Regulation of Toll-like receptor-2 expression by the Gal-lectin of *Entamoeba histolytica*

SRINIVAS J. KAMMANIDIMINTI,* BARBARA J. MANN,† LISA DUTIL,* AND KRIS CHADEE*2

*Institute of Parasitology of McGill University, Macdonald Campus, Ste. Anne de Bellevue, Quebec, Canada; and †University of Virginia, Charlottesville, Virginia, USA

SPECIFIC AIMS

We investigated the regulation of Toll-like receptor 2 expression by Gal-lectin of *E. histolytica* with a view to decipher the signaling events activated by this important parasitic virulent factor and potential vaccine candidate.

PRINCIPAL FINDINGS

1. Gal-lectin induces proinflammatory response from murine macrophage cell line RAW 264.7

Microarray studies were done using an NF-κB-specific gene array with control and Gal-lectin-stimulated macrophages. Gal-lectin up-regulated several cytokines and molecules involved in inflammation. We probed the mechanism of regulation of TLR-2 by Gal-lectin so as to decipher the signaling pathways activated by Gal-lectin.

2. Gal-lectin up-regulates Toll-like receptor 2 mRNA and protein expression in murine macrophage cell line

Gal-lectin stimulated TLR-2 mRNA expression in a dose-dependent fashion. Significant expression (3- to 4-fold) was induced with as little as 250 ng/mL of Gal-lectin; peak response was observed with 1.0 μg/mL.

In a detailed time course experiment with a suboptimum dose of 500 ng/mL of Gal-lectin, TLR-2 mRNA expression was induced by 1 h, peaked at 2 h, and returned to basal levels by 12 h. Surface expression of TLR-2 protein was significantly increased when stimulated with 1.0 μg/mL of lectin for 12 h and was comparable to that stimulated with LPS, demonstrating the potency of Gal-lectin in stimulating TLR-2 mRNA as well as protein expression in macrophages.

3. Polyclonal antiserum and mAbs against the CRR of Gal-lectin inhibit TLR-2 mRNA expression

Anti-Gal-lectin serum inhibited Gal-lectin-induced TLR-2 expression by 90% whereas the LPS-mediated response was unaffected. Preimmune serum did not affect TLR-2 expression. To identify the region of the Gal-lectin that stimulated TLR-2 mRNA expression, we examined the inhibitory effects of a panel of monoclonal antibodies that map to non-overlapping epitopes on the cysteine-rich region (CRR) of Gal-lectin. Three mAbs—1G7 (aa 596-818), 8C12 (aa 895-998), and H85 (aa 1033-1082)—markedly inhibited TLR-2 mRNA expression in response to Gal-lectin (Fig. 1) whereas mAb 3F4 (aa 895-998) and 7F4 (aa 1082-1138) did not. The latter mAbs also acted as internal isotype controls. Thus, aa 596-1082 of the CRR is responsible for stimulating TLR-2 mRNA expression in macrophages.

4. Gal-lectin induced TLR-2 gene transcription

Molecular regulation of TLR-2 gene expression was determined by a PCR-based nuclear run-on assay. A threefold-increase of TLR-2 gene transcription was observed in cells stimulated with Gal-lectin, similar to that of positive control LPS.

5. NF-κB is involved in Gal-lectin-induced TLR-2 mRNA expression

The role of NF-κB in Gal-lectin-induced TLR-2 gene transcription was determined using two NF-κB inhibitors: pyrrolidine dithiocarbamate (PDTC) and SN50 peptide. PDTC inhibited Gal-lectin-induced TLR-2 expression by 64% at 10 μM concentration and completely suppressed TLR-2 expression at 25 μM (Fig. 2A). The specific NF-κB inhibitor SN50 inhibited TLR-2 mRNA induction by 80% (Fig. 2B), confirming it has a role in Gal-lectin signaling. We observed nuclear translocation of the two subunits of NF-κB, p65 and p50, as early as 30 min after Gal-lectin stimulation (Fig. 2C). EMSA checked the DNA binding activity of NF-κB activated by Gal-lectin (Fig. 2D). Gal-lectin treatment significantly increased the DNA binding activity of NF-κB. Specificity was determined by pretreatment of the nuclear extracts with a 50- and 100-fold concentration of cold probe and by mutant NF-κB consensus sequence. Pretreatment of nuclear extracts with cold...
probe inhibited DNA binding by the labeled probe in a dose-dependent fashion whereas pretreatment with mutant probe did not inhibit subsequent binding. Supershift assay using anti-p65 antibody demonstrates the activation of this important subunit in Gal-lectin signaling (Fig. 2E).

6. P38 MAP kinase but not ERK, tyrosine kinase, or PI3 kinase plays a role in Gal-lectin-induced TLR-2 mRNA regulation

By Western blot studies using antibodies against phosphorylated forms of three important MAP kinases (ERK, JNK, and p38), we observed that Gal-lectin activates p38 kinase but not ERK or JNK. Pretreatment of cells with SB203580, a specific inhibitor of the p38 kinase pathway, inhibited TLR-2 mRNA induction by Gal-lectin by 40%; the ERK inhibitor had no effect. To further understand the upstream signaling events in Gal-lectin signaling, we studied the effects of Wortmannin, a PI3 kinase inhibitor, and herbimycin-A, a specific PTK inhibitor on Gal-lectin-induced TLR-2 mRNA expression. Neither significantly affected TLR-2 mRNA expression, which suggests that P38 kinase and tyrosine kinases do not play a role in Gal-lectin-mediated TLR-2 expression.

CONCLUSIONS AND SIGNIFICANCE

This is the first report showing the alteration of innate immune receptor expression during amebic infection. We show that Gal-lectin of *E. histolytica* induces robust TLR-2 expression in murine macrophages. To our knowledge, this is the first purified molecule isolated from a parasite that induces Toll receptor expression in macrophages. Although our study shows that Gal-lectin can stimulate TLR-2 expression, it is premature to hypothesize that it is a ligand for TLR-2. Nonetheless, of the 10 TLRs identified in mammals, TLR-2 appears to be the most promiscuous, and can be activated by a variety of other stimuli. Using a panel of monoclonal antibodies, we identified the region of Gal-lectin that stimulates TLR-2 mRNA expression and protein production. This is of paramount importance in the design...
of a subunit vaccine. No requirement of IFN-γ for TLR-2 protein expression suggests that even unprimed APCs exposed to Gal-lectin can express high levels of this Toll receptor, which may contribute to skewing the immune response to protective Th1 type.

We observed that NF-κB plays a critical role in Gal-lectin-induced TLR-2 expression. Although both Gal-lectin and LPS regulate TLR-2 in an NF-κB-dependent manner, the mechanism used by these molecules differs in several aspects, such as involvement of p38 MAP kinase, degree of DNA binding by activated NF-κB, stability of TLR-2 mRNA transcripts, and kinetics of protein production. Differential post-transcriptional regulation of TLR-2 mRNA might be responsible for these differences. Differential regulation of TLR-2 expression by p38 MAP kinase in response to several stimuli has been reported. We showed for the first time a positive regulation of TLR-2 expression by this kinase, attributing a different functional dimension to this important signaling kinase. This study also contributes to our understanding of amebic pathogenesis and intestinal inflammation. Our observation of up-regulation of TLR-2, via NF-κB, by Gal-lectin is of paramount importance in this context. Several reports reveal an increased expression of TLRs in epithelial cells and lamina propria macrophages during inflammatory conditions. We propose a working model (Fig. 3) for the role of TLR-2 in amebic pathogenesis wherein *E. histolytica* through Gal-lectin enhances the surface expression of TLR-2 in lamina propria mononuclear cell, rendering these cells more responsive to diverse ligands of gut pathogens or even commensals. The consequent activation of TLRs in intestinal epithelium results in a further influx of inflammatory cells and development of colitis, perpetuating uncontrollable intestinal inflammation. Thus, this study provides further support for the current contention of regulating TLR function as an attractive therapeutic target for controlling intestinal inflammation during gut infections with pathogens. This model proposes a role for TLR-2 in the exacerbation of the mucosal inflammation rather than in initiating the pathogenesis.

Figure 3. Hypothetical model for the role of TLR-2 in amebic pathogenesis. Enhanced TLR-2 expression in lamina propria mononuclear cells (LPMNC) by Gal-lectin of *E. histolytica* via NF-κB and p38 pathways will amplify the Toll signaling activated by diverse ligands in gut. The resulting amplified proinflammatory cytokine response from leukocytes in turn can up-regulate Toll receptor expression in epithelial cells. Epithelial cells hitherto unresponsive/hyporesponsive to gut antigens begin responding to diverse Toll ligands. The resulting production of chemokines and cytokines, while helping to signal the adaptive response, also contributes to inflammatory reaction by recruiting phagocytes to the site of tissue damage.