Direct Interactions of Human Natural Killer Cells with *Cryptococcus neoformans* Inhibit Granulocyte-Macrophage Colony-Stimulating Factor and Tumor Necrosis Factor Alpha Production

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Human natural killer (NK) cells and T lymphocytes can bind to and inhibit the growth of the yeast-like organism *Cryptococcus neoformans*. Binding of target cells to NK or T cells also has the potential to modulate cytokine production by the effector cells. In this study, we assessed the ability of *C. neoformans* to modulate NK cell production, or in some cases T-cell production, of granulocyte-macrophage colony-stimulating factor (GM-CSF) or tumor necrosis factor alpha (TNF- α). We found that freshly isolated human NK cells from most individuals make GM-CSF and TNF- α constitutively when cultured in vitro. The addition of *C. neoformans* to T-cell fractions which do not make GM-CSF constitutively did not affect GM-CSF production, but the addition of *C. neoformans* to NK cell fractions significantly reduced the amounts of GM-CSF produced in most NK cell samples. The reduction in the amount of GM-CSF in *C. neoformans*-NK cell cocultures could not be attributed to loss of lymphocyte viability or to *C. neoformans* adsorbing or degrading the cytokine and was dependent on direct contact between the NK cells and cryptococcal cells. GM-CSF was not the only cytokine to be down-regulated. TNF- α production was also diminished when NK cells were incubated with *C. neoformans*. The regulation of both cytokines was at the transcriptional level because GM-CSF and TNF- α mRNA levels were lower in NK cell samples incubated with *C. neoformans* than in NK cell samples incubated without *C. neoformans*. Diminished production of constitutively produced cytokines resulting from the interaction of NK cells with cryptococcal cells has the potential to affect phagocytic cells in the immediate regional environment and to damp the immune response.

Natural killer (NK) cells are considered to have two major functions. One is to directly interact with and kill target cells such as virus-infected cells, tumor cells, other host cells with reduced expression of major histocompatibility complex class I antigens, and some pathogenic microorganisms (37). The second NK cell function is to produce cytokines that can modulate innate and immune responses (34, 38, 39). NK cells can constitutively produce some cytokines such as granulocyte-macrophage colony-stimulating factor (GM-CSF) and can be stimulated to up-regulate the production of those cytokines as well as to produce other cytokines such as gamma interferon (IFN- γ) (1, 7, 33).

Previous studies from our laboratory and others have shown that NK cells as well as T lymphocytes can bind to the pathogenic fungus *Cryptococcus neoformans* and kill the organism (11–16, 21, 22, 28, 29). More recently, Levitz and North have shown that human nonadherent lymphocytes from approximately 60% of the samples that they assessed produced IFN- γ upon incubation for 18 h with *C. neoformans* (23). When the nonadherent lymphocytes were depleted of NK cells or T cells prior to stimulation with *C. neoformans*, the level of IFN- γ production was reduced, suggesting that both NK and T cells have the potential to make IFN- γ when stimulated with *C. neoformans* (23). Using purified populations of human NK or T cells, we have found that NK cells from some individuals produce IFN- γ constitutively, whereas NK cells from other indi-

viduals do not. In about 50% of the human NK cell samples examined, production of IFN- γ could be induced by incubation with *C. neoformans* (unpublished data). Similarly, human T cells from different individuals vary in their ability to produce IFN- γ without a stimulus and after stimulation with *C. neoformans* (unpublished data). Levitz and North (23) also showed that human nonadherent leukocytes could be induced to produce IFN- γ by the NK cell-resistant fungal target *Candida albicans*. Binding of the *Candida albicans* blastoconidia to human NK cells up-regulates the production of two other cytokines, tumor necrosis factor alpha (TNF- α) and GM-CSF (3, 8). We investigated whether the NK-sensitive fungal target *C. neoformans* would similarly modulate the NK cell production of GM-CSF, a cytokine important in host protection (5, 6, 20). After finding that GM-CSF was constitutively produced by human NK cells and that GM-CSF production was down-regulated after stimulation with *C. neoformans*, we assessed the effects of human NK cell interactions with *C. neoformans* on the production of TNF- α , another cytokine that plays a role in host defense against *C. neoformans* (17). Like GM-CSF, TNF- α production was reduced when NK cells were stimulated with *C. neoformans*.

MATERIALS AND METHODS

Reagents. The reagents used in these studies were purchased from the following vendors: RPMI 1640 medium and pooled human serum (type AB) from Gibco Laboratories, Grand Island, N.Y.; fetal bovine serum (FBS) from HyClone Laboratories, Inc., Logan, Utah; penicillin, streptomycin, Ficoll-Hypaque, Percoll, and phorbol myristate acetate (PMA) from Pharmacia, Uppsala, Sweden; nylon wool from Robbins Scientific Corp., Sunnyvale, CA.; fluorescein isothiocyanate-conjugated mouse anti-human CD3 monoclonal antibody (immunoglobulin G1 IgG1), phycoerythrin (PE)-conjugated mouse anti-human CD16 monoclonal antibody (IgG1), PE-conjugated mouse anti-human CD14 monoclonal antibody (IgG2), and fluorescein isothiocyanate-conjugated and PE-conju-

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gated mouse IgG1 and IgG2 isotype control antibodies from Becton-Dickinson, Mountain View, Calif.; the calcium ionophore A23187 from Sigma Chemical Co., St. Louis, Mo.; Tri-reagent from Molecular Research Center, Inc., Cincinnati, Ohio; human GM-CSF and TNF- α enzyme-linked immunosorbent assay (ELISA) kits from Genzyme, Cambridge, Mass.; and GM-CSF primers from ClonTech Laboratories Inc., Palo Alto, Calif. TNF- α primers and probes were designed based on the published sequence of TNF- α (M10988) (31) and were synthesized at the University of Oklahoma Health Sciences Center Molecular Biology Resources Facility.

Preparation of human PBMC and enrichment of lymphocyte subsets. Buffy coats were obtained from normal volunteers at the Sylvan E. Goldman Blood Institute (Oklahoma City, Okla.). Peripheral blood mononuclear cells (PBMC) were isolated by centrifugation on Ficoll-Hypaque at $400 \times g$ for 30 min at room temperature. The PBMC were collected from the interface, washed in 0.0145 M Tris-buffered saline solution (pH 7.6), and resuspended in RPMI 1640 containing 100 U of penicillin per ml and 100 μ g of streptomycin per ml (culture medium [CM]) supplemented with 5% heat-inactivated human type AB serum.

The PBMC were passed over nylon wool columns, and the nylon wool-nonadherent fractions which contained the T and NK cells were further separated by centrifugation on three-step discontinuous Percoll gradients (28). The large granular lymphocyte (LGL) fraction was collected from the interface between the 40 and 50% Percoll, and the T cells were collected from the bottom of the gradient (28). The LGL and T-cell fractions were subjected to flow cytometric analysis after being labeled with fluorochrome-conjugated anti-CD3, anti-CD16, or anti-CD14 or the appropriate isotype control antibodies (28). The LGL fractions were composed of 25 to 40% CD16 $^{+}$ cells, 57 to 73% CD3 $^{+}$ cells, and <1% CD14 $^{+}$ cells, whereas the T-cell fractions were typically 96 to 99% CD3 $^{+}$ cells, 0.2 to 0.4% CD16 $^{+}$ cells, and <0.2% CD14 $^{+}$ cells.

Considering that the LGL population was a mixture of NK (CD16 $^{+}$) and T (CD3 $^{+}$) cells, we performed another fractionation procedure to obtain a more enriched population of NK cells. For this, nylon wool-nonadherent cells were subjected to negative selection with an NK cell isolation kit as specified by the manufacturer (Miltenyi Biotec, Inc., Auburn, Calif.). The NK cell fractions enriched in this way contained 80 to 88% CD16 $^{+}$ cells, 1.5 to 3% CD3 $^{+}$ cells, and 0.04% CD14 $^{+}$ cells as determined by flow cytometric analysis. Throughout this paper, this population of cells is designated NK cells.

C. neoformans. *C. neoformans* 184A (serotype A) (26) and 3501 (serotype D) were cultured in CM-10% FBS at 37°C under 7% CO $_2$ (28). After 24 h in culture, the *C. neoformans* cells were washed three times in CM-10% FBS, counted on a hemacytometer, and adjusted to the desired concentration with CM-10% FBS. Isolate 184A was used in all experiments unless otherwise designated.

Culture conditions for GM-CSF production and quantification of GM-CSF. Suspensions of LGL, NK, or T cells in CM-10% FBS (10 7 cells/ml) were added in 100- μ l volumes to each well of a 96-well plate. To each of four wells containing effector cells was added 100 μ l of one of the following: CM-10% FBS as a background control, *C. neoformans* cells (5×10^6 cells) in CM-10% FBS, or PMA (20 ng/ml) plus the Ca $^{2+}$ ionophore A23187 (1 μ g/ml) as a positive control. The plates were centrifuged at $150 \times g$ for 5 min and incubated under 7% CO $_2$ at 37°C for 48 h. At the end of the incubation period, the viability of the effector cells, as assessed by trypan blue dye exclusion, was similar for all of the conditions used and was greater than 95% in each case. The supernatant from each well was collected and assayed for GM-CSF with a Genzyme GM-CSF ELISA kit as specified by the manufacturer. The percent inhibition of GM-CSF production was calculated as follows: percent inhibition = [(control - test)/control] $\times$ 100; where control is the concentration of GM-CSF in 48-h supernatants from LGL alone and test is the concentration of GM-CSF in 48-h supernatants from LGL plus *C. neoformans*.

Culture conditions for production and quantification of TNF- α . Experiments similar to those done to measure GM-CSF were performed to quantify TNF- α , except that the supernatants were collected at 6 h rather than at 48 h. TNF- α was measured with a Genzyme TNF- α ELISA as specified by the manufacturer.

Culture conditions in Transwell plates. To determine if the LGL had to be in direct contact with the *C. neoformans* cells for GM-CSF production to be affected, 24-well tissue culture-treated Transwell plates (Costar, Cambridge, Mass.) were used. Wells of the Transwell plates have two chambers separated by a polycarbonate membrane with a pore size of 0.45 μ m. The experiments were done by putting the cryptococcal cells (3×10^6 cells/0.4 ml of CM-10% FBS) in the lower chamber and the LGL (6×10^6 cells/0.4 ml of CM-10% FBS) in the upper chamber and incubating the mixture for 48 h under 7% CO $_2$ at 37°C. Additional wells which contained the identical numbers of LGL alone or LGL and cryptococcal cells were included, but in the latter case the LGL and cryptococcal cells were not separated by the membrane.

Total-RNA isolation. Freshly isolated LGL, NK cells, or T lymphocytes were cultured for 6 or 18 h as designated in CM-10% FBS without and with *C. neoformans* or with PMA and the Ca $^{2+}$ ionophore as described above. After the incubation, total RNA was extracted from each sample with Tri-reagent as specified by the manufacturer.

Northern blot analysis for GM-CSF mRNA. To obtain a sufficient amount of RNA for Northern blot analysis, RNA fractions from like samples from two or three individuals were pooled. The RNA had to be pooled because the total number of LGL isolated from any one individual is limited. For each pooled

RNA sample, 10 μ g of RNA was subjected to electrophoresis on a 1.2% agarose-formaldehyde gel and then transferred to a Hybond-N+ membrane (Amersham Life Sciences, Arlington Heights, Ill.). The membranes were probed with three different oligonucleotide probes, which bind to different sites on GM-CSF mRNA, and with an 18S rRNA probe to normalize sample loading of the gels. The three GM-CSF probes were designed based on the GM-CSF mRNA sequence (19) and were antisense oligonucleotides: 3'-GGACGACGAGAAC-CCGTGACA (47 to 67), 3'-GACTTGGACTCATCTCTGTG (159 to 178), and 3'-CTTTGAAGGACACGTTGGGTC (369 to 380). The oligonucleotide probe for human 18S rRNA was designed based on the human 18S rRNA sequence (10) and was 3'-CGTGCCTACTTAGACATGCATGG. A 10-pmol sample of each oligonucleotide probe was labeled with the terminal deoxynucleotidyl transferase labeling system by Promega (Madison, Wis.) and [32 P]dCTP as specified by the manufacturer. After hybridization with a mixture of the 32 P-labeled GM-CSF probes, autoradiograms were developed. The membranes were stripped, probed with the 18S rRNA probe, and again exposed to photographic film. The autoradiograms were scanned on an imaging densitometer, and the data were normalized by computation according to the following formula: density ratio = optical density (OD) of GM-CSF mRNA from lymphocytes alone or lymphocytes plus *C. neoformans*/OD of 18S rRNA from lymphocytes alone or lymphocytes plus *C. neoformans*, respectively.

Semiquantitative RT-PCR for GM-CSF or TNF- α mRNA. To assess the relative amount of GM-CSF or TNF- α mRNA in samples from a single individual, total-RNA preparations from each of the sample groups containing cells from one individual were subjected to reverse transcription (RT) and then PCR. After the total RNA was collected by the method described above, it was used to synthesize cDNA by following the protocol provided by Perkin-Elmer Cetus, Norwalk, Conn., with their reverse transcriptase. Briefly, 1.5 μ g of total RNA from each sample was mixed with 28.5 μ l of reverse transcription mixture as defined by Perkin-Elmer Cetus [PCR buffer II; 5 mM MgCl $_2$, 1 mM each deoxynucleoside triphosphate, 1 U of RNase inhibitor per μ l, 25 U of murine leukemia virus reverse transcriptase per μ l, 2.5 μ M oligo(dT) 16-mer]. The resulting cDNA was subjected to PCR with AmpliTaq DNA polymerase (Perkin-Elmer Cetus) and primers for the target cDNA. The final concentrations of each component in the PCR mixture were as follows: PCR buffer (1 $\times$ concentration), 3 mM MgCl $_2$, 0.2 mM each deoxynucleoside triphosphate, 0.2 U of Taq polymerase per μ l, 0.5 μ M primers, and 0.4 μ g of cDNA. The PCR mixture was subjected to 25 cycles in a Perkin-Elmer Cetus thermocycler. Each cycle consisted of denaturation for 1 min at 94°C, reannealing of primers and fragments for 1.5 min at 56°C, and extension for 1.5 min at 72°C. The primers for human TNF- α were as follows: sense, 5'-CAATCCCTTATTACCCC (829 to 847); antisense, 3'-CTTTAACTGTGTTCACCTGG (1130 to 1149). The expected sizes of the amplified GM-CSF and TNF- α cDNAs were 424 and 321 bp, respectively. The GM-CSF probe used for hybridization to the GM-CSF PCR product was the oligonucleotide GM-CSF (159 to 178), which was one of the three probes used for Northern blot analysis. The TNF- α probe was CACTTCACGACCGTTGGTGATTG (981 to 1003).

Hypoxanthine phosphoribosyltransferase (HPRT), a housekeeping enzyme, was used as an internal standard. To ensure equal loading of each cDNA sample, the total RNA samples were subjected to RT-PCR with primers to amplify HPRT cDNA. The primers for human HPRT were designed according to the published DNA sequence for human HPRT (GenBank accession No. M31642) (18). The primer pair sequences were 5'-AGATGTGATGAAGGAGATGGG (238 to 258) and 3'-CCTTTCTTACAGACATAACACC (464 to 485), and the expected PCR product size was 248 bp. The probe for the HPRT PCR product was 3'-CACTATCTAGGTAAGGATACTAGC (351 to 374). Preliminary experiments showed that when PCR was run for 20 to 30 cycles for GM-CSF and HPRT, the amount of PCR product was proportional to the initial amount of sample cDNA. Consequently, we used 25 cycles for PCR amplifications. The resultant PCR products were subjected to electrophoresis on 1.7% agarose followed by Southern blotting. The specific target DNA on the membrane was detected with reagents in a nonradioactive DNA labeling and detection kit (Boehringer Mannheim, Indianapolis, Ind.). Briefly, 10 pmol of each probe labeled with digoxigenin-11-dUTP was hybridized to DNA on the blots. After hybridization and stringency washes, the blots were washed, blocked, reacted with anti-digoxigenin antibody, treated with a chemiluminescent substrate, and exposed to Lumi-Film chemiluminescent detection film (Boehringer Mannheim) (32). The exposed films were developed and scanned to quantify the target bands, and the results were expressed as follows: density ratio = OD for GM-CSF $_{\text{test}}$ or TNF- α_{test} /O.D. for HPRT $_{\text{test}}$. Similar calculations were made for control samples. The test samples were total RNA from LGL or NK cells plus *C. neoformans*, and the control samples were total RNA from LGL or NK cells alone.

Statistical analysis. Means and standard errors of the means (SEM) were calculated, and a paired Student *t* test was used to analyze the data. Groups were considered to be significantly different when $P \leq 0.05$.

RESULTS

Incubation with *C. neoformans* reduced the ability of LGL to produce GM-CSF. LGL and T-cell fractions obtained from

FIG. 1. Production of GM-CSF by LGL is inhibited by culture with *C. neoformans*. LGL fractions isolated from 14 healthy donors were cultured without (LGL) or with (LGL + Cn) *C. neoformans* 184A for 48 h before the supernatants were removed and assayed for GM-CSF. The assays were done in triplicate, and the bars represent the SEM. (A) Amount of GM-CSF in the culture supernatant. (B) Percent inhibition of GM-CSF production when *C. neoformans* is incubated with the LGL.

buffy coats of 14 different individuals were assessed for their ability to produce GM-CSF over a 48-h period in tissue culture medium alone, with 184A *C. neoformans* cells at an effector-to-target (E/T) ratio of 2:1, or in the presence of a positive stimulant such as PMA plus Ca^{2+} ionophore. Although the ELISA used to detect GM-CSF was capable of detecting as little as 2.5 pg of GM-CSF per ml in the supernatants, none of the 48-h supernatants from the cultured T-cell fractions, whether incubated alone or with *C. neoformans*, had detectable levels of GM-CSF. Each T-cell fraction did produce GM-CSF when stimulated with PMA plus Ca^{2+} ionophore, with a range of 831 to 7,501 pg of GM-CSF/ml; the mean $\pm$ SEM was $4,596 \pm 952$ pg of GM-CSF/ml. These data indicate that the T cells do not constitutively produce GM-CSF when incubated in tissue culture medium for 48 h but can produce GM-CSF when appropriately stimulated. In contrast to the T cells, we found that the LGL fraction from all but one individual produced GM-CSF when incubated in medium alone. The amount of GM-CSF produced by individual LGL samples ranged from 21 to 480 pg/ml with a mean $\pm$ SEM of 164 ± 46 pg of GM-CSF/ml (Fig. 1A). The same LGL samples stimulated with PMA plus Ca^{2+} ionophore produced levels of GM-CSF ranging from 2,341 to 34,913 pg of GM-CSF/ml with a mean $\pm$ SEM of $12,517 \pm 3,175$ pg of GM-CSF/ml. With two exceptions, the LGL samples produced less GM-CSF over a 48-h period when incubated with *C. neoformans* cells (mean $\pm$ SEM = 80.9 ± 33 pg of GM-CSF/ml) than when incubated in medium alone (164 ± 46 pg of GM-CSF/ml; $P = 0.006$) (Fig. 1A).

The concentrations of GM-CSF produced constitutively by LGL varied greatly from individual to individual; therefore, to compare the inhibitory ability of *C. neoformans* in GM-CSF production among samples, we calculated the percent inhibition of GM-CSF production by *C. neoformans*. The data are shown in Fig. 1B. The percent inhibition of GM-CSF production by *C. neoformans* cells interacting with LGL for all but two samples was greater than 10%. There was considerable variation in the percent inhibition of GM-CSF among LGL samples (range, 5 to 100%). However, 10 of the 14 LGL samples incubated with *C. neoformans* showed reductions of 50% or more in GM-CSF production from the amount of GM-CSF produced constitutively. The mean reduction from constitutive

levels in GM-CSF production by the 14 different LGL samples in the presence of *C. neoformans* was $53\% \pm 0.27\%$.

***C. neoformans* does not adsorb or degrade GM-CSF in the culture medium over a 48-h period.** It seemed that the reduction of GM-CSF from control levels in the *C. neoformans*-LGL cocultures could be due to *C. neoformans* yeast cells adsorbing or degrading the GM-CSF after it was produced by the LGL. To assess this possibility, we incubated LGL from two different individuals in medium alone for 48 h and collected the supernatants. The 48-h cell-free LGL supernatants were then incubated with and without *C. neoformans* cells (5×10^6) for an additional 48 h at 37°C under 7% CO_2 before determining the levels of GM-CSF in the supernatants. The GM-CSF concentration in the LGL supernatant from the first individual incubated without *C. neoformans* for 48 h was 116 ± 7.4 pg/ml, and the concentration after incubation with *C. neoformans* under the same conditions was 116 ± 10 pg/ml. The LGL supernatant from the second individual contained 33 ± 4.3 pg of GM-CSF/ml when incubated alone for 48 h and 33.2 ± 8 pg of GM-CSF/ml when incubated with *C. neoformans* for 48 h. These data show that *C. neoformans* cells did not adsorb or degrade GM-CSF in the samples during the 48-h incubation period.

Reductions in GM-CSF from constitutive levels did not occur when the LGL were prevented from making contact with the *C. neoformans* cells. It is well established that NK and T cells must bind to *C. neoformans* cells to inhibit the growth of the organism (13, 28, 30), and so we investigated whether the LGL had to be in direct contact with the *C. neoformans* cells to observe the reduction in GM-CSF production. To test this, Transwell plates were used. In some wells, the cryptococcal cells were separated from the LGL by the membrane dividing the two chambers of the Transwell plate (Transwell LGL/Cn), and in other wells, the cryptococci were put into the same chamber with the LGL (LGL + Cn). We also incubated LGL alone for 48 h in the Transwell plates before collecting and analyzing the culture medium for GM-CSF by ELISA. We found that the GM-CSF concentrations in the medium from LGL-only cultures were similar to the GM-CSF concentrations in the medium from the Transwell LGL/Cn cultures (Fig. 2). In contrast, the concentrations of GM-CSF in the medium from the wells where LGL and cryptococci were incubated together in the same chamber (LGL + Cn) were significantly lower than

FIG. 2. Direct contact between LGL and *C. neoformans* cells is required for reduced GM-CSF production by LGL. LGL were cultured for 48 h in medium alone (LGL), with *C. neoformans* in the same compartment of the Transwell plate (LGL + Cn), or with *C. neoformans* separated by the Transwell plate membrane (Transwell LGL/Cn). The data shown are for LGL from one individual, but they are representative of data from three different experiments with LGL from different individuals for each experiment.

FIG. 3. Analysis of GM-CSF mRNA levels in LGL cultured with and without *C. neoformans*. (A) Representative Northern blot for GM-CSF mRNA in total cellular RNA obtained from LGL or T cells after an 18-h incubation without or with *C. neoformans*. (B) Relative density of GM-CSF mRNA with respect to 18S rRNA from two experiments with LGL from different individuals. (C) Representative Southern blot for GM-CSF after RT-PCR on total cellular RNA obtained from LGL or T cells incubated for 18 h without or with *C. neoformans*. (D) Relative density of GM-CSF with respect to HPRT RT-PCR products for each of the four individual LGL samples assayed.

the concentrations of GM-CSF in the medium from LGL alone or from Transwell LGL/Cn cultures ($P < 0.008$) (Fig. 2).

LGL cultured with *C. neoformans* cells had reduced levels of GM-CSF mRNA compared to LGL cultured alone. Once we were convinced by the data presented above that the direct association of *C. neoformans* with LGL was causing the LGL to produce less GM-CSF than the LGL produced in the absence of cryptococci, we next wanted to know if the LGL-*C. neoformans* interactions were affecting the GM-CSF mRNA levels in the LGL. We used two approaches to the determination of GM-CSF mRNA levels. First, we used Northern blot analysis with total RNA pooled from like samples from two or three blood donors. Second, we performed RT-PCR on total RNA from LGL or highly enriched NK cell fractions from individual donors. LGL, NK cells, or T cells were cultured for 18 h in the absence or presence of *C. neoformans* or PMA plus Ca^{2+} ionophore before the total RNA was extracted. In cases when we pooled RNA from similar samples, the pooling was done after the extraction of the total RNA.

Northern blot analysis demonstrated that there was less GM-CSF mRNA in LGL incubated with *C. neoformans* than in LGL incubated alone for 18 h (Fig. 3A). Density ratios of GM-CSF mRNA/18S rRNA from two different sets of samples (Fig. 3B) confirmed the visual pattern seen on the blots of LGL alone and LGL plus *C. neoformans* (Fig. 3A). T cells did not have detectable levels of GM-CSF mRNA after being cultured alone or with *C. neoformans*; however, large amounts of GM-CSF mRNA was present in T cells incubated for 18 h with PMA plus the Ca^{2+} ionophore (Fig. 3A). As with the T cells stimulated with PMA plus the Ca^{2+} ionophore, LGL and NK

cell-enriched populations stimulated with PMA plus the Ca^{2+} ionophore contained high levels of GM-CSF mRNA (data not shown). Attempts were made to isolate total RNA from *C. neoformans* cultured alone for 18 h by extraction with Tri-reagent and the same method used for isolation of total RNA from the LGL, T cells, and cocultures of *C. neoformans* with LGL or T cells. As can be seen in lane Cn in Fig. 3A, there was no detectable GM-CSF mRNA or 18S rRNA in the extracts from *C. neoformans* alone. We also were unable to detect HPRT mRNA in the extracts from the *C. neoformans* alone (data not shown). Consequently, the *C. neoformans* cells were not contributing RNA of significance to the RNA samples from the lymphocyte-*C. neoformans* cocultures.

Semiquantitative RT-PCR analysis on individual samples of RNA confirmed the results that we had obtained with Northern blot analysis. With samples from four different individuals, total RNA from LGL alone gave a greater amount of RT-PCR product that hybridized with the GM-CSF probe than the amount of RT-PCR product from the LGL cocultured with *C. neoformans* (Fig. 3C and D). A Southern blot containing one of the four samples is shown in Fig. 3C and is representative of the other samples. Density ratios of GM-CSF to HPRT for blots of RT-PCR products from the LGL samples obtained from the four different individuals and incubated without and with *C. neoformans* are shown in Fig. 3C. The data confirm the Northern blot results showing that LGL incubated in medium alone have more GM-CSF mRNA than do LGL incubated with *C. neoformans* cells (compare Fig. 3A and B with Fig. 3C and D). T cells incubated alone or with *C. neoformans* did not have detectable levels of GM-CSF mRNA

FIG. 4. Highly enriched NK cell populations constitutively produce GM-CSF and culture with *C. neoformans* reduces GM-CSF production. Cell samples highly enriched for NK cells from two different individuals were incubated for 48 h in medium alone or with the designated isolate of *C. neoformans*. (A) GM-CSF content of the supernatants were determined by specific ELISA. (B) Representative Southern blot for GM-CSF after RT-PCR on total cellular RNA from NK cells incubated for 18 h without or with the designated isolate of *C. neoformans*. (C) Relative density of GM-CSF with respect to HPRT RT-PCR products in NK cell samples from three different individuals.

(Fig. 3C, lanes T and T + Cn); however, T cells stimulated with PMA plus the Ca^{2+} ionophore had substantial amounts of GM-CSF mRNA (Fig. 3C, lane T + PMA/Ca). Total cellular RNA from LGL and NK cell-enriched populations stimulated with PMA plus the Ca^{2+} ionophore had levels of GM-CSF mRNA (data not shown) equivalent to those from T cells stimulated with PMA plus the Ca^{2+} ionophore (Fig. 3). Extraction of RNA from *C. neoformans* cells alone followed by RT-PCR by the same methods used for quantifying mRNA from LGL and T cells did not result in any product that would hybridize to the GM-CSF or the HPRT probes (Fig. 3C, lane Cn).

Our results obtained with LGL populations that were enriched for NK cells but still contained on average 50% T cells or with highly purified T-cell populations suggested that the NK cells, not the T cells, were the cells producing GM-CSF. However, it was not clear whether the T cells were contributing in some way to the reduction of GM-CSF production by NK cells stimulated with *C. neoformans*. To address this issue, we further enriched for NK cells, thereby reducing the T-cell contamination to 1.5 to 3% before repeating the experiments measuring GM-CSF production in the supernatants and GM-CSF mRNA after culture without and with *C. neoformans*. A reduction in the proportion of T cells from 57 to 73% to 1.5 to 3% in the NK-enriched fractions did not alter the ability of *C. neoformans* to down-regulate GM-CSF production (Fig. 4A) or to reduce the level of GM-CSF mRNA (Fig. 4B and C) in the NK cells. In these experiments with the highly purified NK cells, in addition to stimulating the NK cells with *C. neoformans* 184A, we stimulated the NK cells with a serotype D isolate, 3501. Isolate 3501 induced a reduction in GM-CSF production (Fig. 4A) and in the level of GM-CSF mRNA (Fig. 4B and C).

NK cells cultured with *C. neoformans* cells for 6 h produced less TNF- α and contained less TNF- α mRNA than did NK cells cultured alone. Having found that GM-CSF production was diminished after NK cells interact with *C. neoformans*, we

were interested in whether the interaction of NK cells with *C. neoformans* would affect the production of another cytokine. To determine if another cytokine is down-regulated in response to *C. neoformans*, we needed to measure the levels of a cytokine produced constitutively by NK cells. Since TNF- α is constitutively made by human NK cells, we measured TNF- α levels in supernatants at 6 h after incubating NK cells alone or with *C. neoformans* cells. As seen by the data in Fig. 5A, in 9 of the 10 different NK cell samples examined, there was less TNF- α in the supernatants from NK cells incubated with *C. neoformans* cells than in the supernatants from NK cells incubated in medium alone. NK cell fractions from three different individuals were incubated with and without *C. neoformans* prior to extracting total RNA and performing RT-PCR for TNF- α mRNA. The level of TNF- α mRNA was reduced between 21 to 44% in the three samples from *C. neoformans*-NK cell cocultures compared to that in samples from the same NK cells incubated without *C. neoformans* (Fig. 5B).

DISCUSSION

Our data show that the interaction of the pathogenic fungal target *C. neoformans* with freshly isolated human NK cells from most individuals results in the inhibition of two constitutively produced cytokines, GM-CSF and TNF- α . Modulation of NK cell cytokine production as a result of interactions with fungal target cells has been reported previously; however, in each case, cytokine production was augmented rather than reduced (3, 8, 23). For instance, *Candida albicans*, a fungal target, which binds to but is not killed by NK cells, stimulates human NK cells to produce increased amounts of GM-CSF and TNF- α (3, 8). *C. neoformans* cells have been shown to induce IFN- γ production in human nonadherent lymphocyte populations which contained NK cells (23), and we have found that NK cell fractions from some humans will produce IFN- γ upon stimulation with *C. neoformans* 184A (unpublished data).

FIG. 5. TNF- α production by highly enriched NK cells is inhibited by coculture with *C. neoformans*. (A) NK cell fractions isolated from 10 healthy donors were cultured without (NK) or with (NK + Cn) *C. neoformans* 184A for 6 h before the supernatants were removed and assayed for TNF- α . (B) Southern blot for TNF- α after RT-PCR on total cellular RNA obtained from purified NK cells incubated for 6 h without or with *C. neoformans*. (C) Relative density of TNF- α with respect to HPRT RT-PCR products for each of the three individual NK cell samples assayed. Results shown in panel B are representative of NK cells from three different donors.

Consequently, we were surprised with the results from our initial experiments showing that GM-CSF production was diminished in LGL samples from most individuals after incubation of the LGL with *C. neoformans*. However, after examining GM-CSF production by LGL and highly purified NK cell populations from numerous individuals at the protein and mRNA levels, we were convinced that direct interactions of NK cells with *C. neoformans* result in reduced production of GM-CSF.

The levels of GM-CSF produced by the LGL varied considerably from one individual to another. In spite of this variation, culturing a given population of LGL or highly enriched NK cell population with *C. neoformans* 184A consistently and significantly diminished GM-CSF production relative to the level of GM-CSF produced in the absence of *C. neoformans*. The reduction in GM-CSF production in the presence of *C. neoformans* could not be attributed to a loss of effector cell viability during the 48-h incubation period because 95% of the lymphocytes were viable at the end of the incubation period irrespective of whether *C. neoformans* cells were in the wells. The effector cells were not being inhibited by soluble products produced by the cryptococci, because GM-CSF was produced in the Transwell plates in wells where the NK cells were separated from the cryptococcal cells by a membrane that would allow the exchange of soluble materials (putative toxic products) between the *C. neoformans* chamber and the effector cell chamber. Furthermore, adsorption or degradation of GM-CSF by the cryptococcal cells could be ruled out by two sets of experimental evidence. First, supernatants taken from 48-h cultures of NK cells had similar levels of GM-CSF after being incubated in the absence or presence of *C. neoformans* cells for an additional 48 h. Second, when cryptococcal cells were separated from the NK cells in the Transwell plates by a membrane permeable to soluble components but not cells, there was no reduction in the amount of GM-CSF produced by the NK cells.

Direct contact of *C. neoformans* with NK cells is required for

the reduction in GM-CSF production to occur. This was shown by the fact that LGL incubated alone in the Transwell plates produced similar levels of GM-CSF to those produced by the same NK cell population incubated in Transwell plate wells in which the LGL were separated from the *C. neoformans* cells by a membrane. The LGL populations used in the Transwell plate studies were susceptible to having their GM-CSF production regulated, because when the same NK cells were incubated with *C. neoformans* cells on the same side of the membrane, GM-CSF production was reduced. The need for direct contact between NK cells and cryptococcal cells for down-regulation of GM-CSF production to occur is not surprising. Previous studies from our laboratory and others have shown that mouse and human NK cells bind to cryptococcal cells and that the binding interaction results in killing of the cryptococcal cell (11, 13, 15, 16, 22, 25, 27, 28). The binding of NK cells to cryptococcal cells has the potential to signal the NK cells to perform certain functions, such as exocytosis of granules (11, 13, 15, 16) and/or modulation of cytokine mRNA. Based on the results of the experiments presented here, we can conclude that inhibition of constitutively produced cytokines is another function stimulated by the binding of cryptococcal cells to NK cells.

Down-regulation of GM-CSF production by freshly isolated human NK cells has been reported to occur after NK cells interact with an NK cell-sensitive tumor target, K562 cells, but not after incubation with NK-resistant tumor targets (33). Our findings with NK cells and *C. neoformans* (NK cell-sensitive target), along with the findings of others with NK cells and *Candida albicans* (NK cell-resistant target) (3, 8), are in accord with the observations on NK cell cytokine production after stimulation of human NK cells with NK cell-sensitive and -resistant tumor targets (33). Our Northern blot and RT-PCR results show for the first time that the regulation of GM-CSF production is at the transcriptional level after NK cells bind to an NK cell-sensitive target. From the experiments presented here, we cannot determine whether the interactions of *C. neo-*

formans with NK cells affected the transcriptional regulation of the GM-CSF mRNA or altered the stability of the mRNA. The combined results from the relevant studies stimulate the speculation that NK cell-sensitive targets signal the NK cells to exocytose their granules and simultaneously signal an interruption in cytokine synthesis or blockage in cytokine synthesis in the NK cells conjugated to the target cells. In contrast, NK cell-resistant targets possibly bind to different receptors on NK cells than do NK cell-sensitive targets and therefore do not send the same signals to the NK cells that are being sent by the NK-sensitive target cells. These speculations will have to be evaluated in future investigations.

We were also interested in whether the interaction with the NK cell-sensitive fungal target, *C. neoformans*, would down-regulate the production of other cytokines by NK cells. Since it has been previously reported that interactions of NK cells with K562 target cells cause the NK cells to undergo functional inactivation and cause specific degradation of TNF α mRNA (2, 4), we expected the interactions of NK cells with the cryptococcal target cells to stimulate a reduction in TNF- α . As with GM-CSF production and as predicted by the studies from Brahmi's laboratory (2, 4), we found that interactions of NK cells with *C. neoformans* cells resulted in a reduction in TNF- α in the supernatants and in TNF- α mRNA in approximately 90% of the cases examined. Individual NK cell samples were variable in the amount of TNF- α that they made constitutively. Our findings suggest that the synthesis of cytokines which are being constitutively produced by NK cells at the time of the interaction of the NK cell with an NK cell-sensitive fungal target is likely to be down-regulated.

GM-CSF augments the ability of macrophages to kill *C. neoformans* (5, 6, 20), and TNF- α is required for induction of protection against *C. neoformans* (17). These facts, considered along with our findings that *C. neoformans* down-regulates NK cell production of GM-CSF and TNF- α , suggest that *C. neoformans* interactions with NK cells could have implications with respect to damping the clearance of the organism. One could speculate that in the microenvironment of *C. neoformans*-infected tissue, the presence of NK cells could be both detrimental and favorable to the organism. For instance, NK cells binding to cryptococcal cells could kill the attached organism (12, 14, 16, 22, 24, 28) but, at the same time, down-regulate the production of GM-CSF and TNF- α by the NK cells. Since GM-CSF extends the effector cell function of mature phagocytic cells (9), is an enhancer of antigen-presenting activity of monocytes (35), and is an activator of the antimicrobial activity of mature granulocytes and macrophages (9, 36), a reduction in levels of GM-CSF in the microenvironment at the site of infection very probably translates into a reduction in the ability of the neutrophils and macrophages to eliminate the cryptococci in the region. Considering that TNF- α is necessary for induction of the protective immune response against *C. neoformans*, the reduced levels of TNF- α in tissues where NK cells are interacting with *C. neoformans* could greatly compromise immune protective mechanisms. Such scenarios might give *C. neoformans* an advantage, allowing it to cause a more severe infection in the region. Reduction in GM-CSF production by NK cells after stimulation with *C. neoformans* was not unique to isolate 184A. The serotype D isolate 3501 also induced a reduction in GM-CSF production, suggesting this phenomenon may be induced by other *C. neoformans* isolates as well. Further studies are necessary to determine the relationship between the virulence of a cryptococcal isolate and the ability of the isolate to down-regulate GM-CSF and TNF- α production by NK cells from different individuals. Combined down-regulation of two NK cell-produced cytokines, GM-CSF

and TNF- α , which are important in protection against *C. neoformans*, could give the cryptococci the advantage and be detrimental to the host.

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