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Production of Tumor Necrosis Factor-α and Interferon-γ from Human Peripheral Blood Lymphocytes by MGN-3, a Modified Arabinoxylan from Rice Bran, and Its Synergy with Interleukin-2 In Vitro

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ABSTRACT: Recently, we presented evidence for the role of MGN-3, an enzymatically modified arabinoxylan extracted from rice bran, in potent activation of human natural killer (NK) cell function in vivo and in vitro.[1] In the current study, we examined the mechanism by which MGN-3 elevated NK cytotoxic activity. We did this by testing the action of MGN-3 on the levels of both tumor necrosis factor-α (TNF-α) and interferon-γ (IFN-γ) secretions and MGN-3 function on the expression of key cell surface receptors. Peripheral blood lymphocytes were treated with MGN-3 at concentrations of 0.1 mg/ml and 1 mg/ml, and supernatants were subjected to enzyme-linked immunosorbent assay. Results showed that MGN-3 is a potent TNF-α inducer. The effect was dose-dependent. MGN-3 concentration at 0.1 and 1 mg/ml increased TNF-α production by 22.8- and 47.1-fold, respectively. MGN-3 also increased production of IFN-γ but at lower levels as compared to TNF-α. With respect to key cell surface receptors, MGN-3 increases the expression of CD69, an early activation antigen at 16 hours after treatment. Furthermore, the interleukin-2 receptor CD25 and the adhesion molecule ICAM-1 (CD54) were upregulated after treatment with MGN-3. Treating highly purified NK cells with MGN-3 also resulted in increased levels of TNF-α and IFN-γ secretion in conjunction with augmentation of NK cell cytotoxic function. Furthermore, addition of MGN-3 to interleukin-2-activated NK cells resulted in a synergistic induction of TNF-α and IFN-γ secretion. Overall, our data suggest that MGN-3, a novel biological response modifier, can be used as a safe alternative or as an adjuvant to the existing immunotherapeutic modalities.

KEY WORDS: Arabinoxylan, interleukin-2, MGN-3, natural killer cell, synergy, TNF-α

INTRODUCTION

Natural killer (NK) cells play an important function in the primary host defense against infection and neoplasm, and defective NK cell function has been attributed to the pathogenesis of infectious diseases, such as acquired immunodeficiency syndrome and various malignancies.[2–7] Thus, increase in the NK function or restoration of defective NK function should result in the enhancement of immunity in the diseases mentioned.

Several biological response modifiers (BRMs) of fungal and bacterial origin have been developed for the enhancement of NK cells' anticancer activity. These BRMs include PSK protein bound polysaccharide (Krestin), *Lentinus edodes* mycelia (LEM), and killed streptococcal preparations (OK432), *Corynebacterium parvum,* and bacille Calmette-Guérin.[8–12] Recently, we developed a new BRM called *MGN-3,* an arabinoxylan extracted from rice bran that is treated enzymatically with an extract from shiitake mushrooms. The various biological functions of MGN-3, such as anti–human immunodeficiency virus activity[13] and NK immunomodulatory function[1,14,15] have been demonstrated previously. In this study, we examined the mechanism by which MGN-3 increases NK cell activity; we tested many important properties of NK cell function, such as production of cytokines and cell surface receptors; CD69, an early activation antigen; the interleukin-2 (IL-2) receptor CD25; and the intracellular adhesion molecule–1 (ICAM-1), CD54.

Further studies were designed also to examine possible synergistic immune activating function of MGN-3 in the presence of a low concentration of IL-2 (500 U). Conventional immunotherapeutic modalities, such as IL-2 treatment, are shown to produce undesirable side effects at high doses in cancer patients.[16–18] Further studies are needed to apply this regimen in multiple clinical trials. Thus, MGN-3 might be a safe alternative to the conventional immunotherapeutic regimens used in the activation of immune function in patients suffering from human immunodeficiency virus and malignancies.

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MATERIALS AND METHODS

MGN-3

MGN-3 is an arabinoxylan extracted from rice bran that is treated enzymatically with an extract from shiitake mushrooms. It is a polysaccharide that contains β-1,4-xylopyronase hemicellulose. MGN-3 is commercially known as Biobran (Daiwa Pharmaceutical Co, Ltd., Tokyo, Japan).

Cell Lines and Reagents

K562 target cells were cultured in RPMI 1640 supplemented with 1% penicillin-streptomycin (Life Technologies, Grand Island, NY) and 10% fetal calf serum (Irvine Scientific, Santa Ana, CA).

Recombinant IL-2 was obtained from Hoffman La Roche (Paramus, NJ). Interferon-γ (IFN-γ) was obtained from Peprotech (Piscataway, NJ). NK purification kits were obtained from Miltenyi Biotech (Auburn, CA). FITC-conjugated anti-CD54, anti-CD16, and PE-conjugated anti-CD69 antibodies were obtained from Coulter/Immunotech (Miami, FL). Anti-tumor necrosis factor-α (anti-TNF-α) monoclonal antibodies (mAB) were prepared in our laboratory. Enzyme-linked immunosorbent assays (ELISAs) for TNF-α and IFN-γ have been described.[4]

Purification of Peripheral Blood Lymphocytes and NK Cells

Twenty-five healthy control subjects (12 female, 13 male) were selected for participation in this study. The subjects ranged in age from 17 to 42 years, with a mean of 26 years. Peripheral blood mononuclear cells were isolated as described elsewhere.[4,19] Peripheral blood lymphocytes (PBLs) were obtained after Ficoll-Hypaque centrifugation. Purified NK cells were selected negatively using an NK isolation kit (Miltenyi) and consisted of more than 95% of CD16+ cells, whereas the percentage of CD3+ and CD19+ contaminant cells were 2.3 ± 3.2 and 3 ± 4, respectively.

Culture Conditions

PBLs and highly purified NK cells were cultured overnight with MGN-3 (1–1,000 μg/ml) and IL-2 (500 U/ml) and the combination of MGN-3 and IL-2. The supernatants were removed for ELISA assay, and the cells were washed and used in ^{51}Cr release assay and cell surface staining experiments.

ELISA for TNF-α and IFN-γ

mABs to two distinct epitopes of TNF-α—B154.9.1 and B154.7.1—were provided generously by Dr. G. Trinchieri (Wistar Inst, Philadelphia, PA), and polyclonal rabbit anti-TNF antibodies were prepared in our laboratory. Monoclonal antibody to IFN-γ was purchased from Genzyme (Cambridge, MA), and the polyclonal antibody to IFN-γ was prepared in our laboratory. The ELISA for TNF-α and IFN-γ was described previously.[4] Briefly, wells of ELISA plates were coated with 50 μl of mAB specific for TNF-α and IFN-γ. Plates coated with anti-TNF-α and anti-IFN-γ mABs were kept for 1 day before use, were washed three to four times, and were blocked with ELISA phosphate-buffered saline (PBS) containing 1% bovine serum albumin (BSA) for 30 minutes. Then plates were washed twice, and 50 μl of supernatants from treated NK samples was added to each

well. After overnight incubation at 37°C, plates were washed four times, 50 µl of polyclonal anti-TNF-α and anti-IFN-γ antibodies at 1:1000 dilution was added, and the incubation continued for 2 hours. Alkaline phosphatase–conjugated goat anti–rabbit IgG (Caltag, Burlingame, CA) at a dilution of 1:2000 was added to the plates that were incubated for an additional 2 hours. Finally, the plates were washed and incubated with an alkaline phosphatase substrate (Sigma 104, St. Louis, MO) and read after 2 hours in a titrated Multiskan MCC/240 ELISA reader using a 405-nm filter.

SURFACE STAINING OF NK CELLS

NK cells were washed twice with ice-cold PBS containing 1% BSA and 0.01% sodium azide. Predetermined optimal concentrations of specific mAbs were added to 5×10^4 cells in 50 µl of cold PBS-BSA and were kept on ice for 30 minutes. Thereafter, cells were washed twice in cold PBS-BSA and were brought up in 1:50 dilution of PE-GAM. After 30 minutes of incubation on ice, cells were washed twice and fixed in 1% paraformaldyhyde solution. For staining, cells were incubated with PE-conjugated antibodies for 30 minutes, after which they were washed and fixed as outlined. An Epics C (Coulter Electronics, FL) flow cytometer was used for analysis.

^{51}Cr-Release Assay for Measuring NK Activity

NK activity was measured by ^{51}Cr-release assay. Briefly, 1×10^4 ^{51}Cr–labeled tumor cells in 0.1 ml CM were added to different wells of a 96-well microtiter plate. Effector cells then were pipetted into quadruplicate wells to give effector:target (E:T) ratios of 12:1, 25:1, 50:1, and 100:1. After a 4-hour incubation (at 37°C), the plates were centrifuged (1,400 rpm for 5 minutes), and 0.1 ml of supernatant from each well was collected and counted in a gamma counter (Beckmann G50, Beckmann Instruments, Fullerton, CA). The percentages of isotope released were calculated by the following formula:

$$\% \text{ Lysis} = (\text{Exp. rel.} - \text{sp. rel.}) \times 100/\text{Total rel.} - \text{sp. rel.}$$

where *exp. rel.* is experimental release, *sp. rel.* is spontaneous release, and *total rel.* is total release. Spontaneous release (from target cells was no more than 8% to 10% of total release. Total release was measured by adding 0.1 Triton X-100 (Sigma) to designated wells. Lytic units (LU) were calculated from effector titration curves, with one LU defined as the number of effector cells required to achieve 40% lysis for K562 cells.

Statistical Analysis

The Student's *t* test was performed to determine the significance levels between control and MGN-3-treated cells, and the level of significance was set at a probability equal to .05.

RESULTS

Increase in In Vitro NK Cell Cytotoxicity by MGN-3 and IL-2

PBLs from healthy individuals were treated with MGN-3 in the presence and absence of IL-2 and were examined for NK activity. Figure 1A shows that the addition of MGN-3 at a concentration of 0.5 mg/ml to PBLs significantly increased the cytotoxic function of NK cells ($P < .001$). Furthermore, treatment of PBLs with the combination of IL-2 and MGN-3 resulted in a higher augmentation of NK cell cytotoxicity as compared to treatment with either agent alone. Addition of MGN-3 to highly purified NK cells increased NK cell cytotoxic function (see Figure 1B). Data also show that the level of NK activation is maximized after treatment with IL-2 and that NK activity did not increase further after the combined treatment of MGN-3 and IL-2, as compared to use of either agent alone.

Increase in TNF-α Secretion

Titration Experiments

PBLs were incubated with MGN-3 for 16 hours, and supernatants were recovered and subjected to a specific and sensitive ELISA assay. First, titration experiments were carried out to examine the effect of a wide range of MGN-3 concentrations (1–1,000 µg/ml) on TNF-α production. Figure 2A demonstrates

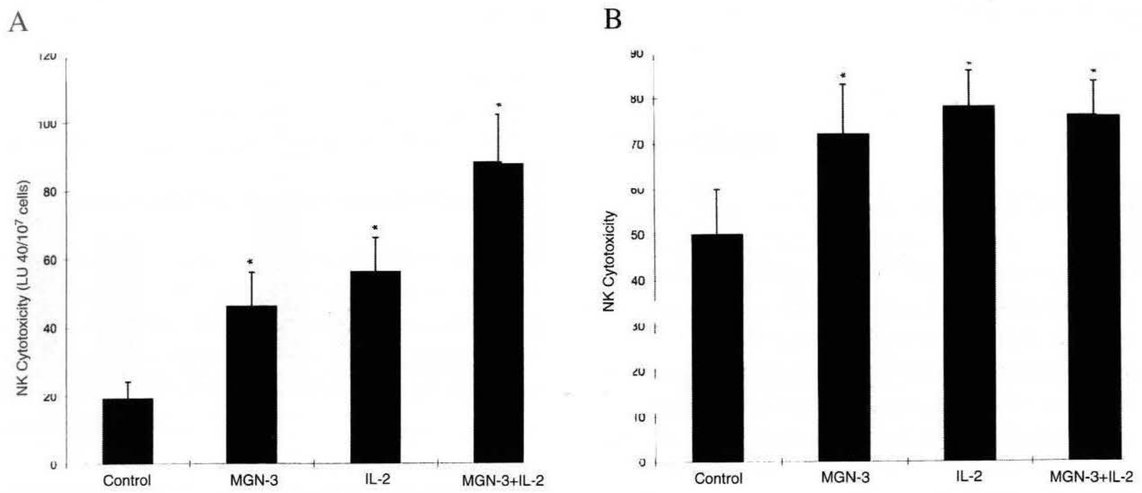


FIGURE 1. (A) Increased cytotoxicity mediated by peripheral blood lymphocytes (PBLs) after treatment with MGN-3 in vitro in the presence or absence of interleukin-2 (IL-2). PBLs from five donors were incubated with MGN-3 (0.5 mg/ml) and interleukin-2 (IL-2; 500 U/ml) for 16 hours, after which they were examined for natural killer (NK) cell activity by ^{51}Cr-release assay. Activity expressed as lytic units at 40%. *$P < .001$. (B) Action of MGN-3 on activity of purified NK cells. Purified NK cells were incubated with MGN-3 (0.5 mg/ml) in the presence or absence of IL-2 (500 U/ml) overnight. Activity examined at effector: target (E:T) ratio of 12:1 M ± standard deviation of five individuals. *$P < .01$.

that MGN-3 is a potent TNF inducer. The effect was dose-dependent. The level of TNF-α did not change at 1 to 10 µg/ml but increased at a concentration of 0.1 mg/ml and maximized at concentration of 1 mg/ml. IL-2 alone had no effect on TNF-α production; however, a synergistic effect of MGN-3 and IL-2 was observed.

Production of TNF-α by PBLs

Subsequently, two concentrations of MGN-3 (1 and 0.1 mg/ml) were selected and applied to 25 donors. Figure 2B shows that addition of MGN-3 to PBLs significantly increased TNF-α secretion in all subjects. The variation among subjects in response toward MGN-3 is clear. Compared to control, untreated samples, subjects treated with 1 mg/ml, could be divided into three groups: G1 (40%; 10 of 25) showed an increase in their TNF-α level up to less than 20-fold; G2 (24%; 6 of 25) showed an increase in TNF-α level between 20- and 100-fold; and G3 (36%; 9 of 25) were highly responsive, with an increase in TNF-α level greater than 100-fold. Further, the data in Figure 2B show that the increase in TNF-α secretion was augmented synergistically in the presence of IL-2 ($P < .001$).

Production of TNF-α by Purified NK cells

Figure 2C depicts data obtained when NK cells were purified and cultured with MGN-3 in the presence or absence of IL-2. Data show a ninefold increase in TNF-α production after treatment with MGN-3 alone at a concentration of 1 mg/ml. IL-2 treatment demonstrated no change in the TNF-α level as compared to controls. TNF-α production did not increase further after combined treatment of MGN-3 and IL-2, as compared to use of either agent alone.

Increase in IFN-γ Secretion

Titration Studies

With respect to IFN-γ secretions, titration experiments were conducted also to examine the effect of a

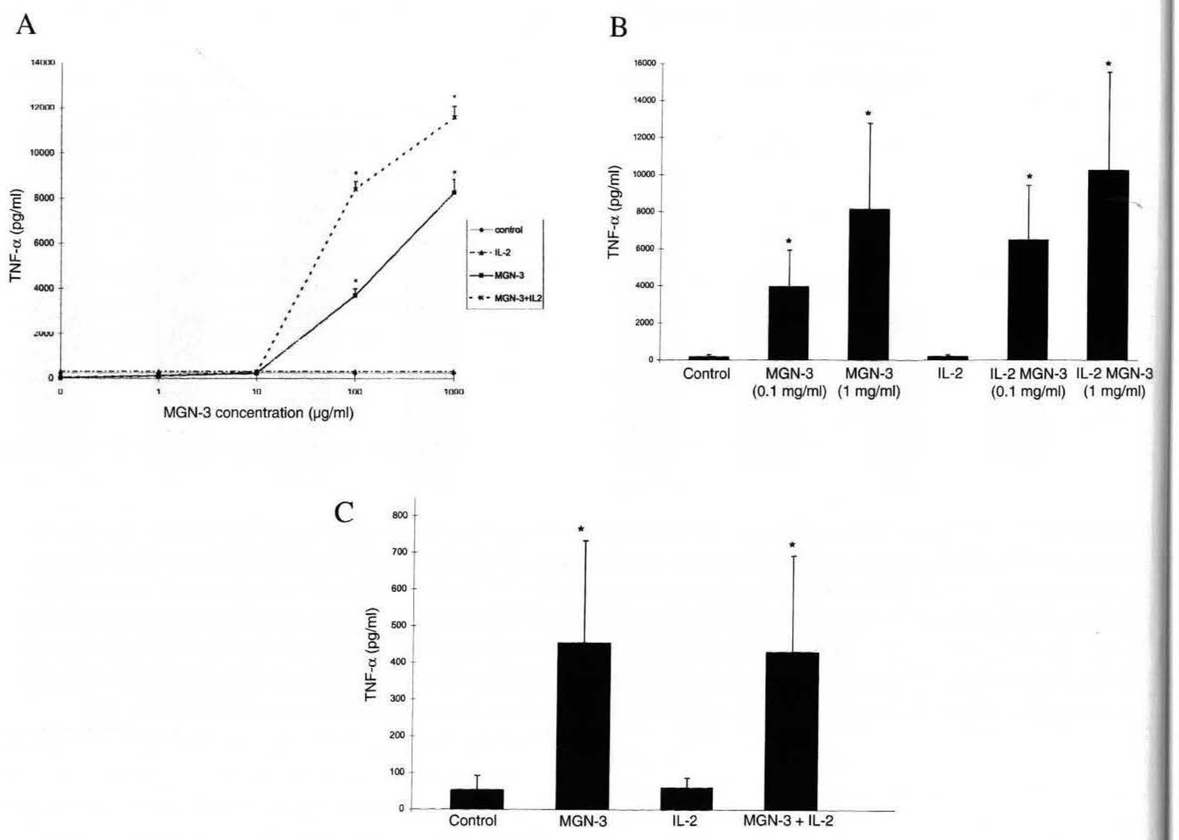


FIGURE 2. **(A)** Titration experiments for tumor necrosis factor-α (TNF-α) production by peripheral blood lymphocytes (PBLs). PBLs were incubated with a wide range of MGN-3 concentrations (1–1,000 μg/ml) in the presence or absence of interleukin-2 (IL-2; 500 U/ml) for 16 hours. Supernatant was harvested and subjected to enzyme-linked immunosorbent assay (ELISA). *$P < .001$. **(B)** Triggering of tumor necrosis factor-α (TNF-α) secretion by PBLs treated with MGN-3. PBLs (5×10^6) were treated with 0.1 mg/ml and 1 mg/ml MGN-3, 500 U/ml IL-2, and the combination of IL-2 and MGN-3. After 16 to 18 hours of incubation, the supernatants were harvested and subjected to TNF-α ELISA. Data of 25 individual donors. *$P < .001$. **(C)** Triggering of TNF-α secretion by purified natural killer (NK) cells treated with MGN-3. NK cells were treated with MGN-3 (1 mg/ml) in the presence or absence of IL-2 (500 U/ml) for 16 hours. Supernatants were harvested and subjected to TNF-α ELISA. Data of three subjects, each tested in triplicate. *$P < .01$.

wide range of MGN-3 concentrations (1–1,000 μg/ml) in the presence and absence of IL-2 on IFN-γ production (Figure 3A). MGN-3 proved to be an IFN-γ inducer in a dose-dependent manner. IFN-γ secretion was noticeable at 1 to 10 μg/ml, was increased further at 0.1 mg/ml, and was maximized at MGN-3 concentration of 1 mg/ml, whereas IL-2 treatment showed no change in IFN-γ level. Furthermore, a synergistic effect was observed when MGN-3 was coupled with IL-2, resulting in a dramatic several-fold increase in IFN-γ production, as compared to use of either agent alone.

Production of IFN-γ by PBLs

Consequently, two concentrations of MGN-3 (0.1 and 1 mg/ml) were applied to PBLs from 14 donors. Figure 3B shows that MGN-3 treatment increased IFN-γ secretion with individual variation among subjects. Addition of MGN-3 to PBL samples in the presence of IL-2 resulted in a synergistic increase in IFN-γ secretion in all subjects. The synergistic effect was MGN-3-dose-dependent: 52-fold induction at MGN-3 concentration of 0.1 mg/ml ($P < .03$) and 66-fold at MGN-3 concentration of 1 mg/ml ($P < .02$).

Production of IFN-γ by Purified NK Cells

Figure 3C shows an increase in IFN-γ secretion after culture of NK cells with MGN-3 at a concentration of 1 mg/ml. Treatment with IL-2 significantly increased IFN-γ level ($P < .01$), and again, a synergistic effect of MGN-3 in the presence of IL-2 was observed clearly ($P < .01$).

Induction of Key Cell Surface Receptors on NK Cells by MGN-3 and IL-2

Upregulation of CD69 Early Activation Antigen

The mechanism by which MGN-3 activated both the cytotoxic and secretory pathways of NK cells was examined. Addition of MGN-3 to PBLs induced significant upregulation of CD69, an early activation marker on NK cells. The induction of CD69 cell surface receptor expression by MGN-3 was higher than those observed by the IL-2 treatment of PBLs (Figure 4A).

Upregulation of IL-2 Receptor Alpha Chain (CD25)

Figure 4B shows significant upregulation of the IL-2 receptor α chain (CD25) expression after treatment of PBLs with MGN-3. The expression of CD25 on MGN-3-treated NK cells was higher than in those induced by IL-2 treatment alone.

Upregulation of CD54 Adhesion Molecule

The addition of MGN-3 to NK cells resulted in the upregulation of CD54, the adhesion molecule. Treatment of NK cells with the combination of IL-2 and MGN-3 augmented the intensity of CD54 expression on NK cells (see Figure 4C).

An upregulation of CD69, CD25, and CD54 also was observed on lymphocytes other than NK cells after MGN-3 treatment but to a lesser extent. Indeed, the levels of CD69 expression on CD4++ T cells were significantly lower than those observed on NK cells, both at the percentage and the mean intensity levels (see Figure 4D).

DISCUSSION

Recently, we tested the ability of MGN-3 to enhance human NK cell activity in both in vivo and in vitro culture conditions.[1,14,15] MGN-3 is composed of denatured hemicellulose, which is obtained by reacting rice bran hemicellulose with multiple carbohydrate-hydrolyzing enzymes from shiitake mushrooms. The main chemical structure of MGN-3 is an arabinoxylan with a xylose in its main chain and an arabinose polymer in its side chain.[1,13] The ability of MGN-3 to activate highly purified NK cells from peripheral blood indicates that the activity is attributed only to the NK cell fraction.

In this study, we analyzed in vitro the possible mechanisms by which MGN-3 enhances NK cell activity; we evaluated cytotoxic and secretory functions of NK cells in the presence of MGN-3. Increase in NK cell cytotoxic activity after treatment with MGN-3 was paralleled with an increase in cytokine secretion. Significant levels of TNF-α secretion were triggered by the presence of MGN-3 in a dose-dependent manner. Researchers have established that TNF-α induces the expression of IL-2Rα.[20–22] Moreover, addition of the anti-TNF-α antibody to IL-2-treated NK cells inhibited IL-2Rα upregulation significantly.[20] The blocking of TNF receptor function by antibodies to p75 and p55 TNF receptors also was shown to inhibit the generation of lymphokine-activated killer cells.[23] Therefore, the induction of TNF-α secretion by MGN-3 might represent the first step in NK cell activation and might be responsible for the observed increases in IL-2Rα (CD25) expression. The increase in IL-2Rα expression by MGN-3 and its association with the IL-2Rβ/γ chain forms the high-affinity binding site for IL-2 (k_d = 10 pM),[24] which results in the observed increase in cellular response to IL-2 binding.

IL-2, in turn, has been shown to upregulate TNF-receptor chains, thus establishing a feedback loop whereby IL-2 will increase TNF-α responsiveness and TNF will augment IL-2 responsiveness.[20] As a result of such interaction, a synergistic response will be obtained when both cytokines are present in the cell cultures. Therefore, observation of a synergistic

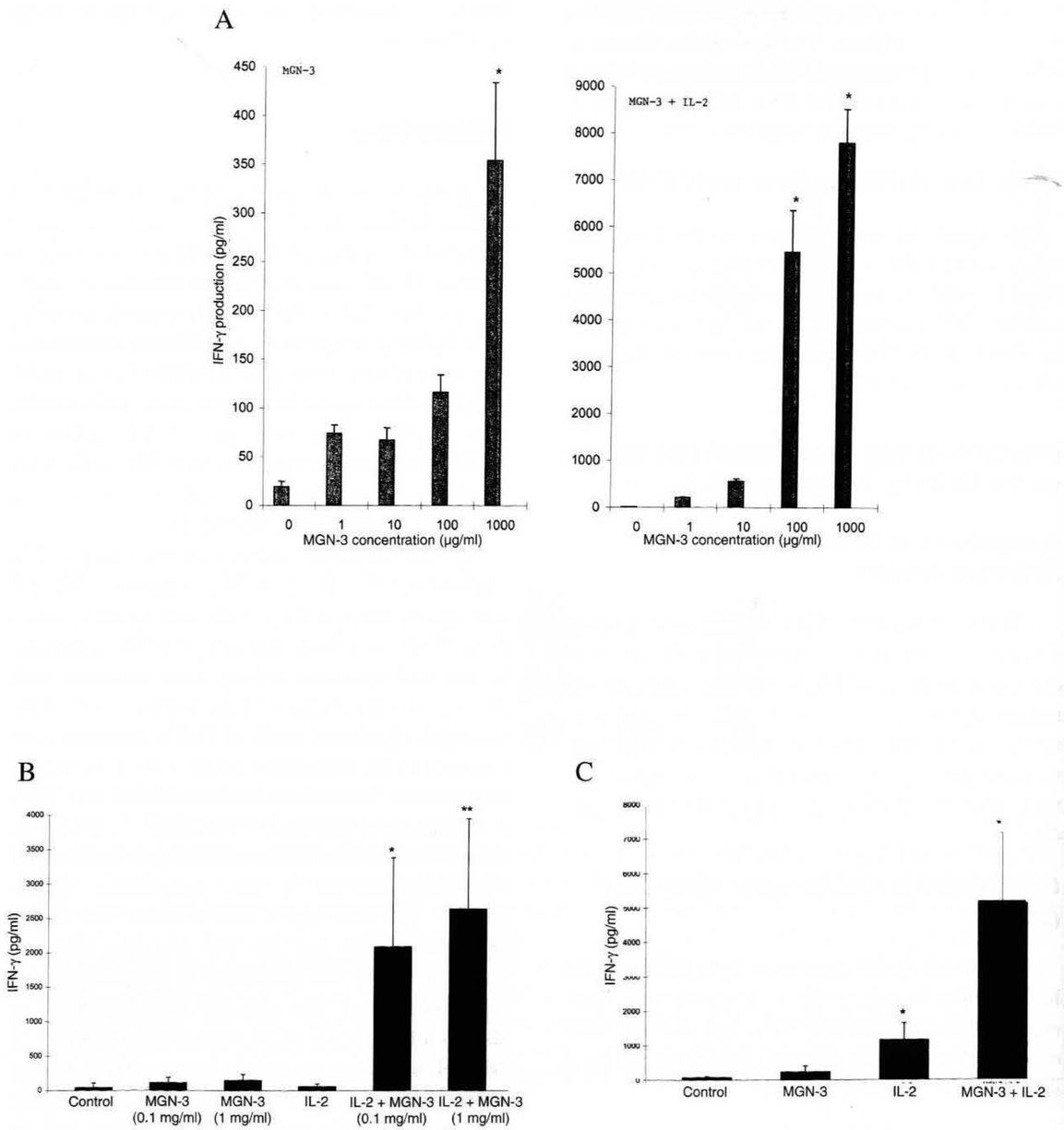


FIGURE 3. **(A)** Titration experiment for interferon-γ (IFN-γ) production by peripheral blood lymphocytes (PBLs). PBLs were incubated with a wide range of MGN-3 (1–1,000 μg/ml) alone or in the presence of interleukin-2 (IL-2; 500 U/ml) for 16 hours. Supernatants were harvested and subjected to enzyme-linked immunosorbent assay (ELISA). *$P < .001$. **(B)** MGN-3-mediated induction of IFN-γ secretion by PBLs. The treatments were conducted as described. After 16 to 18 hours of incubation, supernatants were removed and subjected to IFN-γ ELISA assay. Data from 14 individual donors. *$P < .03$; **$P < .02$. **(C)** Induction of IFN-γ secretion by purified natural killer cells treated with IL-2 and MGN-3. Natural killer cells were treated with MGN-3 at concentrations of 1 mg/ml in the presence or absence of IL-2, as described. The supernatants were harvested and subjected to IFN-γ ELISA. Data of three subjects, each tested in triplicate. *$P < .01$.

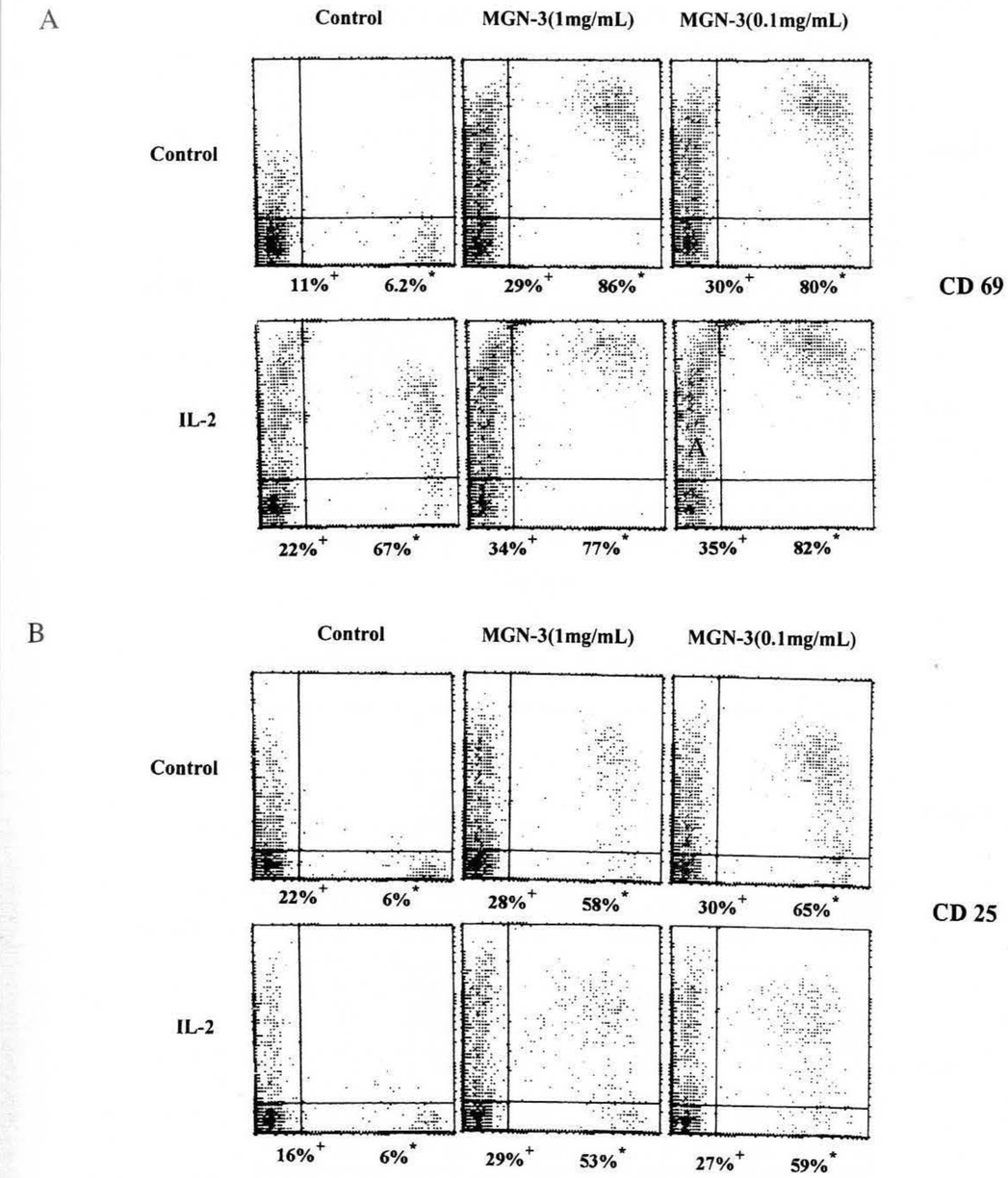
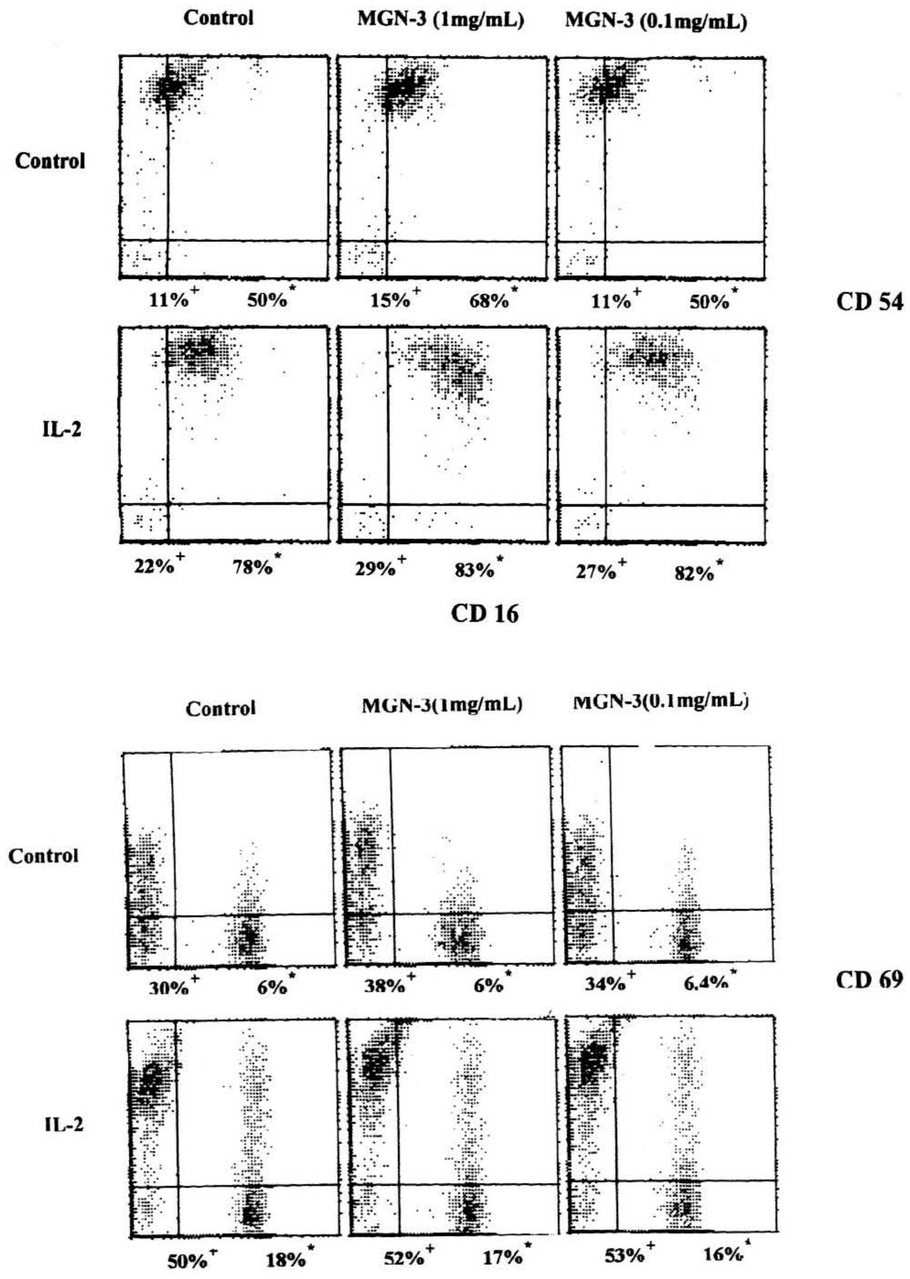


FIGURE 4. Upregulation of CD69, CD25, and CD54 surface receptor expression on natural killer (NK) and T cells by MGN-3 and interleukin-2 (IL-2) treatments. Peripheral blood lymphocytes (PBLs) were treated with MGN-3 (0.1 and 1 mg/ml) and IL-2 (500 U/ml) for 16 to 18 hours. (A) The treated samples were washed and stained with the FITC-conjugated CD16 and PE-conjugated CD69 monoclonal antibodies. The number on the bottom represents the percentage of cells positive for CD69 expression. *Percentage of cells positive for CD69 expression within CD16-positive natural killer cells; +percentage of cells positive for CD69 expression within CD16-negative fraction of peripheral blood mononuclear cells (PBMCs). (B) FITC-conjugated CD16 and PE-conjugated CD25 were used for the staining of the lymphocyte. The numbers on the bottom represent the percentage of positive cells for CD25 expression.



*Percentage of cells positive for CD25 expression within CD16-positive NK cells; +percentage of cells positive for CD25 expression within CD16-negative fraction of PBMCs. (c) Staining with FITC-conjugated CD 16 and PE-conjugated CD54. *Percentage of cells positive for CD54 surface expression within CD16-positive NK cells; +percentage of cells positive for CD54 surface expression within CD16-negative fraction of PBMCs. (d) FITC-conjugated CD4 and PE-conjugated CD69. *Percentage of cells positive for CD69 surface expression within $CD4^+$ fraction of PBMCs; +percentage of cells positive for CD69 surface expression within $CD4^-$ fraction of PBMCs.

function in the presence of IL-2 and MGN-3 is expected, because MGN-3 can trigger significant TNF-α secretion. Addition of IL-2 to MGN-3 at concentrations of 0.1 and 1 mg/ml elevated TNF-α production by 37.5- and 59.3-fold, respectively. In vitro culture of PBLs and highly purified NK cells with either agent alone resulted in a significant increase in NK cell activity. In addition, the synergistic effect of both agents was detected against PBLs but not against highly purified NK cells, because IL-2 treatment alone maximized NK activity.

IFN-γ secretion has been shown to upregulate TNF receptor II (p75) expression, whereas TNF-α exerts its function through both the TNFRII and TNFRI.[22] Induction of TNFRII expression and signaling is important for cellular proliferation and induction of the NFκβ transcription factor.[25] Thus, an intimate relationship among TNF-α, IFN-γ, and IL-2 determines the fate of NK cell activation in MGN-3-treated cells. MGN-3-treated NK cells have increased expression of CD69, an early activation antigen, which has been correlated with increased cytokine secretion by NK cells.[20] TNF-α also plays an important role in the induction of CD69 and CD54 cell surface receptors. CD69 is a 28- to 32-kDa surface homodimer,[26] which is induced rapidly and is phosphorylated in IL-2-activated NK cells.[27] Furthermore, the expression of this antigen correlated well with the levels of cytotoxicity observed in IL-2-stimulated free cells.[20] Moreover, anti-TNF-antibody treatment of IL-2-activated cells resulted in inhibition of CD69 cell surface expression. Thus, TNF-α triggered by MGN-3 might be responsible also for upregulation of CD69 surface expression and for the increase in both cytotoxicity and cytokine secretion. Therefore, activation of TNF-α secretion by MGN-3 plays a central role in the regulation of NK cell activity and function.

Parallel to CD25 and CD69 induction, an upregulation of CD54 (ICAM-1) was observed by MGN-3-treated NK cells. Several lines of evidence suggest a possible role for CD54 in adhesion and cytotoxicity by NK cells.[6,28,29] Indeed, increases in CD54 expression were observed in IL-2-activated PBLs.[30] Thus, increased NK cell cytotoxic function by MGN-3 could be due partly to the ability of MGN-3 to augment the expression of ICAM-1. Treatment of the PBLs with MGN-3 in vivo resulted in a significant increase in effector cell binding capacity to their tumor cell targets.[1]

Though demonstrating a significant increase in TNF-α and IFN-γ production after treatment of PBLs with MGN-3, lower levels of these cytokines were observed when purified NK cells were tested. Although increased activation of CD4+ T cells was observed in the presence of MGN-3, levels of such activation were lower than those obtained by MGN-3-treated NK cells, suggesting that MGN-3 primarily targeted the NK cells, whereas other cells serve as accessory cells for NK activation.

In conclusion, we have presented evidence for the role of MGN-3 in activation of NK cytotoxicity, induction of cytokine production, and upregulation of key NK cellular receptors. Identification and purification of the active fraction in MGN-3, which is responsible for triggering functional activation and upregulation of key surface receptors in NK cells, awaits further investigation.

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