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Filarial Parasites Induce NK Cell Activation, Type 1 and Type 2 Cytokine Secretion, and Subsequent Apoptotic Cell Death

Subash Babu, Carla P. Blauvelt, and Thomas B. Nutman

NK cells are an important source of early cytokine production in a variety of intracellular viral, bacterial, and protozoan infections; however, the role of NK cells in extracellular parasitic infections such as filarial infections is not well-defined. To investigate the role of NK cells in filarial infections, we have used an in vitro model system of culturing live infective-stage larvae (L3) or live microfilariae (Mf) of *Brugia malayi*, a causative agent of human lymphatic filariasis, with PBMC of normal individuals. We found that NK cells undergo early cell activation and produce IFN-γ and TNF-α within 24 h after stimulation with both live L3 and Mf. Interestingly, NK cells also express IL-4 and IL-5 at this time point in response to live Mf but not L3. This is accompanied by significant alterations in NK cell expression of costimulatory molecules and natural cytotoxicity receptors. This activation is dependent on the presence of monocytes in the culture, IL-12, and direct contact with live parasites. The early activation event is subsequently followed by apoptosis of NK cells involving a caspase-dependent mechanism in response to live L3 but not live Mf. Thus, the NK cell-parasite interaction is complex, with filarial parasites inducing NK cell activation and cytokine secretion and finally NK cell apoptosis, which may provide an additional mechanism of down-regulating the host immune response. *The Journal of Immunology, 2007, 179: 2445–2456.*

Natural killer cells are an important component of the innate response to infections and tumors, and they have the ability to provide an early source of inflammatory and regulatory cytokines, and to lyse target cells (1). NK cells typically produce IFN-γ and TNF-α in intracellular infections. The onset of this cytokine production is rapid and occurs well before the onset of the adaptive immune response (2). NK cells have the ability to secrete type 2 cytokines including IL-4, IL-5, and IL-13 (3). In addition, NK cells use perforin- and granzyme-dependent cytolytic pathways to lyse infected or transformed target cells (2). Hence, they play a critical role in the initial defense mechanism against intracellular infections and tumors and also influence Th2 responses. Although the role of NK cells in viral, bacterial, and protozoan infections—and in malignancy—has been extensively delineated (4–8), very little is known about the role played by NK cells in extracellular parasitic infections.

*Wuchereria bancrofti* and *Brugia malayi* are causative agents of lymphatic filariasis, a disease afflicting over 129 million people worldwide. The larval and adult stages of filarial parasites reside in lymph nodes and lymphatics and microfilariae are present in the peripheral circulation. In view of recent data that human NK cells are found in abundance both in inflamed and noninflamed lymph nodes, and because of their presence in peripheral circulation (9, 10), characterization of NK cell interaction with lymph node dwelling or circulating filarial parasites assumes importance. In addition, because of their ability to respond rapidly by cytokine production, NK cells might be postulated to play a crucial early role in responding to this infection. In murine models of filarial infections, it has been demonstrated that NK cells expand in response to filarial infection in normal mice (11) and that they play a profound role in allowing development of adult worms in an immunodeficient environment (12). To study the role of NK cells in filarial infections, we have used an in vitro model of PBMC interaction with live infective-stage larvae (L3) or live microfilariae (Mf) of *B. malayi*. In this study, we have examined NK cell activation, cell surface receptor expression, cytokine production, and cell survival following stimulation with live L3 or live Mf. Our data demonstrate that *B. malayi* L3 and Mf rapidly induce activation of NK cells, alter costimulatory and natural cytotoxicity receptor expression, and induce cytokine production, with L3 inducing NK cell expression of IFN-γ and TNF-α, and Mf inducing NK cell expression of IFN-γ and TNF-α as well as IL-4 and IL-5. We also demonstrate that upon prolonged stimulation with live L3, NK cells undergo caspase-dependent apoptosis.

**Materials and Methods**

**Isolation of PBMC**

Buffy coats and leukopaks obtained from normal blood bank donors at the National Institutes of Health were used as a source of PBMC using a clinical protocol approved by the Institutional Review Board. PBMC were isolated by Ficoll diatrizoate gradient centrifugation (LSM; ICN Biomedicals). Erythrocytes were lysed using ACK lysis buffer (BioSource International). Cells were then washed and cultured in RPMI 1640 (BioWhittaker) supplemented with 20 mM glutamine (BioWhittaker), 10% heat-inactivated FCS (Harlan Bioproducts for Science), and 50 μg/ml gentamicin (Mediatech).

**Live L3 and Mf**

L3 and Mf were obtained from Dr. J. McCall (University of Georgia, Athens, GA). The L3 and Mf were repeatedly washed in RPMI 1640 with antibiotics and cultured at 37°C in 5% CO₂.

**In vitro culture**

Cells were cultured with live L3 (5–10/well) or live Mf (50,000/well) in 24-well tissue culture plates (Corning) at concentrations of 1 × 10⁶ cells/
The numbers of L3 and Mf added were based on previous studies showing optimal activation of PBMC (13). NK cells cultured in IL-2 (100 U/ml) and IL-12 (10 ng/ml; both from R&D Systems) were used as positive controls (14). For neutralization experiments, anti-human IL-12 Ab and mouse anti-human IgG1 isotype control Ab (both from R&D Systems) were used at concentrations of 10 μg/ml. For transwell experiments, 24-well tissue culture inserts were used.

**FIGURE 1.** Early NK cell activation following L3, Mf, and IL-2/IL-12 stimulation. A, Representative flow cytometric dot plot showing gating strategies for NK cell gating. *Left panel,* Lymphocyte gating from the PBMC population using the R1 gate. *Right panel,* Gating on conventional NK cells (R2 gate) using CD56<sup>-</sup>, CD3<sup>-</sup> cells. B, Representative histogram of CD69 and CD71 expression on NK cells (CD56<sup>+</sup>, CD3<sup>-</sup>) following 24-h exposure to live L3 (L3), live Mf (Mf), or IL-2+IL-12 compared with unstimulated (UN) and isotype (Iso) controls. C, Percentage of NK cells expressing CD69 and CD71 in response to live L3, live Mf, or IL-2+IL-12 compared with unstimulated controls. The results are expressed as box plots with the horizontal lines representing the 25th, 50th, and 75th percentiles and the vertical lines representing the 10th and 90th percentiles of the data. Values of p were calculated using the Wilcoxon signed rank test and an asterisk (*) indicates a p value <0.05.
plates containing 3-μm pore size polycarbonate filters on transwells (Corning) were used. Purification of NK cells and depletion of accessory cells CD56+ NK cells were negatively selected by column purification using the NK cell-negative selection kit (Miltenyi Biotec). The NK cells were >96% pure in all our experiments as estimated by flow cytometry. Depletion of B cells or monocytes from PBMC was performed using negative selection kits for monocytes (CD14 beads) or B cells (CD19 beads; Miltenyi Biotec).

Reagents for flow cytometry
Ab used for surface and intracellular flow cytometry were obtained from BD Pharmingen/BD Biosciences except PE-labeled ICOS (eBioscience) and PE-labeled NKp30, NKp44, and NKp46 (Beckman Coulter). The other Abs used in the study were as follows: FITC-labeled anti-human CD69, CD71, and CD56; PE-labeled anti-human CD56, CD94, CD158α, CD158β, CXCR1, CXCR2, CXCR3, CXCR4, CXCR5, CCR6, CCR1, CCR2, CCR3, CCR4, CCR5, CCR6, CCR7, CCR9, CXCR4, 4-1BB (CD137), IFN-γ, TNF-α, IL-8, IL-4, IL-5, and IL-13; and PerCP-labeled anti-human CD3.

Flow cytometry
After 24 h, cells were fixed in 4% paraformaldehyde and permeabilized in PBS/0.1% saponin for intracellular detection of cytokines. Staining of surface markers was done concurrently. Fluorescence was measured on a FACSCalibur (BD Biosciences) using 50,000 gated lymphocytes.

NK cell survival
NK cell survival was assessed by measuring the percentage of propidium iodide (PI)- and annexin V (BD Pharmingen)-positive cells after 72 h of stimulation with live L3 or live Mf. To assess the mechanism of apoptosis, 50 μM z-VAD-fmk (zVAD), a caspase inhibitor (Enzyme Systems Products) was added to wells 30 min to 1 h before addition of L3, zVAD was dissolved in DMSO (Fisher Scientific) and titration of doses ranging from 5 μM to 100 μM was done to assess maximum inhibition of apoptosis.

Statistical analysis
Comparisons were done using the nonparametric Wilcoxon signed rank test. All statistics were performed with StatView 5 (SAS Institute).
A

IFNγ

0.75
1.33
6.03
8.76
19.56

TNFα

0.44
0.58
2.29
3.47
22.43

IL-8

0.69
2.15
5.36
9.14
21.62

Iso
UN
L3
Mf
IL-2/IL-12

B

IFNγ

* * *

Percent NK cells

TNFα

* * *

IL-8

UN L3 Mf IL2+IL12
Results

NK cells are activated in response to live B. malayi L3 and Mf
PBMC were isolated from normal blood bank donors and stimulated for 24 h with live L3, live Mf, or with IL-2 and IL-12. The combination of IL-2 and IL-12 was used as a positive stimulus for activation of NK cells. Flow cytometry was done on cultured PBMC and lymphocytes were gated on the basis of forward and side scatter and NK cells on the basis of CD56 and CD3 expression (Fig. 1A). Expression of activation markers CD69 and CD71 on NK cells (CD56⁺, CD3⁻) was analyzed. As shown in a representative experiment (Fig. 1B), the expression of CD69 and CD71 was up-regulated in NK cells following 24 h of stimulation with L3 and Mf compared with unstimulated controls. This increase was statistically significant for both CD69 (p = 0.009 for L3, Mf, and IL-2 plus IL-12) and CD71 (p = 0.0472 for L3; p = 0.0163 for Mf and IL-2 plus IL-12) in a set of 10 normal individuals (Fig. 1C). These results indicate that NK cells from unexposed individuals undergo rapid activation following exposure to live L3 and Mf.

NK cell expression of costimulatory and natural cytotoxicity receptors

NK cells are known to express costimulatory receptors, whose expression is augmented following activation (15, 16). However, very little is known about the expression patterns of these receptors in infections. Hence, we examined the expression patterns of 4-1BB, OX-40, and ICOS on NK cells following 24 h stimulation with live L3, Mf, or IL-2 plus IL-12. As expected, the percent of NK cells expressing 4-1BB, OX-40, and ICOS was significantly

FIGURE 3. NK cell expression of IFN-γ, TNF-α, and IL-8. A, The percent of NK cells expressing IFN-γ, TNF-α, and IL-8 left unstimulated or following L3, Mf, or IL-2+IL-12 stimulation compared with isotype (Iso) controls depicted as a histogram in a representative individual. The numbers represent the percent of NK cells positive for the respective cytokine. B, The percent of NK cells expressing IFN-γ, TNF-α, and IL-8 summarized as box plots with the horizontal lines representing the 25th, 50th, and 75th percentiles and the vertical lines representing the 10th and 90th percentiles of the data (n = 10). Values of p were calculated using the Wilcoxon signed rank test and an asterisk (*) indicates a p value <0.05.

FIGURE 4. NK cell expression of IL-4 and IL-5. A, The percent of NK cells expressing IL-4 and IL-5 left unstimulated or following L3, Mf, or IL-2+IL-12 stimulation compared with isotype (Iso) controls depicted as a histogram in a representative individual. The numbers represent the percent of NK cells positive for the respective cytokine. B, The percent of NK cells expressing IL-4 and IL-5 summarized as box plots with the horizontal lines representing the 25th, 50th, and 75th percentiles and the vertical lines representing the 10th and 90th percentiles of the data (n = 10). Values of p were calculated using the Wilcoxon signed rank test and an asterisk (*) indicates a p value <0.05.
increased in response to IL-2 plus IL-12. In addition, the percent of NK cells expressing 4-1BB \((p = 0.0018)\), OX-40 \((p = 0.017)\), and ICOS \((p = 0.018)\) was significantly increased in response to live L3, while live Mf induced significant up-regulation of only ICOS-expressing NK cells \((p = 0.0499)\) (Fig. 2A). NK cell activation is associated with increased expression of natural cytotoxicity receptors (e.g., NKp30, NKp44, and NKp46), known to be involved in receptor-ligand mediated target cell killing \((16)\). Upon stimulation of 10 donor PBMC with live L3, live Mf, or IL-2 and IL-12, we observed that the frequency of NK cells expressing NKp44 \((p = 0.0117)\) and NKp46 \((p = 0.0357)\), but not NKp30, was significantly increased upon stimulation with live L3 compared with parasite-unstimulated NK cells. Mf had no effect on NKp30, p44, or p46 receptor expression (Fig. 2B).

**NK cell expression of inhibitory receptors and chemokine receptors**

Because NK cell subsets expressing inhibitory receptors have been reported to be decreased in murine models of filarial infection \((9)\), we examined the response of NK cells expressing the inhibitory receptors CD158a, CD158b, and CD94 to infective-stage larvae. Upon stimulation with live L3, we found no significant alterations \((p > 0.05)\) in the frequency of NK cells expressing CD158a, CD158b, and CD94 in normal individuals \((n = 9)\) compared with L3-unstimulated NK cells (data not shown). Expression of chemokine receptors is pivotal in determining the ability of NK cells to migrate to the site of infection \((17)\). We, thus, examined the expression of a panel of chemokine receptors in response to L3 stimulation. NK cells were found to express CXCR1, CXCR3, CXCR4, CCR3, CCR6, and CCR7 at baseline. No significant alterations in the frequency of NK cells expressing the above chemokine receptors were noted upon stimulation with L3 in normal individuals \((n = 6)\) (data not shown). Little or no expression of CXCR2, CXCR5, CXCR6, CCR1, CCR2, CCR4, CCR5, and CCR9 was found on the surface of NK cells in these same individuals (data not shown).

**Live parasites rapidly induce NK cell expression of IFN-γ and TNF-α**

To assess whether the activation of NK cells (based on cell surface marker expression) translates into a functional response, we measured cytokine production in NK cells following exposure to live L3 and Mf. We measured the frequency of NK cells expressing IFN-γ, TNF-α, and IL-8 by intracellular cytokine flow cytometry \((n = 10)\). As shown in a representative experiment in Fig. 3A, there was an increase in the frequency of NK cells expressing IFN-γ and TNF-α 24 h following stimulation with live L3 and Mf as well as IL-2 plus IL-12. This increase is statistically significant for both IFN-γ \((p = 0.0283\) for L3 and \(p = 0.009\) for Mf and IL-2 plus IL-12) and TNF-α \((p = 0.0283\) for all) as shown in Fig. 3B \((n = 10)\), with there being a geometric mean increase by 3.6-fold for IFN-γ and 6.5-fold for TNF-α in response to live L3; and 5.1-fold for IFN-γ and 6-fold for TNF-α in response to live Mf. Thus, both live L3 and live Mf rapidly induce NK cell differentiation toward type 1 cytokine-expressing cells. IL-2 plus IL-12 stimulation induced significant NK cell production of both IFN-γ and TNF-α in all individuals.

**Live Mf but not live L3 rapidly induce NK cell expression of IL-4 and IL-5**

Patent filarial infections are typically associated with IL-4 and IL-5 dominant responses. Thus, we wanted to determine whether exposure to live filarial parasites would induce differentiation of NK cells into IL-4- and IL-5-expressing NK cells. Indeed, upon 24 h exposure to live Mf but not live L3, the percent of NK cells expressing both IL-4 and IL-5 is augmented as shown in a representative histogram in Fig. 4A. This increase is statistically significant for both IL-4 and IL-5 \((p = 0.0163\) for both) as shown in Fig. 4B \((n = 5)\), with a geometric mean fold increase of 4.2 for IL-4 and 6.9 for IL-5. Thus, the blood-stage Mf but not the early tissue stage L3 are capable of inducing NK cell differentiation into a type 2 cytokine-expressing phenotype.

**Purified NK cells and NK cells separated by transwell do not undergo activation and cytokine expression**

To determine whether the NK cells are interacting with live parasites directly, purified NK cells from PBMC \((n = 5)\) using negative isolation were cultured with live L3 or live Mf in the absence of other cell types. No significant alteration in the surface expression of CD69 or CD71 or in the intracellular expression of IFN-γ or TNF-α was noted, indicating that NK cells require the presence of accessory cells for activation and type 1 cytokine secretion (Fig. 5A). To determine whether direct contact with the parasite was necessary for activation of NK cells, we separated the live L3 or live Mf from PBMC \((n = 5)\) using a transwell system and measured the expression of CD69, CD71, IFN-γ, and TNF-α. The separation of PBMC from live L3 and live Mf led to a significant reduction in the induction of CD69 \((p = 0.0431\) for L3 and 0.0464 for Mf), CD71 \((p = 0.0431\) and 0.0464), IFN-γ \((p = 0.0431\) and 0.0499), and TNF-α \((p = 0.0464\) for both) expression, indicating that proximal contact with live L3 or live Mf was necessary for maximal NK cell activation and cytokine expression (Fig. 5B).

**NK cells require IL-12 or the presence of monocytes in culture for live L3-induced activation and cytokine expression**

Because NK cells require accessory cells in culture for live parasite-induced activation and because IL-12 is a potent activator of NK cells, we hypothesized that accessory cell-induced IL-12 might be required for responses to live parasites. To determine whether IL-12 was required for NK cell activation in response to live L3 or Mf, PBMC \((n = 5)\) were cultured with live L3 or live Mf in the presence of neutralizing anti-IL-12 Ab or isotype control. Although isotype control Ab had no effect on the increased expression of CD69 \((p = 0.0431\) for both L3 and Mf) or CD71 \((p = 0.0431\) for both) on the intracellular expression of IFN-γ \((p = 0.0431\) for both) or TNF-α \((p = 0.0431\) for both), anti-IL-12 Ab abolished the L3- or Mf-induced activation and cytokine expression of NK cells (Fig. 6A). To determine the specific cell subset in the accessory cell population necessary for activation of NK cells, we cultured live L3 with PBMC depleted of monocytes or B cells \((n = 5)\) and measured the expression of CD69, CD71, IFN-γ, and TNF-α. Although depletion of B cells had no effect on activation marker or cytokine expression, depletion of monocytes led to a significant reduction in the induction of CD69 \((p = 0.0431\),
FIGURE 6. NK cells require IL-12 or monocytes in culture for activation and cytokine expression in response to live L3 and Mf. A, The percent of NK cells expressing CD69, CD71, IFN-γ, and TNF-α following stimulation with live L3 (L3) or live Mf (Mf) compared with unstimulated controls (UN) in the presence of anti-IL12 Ab (IL) or isotype control (C). B, The percent of NK expressing CD69, CD71, IFN-γ, and TNF-α in response to live L3 following stimulation with B cell-depleted (B cell-) or monocyte-depleted (Mono-) PBMC. The results are expressed as box plots with the horizontal lines representing the 25th, 50th, and 75th percentiles and the vertical lines representing the 10th and 90th percentiles of the data (n = 5). Values of p were calculated using the Wilcoxon signed rank test and an asterisk (*) indicates a p value <0.05.
CD71 \( (p = 0.0431) \), IFN-\( \gamma \) \( (p = 0.0431) \), and TNF-\( \alpha \) \( (p = 0.0431) \) expression, indicating that monocytes are the accessory cell type necessary for activation of NK cells (Fig. 6B).

**Figure 7.** NK cells undergo apoptosis following 72-h stimulation with live L3. A. The percent of NK cells expressing annexin V following stimulation with L3, Mf, or L3+zVAD depicted as a histogram in a representative individual. B. The percent of NK cells expressing annexin V following 72-h exposure to live L3 (L3) (left panel) or live Mf (middle panel) compared with unstimulated controls (UN) and the percent of NK cells expressing annexin V in the presence (L3+zVAD) or absence (L3) of zVAD following 72-h stimulation with live L3 (right panel). Each line represents a single individual \( (n = 10) \).

**Discussion**

NK cell responses in helminth infections have not been well-delineated. Increased NK cell activity has been reported in human onchocerciasis (18) and trichinellosis (19) and following diethylcarbamazine treatment in lymphatic filariasis (20). Recently, a secreted protein from the human hookworm *Necator americanus* was found to bind NK cells directly and induce IFN-\( \gamma \) production (21). In rodent models of *Trichuris muris* (22), *Schistosoma mansoni* (23), and *Fasciola hepatica* (24), no significant role for NK cells in host immunity has been detected. In a mouse model of *Litomosoides sigmodontis* infection, NK cells were found to be important in host defense (9), while in a SCID model of *B. malayi* infection, NK cells were found to be essential for worm development (10). We have

NK cells exhibit apoptosis following stimulation with live L3 at 72 h

To examine the effect of prolonged exposure to live L3 and Mf, we cultured NK cells with live L3 or Mf for 72 h. At this time point, we found a significant attrition in cell numbers in cultures with live L3 but not in cultures with live Mf or medium controls. Upon staining with PI, we observed an increase in the percentage of NK cells (5.8-fold average) staining positive for PI \( (p = 0.005; n = 10) \). To determine the mechanism of NK cell death, we stained NK cells with annexin V and a representative histogram is shown in Fig. 7A depicting the increase in annexin V staining following culture with live L3 but not with live Mf. A significantly increased percentage of NK cells (3.5-fold average) expressing annexin V \( (p = 0.005; n = 10) \) was found following culture with live L3 but not live Mf indicating that NK cells were undergoing apoptotic cell death (Fig. 7B).

To determine whether the apoptotic cell death was a caspase-mediated event, we added a caspase inhibitor, zVAD, to cells cultured with live L3 at a dose titrated to induce maximal response and found that the frequency of NK cells expressing annexin V was significantly lower \( (p = 0.005) \) in the presence of zVAD, indicating that live L3 induce a caspase-dependent NK cell apoptosis (Fig. 7B).

To determine whether the apoptotic cell death was a caspase-mediated event, we added a caspase inhibitor, zVAD, to cells cultured with live L3 at a dose titrated to induce maximal response and found that the frequency of NK cells expressing annexin V was significantly lower \( (p = 0.005) \) in the presence of zVAD, indicating that live L3 induce a caspase-dependent NK cell apoptosis (Fig. 7B).
NK cell responses to live filariae therefore tried to elucidate the role of NK cells in filarial infections using an in vitro culture system of the human filarial parasite *B. malayi* with PBMC of normal individuals. Because NK cell cytolytic mechanisms would have little, if any, effect on this tissue-dwelling nematode, we have primarily focused on cytokine secretion from NK cells. We have previously shown that the early immune response to live L3 of *B. malayi* in normal blood bank donors is dominated by a proinflammatory Th1 response (13). This response is unlike the chronic immune response observed in infected individuals in an endemic area, who manifest predominantly a Th2 and or regulatory T cell response to parasite Ag (25). In this study, we show that NK cells respond early to live filarial parasites by undergoing up-regulation of activation markers and increased type 1 and/or type 2 cytokine production but subsequently undergo apoptotic cell death.

NK cells are an important component of the rapid-response repertoire of the innate immune system and, as such, respond early to inflammatory stimuli. Using our in vitro model system of culturing PBMC with live parasites, we examined the effect of two different life cycle stages of the filarial parasites on NK cell expression of activating, costimulatory, natural cytotoxicity, inhibitory, and chemokine receptors. Both live L3 and live Mf induce activation of NK cells as manifested by up-regulation of CD69 and CD71; however, live L3, but not live Mf, induced significant effects on the expression of costimulatory and natural cytotoxicity receptors. Very little is known about the expression patterns of costimulatory receptors on NK cells in infectious disease (26). Our findings that the expression of two members of the TNFR family—4-1BB and OX-40—and one member of the B7 family—ICOS—is significantly up-regulated by stimulation with live L3 indicate that augmentation of costimulatory receptor expression is a component of the NK cell activation response to parasite stimulation and could help regulate cytokine secretion. The function of NK cells is regulated by a series of activating or inhibitory receptors. The major activating/trigging receptors are NKp30, NKp44, and NKp46, which are primarily expressed on cells upon activation, mediate cytotoxicity of target cells but also modulate cytokine secretion and apoptosis of NK cells (27). NK cell expression of NKp44 and NKp46 is up-regulated in response to live parasites indicating a potential role for these receptors in NK cell cytokine secretion, survival, and host defense. However, no significant effect was observed on the inhibitory receptors examined, CD158a, CD158b, and CD94. CD158a and CD158b belong to the killer cell Ig-like receptor superfamily, which primarily recognizes HLA-A, -B, and -C (27). These receptors are predominantly involved in NK cytolytic function and not in cytokine secretion; hence, the lack of regulation of these receptors is not surprising. These results are in contrast to the results from an experimental murine infection of *L. sigmodontis*, in which a significant reduction in NK cells expressing inhibitory receptors of the Ly49 superfamily was noted (9). The differences seen between this study and the present study could be related to the marked differences in the host system or parasite species or both. Interestingly, we did not observe any significant alteration in the expression of chemokine receptors by live L3 indicating that regulation of chemokine receptor expression is not absolutely necessary for NK cell responses to inflammatory stimuli.

Classically, NK cell activation was considered to be exclusively dependent on the integration of signals derived from activating and inhibitory receptors upon interaction with ligands on target cells. However, it is becoming increasingly clear that NK cells require signals from accessory cells to respond to pathogens (reviewed in Ref. 4). Accessory cells usually become activated by direct recognition of pathogen-associated molecular patterns and provide both contact-dependent and soluble signals to activate NK cells. Thus, NK cells are known to require dendritic cells, macrophages, or monocytes for activation and cytokine secretion in response to viruses (murine CMV (28), HSV (29), influenza (30)); bacteria (*Listeria monocytogenes* (31), *Staphylococcus aureus* (32), *Mycobacterium bovis* (33)); and parasites (*Plasmodium falciparum* (34), *Trypanosoma cruzi* (35), *Toxoplasma gondii* (36), *Leishmania major* (37)). The accessory cells then serve as a source of soluble and contact-dependent signals to activate NK cells. The most common soluble signals secreted by accessory cells include IL-12 (as evidenced by studies in murine CMV (28), influenza virus (30), *L. major* (37), *P. falciparum* (34), *S. aureus* (32), and *M. bovis* (33)) and type 1 IFNs (important in most viral infections (4)). We investigated the requirements for activation of NK cells by examining whether NK cells can directly interact with the parasites in isolation and whether NK cells (and accessory cells) need to be in proximate contact with live parasites. To address the first issue, we purified NK cells and stimulated them with live filarial parasites and found no significant induction of cytokines in the absence of accessory cells or exogenous type 1 cytokines. To address the second issue, we used transwells to separate PBMC from the live L3 and observed an abolition of induction of cytokines, indicating a requirement for direct contact of NK cells (and accessory cells) with live L3. This finding is in agreement with our earlier report on proinflammatory cytokine production of T cells requiring direct contact with live L3 (13).

We then wanted to identify the specific cell subset and the exogenous cytokines required for NK cell activation. We addressed the first question by depleting either B cells or monocytes (the two major classes of APC in PBMC) and observed that monocytes were required for optimal activation of NK cells. This fits in well with the emerging paradigm that accessory APC interaction with NK cells is a necessary requisite for NK cell activation in response to pathogens. We addressed the cytokine requirement for NK cell activation by neutralizing IL-12 and demonstrating the suppression of NK cell activation in response to live parasites in the absence of IL-12. Thus, we have delineated a model system in which live parasites interact initially with monocytes in a contact-dependent manner to induce IL-12 secretion. Monocytes then interact with NK cells by IL-12 and perhaps other cytokines to induce optimal activation. In this context, it is interesting to note that live L3, but not live Mf, also has a major impact on costimulatory molecules of NK cells indicating that perhaps NK cell-monocyte costimulatory receptor-ligand interaction could contribute additional effects to the L3 interaction.

The most significant finding in our studies was the observation of the ability of live filarial parasites to induce distinct NK cell cytokine secretion patterns. Human NK cells are thought to differentiate into a NK1 or NK2 phenotype analogous to the Th1/Th2 phenotype for T cells, based on their cytokine expression pattern. NK cells that secrete IFN-γ and TNF-α are thought to be NK1 cells, while NK cells that produce IL-4, IL-5, and IL-13 have recently been classified as NK2 cells (3). In our model, both live L3 and live Mf induced IFN-γ and TNF-α secretion, a finding in concordance with a recent report on NK cell induction of IFN-γ by another human helminth, *Necator americanus* (21). Because IFN-γ and TNF-α are known inducers of Nos2 in macrophages, resulting in the production of NO (38), and because NO has been shown to be an important component of the innate defense mechanism against filarial parasites both in vivo and in vitro (39, 40), NK cells might be important in early host defense. Thus, the early
NK cell response to live filarial parasites might facilitate the development of host-resistance mechanisms, which are crucial in the prevention of establishment of infection.

More interestingly, live Mf induces significant production of IL-4 and IL-5 from NK cells. Patent filarial infections (harboring MF) are associated with increased early production of IL-4, a prototypical Th2 cytokine (25). However, the early source of IL-4 in filarial infections is not clear. CD4⁺ T cells, basophils, and NKT cells have all been postulated to play a role in early IL-4 production (41, 42). We provide a new insight to this phenomenon by showing evidence that NK cells could be potential players in the establishment of Th2 responses in patent filarial infections and by extension, Th2 responses in general. Thus, in addition to NKT cells, conventional NK cells might be crucially important cells in the Th2 differentiation in humans. We are currently pursuing the identification of parasite product(s) inducing IL-4 secretion as well as the accessory requirements for IL-4 secretion in human NK cells. Although IL-12, accessory cells, and direct contact are clearly required for MF-induced NK cell activation and type 1 cytokine secretion, it would be interesting to elucidate the requirements for type 2 cytokine secretion. In addition, NK cells also exhibit the propensity to express IL-5 in response to live MF. This suggests that NK cells are a potentially novel source of IL-5 in filarial infections in addition to CD4⁺ T cells and eosinophils (25). The ability of live MF but not live L3 to induce NK2 responses is in concordance with our previous data showing that live L3 predominantly induce proinflammatory Th1 responses from PBMC of unexposed individuals (13). Our data therefore highlight a new role in the propensity to express IL-5 in response to live MF. This suggests that NK cells are a potentially novel source of IL-5 in filarial infections and this could potentially have broader significance in Th2-associated infections and other processes. In addition, the extensive interindividual variation in the NK cell responses, especially NK cell expression of IFN-γ and IL-4, could also contribute to the heterogeneity in clinical manifestations of lymphatic filariasis and hence, the study of the NK cell responses in filarial-infected individuals will be important.

When we extended our analyses to a 72-h time point, we observed a general decrease in cell numbers in cultures with live L3 compared with the medium-alone wells. This effect is not manifest in cultures with live MF. The decrease in NK cell numbers estimated by PI staining appeared to be through apoptosis, as annexin V staining was markedly increased following L3 exposure. More importantly, zVAD, a pan-caspase inhibitor (43), significantly but importantly not completely abolished the induction of cell death, suggesting that caspases—especially caspase-1 and -3—are involved, at least partially in this apoptotic process. This finding adds another dimension to the recent reports demonstrating that filarial parasites can induce cell death in different arms of the immune system (dendritic cells and T cells) (44, 45), thereby facilitating parasite evasion from deleterious host immune responses. Moreover, NK cell up-regulation of natural cytotoxicity and/or costimulatory receptors might contribute to the parasitic modulation of NK cell survival/apoptosis as has been demonstrated in tumor models.

Thus, our studies reveal a role for NK cells in the early immune response to filarial parasites involving both type 1 and type 2 cytokine secretion. This early response is followed by attrition in NK cell numbers through a caspase-dependent apoptosis mechanism, indicating not only a complex interplay between host and parasite, but more importantly that NK cells might play a critical role in determining the outcome of infection in lymphatic filariasis by altering the milieu in which early events shape the T cell subset differentiation pathways.

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Disclosures

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