INFECTION AND IMMUNITY, Nov. 2011, p. 4600–4608 Vol. 79, No. 11
0019-9567/11/$12.00 doi:10.1128/IAI.05419-11

Filarial Lymphatic Pathology Reflects Augmented Toll-Like Receptor-Mediated, Mitogen-Activated Protein Kinase-Mediated Proinflammatory Cytokine Production▽†

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Received 20 May 2011/Returned for modification 30 June 2011/Accepted 17 August 2011

Lymphatic filariasis can be associated with the development of serious pathology in the form of lymphedema, hydrocele, and elephantiasis in a subset of infected patients. Toll-like receptors (TLRs) are thought to play a major role in the development of filarial pathology. To elucidate the role of TLRs in the development of lymphatic pathology, we examined cytokine responses to different Toll ligands in patients with chronic lymphatic pathology (CP), infected patients with subclinical pathology (INF), and uninfected, endemic-normal (EN) individuals. TLR2, -7, and -9 ligands induced significantly elevated production of Th1 and other proinflammatory cytokines in CP patients in comparison to both INF and EN patients. TLR adaptor expression was not significantly different among the groups; however, both TLR2 and TLR9 ligands induced significantly higher levels of phosphorylation of extracellular signal-regulated kinase 1/2 (ERK1/2) and p38 mitogen-activated protein (MAP) kinases (MAPK) as well as increased activation of NF-κB in CP individuals. Pharmacologic inhibition of both ERK1/2 and p38 MAP kinase pathways resulted in significantly diminished production of proinflammatory cytokines in CP individuals. Our data, therefore, strongly suggest an important role for TLR2- and TLR9-mediated proinflammatory cytokine induction and activation of both the MAPK and NF-κB pathways in the development of pathology in human lymphatic filariasis.

Although two-thirds of the 120 million people infected with *Wuchereria bancrofti*, the major causative agent of human lymphatic filariasis, have subclinical infections, ~40 million have other pathological manifestations, including hydroceles (and other forms of urogenital disease), episodic adenolymphangitis, tropical pulmonary eosinophilia, lymphedema, and (in its most severe form) elephantiasis (9, 20). Adult *W. bancrofti* worms reside in the lymphatics and lymph nodes and induce changes that result in dilatation of lymphatics and thickening of the lymphatic vessel walls. *W. bancrofti* and most other filarial parasites harbor *Wolbachia*, an obligate intracellular alphaprotobacterial endosymbiont. Progressive lymphatic damage and pathology potentially result from the summation of the effect of tissue alterations induced by both living and nonliving adult parasites, the host inflammatory response to the parasites and their secreted antigens (Ags), the host inflammatory response to *Wolbachia*, and host responses seen as a consequence of secondary bacterial or fungal infections (10, 26). Thus, inflammatory damage induced by filarial parasites appears to be multifactorial, with endogenous parasite products, *Wolbachia*, and host immune responses all playing important roles.

TLRs are innate immune receptors commonly associated with the initiation of inflammatory processes (14). Upon binding to their cognate ligands, TLRs activate the innate immune response, leading to the production of proinflammatory cytokines and chemokines (13). Studies of animal models of filarial infection and *in vitro* studies in humans have suggested that *Wolbachia*-derived molecules from filarial parasites are key inducers of proinflammatory cytokines (7, 12, 27, 28). Moreover, this inflammatory response to *Wolbachia* has been shown to be mediated primarily through TLR2, TLR4, and TLR6. In addition, with a mouse model of onchocerciasis, a related filarial infection, it was demonstrated that *Wolbachia* interaction with the host innate immune system resulted in development of inflammatory keratitis, a characteristic feature of human onchocercal eye disease (22). Downregulation of TLR on antigen-presenting cells (APCs) and T cells has been shown to be a possible mechanism by which deleterious pathology in clinically asymptomatic filarial infections can be circumvented (23). Indeed, TLR downregulation, both in terms of expression and function, on monocytes and B and T cells appears to be a characteristic feature in patent filarial infections (3, 4). In addition, exposure of human dendritic cells to live filarial parasites has been shown to downregulate the expression and function of TLR3 and TLR4 (24). Finally,

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† Supplemental material for this article may be found at http://iai.asm.org/.

▽ Published ahead of print on 29 August 2011.

TABLE 1. Characteristics of the study population[a]

Characteristic	CP	INF	EN
Median age, yr (range)	40 (20–59)	47.5 (19–73)	25 (20–65)
Gender (no. of subjects M/F)	6/8	7/7	10/4
Lymphedema/elephantiasis	Yes	None	None
ICT card test	Neg	Pos	Neg
W. bancrofti circulating Ag levels (U/ml) (GM range)	4.218 (1.98–26.46)	2,059.073 (136.52–33,125.87)	8 (3.45–28.87)
IgG levels (μg/ml) (GM range)	32.71 (8.09–230.054)	56.54 (9.005–2,174.83)	17.065 (1.55–244.98)
IgG4 levels (ng/ml) (GM range)	13.73 (5.14–363.53)	1,249.45 (19.61–10,122.68)	20.56 (0.42–1,214.18)

[a] M, male; F, female; Neg, negative; Pos, positive.

depletion of *Wolbachia* by doxycycline treatment has been associated with reduction in lymphatic pathology in filaria-infected individuals (8). We have previously shown that enhanced TLR expression is an important feature of chronic lymphatic pathology, with patients with lymphedema exhibiting significantly enhanced expression of TLR2, -4, -7, and -9 mRNA in comparison to asymptomatic, infected patients (2); however, the functional role of this differential TLR expression on immune responses engendered in CP individuals has not been studied.

Hence, we wanted to examine whether the increased expression of TLRs would lead to increased expression of proinflammatory cytokines, which, in turn, could potentially promote pathology development. Moreover, activation of TLRs triggers a series of signaling events leading to activation of the NF-κB pathway and, as a result, induction of inflammatory cytokines (25). This cascade involves recruitment of adaptor molecules (MyD88, TRAM, TRIF, TIRAP, and TRAF6) as well as phosphorylation of MAP kinases, including activation of p38, c-Jun N-terminal kinase (JNK), and ERK1/2 (19). Therefore, we also examined the signaling cascade triggered by the activation of TLRs in filaria-infected and -uninfected individuals. Our study demonstrates that patients with chronic lymphatic pathology (CP)—in contrast to infected patients with subclinical pathology (INF) or uninfected, endemic-normal (EN) individuals—are characterized by an augmented production of proinflammatory cytokines, including Th1 and Th17 cytokines in response to the stimulation of three TLRs: TLR2, -7, and -9.

MATERIALS AND METHODS

Study population. We studied a group of 14 patients with filarial lymphedema (CP), 14 asymptomatic infected (INF) patients, and 14 uninfected endemic-normal individuals (EN) in an area endemic for lymphatic filariasis in Tamil Nadu, South India (Table 1). CP patients were negative for circulating filarial Ag by both the ICT filarial Ag test (Binax, Portland, ME) and the TropBio Og4C3 ELISA (TropBio Pty. Ltd., Townsville, Queensland, Australia), indicating a lack of current active infection. They had undergone treatment with repeated doses of diethylcarbamazine. INF patients tested positive by both the ICT filarial Ag test and the TropBio Og4C3 ELISA as well as by BmA-specific IgG4 and IgG enzyme-linked immunosorbent assays (ELISA). EN patients were negative for circulating filarial Ag by both tests. BmA-specific IgG4 and IgG ELISA were performed exactly as described previously (15). The three groups did not differ significantly in baseline hematological and immunological parameters, including total white blood cell count. All individuals were examined as part of a clinical protocol approved by Institutional Review Boards of both the National Institute of Allergy and Infectious Diseases and the Tuberculosis Research Center, and informed written consent was obtained from all participants.

Reagents. The TLR ligands (Invivogen, San Diego, CA) used were TLR2 ligands, Pam3CysSerLys4 (here Pam3Cys) at 0.5 μg/ml and heat-killed *Listeria monocytogenes* (HKLM) at 10^8 cells/ml; a TLR3 ligand, poly(IC); a TLR4 ligand, ultrapure lipopolysaccharide (LPS), at 0.5 μg/ml; a TLR5 ligand, flagellin *Salmonella enterica* serovar Typhimurium; a TLR2/6 ligand, FSL-1; a TLR7 ligand, imiquimod, at 1 μg/ml; a TLR8 ligand, single-strand RNA 40; and a TLR9 ligand, CpG ODN 2006 (ODN), at 0.5 μM/ml.

Isolation of PBMC and *in vitro* culture. Peripheral blood mononuclear cells (PBMCs) were isolated as described previously (5). PBMCs were cultured in 24-well tissue culture plates (Corning Incorporated, Corning, NY) at concentrations of 5×10^6/well for 24 h. For phosphorylation assays, PBMCs were cultured with TLR ligands for 30 min, following which cell lysate was prepared using the phosphoprotein cell lysis kit (Bio-Rad, Hercules, CA) according to the manufacturer's protocol. The protein concentration was assayed using the Bio-Rad DC protein assay kit. For NF-κB estimation, PBMCs were cultured with TLR ligands for 3 h, and nuclear extracts were prepared using a nuclear extraction kit (Panomics, Milan, Italy) according to the manufacturer's protocol.

Cytokine ELISA. Levels of cytokines in the culture supernatants were measured using the Bioplex multiplex cytokine assay system (Bio-Rad). The cytokines analyzed were gamma interferon (IFN-γ), tumor necrosis factor alpha (TNF-α), interleukin-17A (IL-17A), IL-23, IL-12p70, IL-1β, and IL-6. Net cytokine levels were calculated by subtracting cytokine levels in the medium control for each stimulated condition.

RNA preparation. RNA was isolated from PBMCs following culture with TLR ligands for 24 h. PBMCs were lysed using the reagents of a commercial kit (QIAshredder; Qiagen, Valencia, CA). Total RNA was extracted according to the manufacturer's protocol (RNeasy minikit; Qiagen), and RNA was dissolved in 50 ml 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, Fullerton, CA). Briefly, random hexamers were used to prime RNA samples for reverse transcription using MultiScribe reverse transcriptase.

Real-time RT-PCR. Real-time quantitative reverse transcription (RT)-PCR was performed in an ABI 7500 sequence detection system (Applied Biosystems) using TaqMan assays-on-demand reagents for MyD88, TRAM, TRIF, TIRAP, TRAF6, and an endogenous 18s rRNA control. Relative transcripts were determined according to the manufacturer's protocol.

Detection of phosphorylation and NF-κB activity. The total and phosphoprotein levels for p38 MAPK, ERK1/2, and JNK in cell lysates was determined using the phosphoprotein detection reagent kit and the phospho- and total protein 3-plex kit for the MAP kinases (Bio-Rad). The phosphorylation index was calculated on the basis of the mean fluorescence intensity of the phosphorylated levels after normalizing for total phosphoprotein levels in each sample. NF-κB activation was measured and quantified by an ELISA using a p50 and p65 kit (Panomics).

Pharmacological inhibition of signaling pathways. The pharmacologic inhibitors used were U0126 (ERK1/2 inhibitor) and SB203580 (p38 MAPK inhibitor), both from Calbiochem Biosciences (San Diego, CA). They were reconstituted in sterile, cell-culture grade dimethyl sulfoxide (DMSO) (Sigma-Aldrich, St. Louis, MO). For inhibition experiments, PBMCs from CP patients were first pretreated with 20 μM U0126, 30 μM SB203580, or DMSO alone for 60 min before culture with TLR ligands for 24 h, and supernatants were collected. The efficacy of the inhibitor in terms of inhibition of phosphorylation or activation of a specific molecule was tested independently.

Statistical analysis. Geometric means (GM) were used as the measure of central tendency. Comparisons were made using the Mann-Whitney U test followed by the Holm correction for multiple comparisons or the Wilcoxon signed-rank test. All statistics were performed using GraphPad Prism version 5 for Windows (GraphPad Software, Inc., San Diego, CA).

RESULTS

TLR2, -7, and -9 ligands induce significantly elevated proinflammatory cytokine production in filarial pathology. As previously published, the expression of TLR2, -4, -7, and -9 mRNA was significantly higher in CP compared to INF patients at baseline (15). To examine whether the increased expression of TLRs translates to increased function in terms of production of proinflammatory cytokines in lymphatic filariasis, we stimulated PBMCs from CP, INF, and EN individuals with TLR ligands and measured the levels of IFN-γ, TNF-α, IL-17, IL-23, IL-12, IL-1β, and IL-6 following 24 h of stimulation that revealed several salient features. First, baseline as well as poly(IC) (TLR3), flagellin (TLR5), FSL-1 (TLR2/6), and single-stranded RNA (ssRNA) (TLR8) stimulated levels of proinflammatory cytokines were found to be not significantly different among the three groups (data not shown). Second, the TLR2 ligands Pam3Cys (Fig. 1A) and HKLM (Fig. 1B) induced significantly increased production of IFN-γ, TNF-α, IL-12, IL-1β, and IL-6 but not IL-17 and IL-23 in CP compared with both INF and EN individuals. Third, the TLR4 ligand LPS (Fig. 1C) induced only moderately increased expression of TNF-α and IL-6 in CP patients compared with INF but not with EN patients. Fourth, TLR7 ligand imiquimod (Fig. 1D) induced significantly increased production of IFN-γ, TNF-α, IL-17, IL-23, and IL-6 in CP individuals compared with INF individuals alone. Finally, TLR9 ligand ODN (Fig. 1E) induced significantly increased production of IFN-γ, TNF-α, IL-12, IL-1β, and IL-6 as well as IL-17 and IL-23 in CP individuals compared with both INF and EN individuals. Thus, TLR ligands (especially, Pam3Cys, HKLM, LPS, imiquimod, and ODN) induce a very complex pattern of cytokine induction that is significantly more pronounced in CP patients compared with the other two groups.

TLR ligand stimulation does not alter TLR adaptor gene expression in filarial pathology. Because TLRs are known to recruit specific adaptor molecules to initiate downstream signaling and adaptor molecule expression is known to be controlled at the transcriptional level (19), we measured the mRNA levels of the five major adaptor molecules for TLR signaling: MyD88, TRAM, TRIF, TIRAP, and TRAF6. We failed to observe any significant difference in the expression levels of all of the above adaptors either at baseline (data not shown) or following stimulation with TLR ligands (see Fig. S1 in the supplemental material) among the three groups. Our data therefore suggest that differential production of proinflammatory cytokines is not associated with a significantly altered transcriptional signature of TLR adaptors.

TLR2 and -9 ligands induce enhanced phosphorylation of p38 MAPK and ERK1/2 in filarial pathology. Because phosphorylation of MAP kinases is immediately downstream of TLR activation (19), we examined whether the signaling cascade initiated by TLR ligands in CP individuals is quantitatively different from those of the other two groups. We measured both total and phosphorylated levels of ERK1/2, JNK, and p38 MAPK and estimated the intensity of phosphorylation following stimulation compared to background. As shown in Fig. 2A and B, the TLR2 ligands Pam3Cys and HKLM induced significantly greater phosphorylation of ERK1/2 and p38 MAPK but not JNK in CP individuals compared with INF and EN individuals. Similarly, ODN induced significantly increased phosphorylation of ERK1/2 in CP individuals compared with INF individuals and of p38 MAPK in CP individuals compared to EN and INF individuals (Fig. 2E); however, LPS and imiquimod induced either modest or no significant alteration in the phosphorylation of the above kinases in CP individuals compared with INF or EN individuals (Fig. 2C and D). Thus, activation of the MAPK signaling cascade is differentially induced in those with filaria-associated pathology.

TLR2 and -9 ligands induce increased activation of NF-κB p50 and p65 in filarial pathology. As TLR signaling can lead to activation of NF-κB with increased binding activity of both p50 and p65 subunits (1), we examined whether TLR activation could lead to differential activation of the NF-κB subunits in CP individuals compared with the other two groups. As shown in Fig. 3A, upon stimulation with Pam3Cys, increased binding activity (reflecting activation and increased translocation) of both the p50 and p65 subunits of NF-κB was detected in the PBMC of CP individuals compared with either EN or INF individuals. Similarly, activation with ODN resulted in increased activation of p50 and p65 in CP individuals compared with EN or INF individuals (Fig. 3D); however, stimulation with LPS (Fig. 3B) or imiquimod (Fig. 3C) did not induce differential activation of either p50 or p65 among the three groups. These data suggest that NF-κB activation is an important component of the differential cytokine production characteristically observed with filarial pathology.

TLR2 and -9 ligand-induced proinflammatory cytokine production is dependent on phosphorylation of p38 MAPK and ERK1/2. Because TLR stimulation in CP individuals resulted in increased phosphorylation of p38 MAPK and ERK1/2, we sought to determine whether the proinflammatory cytokine production observed in response to the TLR2 and TLR9 ligands was dependent on these signaling pathways. We therefore utilized pharmacologic inhibitors of p38 MAPK and ERK1/2 activation to inhibit these signaling molecules before stimulating PBMCs from CP individuals with TLR ligands. As shown in Fig. 4A, inhibition of the p38 MAPK pathway resulted in significantly decreased production of IFN-γ, TNF-α, IL-12, and IL-1β in response to Pam3Cys and of IFN-γ and TNF-α in response to ODN. Similarly, as shown in Fig. 4B, inhibition of the ERK pathway resulted in significantly decreased production of IFN-γ, TNF-α, IL-12, and IL-1β in response to Pam3Cys and of IFN-γ, TNF-α, and IL-1β in response to ODN. Thus, p38 MAPK and ERK1/2 pathways play an important role in mediating the proinflammatory cytokine response to TLR ligands in CP individuals.

DISCUSSION

Lymphatic filariasis is a disease characterized by a broad spectrum of clinical manifestations seen among the majority of infected people (17). A subset of individuals with this infection has demonstrable pathology characterized, most notably, by lymphedema, hydrocele, and elephantiasis. The events that lead to the development of lymphatic pathology in filariasis are not fully understood, although the immune responses of the host to the parasite products are believed to play a significant role in determining development of pathology (20). TLRs are important initiators of immune responses through their ability

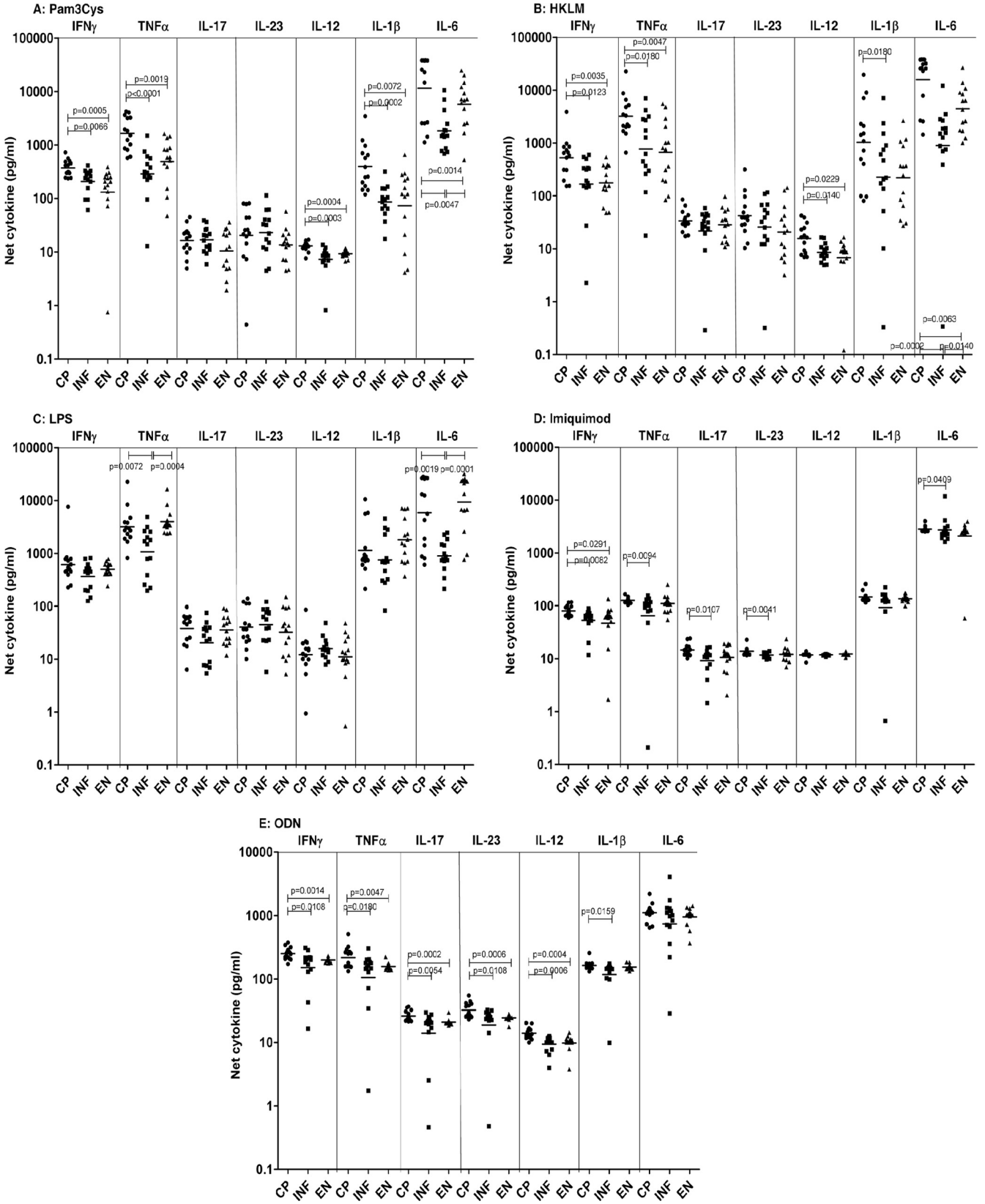
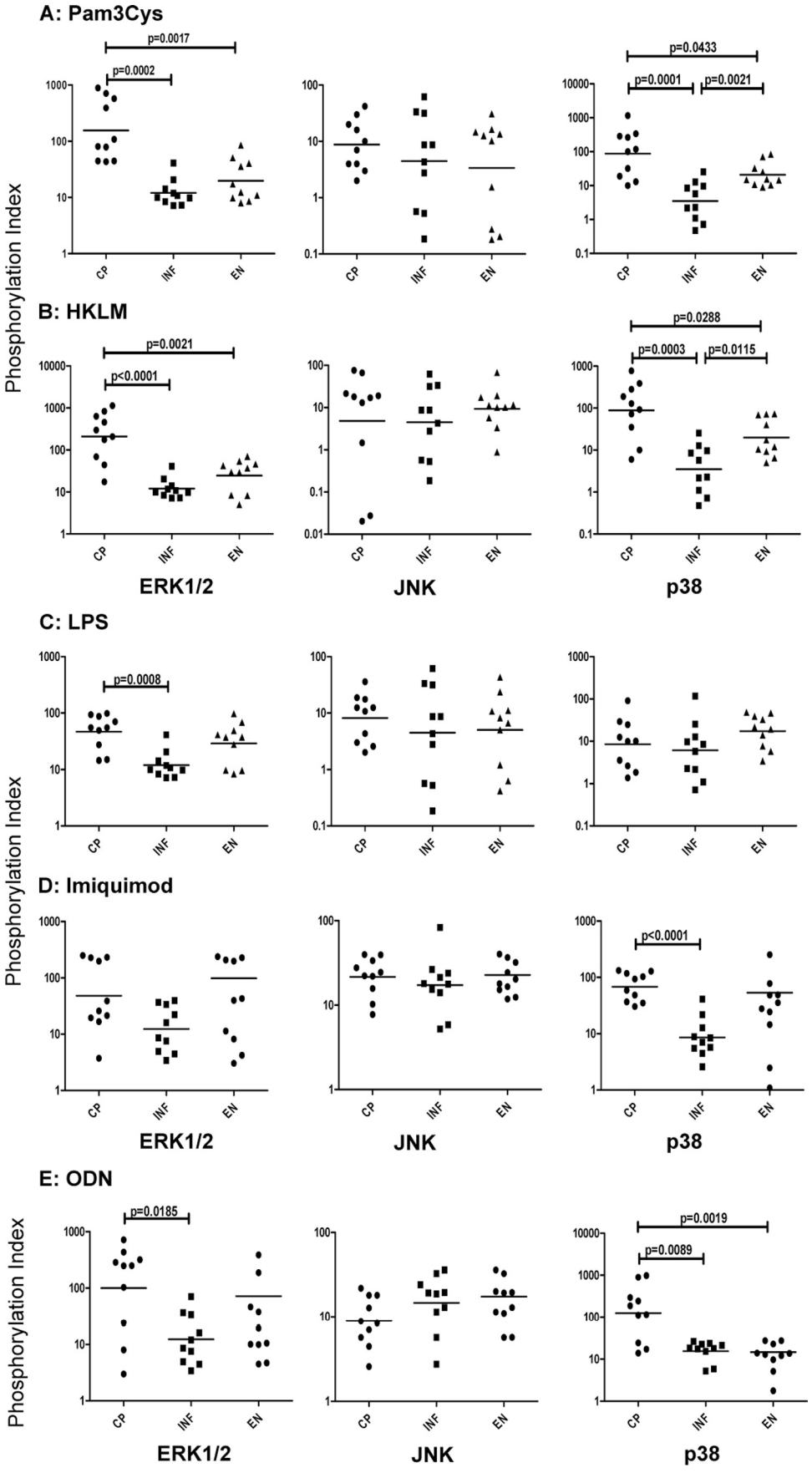


FIG. 1. Enhanced proinflammatory cytokine production in filarial pathology. PBMCs from chronic pathology (CP), infected (INF), and uninfected endemic (EN) individuals ($n = 14$ each) were stimulated with Pam3Cys (A), HKLM (B), LPS (C), imiquimod (D), and ODN (E) for 24 h, and production of IFN-γ, TNF-α, IL-17, IL-23, IL-12, IL-1β, and IL-6 was measured by ELISA. Data are shown as net cytokine production over media control. Each dot represents an individual subject. The horizontal bars are the geometric means (GM).


A: Pam3Cys
p=0.0017
p=0.0002
p=0.0433
p=0.0001
p=0.0021
B: HKLM
p=0.0021
p<0.0001
p=0.0288
p=0.0003
p=0.0115
Phosphorylation Index
CP
INF
EN
ERK1/2
JNK
p38
C: LPS
p=0.0008
D: Imiquimod
p<0.0001
E: ODN
p=0.0185
p=0.0019
p=0.0089

to recognize a variety of microbial products (25). TLR-dependent proinflammatory cascades triggered by microbial products must be tightly regulated to avoid severe pathology (18). In terms of clinical filarial disease, it is thought that this regulation is defective, and the resultant proinflammatory cytokines, in turn, are hypothesized to directly or indirectly (through downstream effects on various angiogenic and lymphangiogenic factors) lead to perturbations in the maintenance and function of the lymphatic endothelial system, resulting in a variety of complications, including lymphatic dilatation and lymphedema (6, 20).

Our findings demonstrate that proinflammatory cytokines are indeed elevated in CP individuals compared with INF or EN individuals in response to specific TLR ligands. Interestingly, the only TLRs that mediate this differential cytokine response are those that have been shown previously to have enhanced expression in CP individuals: TLR2, TLR4, TLR7, and TLR9 (15). This suggests that (i) TLR-mediated release of proinflammatory cytokines is associated with the development of pathology, and (ii) this response clearly demarcates differences between those with overt, clinical pathology and those with asymptomatic or subclinical infection. While a part of the cytokine response to TLR stimulation is clearly blunted in asymptomatically infected individuals, even compared to uninfected individuals, the significantly elevated response seen with CP over not only INF but also EN individuals suggests that heightened proinflammatory cytokine production is a characteristic feature of patients with lymphatic pathology. Even with the proinflammatory responses induced by TLR stimulation in CP individuals, however, distinct patterns of cytokine secretion can be observed. Thus, while TLR2 stimulation resulted predominantly in increased levels of Th1-type proinflammatory cytokines—IFN-γ, TNF-α, and IL-12—TLR7 and -9 ligands also induced elevated levels of Th17-type proinflammatory cytokines IL-17 and IL-23. While TLRs are known inducers of other proinflammatory cytokines, very little is known about the role of TLRs in inducing type 17 cytokines (16). Our data suggest that TLR7 and -9 stimulation leads to IL-17 and IL-23 production and that this specific Th17 response is amplified in the setting of filarial pathology. TLR2 and TLR4 activation on T cells has been shown to induce IL-17 production in mice (21). To our knowledge, this is the first report describing the production of IL-17 directly in response to TLR stimulation in human PBMCs.

In addition to TLR-mediated cytokine production, we examined TLR adaptor usage in filarial pathology. Signaling by all TLRs originates from the conserved Toll–IL-1R domain (19, 25) and recruitment of the common adaptor molecule MyD88 leads to interaction and activation of the IL-1R-associated kinase family members, and subsequent activation of TRAF-6 (TNFR-associated factor-6), results in NF-κB activation through the IκB complex (19). Other Toll–IL-1R domain-containing adaptor molecules include TIRAP/Mal, TRIF, and TRAM (19). These adaptors mediate TLR signaling either alone or in combination with MyD88 and confer the specificity of TLR-mediated inflammatory responses. Recently, it has been shown that MyD88 and TIRAP are the primary adaptor molecules recruited during TLR stimulation by filarial products (12) and that MyD88 gene expression was significantly decreased in dendritic cells exposed to live filarial parasites (24). We examined the gene expression of the above-mentioned TLR adaptors but did not observe any significant difference in the expression of MyD88, TRAM, TRIF, TIRAP, or TRAF-6 at the mRNA level in response to TLR stimulation between the three groups. This suggests that transcriptional regulation of TLR adaptors is not a primary mechanism in the differential cytokine response to TLR stimulation in filarial patients, although regulation at the posttranscriptional level would need to be examined as well to exclude any role for the TLR adaptors.

TLR signaling involves activation of the MAP kinase family of molecules, specifically ERK1/2, JNK, and p38 (25). Enhanced p38 MAPK phosphorylation has been associated with increased TLR-mediated-cytokine responses in malarial infection (11); however, the role of MAP kinases in TLR signaling in other parasitic infections is not known. Therefore, we examined the activation of the three different MAP kinases in response to TLR stimulation in the three groups in which filaria is endemic. Interestingly, we did find that increased phosphorylation of ERK1/2 and p38 but not JNK in response to Pam3Cys and ODN but not LPS or imiquimod was an important feature of filarial pathology patients. This suggests that enhanced activation of the MAP kinase pathway is an important early downstream event in the TLR-mediated cytokine response in filarial pathology. We also examined late (downstream) signaling events of TLR activation by measuring NF-κB activation in response to TLR stimulation in the three groups. Our data clearly demonstrate that Pam3Cys and ODN but not LPS or imiquimod induce significantly enhanced binding activity of both p50 and p65 subunits of NF-κB, which is an indirect measure of NF-κB translocation and activation. Therefore, there appears to be a direct association between the strength of NF-κB activation and the levels of proinflammatory cytokine production in filarial pathology, suggesting that the canonical NF-κB pathway is intrinsically involved in differential TLR-mediated immune responses in lymphatic filariasis. Finally, we examined whether the activation pathways involving ERK1/2 and p38 MAPK are essential for TLR-mediated cytokine induction. Our data with pharmacologic inhibitors of the above-mentioned pathways clearly indicate that the elevated proinflammatory cytokine production in CP patients is dependent on the ERK1/2 and MAPK pathways.

Understanding the pathogenesis of lymphatic filarial disease is of utmost importance, as this could lead to development of

FIG. 2. Enhanced phosphorylation of ERK1/2 and p38 MAPK in filarial pathology. PBMCs from chronic pathology (CP), infected (INF), and uninfected endemic (EN) individuals ($n = 10$ each) were stimulated with Pam3Cys (A), HKLM (B), LPS (C), imiquimod (D), and ODN (E) for 30 min, and the total and phospho-protein levels of ERK1/2, JNK, and p38 MAPK in cell lysates was measured by multiplex phosphorylation ELISA. Data are shown as the phosphorylation index, which is the ratio of phospho-protein to total proteins in the stimulated condition over medium control. Each dot represents an individual subject. The horizontal bars are the geometric means (GM).

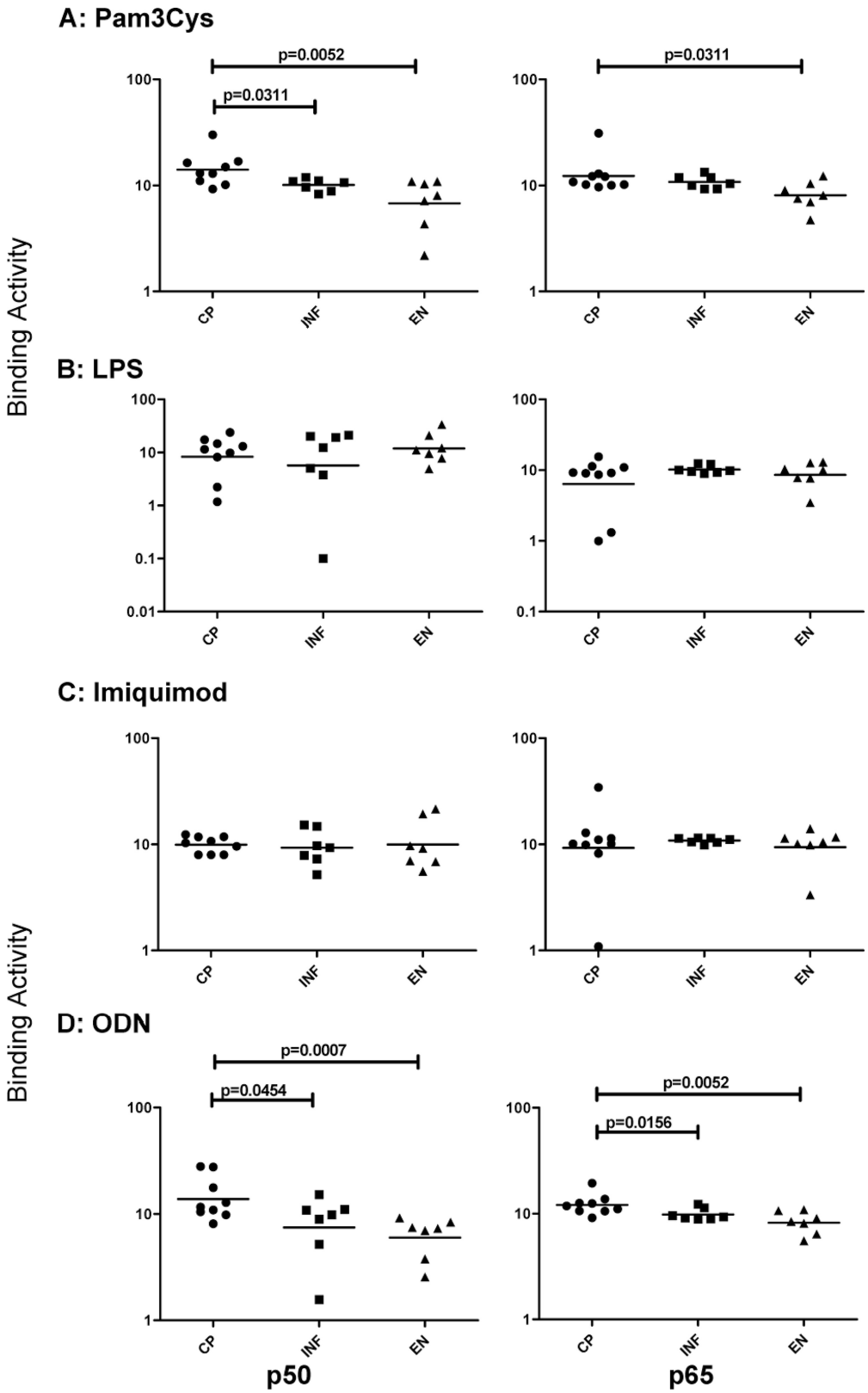


FIG. 3. Enhanced NF-κB activity in filarial pathology. PBMCs from chronic pathology (CP), infected (INF), and uninfected endemic (EN) individuals (n = 7 to 9 each) were stimulated with Pam3Cys (A), HKLM (B), imiquimod (C), and ODN (D) for 3 h, and the binding activity of the p50 and p65 components of NF- κB in nuclear lysates was measured by ELISA. Data are shown as binding activity of p50 and p65 levels in stimulated conditions over medium control. Each dot represents an individual subject. The horizontal bars are the geometric means (GM).

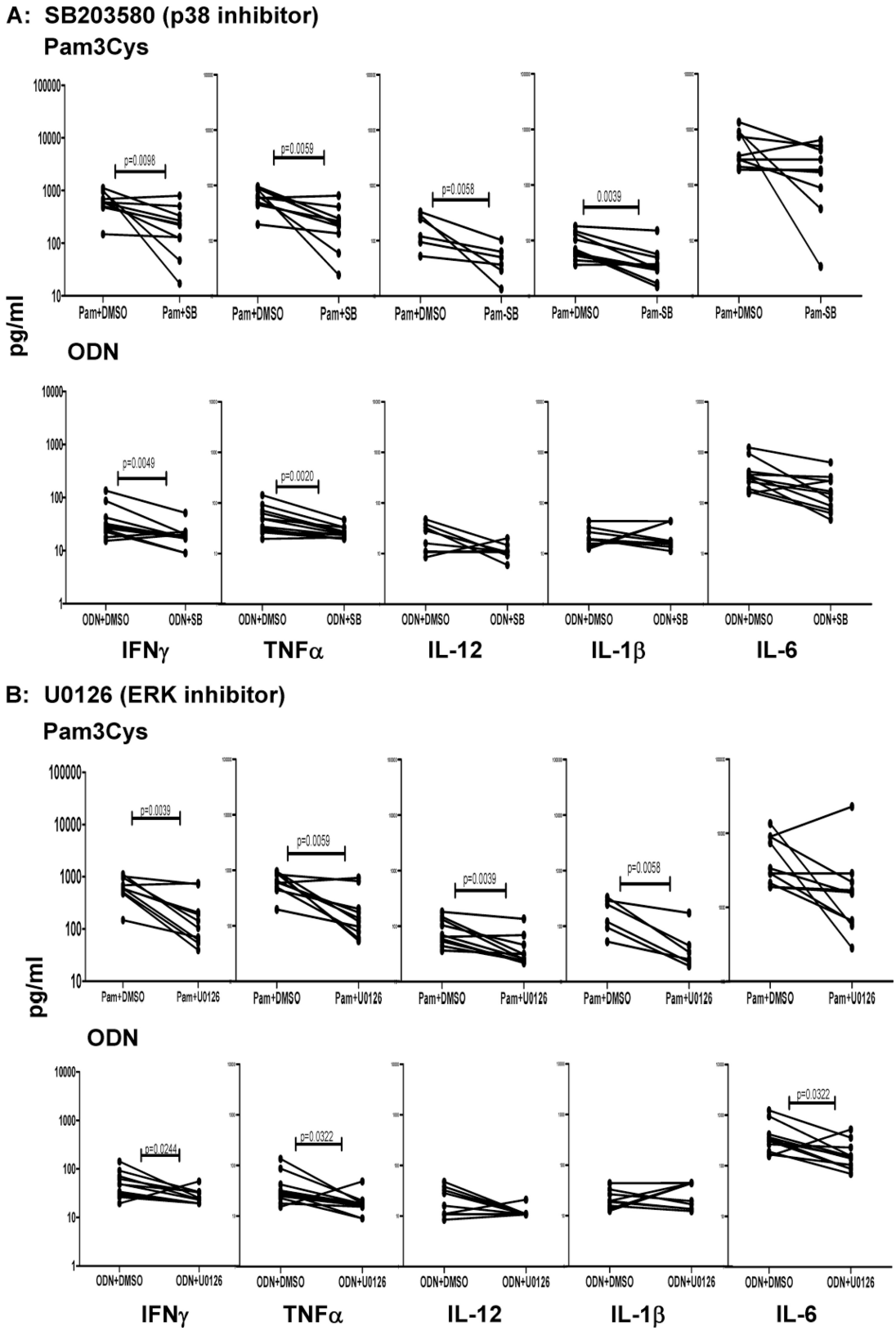


FIG. 4. Decreased proinflammatory cytokine production in filarial pathology by inhibition of p38 MAPK and ERK pathways. PBMCs from chronic pathology (CP) individuals ($n = 10$) were cultured with inhibitors of the p38 (SB203580) (A) and ERK (U0126) (B) pathways for 1 h following stimulation with Pam3Cys or ODN for 24 h, and the production of IFN-γ, TNF-α, IL-12, IL-1 β, and IL-6 was measured by ELISA. Data are shown as net cytokine levels in the presence of inhibitor or DMSO control, with each line representing a single individual.

strategies beyond the supportive care advocated for morbidity control in those with lymphedema. Our study implicates TLR-mediated innate immune responses in the development of lymphatic pathology and unravels the signaling cascade triggered in individuals with demonstrable disease. In addition, the use of TLR ligands as a surrogate to examine immune responses to secondary bacterial or fungal infections—a common occurrence in individuals with lymphedema—has provided an accurate recapitulation of the differential cytokine responses seen with those with subclinical infection and those with disease. While we have not directly addressed the role of *Wolbachia* in the development of lymphatic pathology, the ability of CP patients to mount a differential response to TLR ligands does suggest that *Wolbachia*-mediated innate cytokine responses could potentially have an important effect on pathology. Our study also highlights quantitative differences in the innate immune responses to pathogen-associated molecular patterns in individuals with overt pathology and those with subclinical pathology, emphasizing the fact that infection and disease are not necessarily concomitant in lymphatic filariasis. Further elucidation of the downstream effectors of this TLR-mediated response would provide valuable clues to circumvent the development of pathology and aid in the morbidity management of this debilitating disease.

ACKNOWLEDGMENTS

This work was supported by the Intramural Research Program of the Division of Intramural Research, National Institute of Allergy and Infectious Diseases, National Institutes of Health.

We thank NIAID intramural editor Brenda Rae Marshall for assistance.

We report no potential conflicts of interest.

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Editor: J. F. Urban, Jr.