Cytokine xxx (xxxx) xxx–xxx

Contents lists available at ScienceDirect

Cytokine

journal homepage: www.elsevier.com/locate/cytokine

Short communication

TLR11 or TLR12 silencing reduces *Leishmania major* infection

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ARTICLE INFO

Keywords:
TLR11
TLR12
Anti-leishmanial
Macrophages
Cytokines

ABSTRACT

Toll-like receptors (TLRs) recognize the pathogen-associated molecular patterns (PAMPs) and induce host-protective immune response. The role of the profilin-recognizing TLR11/TLR12 in *Leishmania* infection is unknown. Herein, we report that TLR11/ TLR12 expression increases in virulent *L. major*-infected macrophages but is prevented by miltefosine, an anti-leishmanial drug. While lipohosphoglycan (LPG) increases, LPG or TLR2 blockade prevents, the heightened TLR11/TLR12 expression. LPG-TLR2 interaction triggers MyD88- and TIRAP-mediated signaling enhancing ERK-1/2 activation and increased production of IL-10 that promotes TLR11/TLR12 expression. Profilin expression was higher in the virulent *L. major* and *L. donovani* parasites than that observed in the avirulent parasites. TLR11 or TLR12 silencing reduces parasite burden and increases IFN-γ, but reduces IL-4, production indicating that TLR11 and TLR12 play a pro-leishmanial role.

1. Introduction

Mammalian TLRs recognize PAMPs such as di- or tri-acylated lipopeptides by TLR1, TLR2 and TLR6, lipopolysaccharides by TLR4, flagellin by TLR5, single and double-stranded RNA by TLR3 and TLR7, unmethylated CpG by TLR9 and profilin by TLR11 and TLR12 [1,2]. Upon ligand binding, TLRs recruit adapter molecules such as myeloid differentiation factor 88 (MyD88), TIR (Toll-interleukin 1 receptor) domain containing adaptor protein (TIRAP), TIR domain containing adaptor inducing Interferon-β (TRIF), and TRIF-related adaptor molecule (TRAM) [3]. Signaling intermediates are assembled on the adapters and signal to transcriptionally control pro-inflammatory cytokines, which play major roles in the host-protective immune responses [3]. The TLR-induced critical cytokines are IL-12 and IL-10, the host-protective and disease-promoting correlates, respectively, as IL-12 induces IFN-γ that activates macrophages to kill the intracellular parasite *Leishmania*, but IL-10 exacerbates the infection by inhibiting IFN-γ production and macrophage activation [4–7]. Several TLRs are implicated in *Leishmania* infection [5,8]. TLR2 recognizes the *Leishmania*-expressed lipophosphoglycan (LPG) and signals through MyD88 and TIRAP [9]. TLR2 hetero-dimerization with TLR1 or TLR6 led to pro-leishmanial or anti-leishmanial responses, respectively [10]. Similarly, TLR11-TLR12 hetero-dimerization following *Toxoplasma gondii* binding or recognition of profilin [11] leads to IL-12-dependent immune response that restricts cyst burden in the infected mice [12]. Although *Leishmania* expresses profilin, the ligand for TLR11 and TLR12, the role of these two TLRs in *Leishmania* infection remains unknown. Here, we report that TLR2 engagement with LPG during *L. major* infection alters TLR11 and TLR12 expression, ERK-1/2 activation and IL-10 production. IL-10 increases the expression of TLR11 and TLR12. TLR11 or TLR12 silencing results in host-protective anti-leishmanial Th1 response and *L. major* clearance. These observations uncover a novel TLR network in a model *L. major* infection and reveal an intriguing immunoregulatory framework.

2. Materials and methods

2.1. Reagents

The reagents include antibodies against TLR11 and TLR12 (Imgenex, San Diego, CA), rabbit-IgG (Bio-Rad Laboratories, Hercules, CA), pERK-1/2, total ERK-1/2, β-actin (Santa Cruz Biotechnology, Santa Cruz, CA), LPG (Prof. Salvatore J Turco, University of Kentucky, Lexington), the TLR primers (Integrated DNA Technologies, San Diego,

Abbreviations: HPC, hexadecylphosphocholine; PAMP, pathogen-associated molecular pattern

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http://dx.doi.org/10.1016/j.cyto.2017.10.005
Received 18 July 2017; Received in revised form 29 September 2017; Accepted 2 October 2017

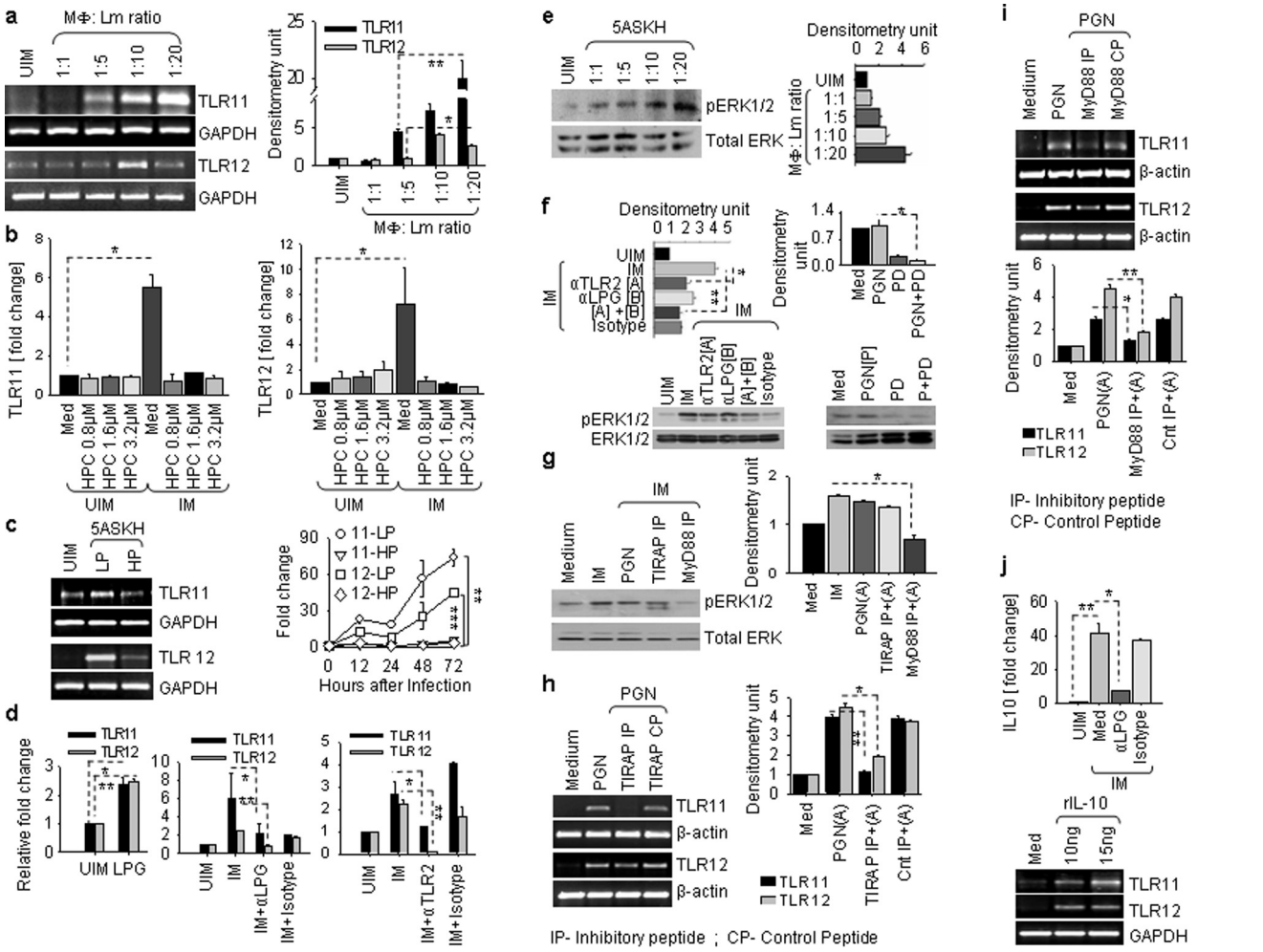

Fig. 1. *L. major* alters TLR11 and TLR12 expression in macrophages. (a) Macrophages- uninfected (UIM) or *L. major*-infected (72 h) at the indicated macrophages: *L. major* ratios- were examined for TLR11 and TLR12 expression by PCR (left; densitometry in the right). (b) Macrophages were infected with *L. major* (IM) at a 1:10 ratio for 66 h followed by miltefosine (HPC) treatment for 6 h and assessed for TLR11 (left) and TLR12 (right) expression. (c) Macrophages were infected with virulent (LP) or avirulent (HP) parasites at a 1:10 ratio for 72 h, followed by TLR11/TLR12 expression (left). TLR11/TLR12 expression in *L. major*-infected macrophages at the indicated time points was observed (right). (d) Macrophages- untreated, 24 h LPG (10 μg/ml)-treated, infected with anti-LPG-treated promastigotes or anti-TLR2-treated-were examined for TLR11/TLR12 expression. (e-g) ERK-1/2 phosphorylation in macrophages infected with different inocculum sizes of *L. major* (e), in presence of anti-TLR2 (10 μg/ml; 2 h, 28 °C) or anti-LPG (1 h, 4 °C; 10 μg/ml/10^6 promastigotes) (f, left), or pretreated with PD098059 (50 μM, 2 h) followed by peptidoglycan (PGN) (5 μg/ml; 15 min) treatment (f, right) or pre-loaded with TIRAP or MyD88 inhibitory peptides (150 μg/ml) (g). (h, i) Macrophages were pre-treated with TIRAP inhibitory peptide (150 μg/ml; 1 h, left; densitometry in the right) (h) and MyD88 inhibitory peptide (150 μg/ml; 1 h, upper; densitometry in the lower) (i), followed by treatment with peptidoglycan (PGN) (5 μg/ml; 8h) and RNA extraction to check TLR11/TLR12 expression. (j) Macrophages (UIM) were infected with anti-LPG pre-incubated *L. major* (IM), as described above. 72 h later, IL-10 expression was assessed (upper). Macrophages were treated with the indicated doses of recombinant IL-10 (rIL-10; 12 h) for assessing TLR11/TLR12 expression (lower). The experiments were performed thrice; the data are shown from one representative experiment. The error bars represent mean ± SEM. $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

CA), TLR ligands- peptidoglycan (PGN) (Alexis, San Diego, CA), the inhibitor or control peptides for MyD88 and TIRAP (Imgenex, San Diego, CA), PD098059 (Merck Millipore (Darmstadt Germany)), anti-cytokine antibodies and standard cytokines (BD-PharMingen, San Jose, CA), TRI-reagent and Miltefosine (Sigma, St Louis, MO).

2.2. Mice, parasites and infection

BALB/c mice (Jackson Laboratories, Bar Harbor, ME) were used following the animal use protocol approved by IACUC and CPCSEA, the regulatory authorities for animal experimentation. Unless otherwise specified, macrophages were BALB/c-derived thioglycolate-elicited peritoneal macrophages. Stationary phase *L. major* (5ASKH) promastigotes were injected (s.c., 2×10^6/mouse) in the mouse foot-pad. Progress of infection was studied weekly. Parasite load in the draining lymph node was assessed.

2.3. Reverse transcriptase PCR and real-time PCR

RNA was extracted using TRI-reagent, cDNA was synthesized and amplified using gene-specific primers (18): GAPDH, F-GAGCCAAACG GGTCATCATC, R-CCTGCTTCACCACCTTCTTG; TLR11, F-CTCCTG AAGGTGAAGGCTTG, R-TTCCACTCCACTTTCCCTTG; TLR12, F-GAGCAGCCGTACCACACTCT, R-CCAAAATTCACATGCACCAC; Profilin, F-CAAGTTCTTCGGCCTCCAGT, R-ACCGCGTGAGCTGTGTTCTT; β-actin, F-GTCCCTGTATGCCTCTGGTC, R-CAAGAAGGAAGGCTGGAAA AG; β-tubulin F-ATGCGTGAGATCGTTTCC, R-GGCGGCCTGCATCAT. Each sample was amplified for mouse glyceraldehyde 3-phosphate dehydrogenase (GAPDH) to ensure equal cDNA input.

Real-Time PCR using SYBR premix TAKARA Clontec (Takara Bio Inc, Shiga, Japan), 20ng of cDNA and gene-specific primers – forward and reverse – was performed: GAPDH, F-CTCATGACCACAGTCCATGC, R-CACATTGGGGGTAGGAACAC; TLR11, F-CTGAAGGTGAAGGCT TGAGGTT, R-CCTGTCATTTCCAATCCCAAA; TLR12 F-GCAGTATTGTTCCTGCCCAT, R-CGCCGCTTATAGTCAAGGTC; Profilin