Interleukin-12 p40- and Fas Ligand-Dependent Apoptotic Pathways Involving STAT-1 Phosphorylation Are Triggered during Infection with a Virulent Strain of *Toxoplasma gondii*

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*Toxoplasma gondii* is an opportunistic intracellular parasite. Infection with the high-virulence *T. gondii* strain RH induces inflammatory cytokine overproduction and uncontrolled apoptosis in lymphoid organs. Here, we show by fluorescent terminal deoxynucleotidyltransferase-mediated dUTP-biotin nick end labeling (TUNEL) assay and binding of fluorescein isothiocyanate-conjugated VAD-FMK, an irreversible pan-caspase inhibitor, that parasite-triggered apoptosis occurs among CD4⁺, CD8⁺, B220⁺, Gr-1⁺, and NK1.1⁺ splenic populations. Caspases 8 and 9 were activated during infection, implicating cell surface death receptors and mitochondria in apoptosis. Induction of apoptosis was absent among all cell populations in both interleukin-12 (IL-12) p40- and Fas ligand (FasL)-negative mice. STAT-1 phosphorylation correlated with onset of apoptosis during infection, but in the absence of IL-12 p40 and functional FasL, activation of this transcription factor failed to occur. The results demonstrate *T. gondii*-induced activation of multiple apoptotic pathways, dependent upon both IL-12 p40 and FasL, that may play a role in the lethal pathology of infection.

*T. gondii* is a widely distributed intracellular parasite of the class Apicomplexa. Acute-stage disease, characterized by dissemination of rapidly multiplying tachyzoites, is followed by chronic infection, during which the parasite forms quiescent cysts in tissues of the central nervous system and skeletal muscle (8). The host mounts a strong type 1 cytokine response to *T. gondii*, with high levels of the proinflammatory mediators interleukin-12 (IL-12), gamma interferon (IFN-γ), and tumor necrosis factor alpha (TNF-α) (11, 47, 55). These cytokines are required for control of infection through mechanisms such as macrophage activation and concurrent nitric oxide production, as well as through the activity of IFN-γ-inducible GTP-binding protein (IGTP) and related genes (57). Nevertheless, the immune response is itself tightly regulated, since excess production of proinflammatory cytokines can result in lethal immunopathology (10, 12, 32, 33, 39).

*T. gondii* has been classified into three lineages, based on restriction fragment length polymorphism analysis of different genes (21, 52). Tachyzoites of the prototypic high-virulence (type I) strain RH are uniformly lethal in mice during acute infection (22, 51). In contrast, low-virulence (type II or III) strains, such as ME49, do not normally kill the host but establish a long-term chronic infection associated with formation of bradyzoite-containing cysts in tissues of the brain and skeletal muscle (2, 20). Type I strains have been associated with cases of human ocular toxoplasmosis and severe congenital disease, suggesting that parasites belonging to this category are also more virulent in humans than type II and III strains (9, 15).

We, and others, recently reported that infection with high-virulence *T. gondii* strains resulted in IFN-γ overproduction and high levels of apoptosis, in contrast to low-virulence infection (10, 39). Thus, mice infected with type I strain RH tachyzoites displayed greatly increased levels of apoptotic cell death in the spleen immediately prior to death. Importantly, this did not appear to be directly attributable to parasite-induced tissue destruction but instead may have been driven by high levels of proinflammatory mediators induced by the RH parasite strain. Therefore, pathology during toxoplasmosis may, in part, be the result of uncontrolled apoptotic death induced by high-virulence strains such as RH.

Execution of programmed cell death (PCD) involves a cascade of proteolytic events characterized by cleavage of a series of procaspasezymogens into active aspartate proteases, or caspases (41, 62). Three signals induce caspase (Casp) activation. One involves death receptor engagement, resulting from an interaction between TNF-α and TNF receptor I (TNFRI), or Fas ligand (FasL) and Fas, and leading to Casp-8 activation (1, 4, 14, 27). A second pathway is initiated by changes in mitochondrial membrane potential. The latter may be induced by the presence of toxic agents, stress, or growth factor deprivation. Cytochrome c, translocated from the inner mitochondrial membrane to the cytoplasm, activates APAF-1, which then cleaves procaspase 9 to the active Casp-9 form (29, 31). Finally, PCD may be triggered by cytolytic T cells. As a result of T-cell receptor engagement, effector cells release the pore-forming molecule perforin and insert caspase-activating granzyme molecules into the target cell cytosol (18, 45, 56). Casp-8 and Casp-9, activated by the above stimuli, continue to cleave a large array of effector caspases (e.g., Casp-3, -6, and -7), which in turn attack a variety of target molecules essential for cellular structural integrity, thus mediating cell death (53).

The importance of mitochondria in PCD is underscored by molecular interactions between antiapoptotic (e.g., Bcl-2, Bcl-XL, and Bcl-w) and proapoptotic (e.g., Bax, Bak, and Bcl-XS)
members of the Bcl-2 family (13, 19). These factors become functional through dimerization in the outer mitochondrial membrane (40, 46). The signaling outcome depends on ratios of pro- and antiapoptotic complexes. Additional complexity is added by the presence of the proapoptotic factor Bid, a Bcl-2 family member which exists as an inactive protein in the cytosome but can be cleaved by Casp-8 into an active fragment (35, 59). Active Bid translocates to the mitochondria and induces cytochrome c release and activation of Casp-9 (61).

In the present study we examined apoptotic pathways triggered during acute infection with high-virulence strain RH tachyzoites. In agreement with others (39), we found that CD4+ and CD8+ T cells, B lymphocytes, NK cells, and granulocytes were induced to undergo PCD. Biochemical analysis of caspase activation suggested that multiple apoptotic pathways were activated by parasite infection. PCD was further examined in a panel of gene knockout mouse strains. Parasite-induced apoptosis continued to occur in p47^phox-/-(Phox+/-) infected mice but was substantially decreased in TNFR1-/-, inducible nitric oxide synthase (iNOS)-/-, IFN-γ/-, and especially IL-12 p40/- and FasL/- strains. Interestingly, we could correlate PCD with the presence of activated signal transducer and activator of transcription STAT-1. We conclude that high-virulence Toxoplasma infection provides a profoundly strong stimulus for PCD, resulting in activation of multiple proapoptotic pathways among different cell types. We hypothesize that global leukocyte apoptosis contributes to early death in mice infected with RH and other high-virulence type I T. gondii strains.

MATERIALS AND METHODS

Mice. B6.129S7-Hspa1bm1Txs (IFN-γ-/-), B6.129-H2b2m1m1 (IL-12 p40-/-), B6.129P2-Not1m1m1 (iNOS-/-), B6Smn.C3H-Tnfα^hm0^ (FasL-/-), and B6.129-Tnfα^hm0^m1Mak (TNFRI-/-/) mice, and C57BL/6J controls, were obtained from The Jackson Laboratory (Bar Harbor, Maine). p47^phox^ (Phox-/-) breeder pairs were kindly provided by S. M. Holland (National Institute of Allergy and Infectious Diseases, Bethesda, Md.) and bred at Cornell University. Female mice 8 to 10 weeks of age were used throughout this study. Mice were housed under specific-pathogen-free conditions in the animal facility of the College of Veterinary Medicine at Cornell University, which is accredited by the American Association for Accreditation of Laboratory Animal Care.

Parasites and infection. Tachyzoites of strain RH were maintained by biweekly passage on human foreskin fibroblast monolayers in fibroblast medium, composed of Dulbecco’s modified Eagle medium (Life Technologies, Gaithersburg, Md.) supplemented with 1% fetal calf serum (Hyclone, Logan, Utah), 100 U of penicillin/ml, and 0.1 mg of streptomycin (Life Technologies)/ml. One thousand tachyzoites were inoculated intraperitoneally (i.p.) into mice. Groups of three mice were sacrificed at 12, 24, and 48 h postinfection and then at 5 and 8 days postinfection, and spleens were harvested for analysis. Unless otherwise indicated, splenocytes from three mice per group were pooled, red blood cells were lysed by using Red Blood Cell Lysis Buffer (Sigma Chemical Co., St. Louis, Mo.), and samples were examined by flow cytometric or Western blot.

Analysis of apoptotic cell populations. For measurement of early apoptosis, freshly isolated splenocytes were resuspended at 10^7 cells/ml in complete medium consisting of Dulbecco’s modified Eagle medium (Life Technologies) supplemented with 10% fetal calf serum (Hyclone), 1 mM sodium pyruvate, 0.1 mM nonessential amino acids, 30 mM HEPES, 100 U of penicillin/ml, 0.1 mg of streptomycin/ml, and 50 mM 2-mercaptoethanol (supplements from Life Technologies). The irreversible pan-caspase inhibitor peptide VAD-FMK, conjugated to fluorescein isothiocyanate (FITC; Promega, Madison, Wis.), was added at a final concentration of 10 μM. After a 20-min incubation at 37°C, splenocytes were washed, Fc receptors were blocked with 10% normal mouse serum (Jackson ImmunoResearch Laboratories, West Grove, Pa.), and cells were stained for surface markers with monoclonal phycoerythrin (PE)-conjugated antibodies (Ab) directed against mouse CD4, CD8, NK1.1, Gr-1 (Ly-6G) (all from BD PharMingen, San Diego, Calif.), and B220 (Caltag Laboratories, Burlingame, Calif.). Alternatively, for late-phase apoptosis, freshly isolated splenocytes were fixed in 3.7% formalin, permeabilized, and subjected to terminal deoxynucleotidyl transferase (TdT)-mediated labeling of nick-end DNA strands with FITC-conjugated dUTP (TUNEL) by using a commercially available kit and following the manufacturer’s protocol (Boehringer Mannheim, Indianapolis, Ind.). Cells were then surface stained as above. All flow cytometric data were acquired on a FACScalibur flow cytometer and analyzed with CellQuest software (Becton Dickinson Immunocytometry Systems, San Jose, Calif.).

Western blotting. Cells (10^6 per sample) were lysed in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer, composed of 62.5 mM Tris-HCl (pH 6.8), 2% (wt/vol) SDS, 10% glycerol, 50 mM dithiothreitol, and 0.01% (wt/vol) bromphenol blue. To facilitate gel loading, DNA in the lysate was fragmented by sequential passage through 18-, 23-, and 27-gauge needles. Samples (20 μl) were subjected to electrophoresis on an SDS-PAGE gel (10 or 12% polyacrylamide) under reducing conditions and were transferred to nitrocellulose membranes. After transfer, membranes were blocked for 2 h at room temperature with 1× Tris-buffered saline plus 5% nonfat dry milk and were incubated overnight at 4°C with the recommended dilution of primary Ab to the full-length and cleaved forms of poly(ADP-ribose) polymerase (PARP), Casp-9 (Cell Signaling, Beverly, Mass.), Casp-8 (BD PharMingen), and the total and phosphorylated forms of STAT-1 (Cell Signaling). Bound Ab were detected with a horseradish peroxidase-conjugated secondary Ab and an enhanced chemiluminescence system (LumiGLO; Cell Signaling).

RESULTS

T. gondii triggers apoptosis in multiple cell types. We previously reported that after initial cell proliferation, all splenocyte populations decreased to levels at or below those of noninfected animals. This suggested that cell death may be a widespread phenomenon during RH infection. We therefore sought to identify which spleen cell populations were undergoing apoptosis. Freshly isolated splenocytes from noninfected or 5-day-RH-infected animals were loaded with the fluorescently-labeled irreversible pan-caspase inhibitor VAD-FMK–FITC, surface stained for membrane markers (CD4, CD8, B220, Gr-1, and NK1.1), and analyzed by flow cytometry. We determined that apoptotic cell death, detected by the presence of activated caspases to which the inhibitor bound, was not limited to one splenic population but was triggered in CD4+ and CD8+ T cells, B cells, NK cells, and granulocytes (Fig. 1A and B). We obtained similar results at 7 days post-infection using fluorescent TUNEL reagents (Fig. 1C and D). No TUNEL-positive cells were detected at 5 days post-infection (data not shown), a time when caspase activation was maximal. This difference likely relates to the fact that caspase activation precedes DNA degradation during progression of PCD.

We previously showed the presence of a major population of TUNEL-positive cells in the spleens of mice infected for 8 days with the high-virulence type I T. gondii strain RH (10). In order to further confirm that PCD was activated, we assayed for the presence of cleaved PARP. Intact PARP is a crucial 116-kDa enzyme involved in multiple cell survival-related functions, including DNA repair (49). PARP cleavage by activated effector caspases, an event specific to apoptosis but not necrosis, renders the enzyme no longer functional (7, 30). To assess PARP cleavage after T. gondii infection, we examined cell lysates obtained at given time points from the spleens of mice inoculated i.p. with 10^7 tachyzoites of the high-virulence strain RH. Western blot analysis of lysates, using an Ab recognizing both full-length and cleaved PARP peptides, showed the appearance of the diagnostic p89 fragment, indicative of apoptosis at 5 and 8 days postinfection (Fig. 2A).

Infection results in activation of multiple apoptotic path-
Apoptosis induced through death receptors, such as Fas or TNFRI, leads to activation of Casp-8. However, Casp-9 is activated by the mitochondrially induced pathway of PCD, after release of cytochrome c and APAF-1 activation. This is linked to loss of mitochondrial membrane potential resulting from stress and inflammation. Therefore, in order to assess if either the receptor-mediated or the mitochondrially induced pathway was activated by the parasite, we examined levels of the proforms (inactive) and cleaved forms (active) of Casp-8 and Casp-9.

Remarkably, we found a rapid degradation of the pro-Casp-8 protein to undetectable levels, as early as 48 h postinfection (Fig. 2B). This result suggested a receptor-mediated induction of caspase activation. We also found that the levels of pro-Casp-9 (p49) diminished during the course of infection. Cleavage at Asp368, resulting in the p39 peptide, is due to Casp-3 activity, and the p37 Casp-9 fragment results from self-cleavage at Asp353 (31). For reasons presently unclear, the latter band appeared only at 5 days postinfection, fading by 8 days. Activation of Casp-9 suggests a role for the mitochondrial pathway of PCD during *T. gondii* infection.

We next assessed apoptosis in a panel of gene knockout mouse strains undergoing acute RH infection. Western blot analysis for the presence of degraded PARP showed a strong p89 band in samples from infected wild-type (WT) and Phox−/− mice. However, PARP degradation was visibly less in samples from infected TNFRI−/− mice, greatly decreased in samples from infected iNOS−/− and FasL−/− mice, and absent in samples from IL-12 p40−/− mice (Fig. 3).

Because several of the infected knockout mouse strains displayed lower levels of PARP degradation, we sought to determine if, in a given mouse strain, a specific splenic population would lack apoptosis. We therefore incubated splenocytes from infected mice with VAD-FMK–FITC, surface stained for membrane markers, and compared data to those obtained from noninfected samples (Fig. 4) and WT controls (see Fig. 1B). All cells from Phox−/− mice that were examined displayed apoptosis, similarly to cells from WT mice. Splenocytes corresponding to samples with reduced PARP degradation showed lower proportions of caspase-positive cells after infection than were observed in samples from Phox−/− and WT mice. Cells from IL-12 p40−/− mice exhibited the lowest levels of inhibitory peptide binding, consistent with the total lack of PARP degradation shown in Fig. 3. Notably, for any given gene knockout mouse strain, levels of apoptosis were equivalently affected among all cell types.

**PCD is correlated with STAT-1 activation.** In previous studies, we and others found that T cells from mice infected with high-virulence strains of *T. gondii* secrete excessively high levels of proinflammatory cytokines such as IFN-γ (10, 39). We hypothesized that these proinflammatory signals may trigger apoptosis in spleens of infected mice. Because IFN-γ signals through STAT-1 (37), we examined its activation during the course of acute *T. gondii* infection. We found high levels of activated STAT-1 (detected as phospho-STAT-1) at 5 and 8
days postinfection (Fig. 5A), time points corresponding to the presence of degraded PARP (Fig. 2A). We then determined if STAT-1 activation in the infected gene knockout mice predicted the onset of apoptosis as determined by PARP degradation. Indeed, activated STAT-1 was detected at high levels in WT and Phox−/− samples, was present at substantially lower levels in cells from infected TNFRI−/−, iNOS−/−, and FasL−/− mice, and was undetectable in IL-12 p40−/− and IFN-γ−/− spleen cell lysates (Fig. 5C).

DISCUSSION

Immune stimulation with a single antigen, or during infection with a pathogen, induces antigen-specific lymphocyte proliferation, as well as production of cytokines and antibodies. The initial response is later down-regulated by diverse mechanisms. Activation-induced cell death eliminates antigen-responsive cells by apoptosis and has been shown to occur during protozoan infection (1, 28, 42). Another mechanism regulating the immune response is production of modulatory cytokines (e.g., IL-10 and transforming growth factor β), which counteract the pathological effect of excessive proinflammatory factors (12, 16, 25, 26, 50). In previous work comparing mice infected with low- and high-virulence T. gondii strains, we and others determined that in the latter case lethality is associated with excessive levels of proinflammatory cytokines such as IFN-γ and extensive splenic lymphocyte death (10, 39). Therefore, we hypothesized that splenic apoptosis during acute toxoplasmosis is induced by the high levels of proinflammatory cytokines present, and we sought to define the pathways involved.

Here, we first show that apoptosis is a generalized event in the spleen after RH infection, in that all lymphocyte populations studied have increased levels of apoptosis (Fig. 1). Similar phenomena have previously been described for secondary lymphoid organs after either T. gondii or Plasmodium chabaudi chabaudi infection (17, 34) and also for nonlymphoid organs such as the liver after Entamoeba histolytica infection (48). Nevertheless, the present results demonstrate a strikingly widespread induction of PCD during T. gondii infection. Thus, cells of both lymphoid and myeloid lineages were triggered by the parasite to undergo apoptosis during acute infection. While RH infection induces massive splenic apoptosis, the proportion of infected cells does not rise above 2% (10). This strongly argues against a direct effect of Toxoplasma in inducing apoptosis, suggesting instead an immunopathological basis underlying parasite-induced PCD.

Both the Fas-FasL and TNF-α–TNFRI pathways may be activated in the end phases of the immune response, in order to modulate excessive responses with possible pathological outcomes and to remove antigen-specific lymphocytes after microbial clearance (6). Indeed, these pathways have been implicated in down-regulating the inflammatory cell infiltrates at the

FIG. 2. T. gondii induces degradation of PARP and cleavage of Casp-8 and Casp-9. At the indicated times following i.p. injection of 10⁵ strain RH tachyzoites into C57BL/6J mice (WT), spleens were collected, cells were lysed, and lysates were subjected to SDS-PAGE and Western blot analysis. (A) The presence of caspase-cleaved PARP was detected with an Ab recognizing both the full-length enzyme and its cleaved fragment. The full-length (p116) and cleaved (p89) PARP molecules are indicated by arrows. (B) An Ab directed against the pro-Casp-9 peptide detects a band that decreases in intensity by 24 h postinfection. Two active forms of Casp-9 are found at 5 days postinfection. The upper band, p39, results from pro-Casp-9 and its cleaved fragment. The full-length (p116) and cleaved (p89) PARP was detected with an Ab recognizing both the full-length enzyme and...
site of lesion during *Leishmania major* and *Trypanosoma cruzi* infections (3, 5, 24, 36, 58). We show here that infected FasL/H11002 and TNFRI/H11002 mice display low levels of PARP degradation (Fig. 3) and that splenocytes bind low levels of caspase inhibitor, relative to those in infected WT animals (Fig. 4). This indicates that both pathways are involved in *Toxoplasma*-induced apoptosis. Furthermore, Casp-8 undergoes remarkably rapid degradation in splenocytes from infected WT mice (Fig. 2B).

**FIG. 4.** Reduced apoptosis in infected gene knockout mice occurs uniformly among all splenic populations. Mice were infected i.p. with $10^7$ RH tachyzoites, and splenocytes were analyzed 6 days later in comparison with cells from noninfected animals. Freshly isolated cells were incubated with VAD-FMK-FITC and were PE surface stained for cell-specific markers. Graphs represent summaries of the fluorescence-activated cell sorter data. Open bars, samples from noninfected mice; solid bars, samples from infected animals. This experiment was repeated twice with similar results.

**FIG. 5.** STAT-1 activation correlates with the induction of apoptosis during *Toxoplasma* infection. Spleen cell lysates were prepared after i.p. infection with $10^7$ RH tachyzoites and were subjected to Western blot analysis using Ab directed against activated STAT-1 (A and C) and total STAT-1 (B and D). (A and B) Samples from WT mice obtained at the indicated time points. (C and D) Cell lysates from the indicated noninfected (N) and 6-day-postinfection (I) knockout mice were analyzed. The Ab used recognizes an epitope common to both the STAT-1α (p91) and STAT-1β (p84) splice variants; thus, STAT-1 appears as a dimer in these blots. Data are representative of four (panels A and B) and two (panels C and D) experiments independently performed.
We conclude that *T. gondii* triggers extracellular pathways of apoptosis through both TNF-α–TNFRI and Fas–FasL interactions.

A role for the mitochondrially induced apoptotic pathway is suggested by our finding that Casp-9 is activated during acute *T. gondii* infection. This pathway may be activated as a response to stress, and a candidate mediator would be nitric oxide, since iNOS−/− mice display greatly reduced levels of apoptosis (44). Nevertheless, because the mitochondrial pathway may be triggered by Casp-8 through the activity of Bid (Fig. 1) (35), it is possible that Casp-9 activation is mediated by Fas-FasL and TNF-α–TNFRI engagement. Arguing against the latter, we did not detect Bid activation at any point during the acute phase of *T. gondii* infection (data not shown).

We report here for the first time in an in vivo parasite infection model the tight correlation between apoptosis and STAT-1 activation (Fig. 5). Thus, STAT-1 phosphorylation occurred in WT animals concurrently with the timing of apoptosis, and STAT-1 activation was reduced or absent in those knockout mouse strains in which levels of apoptosis were reduced. Activation of STAT-1, the only known signal transducer and activator of transcription responding to IFN-γ, has been linked to apoptosis in the cases of ischemia-induced cell death in cardiomyocytes and keratinocyte apoptosis after epithelial injury in vivo, while STAT-1-null mice showed resistance to apoptotic stimuli (38, 54). In vitro studies on the mechanisms by which STAT-1 activation may trigger apoptosis have linked the molecule to induction of Casp-1 and Fas-FasL, but the precise mechanisms involved remain obscure (54, 60). The studies reported here suggest that protozoan infection triggers apoptosis in multiple cell types through multiple pathways and that STAT-1 activation is correlated with the presence of PCD. While IFN-γ serves as the major activator of STAT-1, other molecules such as IL-6 and IL-10, as well as platelet-derived growth factor and epidermal growth factor, may activate this transducing molecule (37, 43). Thus, it is possible that the lack of apoptosis in infected FasL−/− and iNOS−/− animals points to a direct role for STAT-1 in PCD triggered through Fas and nitric oxide, although the mechanism linking STAT-1 with FasL and iNOS is not presently clear. This may be an event independent of IFN-γ, as cultures of splenocytes from infected FasL−/− and iNOS−/− animals harbored high levels of IFN-γ (data not shown). Our laboratory is currently further investigating the role of STAT-1 during *T. gondii* infection.

Apoptosis has previously been linked to acute *T. gondii* infection (28). Recent studies have demonstrated induction of PCD during high-virulence infection (10, 39). Induction of apoptosis has also been reported during ocular toxoplasmosis, and in Peyer’s patch CD4+ T cells in orally infected mice (23, 34). The latter effect was dependent on IFN-γ and was linked to FasL up-regulation. Therefore, it is possible that IFN-γ exerts its proapoptotic effect at least partly through up-regulation of this death receptor.

Why *T. gondii* would induce such a generalized cell death is not clear. In the context of the mouse, infection with RH and other type I parasite strains is uniformly lethal, and dysregulated apoptosis driven by overproduction of inflammatory mediators may contribute to early death in this model. Inasmuch as human infection with type I strains is not lethal, it is possible that induction of apoptosis would serve as a parasite defense strategy temporarily disabling the immune system and allowing the establishment of long-term infection.

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