Endogenous Production of Interleukin 15 by Activated Human Monocytes Is Critical for Optimal Production of Interferon-γ by Natural Killer Cells In Vitro

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Abstract

Natural killer (NK) cells are large granular lymphocytes that constitutively express functional IL-2 receptors. We have shown that recombinant human IL-15 uses the IL-2 receptor to activate human NK cells and can synergize with recombinant human IL-12 to stimulate NK cell production of IFN-γ in vitro. IFN-γ production by NK cells is critical in the prevention of overwhelming infection by obligate intracellular microbial pathogens in several experimental animal models. Herein, we demonstrate that human monocytes produce IL-15 protein within 5 h of activation with LPS. Using an IL-15-neutralizing antiserum in a coculture of LPS-activated monocytes and NK cells, we demonstrate that monocyte-derived IL-15 is critical for optimal NK cell production of IFN-γ. Endogenous IL-15 activates NK cells through the IL-2 receptor, and with endogenous IL-12, regulates NK cell IFN-γ production after monocyte activation by LPS. These in vitro studies are the first to characterize a function for endogenous IL-15, and as such, suggest an important role for IL-15 during the innate immune response. IL-15 may be an important ligand for the NK cell IL-2 receptor in vivo. (*J. Clin. Invest.* 1995. 96:2578–2582.)

Key words: interleukin-15 • interleukin-12 • interferon-γ • natural killer cell • monocyte

Introduction

Natural killer (NK)[1] cells are large granular lymphocytes that are capable of IFN-γ production in response to infection. This effector function does not require prior sensitization, suggesting that NK cells may play an important role in the early innate immune response to microbial invasion (1, 2). Indeed, it has been shown in a number of in vitro and in vivo experimental systems that NK cell production of IFN-γ is critical for the control of several intracellular pathogens. This phenomenon has been best examined by studying monocytes infected with *Listeria monocytogenes, Toxoplasma gondii,* and *Leishmania major* (reviewed in reference 3).

In the case of infection with *Listeria monocytogenes* or *Toxoplasma gondii,* the production of both TNF-α and IL-12 by infected monocytes has been shown to be critical in the induction of IFN-γ secretion by NK cells (4–6). This response was found to be markedly enhanced in the presence of exogenous IL-2, and at times was inefficient in the absence of IL-2 (4, 7). While NK cells constitutively express a functional IL-2 receptor (IL-2R) (8, 9), neither monocytes nor NK cells make IL-2, which suggests that either IL-2 produced by antigen-activated T cells or other cytokines may also be important for the production of IFN-γ by NK cells in vivo (10, 11). Recently, Grabstein et al. have cloned IL-15 (12), which, while lacking any sequence homology to IL-2, can activate human NK cells via components of the IL-2R and can combine with recombinant human (rh) IL-12 to significantly potentiate NK cell production of IFN-γ in vitro (13). Further, monocytes produce abundant transcript for IL-15 upon activation with LPS (12), an event that occurs within the same time frame as monocyte secretion of IL-12 (14).

In the present report, we show that activated human monocytes produce IL-15 at the protein level, and we demonstrate that endogenous production of IL-15 by LPS-activated monocytes is critical for optimal human NK cell production of IFN-γ in an in vitro coculture system.

Methods

Reagents and antibodies. Purified LPS from *Escherichia coli* strain 0127:B8 was purchased from Sigma Chemical Co. (St. Louis, MO). Rabbit anti-human (hu) IL-15 antiserum and rabbit anti-huIL-2 antiserum were obtained from Pepro Tech, Inc. (Rocky Hill, NJ) and were produced after serial immunization of rabbits with purified rhIL-15 and rhIL-2, respectively. A rabbit anti-murine IL-3 antiserum (Pepro Tech, Inc.) was used as a nonreactive control. All three antisera were purified by ammonium sulfate precipitation followed by ion exchange chromatography and were used at a concentration of 5 μg/ml unless otherwise specified. The anti-huIL-15 antiserum was tested for specificity in a $CD56^{bright}$ NK cell proliferation assay and $CD56^{+}$ NK cell IFN-γ production assay (13), and was found to neutralize the activity of rhIL-15 but did show any cross-reactivity with rhIL-2, rhIL-1β, rhTNF-α or rhIL-12. At a concentration of 5 μg/ml, anti-huIL-15 antiserum neutralized ~ 75% of the bioactivity induced by 10 ng/ml rhIL-15 in $CD56^{bright}$ NK cells. The anti-huIL-2 antiserum did not cross-react with rhIL-15, and the anti-murine IL-3 antiserum did not cross-react with rhIL-2 or rhIL-15. The anti-huIL-15 mAbs (IgG_1), M110 and M111 (Immunex Corp., Seattle WA), neutralized the activity of IL-15 but not IL-2 or IL-4 in a cytotoxic T lymphocyte line 2 cell proliferation assay (12, 15, 16). Neutralizing rabbit anti-huIL-12 antiserum was kindly provided by Dr. Stanley Wolf (Genetics Institute, Andover, MA) and was used at a concentration of 5 μg/ml. TU27 is an anti-huIL-2Rβ mAb which was kindly provided as sterile mouse ascites by Dr. Kazuo Sugamura

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1. *Abbreviations used in this paper:* HAB, human AB; hu, human; NK, natural killer; R, receptor; rh, recombinant human.

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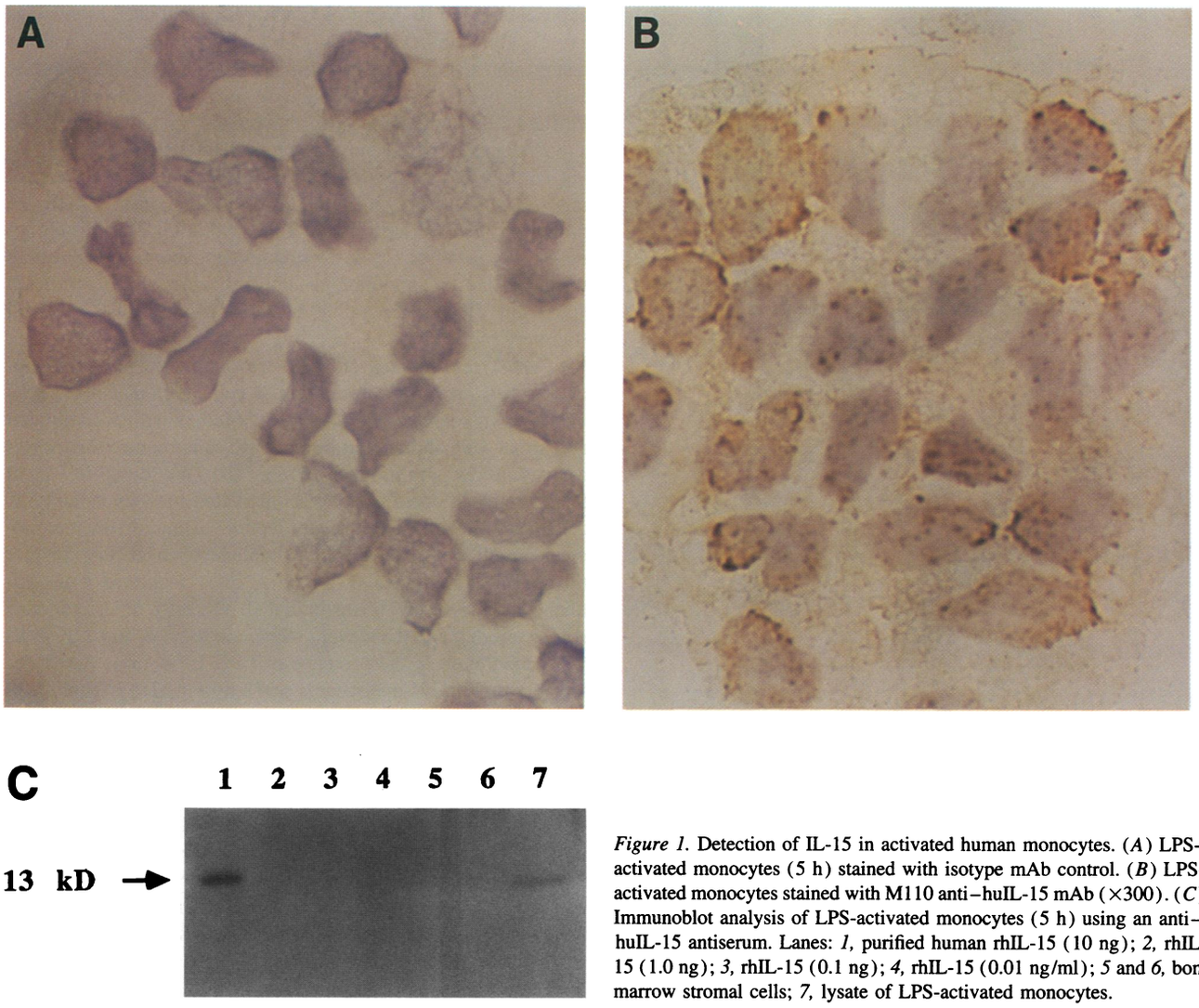

Figure 1. Detection of IL-15 in activated human monocytes. (*A*) LPS-activated monocytes (5 h) stained with isotype mAb control. (*B*) LPS-activated monocytes stained with M110 anti-huIL-15 mAb (×300). (*C*). Immunoblot analysis of LPS-activated monocytes (5 h) using an anti-huIL-15 antiserum. Lanes: *1,* purified human rhIL-15 (10 ng); *2,* rhIL-15 (1.0 ng); *3,* rhIL-15 (0.1 ng); *4,* rhIL-15 (0.01 ng/ml); *5* and *6,* bone marrow stromal cells; *7,* lysate of LPS-activated monocytes.

(Ajinomoto Co., Kawasaki, Japan) (17). Reagents used for the immunomagnetic depletion of PBMC have been described (18).

Isolation of human NK cells and monocytes. $CD56^+CD3^-$ human NK cells were purified from fresh leukopacs (American Red Cross, Buffalo, NY) by cell sorting (≥ 97% purity) as previously described (18). Human monocytes from the same donors were isolated after a 2-h plastic adherence as described by D'Andrea et al. (19). To remove the adhered monocytes, the plastic plate was repeatedly sprayed with a 25-gauge needle attached to a 12-cm^3 syringe containing cold RPMI 1640 medium. Cells were always found to be > 95% $CD14^+$ by flow cytometry.

Detection of IL-15 protein by immunohistochemistry. For immunohistochemical and Western blot analysis, purified human monocytes were incubated for 5 h in the presence or absence of LPS (5 μg/ml). Cells for staining were resuspended in medium containing 10% human AB (HAB) serum and 50% human albumin, cytocentrifuged onto silane-coated slides (Oncor Inc., Gaithersburg, MD), and placed into fixative (2% paraformaldehyde, 0.1 M lysine, 0.1 M Na_2PO_4, 10 mM $NaIO_4$) for 15 min at 4°C. Cytospin preparations were next treated with blocking solutions as described (20), followed by incubation with anti-huIL-15 mAb (M110) or an isotype control mAb (Sigma). Bound primary mAbs were then detected using a biotinylated goat anti-mouse IgG avidin-horseradish peroxidase complex and diamino benzidine substrate, followed by counterstaining and mounting as described (20).

Detection of IL-15 protein by Western blot and ELISA. Lysates from human cells were washed, lysed, quantitated, equally loaded (100 μg/lane), separated by SDS-PAGE, and transferred to nitrocellulose as previously described (20). Purified rhIL-15 (Pepro Tech, Inc.) was used as a positive control. Preblocked nitrocellulose sheets were first incubated with 1 μg equivalent rabbit anti-huIL-15 antiserum (Pepro Tech, Inc.) in TTBS (0.9% NaCl, 100 mM Tris base, 0.1% Tris base, 0.1% Tween 20, 1% BSA, pH 7.5) for 1 h at room temperature, and then biotin-conjugated secondary antibody for 20–30 min at room temperature, followed by an avidin-horseradish peroxidase complex (Vector Laboratories, Inc., Burlingame, CA). IL-15 protein was detected by using detection reagent for 1 min (ECL; Amersham Corp., Arlington Height, IL). The blot was then drained of excess detection reagent and exposed to x-ray film for 30 s. The ELISA for huIL-15 was performed as previously described (20), except that the anti-huIL-15 mAb (M111) was used as a capture antibody and rabbit anti-huIL-15 antiserum was used as the secondary detection antibody. Standards for the ELISA were prepared by serial dilution of rhIL-15 in RPMI 1640 supplemented with 10% HAB serum. Sensitivity of the huIL-15 ELISA is 10 pg/ml. The ELISA for huIL-12 (R & D Systems, Inc., Minneapolis, MN) has a sensitivity of 5 pg/ml and was used according to the manufacturer's instructions.

Coculture of human NK cells and monocytes and measurement of IFN-γ production. After isolation, 10^5 NK cells and/or 10^5 monocytes were plated in 96-well U-bottomed plates in 200 μl of medium containing 10% HAB. 5 μg/ml LPS or an equal volume of PBS was added

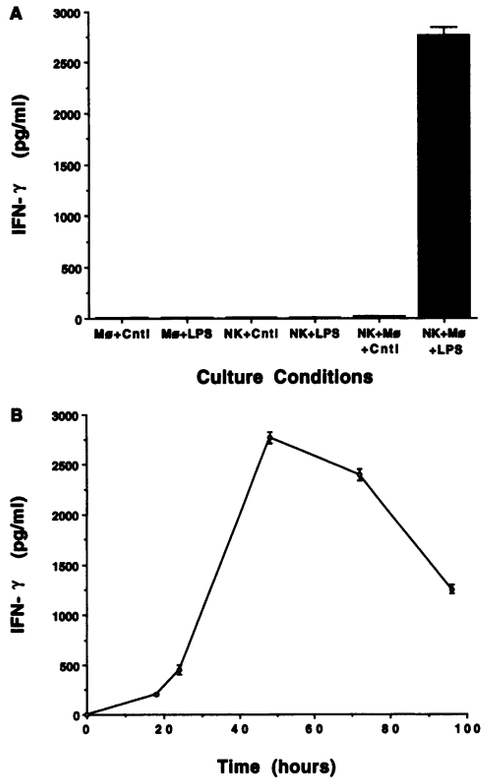

Figure 2. LPS stimulation induces IFN-γ production in cocultures of human monocytes and NK cells. (*A*) Monocytes (*Mφ*) and/or NK cells were isolated from the fresh blood of normal donors, purified, and cultured at a density of 10^5 cells/well in 200 μl of 10% HAB, in the absence or presence of LPS (5 μg/ml). After 72 h of culture, supernatants from each culture condition were harvested and analyzed for IFN-γ protein production by ELISA. *Cntl,* control. (*B*) Time course of IFN-γ protein production by LPS-activated cocultures of monocytes and NK cells. Culture supernatants were harvested at the time intervals indicated on the x-axis, frozen at −70°C, and subsequently assayed simultaneously for IFN-γ protein by ELISA. All results represent the mean of duplicate wells ±SEM and are representative of three sets of experiments with similar results.

to cocultures immediately after plating. Cells were incubated at 37°C in a CO_2 incubator for 72 h, at which time supernatants were harvested and assayed by ELISA for the presence of human IFN-γ (GIBCO BRL, Gaithersburg, MD). Results represent the mean of duplicate wells ±SEM. In one series of experiments, culture supernatants were harvested at sequential intervals over a period of 96 h. In cytokine neutralization studies, antisera or mAbs were added to cocultures at the concentrations indicated above 1 h before the addition of LPS. In one set of experiments, supernatants from cocultures of LPS-activated monocytes and NK cells were collected after 12, 24, and 48 h of culture and assayed by ELISA for their content of IL-12 and IL-15.

Statistical analysis. Data are expressed as a mean±SEM for the indicated number of experiments. Differences between paired experimental data were assessed with Student's *t* test. Statistical testing was performed using STATview (Brain Power Inc., Calabasas, CA) on a Macintosh Quadra 650 computer with $P < 0.05$ defined as a significant difference.

Results

Endogenous production of IL-15 protein by activated human monocytes. Grabstein et al. have shown that fresh human PBMC express minimal IL-15 mRNA, while LPS-activated monocytes express abundant levels of IL-15 transcript. Importantly, neither resting nor activated $CD2^+$ lymphocytes (i.e., T cells and NK cells) showed evidence of significant IL-15 transcript (12). Purified LPS-activated human monocytes were therefore fixed and evaluated for IL-15 protein by immunohistochemical staining with an anti-huIL-15 mAb (M110) or an isotype control mAb. Results shown in Fig. 1, *A* and *B,* demonstrate huIL-15 protein production after 5 h of LPS activation. Resting monocytes were negative for IL-15 protein (not shown). The results were verified by immunoblot analysis using an anti-huIL-15 antiserum distinct from that used for immunohistochemical staining (Fig. 1 *C*).

Coculture of human NK cells with activated human monocytes results in IFN-γ production. T cells and NK cells are the only known sources of IFN-γ (21). Studies with recombinant cytokines have shown that human NK cells require monocyte-derived products to produce IFN-γ (13). We therefore sought to determine if coculture of LPS-activated monocytes and NK cells would result in IFN-γ production. Alone, neither monocytes nor NK cells produced detectable levels of IFN-γ in either the presence or absence of LPS (Fig. 2 *A*). Coculture of unstimulated monocytes with unstimulated NK cells did not induce IFN-γ production. However, when monocytes and NK cells were cocultured in the presence of LPS, IFN-γ was easily detectable in culture supernatants at 72 h (Fig. 2 *A*). Thus, LPS activation of human monocytes induces IFN-γ production in NK cells.

We next measured the kinetics of IFN-γ production during coculture of LPS-activated monocytes and NK cells. IFN-γ was detected in culture supernatants 18 h after the addition of LPS, peaked in concentration at 48 h, and underwent a significant decline by 96 h (Fig. 2 *B*). A fourfold increase in the number of monocytes per well in these cocultures resulted in a 300% increase in IFN-γ protein production (not shown). The actual production of IFN-γ in cocultures of LPS-activated monocytes and NK cells varied approximately fivefold between different donors.

Endogenous IL-15 production by human monocytes is critical for optimal NK cell IFN-γ production. The kinetics and quantity of IL-15 production in this coculture system were determined by ELISA and compared to IL-12 protein production in the same culture. As can be seen in Fig. 3 *A,* significant levels of IL-15 and IL-12 are produced within 24 h of LPS activation. We next investigated whether endogenous IL-15 production by LPS-activated human monocytes was contributing to IFN-γ secretion by human NK cells. Cocultures of LPS-activated monocytes and NK cells were treated with neutralizing anti-huIL-15 antiserum or control antiserum. Culture in the presence of neutralizing anti-huIL-15 antiserum resulted in a 77% decrease in IFN-γ production as compared to a control antiserum (Fig. 3 *A*). IFN-γ production could also be partially inhibited in the presence of an anti-IL-2Rβ mAb (68% reduction, Fig. 3 *B*), while an anti-huIL-2 neutralizing antiserum had no significant effect (Fig. 3 *C*). It is noteworthy that while endogenous IL-15 is required for optimal IFN-γ production, complete abrogation was never achieved with higher concentrations of anti-huIL-15 antiserum or anti-huIL-2Rβ mAb (data not shown). This suggests that suboptimal amounts of IFN-γ can be produced by NK cells in the presence of other monocyte-derived cytokines (4–6). In data not shown, complete neutralization of huIL-12 alone reproducibly abolished IFN-γ produc-

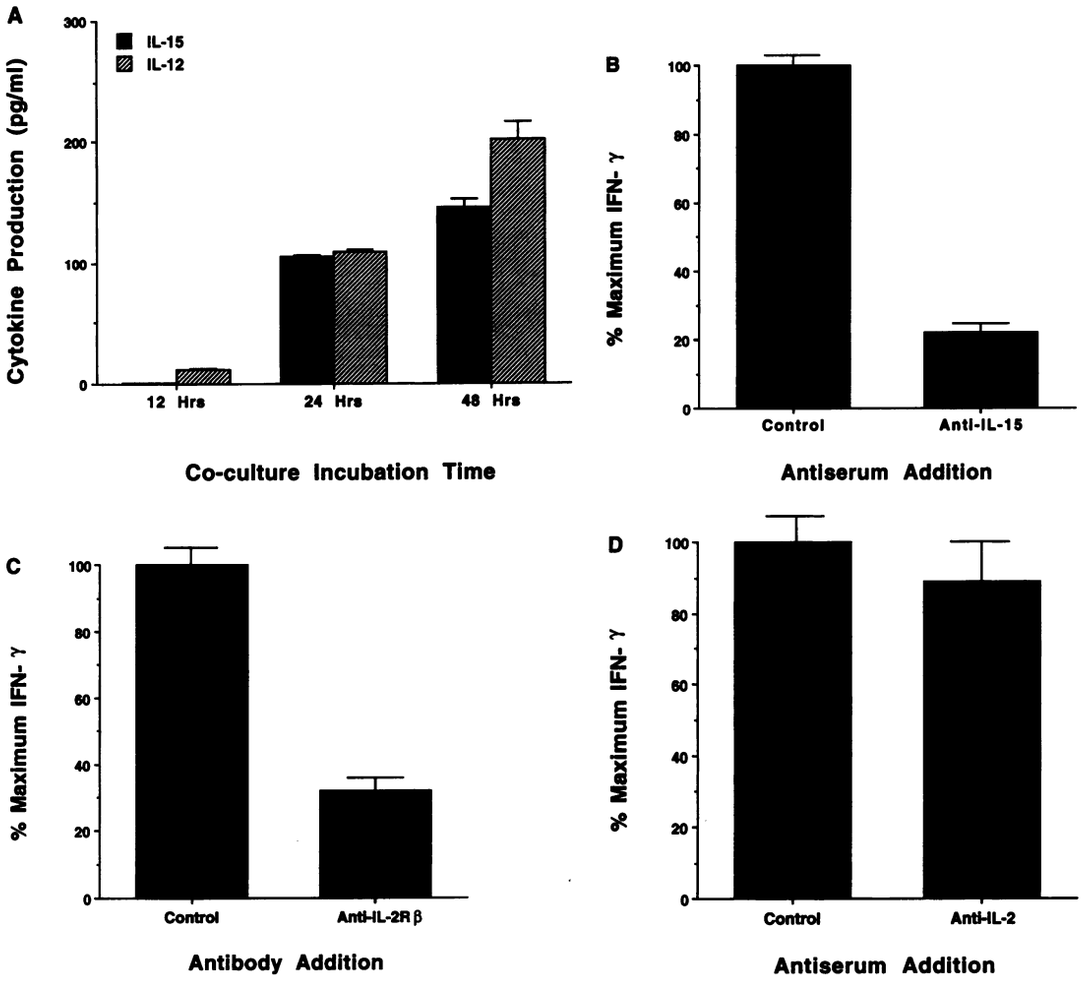

Figure 3. Endogenous production of huIL-15 is required for optimal production of IFN-γ. Cocultures of 10^5 monocytes and 10^5 NK cells were activated with LPS (5 μg/ml). (*A*) Kinetics and quantitation of endogenous huIL-15 and huIL-12 protein production. Culture supernatants were harvested at 12, 24, and 48 h and analyzed for huIL-15 and huIL-12 production by ELISA. Results represent the mean of duplicate wells ±SEM. (B) Neutralization of endogenous huIL-15. The addition of anti-huIL-15 antiserum to cocultures resulted in a 77.2%±4.1 reduction in IFN-γ production. This result is representative of 12 independent experiments ($P < 0.0005$). (C) Effect of blocking IL-2Rβ. The addition of anti-IL-2Rβ antibody to cocultures resulted in a 68%±4.2 reduction in IFN-γ production and is representative of three independent experiments ($P < 0.05$). (D) Neutralization of endogenous huIL-2. The addition of anti-huIL-2 antiserum to cocultures resulted in an 11%±13.9 reduction in IFN-γ production and is representative of four independent experiments ($P > 0.1$).

tion in this coculture system (94.3%±2.4 reduction, $P < 0.0005$ for $n = 5$ experiments).

Discussion

In the present report we demonstrate that human monocytes produce IL-15 protein within 5 h of activation by LPS. While LPS activation of either monocytes or NK cells failed to produce detectable IFN-γ, coculture of monocytes and NK cells in the presence of LPS resulted in the production of significant amounts of IFN-γ. By performing these experiments in the presence of an antiserum that neutralizes IL-15, we have demonstrated that the endogenous production of IL-15 by human monocytes is required for the optimal production of IFN-γ by NK cells. The induction of NK cell IFN-γ synthesis by activated monocytes was also partially inhibited by an anti-IL-2Rβ mAb, whereas an anti-IL-2 neutralizing antisera had no effect. This finding supports our previous observation that rhIL-15 interacts with the β subunit of the IL-2R to activate human NK cells (13).

Other combinations of endogenously produced monocyte-derived cytokines can induce IFN-γ production by NK cells in the absence of IL-15, including IL-12, TNF-α, and IL-1β (4–6, 14). Indeed, the combination of rhIL-12 and rTNF-α induces suboptimal IFN-γ production in purified human NK cells compared with rhIL-12 plus rhIL-15 (Carson, W. E., unpublished observations). Thus, rhIL-12 can combine with either rhIL-15 or rhTNF-α to induce IFN-γ secretion by NK cells, whereas the combination of rhIL-15 and rhTNF-α is not effective (13). IL-12 therefore appears to be the pivotal cytokine in the NK cell IFN-γ response, but costimulation with IL-15 is required for optimal production. Further, just as some pathogens evade eradication by their failure to induce TNF-α or IL-12 secretion by monocytes (22, 23), others may eventually be found to evade detection by not inducing IL-15 secretion.

IFN-γ remains the prototypic monocyte activation factor

for virtually all antimicrobial and antiparasitic activities (24) and is only produced by T cells and NK cells (21). NK cells appear to be critical in the early defense against many of these pathogens by supplying IFN-γ after activation by monocyte-derived cytokines such as TNF-α and IL-12. Our work suggests that IL-15 is an important monocyte-derived cytokine required for optimal production of IFN-γ by human NK cells. As such, it is likely to have a significant role during the innate immune response to certain infections in vivo. Its production by activated human monocytes provides additional insight as to why NK cells constitutively express a functional IL-2 receptor.

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