

*Trypanosoma cruzi* is the etiologic agent of Chagas' disease, a parasitic disease of enormous importance in Latin America. Herein we review the studies that revealed the receptors from innate immunity that are involved in the recognition of this protozoan parasite. We showed that the recognition of *T. cruzi* and its components occurs through Toll-like receptors (TLR) 2/CD14. Further, we showed *in vivo* the importance of the myeloid differentiation factor (MyD88), an adapter protein essential for the function of TLRs, in determining the parasitemia and mortality rate of mice infected with *T. cruzi*. We also discuss the implications of these findings in the pathophysiology of Chagas' disease.

**Key words:** *Trypanosoma cruzi*, Toll-like receptors, host defense, innate immune response, inflammation, glycosylphosphatidylinositol

## ***Trypanosoma cruzi* and its components as exogenous mediators of inflammation recognized through Toll-like receptors**

Marco A. Campos<sup>1,CA</sup> and Ricardo T. Gazzinelli<sup>1,2</sup>

<sup>1</sup>Laboratory of Immunopathology, Centro de Pesquisas René Rachou, FIOCRUZ, Av. Augusto de Lima 1715, Barro Preto, 30190-002 Belo Horizonte, MG, Brazil and <sup>2</sup>Department of Biochemistry and Immunology, Federal University of Minas Gerais, Belo Horizonte, MG, Brazil

<sup>CA</sup>Corresponding author

Tel: +55 31 3295 3566

Fax: +55 31 3295 3115

E-mail: marcoasc@cpqrr.fiocruz.br

## **Introduction**

Over 100 years ago, Ilya Metschnikoff<sup>1</sup> reported that the phagocytes were responsible for the innate immune response. At the same time, Paul Ehrlich reported the role of antibodies in the acquired immune response.<sup>2</sup> In 1909 both researchers received the Medicine Nobel Prize for their contributions, which had major implications in the understanding of how the immune system works. Since then, the acquired immune response, present only in vertebrates, has been the object of extensive research, resulting in vast knowledge in this field of the immunology.<sup>3</sup> On the other hand, the innate immune response, present in all live organisms, was largely neglected and until recently was only poorly understood.

By definition, inflammation is a sum of increased flow in local blood vessels and increased vascular permeability, plus the release of substances at the site of inflammation causing pain and sometimes loss of function of determined vascularized tissues.<sup>4</sup> The inflammation is largely influenced by the innate compartment of the immune system, for a long time considered an 'unspecific' response of the host to an internal or external stimulus.<sup>4</sup> For instance, cytokines are endogenous mediators of inflammation, whereas an exogenous microorganism or their molecules that

induce the production of cytokines are considered exogenous mediators of inflammation.<sup>4</sup>

## **The discovery of Toll and Toll-like receptors**

In 1985 Anderson *et al.*<sup>5,6</sup> reported that the Toll receptors were responsible for the establishment of dorso-ventral polarity in the *Drosophila* embryos. Eleven years later, Lemaitre *et al.*<sup>7</sup> relate Toll receptors with the innate immune response of *Drosophila* to fungi infection. Following that, Medzhitov *et al.*<sup>8</sup> and Rock *et al.*<sup>9</sup> described receptors related to the Toll *Drosophila* receptors in mammals, and named them Toll-like receptors (TLRs). There are 10 TLRs described in mammals to date.<sup>8,9</sup> In a broad sense the TLRs were included in a group of receptors from the innate immune system that are denominated pattern recognition receptors (PRR). The PRR recognize pathogen-associated molecular patterns (PAMPs), which are microbial targets of the innate immune system. A PAMP is a highly conserved molecule that is expressed by a class of microorganisms and not by host cells, and thus allows the discrimination of the invasive organism from self-tissues by the host innate immune system. Soon after the discovery of various TLRs, a list of counterpart agonists was identified.

**Table 1.** Toll-like receptors and their microbial agonists

Author	Reference	Year	Agonist	TLR
Poltorak <i>et al.</i>	10	1998	LPS ( <i>Escherichia coli</i> )	4
Lien <i>et al.</i>	11	2000	LPS ( <i>E. coli</i> )	4
Werts <i>et al.</i>	12	2001	LPS ( <i>L. interrogans</i> )	2
Campos <i>et al.</i>	13	2004	LPS and lipid A ( <i>B. abortus</i> )	4
Lien <i>et al.</i>	14	1999	Lipoprotein/lipopeptide, ara-lipo-arabinomannan, <i>S. aureus</i>	2
Takeuchi <i>et al.</i>	15	1999	Peptidoglycan, LTA	2
Hemmi <i>et al.</i>	16	2000	CpG DNA	9
Kurt-Jones <i>et al.</i>	17	2000	Respiratory syncytial virus molecules	4
Hayashi <i>et al.</i>	18	2001	Bacterial flagelin	5
Alexopoulou <i>et al.</i>	19	2001	Double-stranded RNA	3
Campos <i>et al.</i>	20	2001	<i>T. cruzi</i> GPI	2

LPS, lipopolysaccharide; LTA, lipoteichoic acid; GPI, glycosylphosphatidylinositol.

Table 1 presents a list of microbial molecules that act as TLR agonists.<sup>10–20</sup>

Ozinsky *et al.*<sup>21</sup> have shown that dimerization between TLR2 and TLR6 is necessary so the host cells can effectively respond to peptidoglycan. Later there was a second report showing that TLR2 may also dimerize with TLR1.<sup>22</sup> Further, Adachi *et al.*<sup>23</sup> described that with the disruption of the myeloid differentiation factor 88 gene (MyD88) the functions of IL-1 and IL-18 were lost, and Kawai *et al.* reported unresponsiveness of MyD88-deficient mice to endotoxin.<sup>24</sup> Then it was clear that this adapter protein is very important to the function of most, if not all, TLRs.<sup>22</sup>

### Recognition of *Trypanosoma cruzi* parasites through TLRs

*T. cruzi* is the etiologic agent of Chagas' disease, which infects 18 million people in Latin America. The resistance to the existent drugs against this disease is growing and there is no vaccine against *T. cruzi* infection. The symptoms of the *T. cruzi*-infected people could vary from one region to another, but they can have cardiac, gastrointestinal or neurological disturbs. Up to 25% from chagasic patients may develop cardiac, oesophageal and colonic irreversibly pathology. It is estimated that 120 million people of Latin America are at risk of contracting the infection (<http://www.who.int/tdr/diseases/chagas/direction.htm>).

The *T. cruzi* recognition and activation of the innate immune system during infection with this protozoan parasite could have two main consequences: first, it would be important for the control of the parasite in the early phase of infection; and, second, it could promote an excessive inflammatory process, through the induction of pro-inflammatory cytokines and chemokines, leading to the characteristic symptoms of acute Chagas' disease; like, for example, fever, splenomegaly and myocar-

ditis (<http://www.who.int/tdr/diseases/chagas/diseaseinfo.htm>).

### *T. cruzi* components responsible for the activation of the innate immune response

In 2001, we reported the first example of TLR recognizing a parasite molecule. We described that *T. cruzi*-derived glycosylphosphatidylinositol (GPI) anchors linked to the surface mucin-like glycoproteins and free GPI anchors named glycoinositolphospholipids (GIPLs) were recognized through TLR2/CD14, suggesting that these parasite glycolipids may be a PAMP associated with protozoan parasites.<sup>20</sup> *T. cruzi*-derived GPI anchors and GIPLs were previously shown to have immunostimulatory and immunoregulatory properties.<sup>25–27</sup> The purified GPI anchors derived from *T. cruzi* tripomastigotes were shown to be potent inducers of nitric oxide, tumor necrosis factor (TNF) $\alpha$  and interleukin (IL)-12 by macrophages, in concentrations ranging from 1 to 10 nM.<sup>20</sup> The ceramide containing GIPLs were also shown to activate macrophages (in the range of  $\mu$ M).<sup>28</sup>

*In vitro*, using stable transfected Chinese hamster ovary cells with a gene reporter, and *ex vivo*, in macrophages of TLR2 knockout (KO) mice, we showed that the recognition of *T. cruzi*-derived GPI anchors (and live parasites) was through TLR2-CD14.<sup>20</sup> The ability to trigger TLR2/CD14 from most *T. cruzi*-derived GPI anchors was in the range of 0.1–1.0  $\mu$ M. Further, the GPI anchors containing extra galactose residues in the glycan core and unsaturated fatty acids in the sn-2 position of the alkylacylglycerolipid showed activity in the range of 1–10 nM. This activity was essential for the induction of IL-12, TNF $\alpha$  and NO.

### Macrophage signaling and hypothetical functions of TLRs during infection with *T. cruzi*

It was also shown in our laboratory that the *T. cruzi*-derived GPI anchors trigger in macrophages the

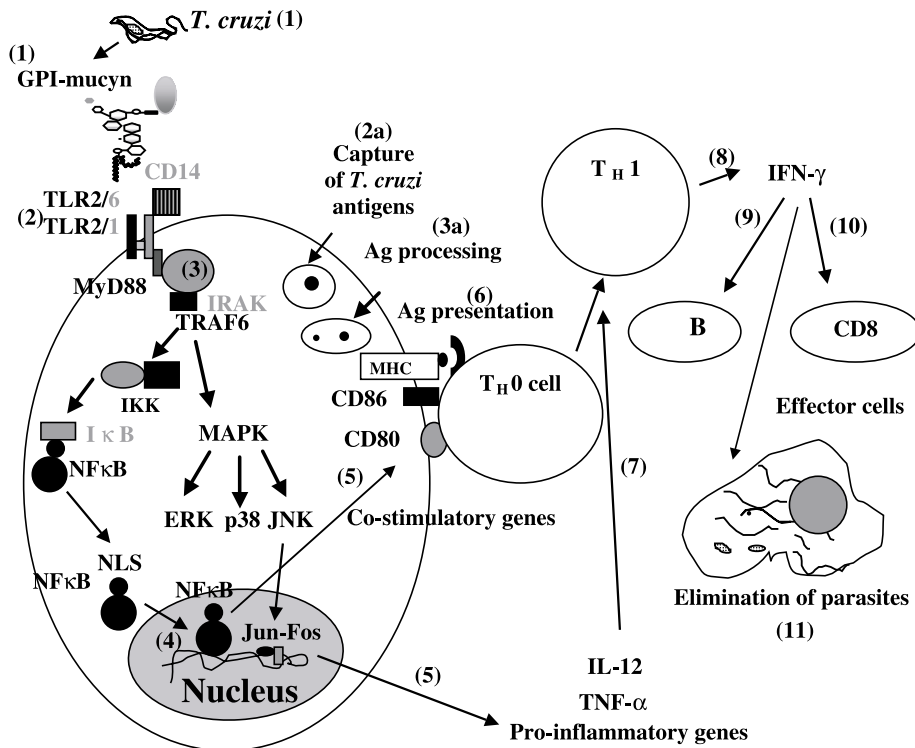


FIG. 1. Cell signaling and hypothetical functions of TLRs during infection with *T. cruzi*. After the external TLR domain recognizes *T. cruzi* and/or its molecules (step 1), the Toll/IL-1 receptor (TIR) domain (step 2) transduces the signal, which is first received by the MyD88 adaptor protein and then occurs a cascade (step 3) via IRAK, TRAF-6, IKK, IκB degradation, NFκB driving to the nucleus (step 4) and activating genes like IL-12 and costimulatory CD80, CD86 and CD40 (step 5). Another via after TRAF-6 is a cascade via MAPK, activating the Jun-Fos heterodimer, which drives to the nucleus, binding DNA and activating transcription. At the same time, *T. cruzi* antigens are captured (step 2a), processed (step 3a) and presenting to T<sub>H</sub>0 cells (step 6), in an interaction from MHC with costimulatory molecules, to obtain an effective antigen presentation. Following, the IL-12 drives the response to T<sub>H</sub>1 (step 7), which induces interferon (IFN)-γ (step 8), which stimulates B cells (step 9) to make antibodies and induces CD8 (step 10) to eliminate the parasites (step 11).

phosphorylation of mitogen-activated protein kinases (MAPKs) as well as IκB.<sup>29</sup> We propose that during the initial steps of infection with *T. cruzi*, molecules such as the parasite-derived GPI anchors stimulate cells from the host innate immune system, like dendritic cells or macrophages (step 1 from Fig. 1), through the external receptors, like TLR2 (step 2). Today it is accepted that TLR2 works in dimer,<sup>30</sup> with TLR6 or with TLR1.<sup>21</sup> Then, the TLR2/TLR6 or TLR2/TLR1 dimer transduces the signal to MyD88, which recruits IL-1-receptor-associated kinase (IRAK) (step 3), which in turn activates TNF-receptor associated factor 6 (TRAF-6) that activates the MAPK pathway,<sup>31</sup> and drives the heterodimer Fos-Jun to the nucleus, activating the AP-1 complex and activating gene transcription.<sup>32,33</sup> The TRAF-6 will also be responsible for phosphorylation of and ubiquitination of the IκB, leaving the nuclear localization signal of NFκB free, and then driving this transcription factor to the nucleus<sup>34</sup> (step 4). As a consequence, costimulatory and proinflammatory genes are activated<sup>35</sup> (step 5). At the same time, antigens of *T. cruzi* are captured (step 2a) and processed (step 3a). After that, the innate and adaptive system interacts, with the costimulatory molecules CD80, CD86 and CD40 working together with the MHC complex, to make the antigen

presentation functional (step 6). Then, the cytokine IL-12 works to drive the T<sub>H</sub>0 to T<sub>H</sub>1 (step 7), which induces interferon-γ (step 8), which in turn stimulates the effector cells lymphocyte B (step 9) and CD8 (step 10) to produce effective antibodies and destroy the pathogen, respectively (step 11).

### The *in vivo* role of TLRs during infection with *T. cruzi*

*In vivo*<sup>36</sup> we observe no major difference between parasitemia of TLR2 KO and wild-type (WT) mice infected with *T. cruzi*. Further, we observe no difference also in mortality between the infected WT and TLR2 KO mice. In experiments performed with MyD88 KO mice, however, we could show that these mice were more susceptible to *T. cruzi*, as we observed higher parasitemia (40,000 tripomastigotes/5 μl MyD88<sup>-/-</sup> × 10,000 tripomastigotes/5 μl WT) and greater mortality (100% MyD88<sup>-/-</sup> × 38% WT, by the 16th day post-infection), as compared with WT mice.

When we stimulated the macrophages from TLR2 KO or MyD88 KO mice with *T. cruzi*-derived GPI anchors, we observed that the production of the cytokines IL-12 and TNFα and also of nitrite were

completely abrogated, as compared with WT mice. However, when we used the whole parasite as stimulus, we observed a lack of response in macrophages from MyD88 KO mice, but only partial inhibition of cytokines and no effect on nitric oxide release by macrophages from TLR2 KO mice. We also showed that the cytokine production by spleen cells as well as serum levels of cytokines were greatly reduced in MyD88 KO mice, but not in TLR2 KO mice, infected with *T. cruzi*, as compared with infected WT mice. Therefore, we conclude that the host innate immune response to *T. cruzi* infection requires MyD88, but clearly involves other TLR or PRR, in addition to TLR2.

More recently, Oliveira *et al.*<sup>37</sup> observed that *T. cruzi*-derived GIPL ceramide, in high concentration, could activate mice response cells through TLR4 *in vitro* and *in vivo*. Further, it was reported that *T. cruzi* DNA stimulates macrophage to express IL-12, TNF $\alpha$  and nitric oxide.<sup>38</sup> The receptor involved on macrophage and dendritic cells activation by *T. cruzi* DNA was not defined. Nevertheless, it is reasonable to assume that this activation process may occur through TLR9, which is activated by unmethylated CpG motifs of bacteria DNA.<sup>16</sup> This hypothesis is supported by the fact that *T. cruzi* DNA has high CG contents, and that the activity of parasite DNA was blocked by digestion with DNase as well as by DNA treatment with methylase.<sup>36</sup>

## Conclusion

Our results suggest an important role of TLR signaling pathway in the innate immune response to *T. cruzi*. While we found that MyD88 is an essential molecule for activation of innate immune system during *T. cruzi* infection, our findings suggest that more than one TLR (or PRR) may be involved in this response.

Defining the role of TLRs in the pathophysiology of infection with *T. cruzi*, as well as the characterization of the *T. cruzi*-derived TLR agonists (e.g. GPI anchors and DNA), may lead us to develop more effective adjuvants to be employed in vaccines, aiming to elicit protective immunity against Chagas' disease. In addition, drugs that interfere with the TLR signaling pathways may be proven useful to protect individuals of excessive activation of cells from the immune system, and inflammation and consequent pathological effects observed during acute phase Chagas' disease.

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## References

- Metschnikoff I. Ueber eine Sprosspilzkrankheit der Daphnien. Beitrag zur Lehre über den Kampf der Phagozyten gegen Krankheitserreger. *Archiv pathol Anat Physiol klin Med* 1884; **96**: 177–195.
- Erlach P. Experimentelle Untersuchungen über Immunität, I. Ueber Ricin, II. Ueber Abrin. *Deutsche Med Wchnschr* 1891; **976**: 1218–1231.
- Janeway CA, Jr. Medzhitov R. Innate immune recognition. *Annu Rev Immunol* 2002; **20**: 197–216.
- Larsen GL, Henson PM. Mediators of inflammation. *Ann Rev Immunol* 1983; **1**: 335–359.
- Anderson KV, Jurgens G, Nusslein-Volhard C. Establishment of dorsal-ventral polarity in the Drosophila embryo: genetic studies on the role of the Toll gene product. *Cell* 1985; **42**: 779–789.
- Anderson KV, Bokla L, Nusslein-Volhard C. Establishment of dorsal-ventral polarity in the Drosophila embryo: the induction of polarity by the Toll gene product. *Cell* 1985; **42**: 791–798.
- Lemaître B, Nicolas E, Michaut L, Reichhart JM, Hoffmann JA. The dorso-ventral regulatory gene cassette Spatzle/Toll/Cactus controls the potent antifungal response in Drosophila adults. *Cell* 1996; **86**: 973–983.
- Medzhitov R, Preston-Hurlburt P, Janeway CA, Jr. A human homologue of the Drosophila toll protein signals activation of adaptive immunity. *Nature* 1997; **388**: 394–397.
- Rock FL, Hardiman G, Timans JC, Kostelevin RA, Bozon JF. A family of human receptors structurally related to Drosophila Toll. *Proc Natl Acad Sci USA* 1998; **95**: 588–593.
- Poltorak A, He X, Smirnova I, *et al.* Defective LPS signaling in C3H/HeJ and C57BL/10ScCr mice: mutations in TLR4 gene. *Science* 1998; **282**: 2085–2088.
- Lien E, Means TK, Heine H, *et al.* Toll-like receptor 4 imparts ligand-specific recognition of bacterial lipopolysaccharide. *J Clin Invest* 2000; **105**: 497–504.
- Werts C, Tapping RI, Mathison JC, *et al.* Leptospiral lipopolysaccharide activates cells through a TLR2-dependent mechanism. *Nat Immunol* 2001; **2**: 346–352.
- Campos MA, Rosinha GMS, Almeida IC, *et al.* The role of toll-like receptor 4 in induction of cell-mediated immunity and resistance to *Brucella abortus* infection in mice. *Infect Immun* 2004; **72**: 176–186.
- Lien E, Sellati TJ, Yoshimura A, *et al.* Toll-like receptor 2 functions as a pattern recognition receptor for diverse bacterial products. *J Biol Chem* 1999; **274**: 33419–33425.
- Takeuchi O, Hoshino K, Kawai T, *et al.* Differential roles of TLR2 and TLR4 in recognition of Gram negative and Gram positive bacterial cell wall components. *Immunity* 1999; **11**: 443–451.
- Hemmi H, Takeuchi O, Kawai T, *et al.* A Toll-like receptor recognizes bacterial DNA. *Nature* 2000; **408**: 740–745.
- Kurt-Jones EA, Popova L, Kwinn L, *et al.* Pattern recognition receptors TLR4 and CD14 mediate response to respiratory syncytial virus. *Nat Immunol* 2000; **1**: 398–401.
- Hayashi F, Smith KD, Ozinsky A, *et al.* The innate immune response to bacterial flagellin is mediated by Toll-like receptor 5. *Nature* 2001; **410**: 1099–1103.
- Alexopoulou L, Holt AC, Medzhitov R, Flavell RA. Recognition of double-stranded RNA and activation of NF $\kappa$ B by Toll-like receptor 3. *Nature* 2001; **413**: 432–438.
- Campos MA, Almeida IC, Takeuchi O, *et al.* Activation of Toll-like receptor-2 by glycosylphosphatidylinositol anchors from a protozoan parasite. *J Immunol* 2001; **167**: 416–423.
- Ozinsky A, Underhill DM, Fontenot JD, *et al.* The repertoire for pattern recognition of pathogens by the innate immune system is defined by cooperation between toll-like receptors. *Proc Natl Acad Sci USA* 2000; **97**: 13766–13771.
- Takeda K, Kaisho T, Akira S. Toll-like receptors. *Annu Rev Immunol* 2003; **21**: 335–376.
- Adachi O, Kawai T, Takeda K, *et al.* Targeted disruption of the MyD88 gene results in loss of IL-1 and IL-18-mediated function. *Immunity* 1998; **9**: 143–150.
- Kawai T, Adachi O, Ogawa T, Takeda K, Akira S. Unresponsiveness of MyD88-deficient mice to endotoxin. *Immunity* 1999; **11**: 115–122.
- Camargo MM, Almeida IC, Pereira MES, Ferguson MA, Travassos LR, Gazzinelli RT. Glycosylphosphatidylinositol anchored mucin-like glycoproteins isolated from *Trypanosoma cruzi* tripomastigotes initiate the synthesis of proinflammatory cytokines by macrophages. *J Immunol* 1997; **158**: 5980–5991.
- Almeida IC, Camargo MM, Procopio DO, *et al.* Highly-purified glycosylphosphatidylinositols from *Trypanosoma cruzi* are potent proinflammatory agents. *EMBO J* 2000; **19**: 1476–1485.
- Almeida IC, Gazzinelli RT. Proinflammatory activity of glycosylphosphatidylinositol anchors derived from *Trypanosoma cruzi*: structural and functional analysis. *J Leuk Biol* 2001; **70**: 467–477.
- Freire-de-Lima CG, Nunes MP, Corte-Real S, Soares MP, Previato JO, Mendonça-Previato L, Dos Reis GA. Proapoptotic activity of a *Trypanosoma cruzi* ceramide-containing glycolipid turned on in host macrophages by IFN $\gamma$ . *J Immunol* 1998; **161**: 4909–4916.
- Roport C, Almeida IC, Closel M, Travassos LR, Ferguson MA, Cohen P, Gazzinelli RT. Requirement of MAP kinases and I $\kappa$ B phosphorylation for

- induction of proinflammatory cytokines by macrophages indicates functional similarity of receptors triggered by glycosylphosphatidylinositol anchors from parasitic protozoa and bacterial LPS. *J Immunol* 2001; **166**: 3423–3431.
30. Zhang H, Tay PN, Cao W, Li W, Lu J. Integrin-nucleated Toll-like receptor (TLR) dimerization reveals subcellular targeting of TLRs and distinct mechanisms of TLR4 activation and signaling. *FEBS Lett* 2002; **532**: 171–176.
  31. Sabroe I, Read RC, Whyte MKB, Dockrell DH, Vogel SN, Dower SK. Toll-like receptors in Health and Disease: Complex questions remain. *J Immunol* 2003; **171**: 1630–1635.
  32. Kerppola T, Curran T. Transcription. Zen and the art of Fos and Jun. *Nature* 1995; **373**: 199–200.
  33. Campos MAS, Kroon EG, Gentz R, Ferreira PCP. Protein domains involved in nuclear transport of Fos. *Cell Biol Int* 1999; **23**: 81–88.
  34. Hunter T, Karin M. The regulation of transcription by phosphorylation. *Cell* 1992; **70**: 375–387.
  35. Kaisho T, Akira S. Dendritic-cell function in Toll-like receptor and MyD88-knock out mice. *Trends Immunol* 2001; **22**: 78–83.
  36. Campos MA, Clozel M, Valente EP, *et al.* Impaired production of proinflammatory cytokines and host resistance to acute infection with *Trypanosoma cruzi* in mice lacking functional myeloid differentiation factor 88. *J Immunol* 2004; **172**: 1711–1718.
  37. Oliveira AC, Peixoto JR, Arruda LB. TLR4 functional expression confers proinflammatory responsiveness to *Trypanosoma cruzi* glycosylphospholipids and higher resistance to infection with *T. cruzi* from *Trypanosoma cruzi* (submitted)
  38. Shoda LK, Kegerreis KA, Suarez CE, *et al.* DNA from protozoan parasites *Babesia bovis*, *Trypanosoma cruzi*, and *T. brucei* is mitogenic for B lymphocytes and stimulates macrophage expression of interleukin-12, tumor necrosis factor alpha, and nitric oxide. *Infect Immunol* 2001; **69**: 2162–2171.

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