Toll-Like Receptor- and Filarial Antigen-Mediated, Mitogen-Activated Protein Kinase- and NF-κB-Dependent Regulation of Angiogenic Growth Factors in Filarial Lymphatic Pathology

Subash Babu,a,b R. Anuradha,a N. Pavan Kumar,a P. Jovvian George,a V. Kumaraswami,c and Thomas B. Nutmanb

National Institutes of Health-International Center for Excellence in Research, Chennai, India; Laboratory of Parasitic Diseases, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, Maryland, USA; and Tuberculosis Research Center, Chennai, India

Filarial lymphatic pathology is of multifactorial origin, with inflammation, lymphangiogenesis, and innate immune responses all playing important roles. The role of Toll-like receptors (TLRs) in the development of filarial pathology is well characterized. Similarly, the association of pathology with elevated levels of plasma angiogenic factors has also been documented. To examine the association between TLR function and the development of lymphangiogenesis in filarial infections, we examined TLR- and filarial antigen-induced expression and production of various angiogenic growth factors. We demonstrate that TLR ligands (specifically TLR2, -3, and -5 ligands) induce significantly increased expression/production of vascular endothelial growth factor A (VEGF-A) and angiopoietin-1 (Ang-1) in the peripheral blood mononuclear cells of individuals with lymphatic pathology (CP individuals) compared to that in cells of asymptomatic infected (INF) individuals. Similarly, filarial antigens induce significantly enhanced production of VEGF-C in CP compared with INF individuals. TLR2-mediated enhancement of angiogenic growth factor production in CP individuals was shown to be dependent on mitogen-activated protein kinase (MAPK) and NF-κB signaling, as pharmacologic inhibition of either extracellular signal-regulated kinase 1/2 (ERK1/2), p38 MAPK, or NF-κB signaling resulted in significantly diminished production of VEGF-A and Ang-1. Our data therefore strongly suggest an important association between TLR signaling and lymphangiogenesis in the development of pathology in human lymphatic filariasis.
lymphedema and in those with subclinical (or asymptomatic) infection. Our findings reveal an important association between TLR- and filarial antigen-mediated production of VEGF-A, VEGF-C, and Ang-1 and the presence of overt filarial lymphatic pathology.

**MATERIALS AND METHODS**

**Study population.** All individuals were examined as part of a clinical protocol to study the natural history of lymphatic filariasis and associated immune responses, approved by the Institutional Review Boards of both the National Institute of Allergy and Infectious Diseases and the Tuberculosis Research Center and registered at http://www.clinicaltrials.gov (trial NCT00001230). Informed written consent was obtained from all participants.

We studied a group of 34 unrelated patients with filarial lymphedema (CP patients) and 34 asymptomatic infected (INF) patients in an area in Tamil Nadu, South India, where lymphatic filariasis is endemic (Table 1). The first 42 subjects (equally divided between the CP and INF groups) were studied prospectively for responses to TLR agonists. For 10/21 CP subjects, there were sufficient numbers of cells to perform studies on pharmacological inhibition of cell signaling. The remaining 26 subjects (equally divided between the INF and CP groups) were then studied for their responses to filarial antigen.

CP patients were circulating filarial antigen negative by both the ICT filarial antigen test (Binx, Portland, ME) and the TropBio Og4C3 enzyme-linked immunosorbent assay (ELISA) (TropBio Pty. Ltd., Townsville, Queensland, Australia), indicating a lack of current active infection. VEGF levels were undetectable, and Ang-1 and Ang-2 were produced at levels of 261.9 pg/ml in CP individuals versus 129.4 pg/ml in INF individuals. As shown in Fig. 1a, we did not detect phosphorylation of Ang-1 and Ang-2 in PBMC preparations from CP individuals, patients with filarial pathology; INF individuals, patients with asymptomatic filarial infection.

**RESULTS**

Baseline expression of angiogenic growth factors is not significantly altered in filarial pathology. Because expression of VEGF by PBMCs is not well defined, we first examined the expression patterns of VEGF-A, -B, -C, -D in PBMCs by real-time RT-PCR. We found that PBMCs expressed detectable levels of all of the VEGF genes examined. Next, we compared the baseline expression of all of the genes examined in CP and INF individuals. While VEGF-D was undetectable, VEGF-A, -B, -C, and -D were detectable at levels of 261.9 pg/ml in CP individuals versus 129.4 pg/ml in INF individuals (Fig. 1a). Baseline expression of VEGF-A, -B, and -D by PBMCs in CP and INF individuals and found that only Ang-2 was detected at significantly higher levels in CP individuals than in INF individuals (GM of 261.9 pg/ml in CP individuals versus 129.4 pg/ml in INF individuals; P = 0.0391). Thus, baseline expression and/or production of VEGF and Ang-2 is not significantly altered in filarial pathology.

**TABLE 1 Characteristics of the study population**

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Value or descriptiona</th>
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<tbody>
<tr>
<td>CP individuals</td>
<td>INF individuals</td>
</tr>
<tr>
<td>(n = 34)</td>
<td>(n = 34)</td>
</tr>
<tr>
<td>Median age (range)</td>
<td>48 (20–60) 44 (15–73)</td>
</tr>
<tr>
<td>Gender (no. of males/no. of females)</td>
<td>18/16 21/13</td>
</tr>
<tr>
<td>Lymphedema/elephantiasis</td>
<td>Yes No</td>
</tr>
<tr>
<td>ICT card test</td>
<td>Negative Positive</td>
</tr>
<tr>
<td>W. bancrofti circulating antigen level (U/ml) (GM range)</td>
<td>&lt;32b 2,814 (132–32,768)</td>
</tr>
<tr>
<td>IgG level (mg/ml) (GM range)</td>
<td>44.32 (10.6–395) 58.12 (5.1–3134)</td>
</tr>
<tr>
<td>IgG4 level (ng/ml) (GM range)</td>
<td>165.2 (2.1–654.8) 5,238 (98–36,836)</td>
</tr>
</tbody>
</table>

a CP individuals, patients with filarial pathology; INF individuals, patients with asymptomatic filarial infection.

b Below the limit of detection.
of angiogenic growth factors (with the exception of Ang-2) is not significantly elevated in CP individuals compared with INF individuals.

**TLR ligand stimulation induces increased expression of VEGF-A mRNA in filarial pathology.** Because the effect of TLR stimulation on VEGF gene expression in PBMCs is not known, we examined the mRNA levels of VEGF-A, -B, -C, and -D in response to TLR2, -3, -4, -5, and -9 agonists. While the expression levels of VEGF-B (Fig. 2b), VEGF-C (Fig. 2c), and VEGF-D (Fig. 2d) were not significantly different between CP and INF individuals, we detected significantly increased expression of VEGF-A mRNA in response to the TLR2 ligands Pam3Cys (GM change of 3.4-fold in CP individuals versus 1.6-fold in INF individuals; \( P = 0.0021 \)) and HKLM (GM change of 3.9-fold versus 1.6-fold; \( P = 0.0031 \)), the TLR5 ligand flagellin (GM change of 2.9-fold versus 1.5-fold; \( P = 0.0054 \)), and the TLR9 ligand CpG ODN 2006 (GM change of 0.83-fold versus 0.42-fold; \( P = 0.0009 \)) in CP individuals compared with INF individuals (Fig. 2a). Our data therefore demon-

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**FIG 1.** Baseline levels of VEGF mRNA and VEGF and angiopoietin proteins in filarial pathology. PBMCs from individuals with chronic pathology (CP) and from infected (INF) individuals (\( n = 21 \) each) were cultured with medium alone for 24 h, and mRNA expression of VEGF-A, -B, -C, and -D (by RT-PCR) (a) and protein expression of VEGF-A and -C and Ang-1 and -2 (by ELISA) (b) were measured. Data are shown as relative transcript levels (a) or baseline protein levels (b). Data are shown in the form of bar graphs depicting the geometric means + 95% confidence intervals.
strate that altered expression of VEGF-A mRNA is associated with filarial lymphatic pathology.

**TLR ligand stimulation induces increased production of VEGF-A and Ang-1 in filarial pathology.** Because TLR stimulation resulted in increased expression of VEGF gene expression, and because baseline production of Ang-2 was elevated in CP individuals, we next examined the protein levels of VEGF-A and -C as well as Ang-1 and Ang-2 in response to TLR2, -3, -4, -5, and -9 ligands. While the expression levels of VEGF-C (Fig. 3b) and Ang-2 (Fig. 3d) were not significantly different between CP and INF individuals, we detected significantly increased production of VEGF-A in response to the TLR2 ligands Pam3Cys (GM of 64 pg/ml in CP individuals versus 4.4 pg/ml in INF individuals; \( P = 0.0018 \)) and HKLM (GM of 61.4 versus 6.9 pg/ml; \( P = 0.0082 \)), the TLR3 ligand poly(IC) (GM of 528.3 versus 101.5 pg/ml; \( P = 0.0257 \)), and the TLR5 ligand flagellin (GM of 1,051 versus 84 pg/ml; \( P = 0.0107 \)) in CP individuals compared with INF individuals (Fig. 3a). Similarly, we noted significantly increased pro-
roduction of Ang-1 in response to the TLR2 ligands Pam3Cys (GM of 211.2 pg/ml in CP individuals versus 13.9 pg/ml in INF individuals; \( P = 0.0065 \)) and HKLM (GM of 116 versus 16.5 pg/ml; \( P = 0.0413 \)), the TLR3 ligand poly(IC) (GM of 7.7 versus 1.7 pg/ml; \( P = 0.0447 \)), and the TLR5 ligand flagellin (GM of 135 versus 6.6 pg/ml; \( P = 0.0240 \)) in CP individuals compared to INF individuals (Fig. 3c). Our data therefore demonstrate that altered protein levels of VEGF-A and Ang-1 are associated with filarial lymphatic pathology.

**Filarial antigen stimulation induces increased production of VEGF-A and -C during filarial pathology.** Because TLR stimulation resulted in increased production of VEGF-A and Ang-1, we wanted to examine whether filarial antigens could also induce production of angiogenic growth factors and whether such production was associated with the outcome of filarial infection. While the levels of VEGF-A (Fig. 4A), Ang-1 (Fig. 4C), and Ang-2 (Fig. 4D) were not significantly different between CP and INF individuals upon stimulation with either BmA or Mf antigen, we detected significantly increased production of VEGF-C in response to both BmA (GM of 99.1 versus 12.3 pg/ml; \( P = 0.0276 \)) and Mf (GM of 121.6 versus 16.2 pg/ml; \( P = 0.0108 \)) antigens in CP individuals compared with INF individuals (Fig. 4B). Our data therefore suggest that production of angiogenic growth factors associated with filarial lymphatic pathology could also be induced directly by filarial antigens.
TLR2 ligand-induced VEGF-A and Ang-1 production is dependent on phosphorylation of p38 MAPK and ERK1/2 and on NF-κB activation. Because TLR stimulation has been shown to signal by phosphorylation of p38 MAPK and ERK1/2 and through activation of NF-κB (20), we sought to determine whether the angiogenic growth factor production observed in response to TLR2 agonists was dependent on these signaling pathways. We therefore utilized pharmacologic inhibitors of p38 MAPK, ERK1/2, and NF-κB activation to inhibit these signaling molecules before stimulating PBMCs from CP individuals with Pam3Cys. As shown in Fig. 5A, inhibition of the p38 MAPK, ERK1/2, and NF-κB pathways resulted in significantly decreased production of VEGF-A in response to Pam3Cys (P = 0.0020 for all). Similarly, as shown in Fig. 5B, inhibition of the above pathways resulted in significantly decreased production of Ang-1 in response to Pam3Cys (P = 0.0020 for p38 and ERK inhibition; P = 0.0195 for NF-κB inhibition). Thus, the p38 MAPK and ERK1/2 pathways, as well as NF-κB activation, play an important role in mediating the angiogenic growth factor response to TLR ligands in CP individuals.

DISCUSSION
Among the 120 million people worldwide who are infected with lymphatic filarial parasites, a subset exhibit demonstrable clinical pathology, characterized most notably by lymphedema, hydrocele, and elephantiasis (18). The events that lead to development
of lymphatic pathology in filariasis are not fully understood, although host-parasite interactions, parasite products, and opportunistic secondary infections are all believed to play significant roles in determining the development of pathology (21). TLRs are important initiators of immune responses through their ability to recognize a variety of microbial products (24), but if they are unregulated, TLR-dependent proinflammatory cascades can also escalate to cause severe pathology (19). It has been shown previously that TLR-dependent proinflammatory cytokine production—most notably of Th1 and Th17 cytokines—is strongly associated with chronic pathology in lymphatic filariasis (1).

A potential mechanism by which proinflammatory cytokines could mediate lymphatic damage is through their ability to induce production of various angiogenic and lymphangiogenic factors. This, in turn, could lead to perturbations in maintenance and function of the lymphatic endothelial system, resulting in a variety of complications, including lymphatic dilatation and lymphedema (5, 21). Indeed, the presence of elevated levels of lymphangiogenic factors has been shown to be associated with the severity of lymphatic pathology (3, 8, 9); however, no data are available on whether there is a direct association between TLR stimulation and angiogenic growth factor production in filarial pathology. In addition, filarial antigens themselves have been shown to induce proliferation and differentiation of human lymphatic endothelial cells in vitro (4). The VEGFs and angiopoietins are important regulatory growth factors for vascular and lymphatic endothelial function (17). While the VEGF family of proteins can promote both angiogenesis and lymphangiogenesis by interacting with
cognate receptors (VEGFR-1, -2, and -3) on endothelial cells, Ang-1 and Ang-2 can interact with Tie-2 receptors and contribute to maturation and stabilization of the vasculature (17, 27). Although Ang-1 is implicated primarily in vascular stabilization, it has been shown to cause endothelial cell proliferation and differentiation (27). There is a dearth of literature on the different cell populations that can produce angiogenic growth factors per se, with even more limited data on the nature of this response to TLR stimulation. We therefore first established that PBMCs do have the capacity to produce these growth factors, although the exact cell population(s) that is responsible for the production of these factors is under investigation. Next, our examination of TLR stimulation demonstrated clearly delineated responses in individuals with overt clinical pathology compared with asymptomatic in-
fected individuals. We have previously shown that expression as well as function (in the form of activation and cytokine produc-
tion) of TLR2 is significantly enhanced in CP individuals com-
pared with INF individuals (1). Here we demonstrated that TLR2
stimulation also leads to significantly enhanced VEGF-A and
Ang-1 production, further confirming the importance of TLR2 in
development of clinical filarial pathology. In addition, we also
implicate a role for TLR3 and TLR5 in the activation of the angio-
genic vascular network that might underlie the development of
lymphatic complications in lymphedema. We can envision a cou-
ple of mechanisms (not mutually exclusive) by which TLR stim-
ulation could influence angiogenic growth factor production in
PBMCs: (i) a direct effect on VEGF and angiopoietin production and/or (ii) an indirect effect due to TLR-induced proinflam-
matory cytokine production. Proinflammatory cytokines, including
tumor necrosis factor alpha (TNF-α) and IL-17, are known induc-
ers of VEGF proteins (13), and hence an indirect effect of TLR
stimulation on angiogenic growth factor production cannot be
excluded.

TLR signaling involves activation of the MAPK family of mol-
ecules, specifically ERK1/2, Jun N-terminal kinase (JNK), and p38
(24). Enhanced p38 MAPK and ERK1/2 phosphorylation, as well
as NF-κB activation, has been associated with increased TLR-me-
diated cytokine responses (20). Therefore, we examined the roles
of these MAP kinases as well as NF-κB activation in the observed
immune response to TLR stimulation in CP individuals. Our data
obtained using pharmacologic inhibition of the above-mentioned
pathways clearly indicate that the elevated angiogenic growth fac-
tor production in CP patients is dependent on the ERK1/2 and
MAPK pathways as well as on NF-κB activation. This provides a
mechanistic framework in which TLR (and perhaps filarial anti-
gen) stimulation could promote lymphangiogenesis that ulti-
mately results in lymphatic pathology. While the use of TLR li-
gands in this study serves as a useful model for studying the effects of
filarial products and bystander antigens on the innate immune
response, it does not recapitulate the exact effects of parasite stim-
ulation. Hence, we also examined whether filarial antigens of the
relevant life cycle stages (adult worms and Mf) in humans have any
effect on angiogenic growth factor production. We demonstrated
that filarial antigens induce differential production of VEGF-C, illus-
trating certain similarities and (perhaps) differences between filarial
antigen and TLR stimulation. The fact that filarial antigens induce
significantly elevated levels of VEGF-C also strengthens previous data
suggesting that this important angiogenic/lymphangiogenic factor
plays a crucial role in lymphatic pathology. In addition, preliminary
data indicate that TLR- and filarial antigen-induced VEGF produc-
tion is also significantly higher in CP individuals than in uninfected
individuals in areas where the disease is endemic (data not shown),
reinforcing the importance of these factors in pathogenesis.

Inhibition of the VEGF-VEGFR and Ang-Tie systems is gain-
ing importance in the fields of tumor immunology, vascular dis-
eases, and other inflammatory disorders in terms of maximizing
the efficacy of anti- and proangiogenic therapies (7). Our data
suggest that targeting of these pathways, as well as new treatment
regimens involving TLR modulation, could potentially provide
new avenues in ameliorating pathology in chronic lymphatic fi-
lariasis. In addition, our data also reveal a new link between innate
immune responses (in the form of TLR activation) and angiogenic/
lymphangiogenic pathways (in the form of elevated growth factors), thereby providing additional insight into the pathways connecting the immune system and normal physiologic processes such as lymphatic circulation.

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REFERENCES