocytes synergize with cytokines to promote IFN- γ expression by CD56^{bright} NK cells

We were interested in the possibility that monocytes were able to reciprocally influence the function of NK cells. Again, preliminary experiments were performed using pure populations of monocytes and CD56^{dim} and CD56^{bright} NK cells separated from the peripheral blood of normal individuals. The majority of these peripheral NK cells did not express CD69. CD14⁺ cells were added to autologous CD56^{dim} or CD56^{bright} NK cells in the absence or the presence of IL-12, IL-15, and IL-18, and the effect on IFN- γ expression by the NK cells was assessed. A representative example of results is shown in Fig. 5A. As previously reported, in the presence of monokines only a small proportion of CD56^{dim} NK cells expressed IFN- γ , whereas a larger proportion of CD56^{bright} NK cells expressed this cytokine. Addition of monocytes led to a small increase in the number of CD56^{dim} NK cells expressing IFN- γ and a more marked increase in the number of CD56^{bright} NK cells expressing this cytokine. No effect was seen when the experiments were performed in the absence of added cytokines (data not shown). Similar results were obtained from experiments performed using cells separated from blood taken from four further healthy individuals (Fig. 5B). The effect of monocytes in promoting IFN- γ expression by CD56^{dim} NK cells did not reach significance ($p = 0.07$), whereas the effect of monocytes on CD56^{bright} NK cells clearly did ($p < 0.001$).

Monocytes synergize with cytokines to promote IFN- γ expression by NK cells at sites of inflammation

To determine the possible relevance of these findings to the inflammatory processes occurring in our patients, we isolated NK

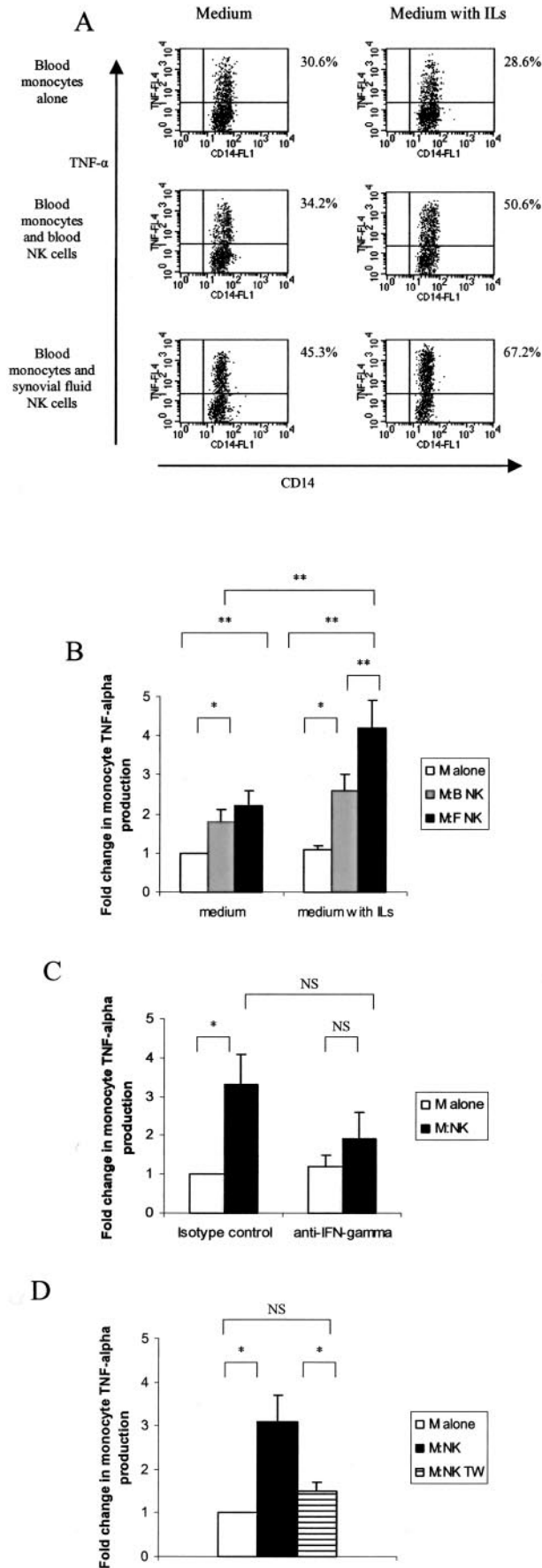


FIGURE 4. NK cells from inflamed sites amplify monocyte TNF- α production. CD14⁺ monocytes (M) and NK cells were isolated from paired blood (B) and inflamed fluid (F) samples and cocultured at a 1:1 ratio in

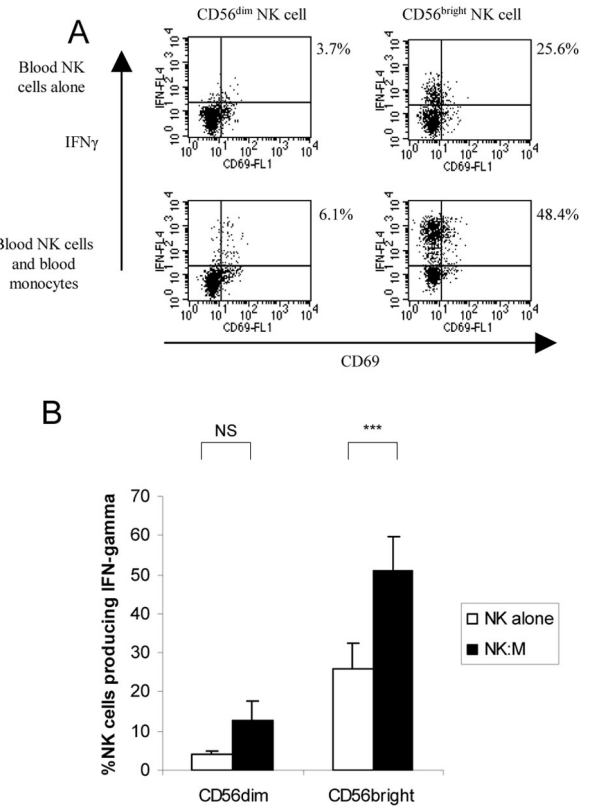


FIGURE 5. Monocytes synergize with cytokines to promote IFN- γ expression by CD56^{bright} NK cells. NK cell subsets and CD14⁺ monocytes (M) were isolated from healthy donor blood by FACS sorting and cocultured at a 1:1 ratio in complete medium in the presence of IL-12, IL-15, and IL-18. NK cells (CD56⁺CD3⁻) were then analyzed for IFN- γ production and CD69 expression by flow cytometry. **A**, Representative experiment showing that addition of CD14⁺ cells to NK cells leads to increased IFN- γ production. Values indicate the percentage of NK cells producing IFN- γ . **B**, Summary of experiments from five donors showing the effect of monocytes on NK cell IFN- γ production. Values indicate the mean (SEM) percentage of NK cells producing IFN- γ . ***, $p < 0.001$.

cells and monocytes from paired samples of blood and inflammatory fluid. A representative experiment in which cells were separated from the blood and synovial fluid of a patient with rheumatoid arthritis is shown in Fig. 6A. In the presence of supplementary monokines, addition of monocytes to NK cells led to an increase in NK cell expression of IFN- γ . In the absence of monokines, the effect of adding monocytes to NK cells was minimal (data not shown). Four additional experiments were performed and gave similar results; monocytes were able to synergize with monokines

complete medium in the absence or the presence of IL-12, IL-15, and IL-18 (designated ILs). Monocytes were then analyzed for intracellular TNF- α expression. **A**, Representative experiment from a patient with rheumatoid arthritis showing that addition of NK cells, particularly those from synovial fluid, leads to increased monocyte TNF- α production. Values indicate the percentage of CD14⁺ cells producing TNF- α . **B**, Summary of five separate experiments (synovial fluid, $n = 3$; pleural fluid, $n = 2$) performed in medium alone or medium supplemented with the monokines. Values indicate the mean (SEM) fold change in monocyte TNF- α from baseline. **C**, In five additional experiments the effect of IFN- γ blockade on the capacity of fluid NK cells, cultured in the presence of ILs, to activate monocytes was examined. **D**, In seven additional experiments cell:cell contact between fluid NK cells, cultured in the presence of ILs, and monocytes was prevented by a Transwell (TW) plate. *, $p < 0.05$; **, $p < 0.01$.

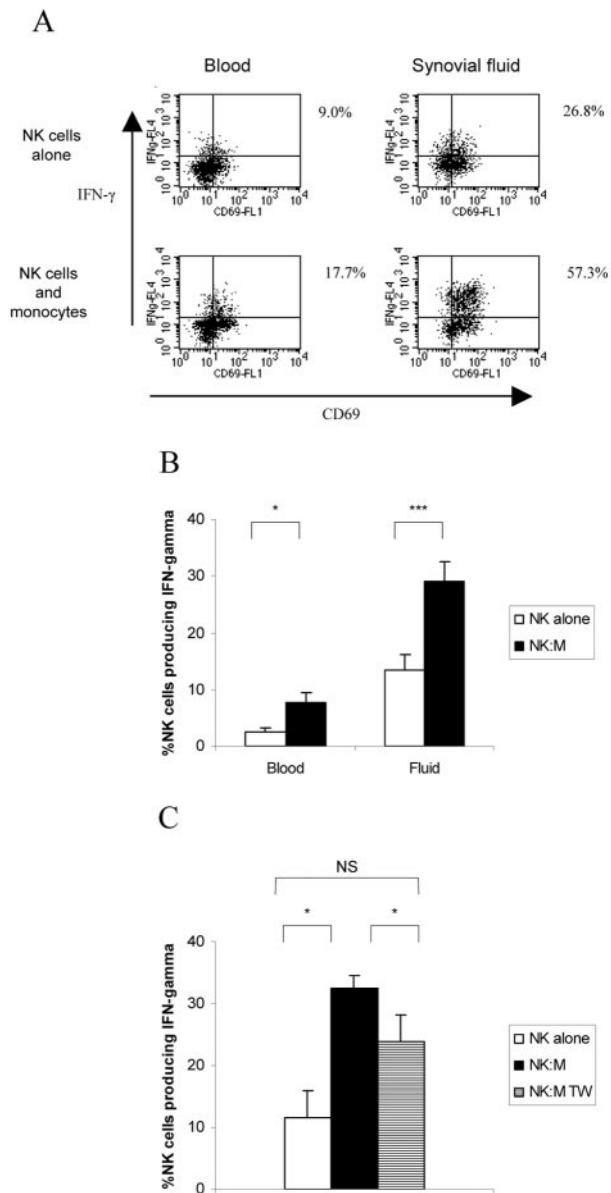


FIGURE 6. Monocytes synergize with cytokines to promote IFN- γ expression by NK cells at sites of inflammation. CD14⁺ monocytes (M) and NK cells were isolated from paired blood and inflamed fluid samples (synovial fluid, $n = 3$; pleural fluid, $n = 2$). NK cells were stimulated with IL-12, IL-15, and IL-18 and were cultured in the absence or the presence of CD14⁺ cells. In some experiments cell:cell contact between stimulated NK cells and monocytes was prevented by a Transwell (TW) plate. NK cells (CD56⁺, CD3⁻) were then analyzed for IFN- γ production and CD69 expression by flow cytometry. **A**, Representative experiment showing that addition of CD14⁺ cells to NK cells leads to increased IFN- γ production. Values indicate the percentage of NK cells producing IFN- γ . **B**, Summary of results from five experiments showing the effect of monocytes on NK cell IFN- γ production. **C**, Summary of results from three separate donors showing the effect of cell:cell separation by a Transwell (TW) plate on inflammatory fluid NK cell IFN- γ production. Values indicate the mean (SEM) percentage of NK cells producing IFN- γ . *, $p < 0.05$; ***, $p < 0.001$.

in promoting IFN- γ expression by NK cells, particularly by those NK cells found within inflammatory fluid (Fig. 6B). CD14⁺ cells from both blood and inflammatory fluid had a similar synergistic effect on NK cells (data not shown). We investigated the effect of preventing contact between NK cells and CD14⁺ cells isolated

from inflammatory fluid using a Transwell plate in three experiments and found that this significantly reduced the effect of the monocytes on the NK cells (Fig. 6C).

Monocyte cell surface molecules may engage with a range of receptors and coreceptors on NK cells to modulate NK cell cytokine expression. We have found NKG2D to be highly expressed on CD56^{bright} NK cells (our unpublished observations). To examine the possible involvement of NKG2D and other members of the NKG2 family of receptors in the interaction between monocytes and NK cells, we performed experiments in which we cultured synovial fluid NK cells with monokines in the absence or the presence of monocytes without or with Abs specific for NKG2D, HLA class I, or CD94. Anti-NKG2D did not prevent monocytes from synergizing with monokines to promote IFN- γ expression by NK cells (data not shown). In the presence of either W6/32 or anti-CD94, IFN- γ production by NK cells cultured alone rose, raising the possibility that NK cells inhibit each other via recognition of HLA-E and possibly other HLA class I molecules. Under these conditions, we were unable to consistently demonstrate a further increase in IFN- γ production by NK cells by addition of monocytes (data not shown).

Discussion

These experiments show that a subset of NK cells is enriched at sites of inflammation. These cells have the phenotypic characteristics of CD56^{bright} NK cells; many express CD69, suggesting activation, and they produce IFN- γ after stimulation with low concentrations of monokines. These NK cells are found consistently in exudative pleural fluid, inflamed synovial fluid, and synovial tissue and are also present in inflamed peritoneal fluid, all regardless of disease etiology. Within synovial tissue we found that NK cells are preferentially located in the lymphocytic infiltrates in the sublining layer of rheumatoid synovial tissue (our unpublished observations).

Accumulation of these NK cells at sites of inflammation may reflect preferential recruitment, local differentiation, local proliferation, or selective survival. The observation that CD56^{bright} NK cells and CD56^{dim} NK cells differ in their pattern of expression of chemokine receptors and adhesion molecules is consistent with the idea that the two subsets of cells home to different sites (5). CD56^{bright} NK cells express CCR5, and this may influence their capacity to home to inflammatory sites where relatively high concentrations of MIP-1 α , MIP-1 β , and RANTES may be present (13).

A recent publication suggests that CD56^{dim} NK cells may be stimulated to differentiate into CD56^{bright} NK cells by cytokines (9), and this lends some support to the second idea; local differentiation of CD56^{dim} NK cells to CD56^{bright} NK cells at sites of inflammation may occur. We found CD69 expressed on many of the NK cells at sites of inflammation, supporting the idea that they have been activated locally. We have sorted pure populations of CD16⁺/CD56^{dim} NK cells and have cultured them in monokines including IL-12 or in inflammatory fluid. This resulted in increased CD56, CD94, and CD69 expression, but did not otherwise reproduce the phenotype of the CD56^{bright} NK cells (our unpublished observations). Additional studies are required to resolve the uncertainties surrounding the developmental relationship between CD56^{dim} and CD56^{bright} NK cells. Microarray analysis of gene expression in different NK cell subsets has shown few differences between the two NK cell subsets in peripheral blood, suggesting that they are closely related in ontogeny (14).

We were interested in determining the role that the CD56^{bright} NK cells might play at sites of inflammation and investigated the

possibility that CD56^{bright} NK cells interact with other mononuclear cells to promote inflammation. The experiments showed that NK cells were capable of engaging with monocytes in a reciprocal activatory fashion.

The activatory effect of monocytes on CD56^{bright} NK cells was clear and consistent when cells were isolated from peripheral blood of healthy donors and from patients with inflammatory disease. CD56^{bright} NK cells at inflammatory sites are often activated and express CD69. Similarly, CD14⁺ mononuclear cells localized to inflammatory sites will differentiate and show some phenotypic and functional differences from their circulating counterparts. Experiments to further investigate the interaction between CD14⁺ monocytes and NK cells were therefore performed using cells from inflammatory sites. In this way we were able to show that contact between monocytes and NK cells was required to induce the maximal effect. Many different ligand:receptor interactions might allow monocytes to stimulate CD56^{bright} NK cells. Activatory signals might be delivered via members of the C-type lectin family of receptors, specifically NKG2D or CD94/NKG2C. Stimulation of NK cells by monocytes via NCRs is also plausible (9, 15, 16). Costimulation via a range of receptors, including 2B4 and CD27, may influence monocyte:NK cell interactions. The KIRs and CD16 are not highly expressed on CD56^{bright} NK cells; therefore, these molecules are less likely candidates. We performed preliminary experiments to investigate the possible importance of the NKG2 family of receptors in the interaction. We found high levels of expression of NKG2D on CD56^{bright} NK cells, but in experiments using Abs to block NKG2D, were unable to confirm a role for this molecule in the interaction we demonstrated *in vitro*, probably reflecting the observation that *ex vivo* monocytes do not express the relevant ligands (17, 18). Interpretation of experiments performed using Abs specific for CD94 or HLA class I molecules was complicated by the fact that the Abs themselves stimulated isolated NK cells to express IFN- γ . Further investigation of the molecular basis for the contact-dependent effect of monocytes on NK cells is required. We note that the use of Transwells did not usually completely abrogate the effect of the monocytes, and we do not exclude the possibility that soluble factors also contribute to the effect.

The reciprocal, activatory effect of NK cells on monocytes was more subtle, particularly when experiments were performed with cells isolated from peripheral blood of healthy individuals. Nevertheless, we found that after culture with peripheral NK cells, particularly CD56^{bright} NK cells, a small proportion of peripheral monocytes expressed TNF- α . The effect was usually more obvious when experiments were performed using NK cells from sites of inflammation, although there was variability between donors. One should note that in our protocol, brefeldin A was added to the cultures after the first 4 h to facilitate intracellular staining. This would limit the amount of IFN- γ released into the medium and makes it unlikely that the effect we demonstrated was mediated via IFN- γ alone. Consistent with this, the addition of an anti-IFN- γ Ab to the cells only partially and variably reduced the effect of the NK cells on the monocytes, and the result did not reach statistical significance. In contrast, we were able to demonstrate that contact between NK cells and monocytes was required to achieve the full effect of NK cells on monocytes. Additional experiments are required to identify the ligand and receptors involved in this interaction.

This interaction may be particularly relevant when considering the pathogenesis of chronic inflammatory arthritis. In many of these conditions, and in rheumatoid arthritis in particular, production of TNF- α by cells of the monocyte lineage plays a pivotal role in promoting inflammation (19, 20). Uncertainty still exists as to

the mechanisms by which cells of the monocyte lineage are stimulated to produce TNF- α in these diseases. Cytokine-stimulated lymphocyte preparations have been shown to activate monocytes in a contact-dependent manner, and T cells have been the focus of additional investigation of this interaction (21–23). However, in chronic inflammatory disorders such as rheumatoid arthritis, T cells are hyporesponsive, with depressed responses to both recall and mitogenic stimuli, poor helper functions, and reduced autologous mixed lymphocyte responses (24). The data we present in this study suggest that NK cells are likely to account for some of the previously reported effects of activated lymphocytes on monocytes.

Our results have some similarities with the recently described interaction between NK cells and dendritic cells. NK cells stimulate TNF- α and IL-12 secretion by dendritic cells and promote dendritic cell maturation, thereby supporting the transition from innate to adaptive immunity (15, 25, 26). These effects are partly dependent on cell:cell contact and IFN- γ production (25). The differential effect of CD56^{bright} NK vs CD56^{dim} NK cells on dendritic cells has yet to be described.

Overall, this work shows that CD56^{bright} NK cells accumulate in inflammatory lesions and are capable of engaging in a reciprocal, contact-dependent, activatory interaction with monocytes. We suggest that this interaction is important in the maintenance of inflammation in chronic inflammatory diseases such as rheumatoid arthritis.

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