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Proinflammatory Cytokines Dominate the Early Immune Response to Filarial Parasites

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Although the early human immune response to the infective-stage larvae (L3) of Brugia malayi has not been well-characterized in vivo (because of the inability to determine the precise time of infection), the consensus has been that it must involve a predominant Th2 environment. We have set up an in vitro system to study this early immune response by culturing PBMC from unexposed individuals with live L3 of B. malayi. After 24 h of culture, T cell responses were examined by flow cytometry and by quantitative real-time RT-PCR for multiple cytokines. T cells were activated early following exposure to L3 as indicated by up-regulation of surface markers CD69 and CD71. The frequency of T cells expressing proinflammatory Th1 cytokines (IFN-γ, TNF-α, GM-CSF, IL-1α, and IL-8) but not Th2 cytokines (IL-4, IL-5, IL-6, IL-10, and IL-13) was significantly increased in response to L3. This T cell response occurred in both the CD4 and CD8 T cell compartment and was restricted to the effector/memory pool (CD45RO+). This T cell response was not due to LPS activity from the parasite or from its endosymbiont, Wollbachia; moreover, it required the presence of APC as well as direct contact with live L3. Real-time RT-PCR analysis of multiple cytokines in the T cells confirmed the increased expression of proinflammatory Th1 cytokines. Up-regulation of these cytokines suggests that the primary immune response to the live infective stage of the parasite is not predominantly Th2 in nature but rather dominated by a proinflammatory response. The Journal of Immunology, 2003, 171: 6723–6732.
Live L3 and L3 Ag

*B. malayi* live L3 isolated from infected *Aedes aegypti* mosquitoes were provided by Dr. J. McCall (University of Georgia, Athens, GA). The L3 were then incubated in a six-well plate (Corning, Acton, MA) in RPMI 1640 plus penicillin/streptomycin/amphotericin B, 200 mM L-glutamine, and gentamicin for 4 h. For that 37° C, Polymyxin B was obtained from Sigma-Aldrich (St. Louis, MO). Bacterial carbonate 24-well tissue culture plates (Corning) at concentrations of 100,000 cells/well. The Ab used in the study were as follows: FITC-labeled anti-human CD69, CD71, CD45RA, and CD4; PE-labeled anti-human CD25, CD62 ligand (CD62L), CD3, IFN-γ, TNF-α, GM-CSF, IL-1α, IL-8, IL-4, IL-5, IL-6, IL-10, and IL-13; PerCP-labeled anti-human CD3 and CD8; PE-Cy5-labeled anti-human CD45RO; and allophycocyanin-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, San Jose, CA). Total RNA was extracted according to the manufacturer’s protocol (RNaseasy Mini Kit; Qiagen), and RNA was dissolved in 50 μl of RNase-free water.

cDNA synthesis

RNA (1 μg) was used to generate cDNA using TaqMan Reverse Transcription Reagents according to the manufacturer’s protocol (Applied Biosystems, Foster City, CA). Briefly, random hexamers were used to prime RNA samples for reverse transcription using MultiScribe Reverse Transcriptase.

Real-time PCR

Real-time quantitative PCR was performed in an ABI 7700 sequence detection system using the TaqMan Cytokine Gene Expression Plate 1 (Applied Biosystems). This plate consists of a MicroAmp Optical 96-well reaction plate arranged into 12 columns, one for each cytokine in the assay. Each column is made up of eight identical wells containing TaqMan primers and probes for one human cytokine mRNA and an 18S ribosomal endogenous control. The system uses two dye layers to detect the presence of target and control sequences. The TaqMan dye layer yields the results for quantification of the cytokine target mRNA, and the VIC dye layer yields the results for quantification of the 18S ribosomal RNA endogenous control. The cytokine targets examined were IL-1α, IL-1β, IL-2, IL-4, IL-5, IL-8, IL-10, IFN-γ, and TNF-α. Reaction mixtures for the real-time PCR had a final volume of 50 μl consisting of 5 μl of cDNA and 25 μl of the master mix. Amplification conditions were: min at 50°C, 10 min at 95°C and 40 cycles of 15 s at 95°C and 1 min at 60°C. The endpoint used in real-time PCR quantification, CT, is defined as the PCR cycle number that crosses the signal threshold. CT values range from 0 to 40, with the latter number assumed to represent no product formation. Quantification of gene expression was performed using the comparative CT method (Sequence Detector User Bulletin 2; Applied Biosystems) and reported as the fold difference relative to the housekeeping gene. To calculate the fold change (increase or decrease), the CT of the housekeeping gene (18S RNA) was subtracted from the CT of the target gene to yield the ΔCT. Change in expression of the normalized target gene as a result of an experimental manipulation (L3 exposure) was expressed as 2^ΔΔCT where ΔΔCT = ΔCT samples − ΔCT controls.

**Statistical analysis**

Comparisons were done using the nonparametric Wilcoxon signed rank test. All statistics were performed with StatView 5 (SAS Institute, Cary, NC).

**Results**

Early activation of T cells following exposure to live L3 and L3 Ag

PBMC were isolated from normal blood bank donors and cultured in the presence of live L3 or L3 Ag. Twenty-four hours following exposure, the PBMC were stained with mAb against CD3 and activation markers CD69, CD71, CD25, and CD62L and analyzed by flow cytometry. Analysis of CD3+ cells in a set of five individuals revealed an increase in the frequency of T cells expressing the markers CD69 and CD71 at this early time point following exposure to both live L3 and L3 Ag when compared with those in unstimulated controls (Fig. 1). No difference was observed in the frequency of T cells expressing CD25 or CD62L (data not shown). Up-regulation of surface expression of CD69 and CD71 indicates that both live L3 and L3 Ag are capable of activating T cells from unexposed individuals.

Proinflammatory Th1 cytokines dominate the early T cell response to live L3

To examine whether this early T cell activation translates into a functional response, the frequency of Th1 (IFN-γ, TNF-α, GM-CSF, IL-8, and IL-1α) and Th2 (IL-4, IL-5, IL-6, IL-10, and IL-13) -related cytokine-expressing T cells was assessed 24 h following incubation with live L3 or L3 Ag in 12–15 normal blood bank donors (Fig. 2).

As seen in a representative experiment (Fig. 2A), there was an increase in the frequency of T cells expressing IFN-γ, TNF-α, GM-CSF, IL-8, IL-1α, and IL-6 but not IL-4, IL-5, IL-10, and IL-13. Analysis of the response in each of the 12–15 individuals indicates that the frequency of T cell expression of IFN-γ (p = 0.005), TNF-α (p = 0.003), GM-CSF (p = 0.007), IL-1α (p = 0.028), and IL-8 (p = 0.014) was significantly increased following exposure to live L3 (Fig. 2B), whereas those of the Th2-related cytokines including IL-4 (p = 0.67), IL-5 (p = 0.23), IL-6 (p = 0.14), IL-10 (p = 0.06), and IL-13 (p = 0.13) was not altered (Fig. 2B). We also examined the intracellular expression of RANTES and MIP-1α in T cells following exposure to live L3 but were unable to detect their expression (data not shown).

Of interest, while both live L3 and L3 Ag up-regulated surface expression of activation markers CD69 and CD71 on T cells, L3 Ag had no effect on cytokine-expression frequencies (data not shown). Thus, it appears that the presence of active, live L3 and/or
the release of excretory/secretory Ag is essential for induction of cytokines in T cells.

**Both CD4 and CD8 T cells express proinflammatory cytokines**

Because both CD4 and CD8 T cells are known sources of proinflammatory Th1 cytokines (17), we assessed which of these subsets was responsible for the increased frequency of expression of cytokines following stimulation with the live nematode larvae. Twenty-four hours following PBMC exposure to live L3, CD4 and CD8 T cells were examined for expression of IFN-γ, TNF-α, GM-CSF, IL-8, IL-1α, IL-4, IL-5, IL-6, IL-10, and IL-13. Both CD4 and CD8 T cells showed a significant increase in expression of IFN-γ, TNF-α, GM-CSF, IL-1α, and IL-8 in response to live L3 (Fig. 3). No significant alteration in the expression of IL-4, IL-5, IL-6, IL-10, and IL-13 was observed (data not shown). These data indicate that efficient priming of both CD4 and CD8 T cells occurs early following exposure to live L3.

To examine whether proinflammatory cytokine induction occurred in the naive T cell (CD4⁺CD45RA⁺) compartment or the effector/memory (CD4⁺CD45RO⁺) compartment, coincident surface expression of CD4 and CD45RA or CD45RO and intracellular expression of various cytokines was assessed (Fig. 3B). As seen, expression of IFN-γ, TNF-α, GM-CSF, IL-1α, and IL-8 occurred only in the CD4⁺CD45RO⁺ subset and not in the CD4⁺CD45RA⁺ subset. Moreover, cytokine expression was not detected in the CD4⁺CD45RA⁺CD45RO⁺ subset, and we did not observe any increase in the number of CD4⁺CD45RO⁺ cells under L3-stimulated as compared with unstimulated conditions. Thus, the CD45RO⁺ T cell subset (effector/memory population) in these naive individuals is responsible for expression of Th1 cytokines.

**The induction of proinflammatory cytokines is not LPS-mediated**

_B. malayi_ is a filarial parasite that harbors the intracellular rickettsia-like bacterial endosymbiont Wolbachia (18). Because Ag extracts from different life-cycle stages of the parasite are known to stimulate proinflammatory cytokine release through an LPS-like effect of the endosymbiont (19), we cultured PBMC with live L3 in the presence or absence of 10 μg/ml polymyxin B, a known LPS inhibitor (20). Fig. 4A demonstrates that the presence of polymyxin B had no effect on induction of proinflammatory cytokines. As seen, a statistically significant increase in all the proinflammatory Th1 cytokines was detected in response to live filarial L3 in both the presence and absence of polymyxin B. In addition, when LPS was blocked using rBPI in cultures of live L3-exposed PBMC, the production of proinflammatory cytokines was not significantly inhibited compared with that in cultures of live L3-exposed PBMC without rBPI (21), as seen in Fig. 4B. Finally, because LPS or LPS-like molecules of Wolbachia exert their effect through TLR4 and/or TLR2 (22), we incubated PBMC with blocking Ab to TLR4 and TLR2 1 h before addition of live L3. TLR blockade had no inhibitory effect on L3 induction of proinflammatory cytokines (Fig. 4C).

**T cells require the presence of APC to express proinflammatory cytokines**

The rapid induction of cytokines in naive individuals raises the possibility that live L3 might exert a mitogenic (nonspecific) effect on T cells. By culturing purified T cells with live L3 alone or in the presence of APC, we have shown that APC are absolutely required for induction of T cell expression of IFN-γ (p = 0.027), TNF-α (p = 0.046), GM-CSF (p = 0.027), IL-1α (p = 0.046), and IL-8 (p = 0.046) (Fig. 5). In addition, we examined the role of TCR-MHC interaction in the proinflammatory response by blocking activation of T cells using an anti-HLA-DR mAb (23). Fig. 3 demonstrates that expression of IFN-γ (p = 0.0277), TNF-α (p = 0.0277), and IL-8 (p = 0.0277) was significantly inhibited in the presence of anti-HLA-DR Ab but not with isotype control Ab.
FIGURE 2. T cell expression of Th1- and Th2-related cytokines. A. One representative flow cytometry plot of CD3 expression on the x-axis and Th1-related cytokine on the y-axis in the unstimulated condition (upper panels) or following stimulation with L3 (lower panels). The numbers within the plots represent the percent of T cells positive for the respective cytokine. B. One representative flow cytometry plot of CD3 expression on the x-axis and Th2-related cytokine on the y-axis in the unstimulated condition (upper panels) or following stimulation with L3 (lower panels). The numbers within the plot represent the percent of T cells positive for the respective cytokine. C. The percent of T cells expressing the different cytokines in the unstimulated condition (Un) or following stimulation with L3 (L3). Each line represents a single individual (n = 12-15).
Thus, induction of proinflammatory cytokines requires Ag presentation on MHC class II molecules and is not a TCR-independent process.

**T cell expression of cytokines requires physical contact with live L3**

Either the excretory/secretory Ag actively synthesized by live L3 and/or physical contact with live L3 could be responsible for activating T cells. To distinguish between these possibilities, we used 3-μM pore filters on transwells to physically separate the L3 from PBMC (Fig. 6). As seen, direct contact between live L3 and CD4+ T cells (and, to a small degree, CD8+ cells) was necessary for optimal cytokine expression.

**Real-time RT-PCR analysis of T cell cytokine gene expression**

To provide independent confirmation of the data obtained by intracellular cytokine flow cytometry, real-time RT-PCR analysis of cytokine mRNA expression in T cells purified from PBMC following L3 stimulation was examined. Using three different sets of pooled RNA, expression of nine cytokine mRNA and an endogenous control from unstimulated and L3-stimulated T cells was measured. As seen in Fig. 7, there were major increases in the expression of IFN-γ and TNF-α, with more moderate increases of IL-1α, IL-1β, and IL-8 in response to live L3.

**Discussion**

The study of human immune responses to filarial parasites in vivo has primarily focused on the adult and microfilarial stages of the parasite (4). Very little is known about immune responses to the larval stages of the parasites because of the inability to determine the exact time of infection. The few studies that examined such early filarial infections have been on deliberately infected human volunteers, transmigrants from a nonendemic to an endemic area, expatriate travelers to or long-term residents of a filaria-endemic area.
area, and military troops stationed in areas of high transmission (4). These acute infections were found to be associated with a mixed Th1/Th2 cytokine profile (high IL-2, IFN-γ, IL-4, and IL-5) (24–27). But even in these individuals, it is difficult to dissect the nature of the in vivo immune response to the L3 stage of the parasite. Therefore, we attempted to use an in vitro culture system of human PBMC with live L3 of *B. malayi* as a model to study human immune responses to larval stages of filarial parasites. We observed several salient features in our in vitro model system: 1) the early activation of T cells and 2) a predominant Th1 response to infective-stage larvae.

Traditionally, immune responses to extracellular helminth parasites have been considered to be Th2 in nature (28). This inference is based primarily on animal models of helminth infections.

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**FIGURE 4.** Proinflammatory Th1 cytokine induction is not inhibited by (A) polymyxin B, (B) rBPI or (C) neutralizing Ab to TLR2 or TLR4. A, The percent of T cells expressing the different cytokines under unstimulated (Un), live L3 (L3)-stimulated, or live L3 plus polymyxin B (L3 + P)-stimulated conditions. Each line represents a single individual (*n* = 4). The percent of T cells expressing cytokines under L3-stimulated and L3 + P-stimulated conditions was significantly higher (*p* < 0.05) than unstimulated for all cytokines studied. B, The percent of T cells expressing the different cytokines under Un, L3-stimulated, or live L3 plus rBPI (L3 + rBPI)-stimulated conditions. Each line represents a single individual (*n* = 5). The percent of T cells expressing cytokines under L3-stimulated and L3 + rBPI-stimulated conditions was significantly higher (*p* < 0.05) than unstimulated for all cytokines studied except GMCSF. C, The percent of T cells expressing the different cytokines under Un, L3 plus Ab to TLR4 and TLR2 (L3 + anti-TLR)-treated, or L3 plus isotype control Ab-treated (L3 + C) conditions. The percent of T cells expressing cytokines under the Ab-treated conditions was significantly higher (*p* < 0.05) than unstimulated for all cytokines studied except GMCSF.
FIGURE 5. T cells require APC and Ag presentation for expression of Th1 cytokines. A, One representative flow cytometry plot of CD3 expression on the x-axis and the respective cytokine on the y-axis. Data are shown for purified T cells cultured with live L3 (upper panels) or purified T cells and adherent cells cultured with L3 (lower panels). The numbers within the plot represent the percent of T cells positive for the respective cytokine. B, The percent of T cells expressing the different cytokines in purified T cells cultured with L3 (T) or T cells cultured with adherent cells and L3 (T + AC). Each line represents a single individual (n = 6). C, The percent of T cells expressing different cytokines under live L3 plus isotype control Ab (L3 + C)-treated or live L3 plus anti-HLA-DR Ab (L3 + anti-HLA-DR)-treated conditions. Each line represents a single individual (n = 5).
and, to be more specific, murine models of infection. Human studies also suggest that Th2 cytokines dominate the immune responses seen in chronic, longstanding helminth infections (29). For example, cross-sectional studies in filaria-infected humans have shown that PBMC stimulation with filarial Ag or polyclonal activators results in IL-4, IL-5, and IL-10 production (30, 31) with concomitant inhibition/suppression of IFN-γ responses (32). It must be pointed out, however, that the focal point of these studies was the adult and microfilarial stages of the parasite and also that the infected humans had presumably been exposed to multiple lifecycle stages already. The present study is thus, to our knowledge, the first to concentrate on human immune responses to live L3 larvae and, more important, to examine the response of naive (unexposed, uninfected) individuals.

First, early activation of T cells was observed among filaria-naive individuals in response to live L3 and L3 Ag. Activation of naive T cells normally requires a time period of 3–5 days. We observed, however, that T cell activation occurred as early as 24 h after L3 stimulation, suggesting either that the donors had all been exposed to filarial or related infections or, more likely, that cross-reactive effector/memory T cells exist in the naive individuals that are rapidly induced by the Ag stimuli in L3. This first possibility is unlikely, as the sera were from individuals without travel to filaria-endemic regions of the world and who were negative for circulating filarial Ag (data not shown). The second possibility is all the more likely in light of our experiments showing that the functional T cell response is confined to the effector/memory subset of T cells in these filaria-naive individuals. The existence of such extensive cross-reactivity raises many interesting questions about the importance of these responses in resistance to infectious disease.

The second salient observation in this study was a surprising one: the presence of a predominantly Th1 response to live L3. The Th1 or Th1-related cytokines significantly augmented in our experiments are also, for the most part, proinflammatory in nature. The implication from this observation is that T cells from naive individuals might mount an early proinflammatory cytokine response to filarial parasites in vivo. Because one of the major clinical manifestations of lymphatic filariasis is a poorly understood acute syndrome characterized by episodes of fever, adenolymphangitis, and/or other constitutional symptoms, the mediators underlying this clinical manifestation may involve such cytokines as TNF-α (33). Our results suggest that the release of proinflammatory cytokines from T cells of individuals encountering L3 larvae for the first time (or perhaps even subsequent times) could mediate the clinical spectrum of acute filariasis.

Dissection of the cytokine response to live L3 reveals that both CD4 and CD8 T cells are responsible for secretion of IFN-γ, TNF-α, GM-CSF, IL-1α, and IL-8. The increased T cell cytokine production is seen at both the protein and mRNA levels. Early activation of CD8 T cells in response to the extracellular parasite implies a rapid cross-priming event possibly mediated by monocytes. This inference is further strengthened by the observation that host T cells require the presence of APC for cytokine expression. For optimal activation and cytokine expression, T cells required live L3 to be in proximate contact with PBMC. One explanation for this requirement is that the active motility exhibited by the larval parasites and/or parasite-cell contact is necessary to activate monocytes into becoming efficient APC for T cells. In addition, our results using HLA-DR blockade demonstrate that Ag presentation is mandatory for T cell induction of cytokines. Studies in murine models of filariasis indicate that a proinflammatory response is induced in macrophages by the Wolbachia endosymbiont of filarial parasites (34); however, our LPS inhibition studies with both polymyxin B (20) and rBPI (21) and the studies using neutralization of TLR4 and TLR2, known to completely inhibit effects of LPS-like molecules (22), exclude a significant role for Wolbachia in the proinflammatory T cell response observed in this in vitro system. Moreover, the use of live L3 (rather than Ag) increases the likelihood that the intracellular Wolbachia are unlikely to provide a source of LPS.

The association of an inflammatory Th1-like response in naive individuals with live L3 stimulation could be significant in several ways. Studies in endemic populations suggest that truly endemic
normal individuals mount a Th1-like antifilarial immune response (25, 35). Thus, mounting an appropriate Th1 response to the L3 stage of the parasite could be the mechanism by which endemic normal individuals remain infection free. Another intriguing hypothesis suggests that the induction and release of Th1 cytokines assist growth and development of filarial larvae (14). Our results demonstrate that live L3 elicit a Th1-like inflammatory response from PBMC, suggesting perhaps that the filarial parasites have evolved mechanisms to induce a proinflammatory Th1 cytokine milieu to facilitate their growth and development.

The absence of a Th2 response in our in vitro stimulation studies is fascinating. It is known from kinetic studies in natural host-parasite relationships that cytokine responses tend to fluctuate over time, resulting in periods of polarization and nonpolarization (36). In bovine onchocerciasis, the initial cytokine response in cattle is the elevation of IFN-γ preceding the elevation of IL-4 (37). Similarly, in an experimental model of loiasis, induction of IL-12 was shown to precede induction of IL-10 (38). Finally, in the murine model of schistosomiasis, it has been elegantly shown that the larval stages of the parasite induce a Th1 response, which is subsequently down-regulated and replaced by a Th2 response to the adult and egg stages of the parasite (39). Thus, it is entirely possible that had we performed kinetic studies of cytokine polarization in our system, we would have observed the frequency of Th2 cells increasing at a later time point. We are currently performing time-course studies to examine this possibility.

In conclusion, our results indicate that the early immune response to live L3 of filarial parasites is dominated by early activation of and proinflammatory cytokine production by T cells in naive individuals. This inflammatory T cell response could be the basis for the acute manifestations of lymphatic filariasis. In addition, this inflammatory response might form an important component of host resistance to the helminth infection. Our results indicate that the general paradigm of Th2 immune responses to extracellular parasitic infections is not universally applicable and depends on the type of extracellular parasite and its life-cycle stage. Finally, our observation that naive individuals mount a rapid immune response to live parasites implies that host immune responses to infectious agents are influenced by the pre-existing cross-reactive repertoire of T cells. This finding holds important implications for vaccine development strategies. Thus, the observation that live L3 of filarial parasites induce early activation of Th1 cells in naive individuals is a hallmark finding of profound significance in terms of understanding the pathogenesis of infection as well as host-parasite relationships. Further investigation of this early T cell response to filarial nematodes should reveal clues toward manipulating the host immune response to establish resistance to infection.

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References


