Anti-apoptotic and inflammatory responses induced by *Toxoplasma gondii* infection are regulated distinctively by members of the host TLR family

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**Abstract**

Infection with *Toxoplasma gondii* results in significant changes in host cell transcription, particularly among genes affecting the immune response and prevention of apoptosis. The parasite-induced resistance to multiple apoptotic triggers has been well documented in immune and non-immune cells yet the underlying mechanisms for this phenomenon remain unclear. The role of the host Toll-like receptor (TLR) family has been evident in the immune response to *T. gondii*, however, there is little knowledge of the TLR signaling pathways regulating the anti-apoptotic response. The goal of this study was to examine whether the anti-apoptotic and pro-inflammatory responses elicited by *T. gondii* infection are regulated selectively by different members of the TLR family. Macrophages from knockout mice lacking different components of TLR signaling were infected with *T. gondii* and the induction of apoptosis was examined by activation of caspase-3 following stimulation of the death receptor pathway. Profiles of inflammatory cytokine expression in infected cells were determined by RT-PCR and ELISA. The results suggest that Toll-like receptor (TLR) domain-containing adaptors are expendable in the prevention of apoptosis by infection. On the contrary, TRadaptor molecules are essential for the activation of inflammatory cytokines in infected cells. The results reflect the capability of *T. gondii* to induce an anti-apoptotic phenotype in the absence of components of the TLR pathway that are critical regulators of a robust immune response. The selective modulation of immune and pro-survival functions by distinct members of the TLR family represents a complex level of control of host functions by *T. gondii* that might extend to other intracellular parasites.

**Introduction**

*Toxoplasma gondii* an obligate intracellular protozoan parasite capable of establishing life-long chronic infection in the host. Adaptation of the parasite to infected cell is characterized by the modulation of signal transduction components that regulate the immune response and apoptosis (1). The resistance of *T. gondii*-infected cell to apoptosis simulates the induction of anti-apoptotic genes dependent on the activation of the host transcription factor NF-κB (2). Signaling to NF-κB by pathogens occurs via recognition of pattern-associated molecular patterns by TLRs and recruitment of TIR domain-containing adaptor molecules (3). Recent studies have identified the types of TLRs involved in host resistance to *T. gondii* (4), yet little is known about the TLR-mediated mechanisms leading to the anti-apoptotic phenotype. Previous work reported that infection with *T. gondii* results in temporal control and selective activation of host genes through the participation of both host pro-apoptotic and anti-inflammatory upstream of NF-κB (5). We postulate that different subsets of TLRs and TIR adaptor molecules provide an additional level of control of selective gene expression during infection. Accordingly, the modulation of pro-survival factors and cytokines in the host cell via distinct members of the TLR family may govern the extent of anti-apoptotic responses elicited by *T. gondii* infection.

To gain a better understanding of the distinctive roles of TLRs in anti-apoptotic and inflammatory responses induced by *T. gondii*, studies were performed with TLR knockdown macrophage cell lines available at the Bodefand and Emerging Infections Research Resources Repository (BEI Resources). Use of these cell lines enables the investigation of immune mechanisms against pathogens as knockout for many of the TLRs and adaptor molecules is not always widely available. Emphasis was placed on TLRs previously reported to play roles in the host response to *T. gondii* infection (TLR2 and TLR4) (6) and on a subset of adaptor molecules involved in the late activation of NF-κB (TRIF and TRAM) (7). Our results provide support for distinctive roles among members of the host TLR family in the promotion of anti-apoptotic and inflammatory responses induced by *T. gondii* infection.

**Materials and Methods**

**Cell cultures.** Murene macrophage cell lines were derived using primary bone marrow cells from wild type, TLR2−/−, TLR4−/−, MAL−/−, TRIF−/−, and TRAM−/− mice in a C57Bl/6 background. The primary bone marrow cells were immortalized by infection with the extracellular replication-deficient retrovirus J2 (8). BEI Resources catalog numbers of the cell lines are listed in Table 1.

**Parasite strain.** The B7 clone of *Toxoplasma gondii* ME49 (BEI catalog # NR-10150) was maintained in human foreskin fibroblasts (ATCC CRL-1634) (9).

**Apoptosis protocol.** Macrophages were seeded in 6 well plates at 1 x 10⁶ cells/well. Following an overnight incubation, cells were infected with *T. gondii* at a multiplicity of infection (m.o.i) of 5 for 1 h and incubated for 12 h. Apoptosis was induced by using 25 ng/ml of recombinant murine TNF-α (R&D Systems®) in the presence of 10 μg/ml of recombinant murine IFN-γ (Sigma®). Induction of apoptosis was conducted for 12 h.

**Flow cytometry.** Macrophages were harvested by scraping and centrifugation and washed three times in PBS. Cells were fixed in 3% paraformaldehyde for 15 minutes and permeabilized in PBS containing 0.1% Triton X-100 for 5 minutes. Immunostaining was performed with rabbit antibodies against activated-caspase-3 (1:1000 dilution; Cell Signaling®) and mouse monoclonal antibodies against the adaptor molecules TRAM (1:1000 dilution; Cell Signaling®) and mouse monoclonal antibodies against the death receptor DR5. Following an overnight incubation, cells were infected with *T. gondii* at a multiplicity of infection of 5:1 and incubated for 12 h. Apoptosis was induced using 25 ng/ml of recombinant murine TNF-α and incubated for 12 h. Apoptosis was induced by using 25 ng/ml of recombinant murine TNF-α (R&D Systems®) in the presence of 10 μg/ml of recombinant murine IFN-γ (Sigma®).

**Results**

**Fig. 1. Roles of host TLRs and TIR adaptors in the inhibition of apoptosis by *T. gondii*.**

**Fig. 2. Roles of host TLRs and TIR adaptors in the *T. gondii*-mediated activation of anti-apoptotic and pro-inflammatory cytokines**

**Fig. 3. Chemokine secretion profiles among WT, TLR KO, and TIR adaptor KO macrophages in response to *T. gondii* infection**

**Fig. 4. The requirements for different members of the TLR family for the induction of anti-apoptotic gene expression by *T. gondii* infection.**

**Conclusions**

- Components of TRIL2 and TRIL4 signaling are involved in establishing a robust anti-apoptotic response in *T. gondii*-infected macrophages.
- Expression profiles of additional anti-apoptotic effectors are required to correlate the decreased resistance to apoptosis observed in macrophages deficient in TRIL2 and TRIL4.
- A lack of individual members of the TLR family results in divergent gene expression profiles in response to *T. gondii* infection. Contrary to anti-apoptotic gene subsets of inflammatory cytokines display a greater dependency on the integrity of TLR signaling components in the infected host cell.

**References**