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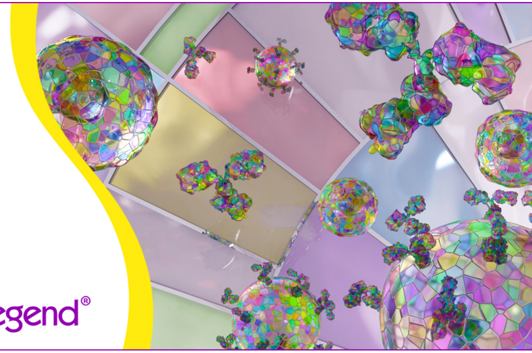

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Acute Toxoplasmosis Leads to Lethal Overproduction of Th1 Cytokines[1]

Dana G. Mordue,* Fernando Monroy,[2]* Marie La Regina,[†] Charles A. Dinarello,[‡] and L. David Sibley[3]*

Virulence in *Toxoplasma gondii* is strongly influenced by the genotype of the parasite. Type I strains uniformly cause rapid death in mice regardless of the host genotype or the challenge dose. In contrast, the outcome of infections with type II strains is highly dependent on the challenge dose and the genotype of the host. To understand the basis of acute virulence in toxoplasmosis, we compared low and high doses of the RH strain (type I) and the ME49/PTG strain (type II) of *T. gondii* in outbred mice. Differences in virulence were reflected in only modestly different growth rates in vivo, and both strains disseminated widely to different tissues. The key difference in the virulent RH strain was the ability to reach high tissue burdens rapidly following a low dose challenge. Lethal infections caused by type I (RH) or type II (PTG) strain infections were accompanied by extremely elevated levels of Th1 cytokines in the serum, including IFN-γ, TNF-α, IL-12, and IL-18. Extensive liver damage and lymphoid degeneration accompanied the elevated levels of cytokines produced during lethal infection. Increased time of survival following lethal infection with the RH strain was provided by neutralization of IL-18, but not TNF-α or IFN-γ. Nonlethal infections with a low dose of type II PTG strain parasites were characterized by a modest induction of Th1 cytokines that led to control of infection and minimal damage to host tissues. Our findings establish that overstimulation of immune responses that are normally necessary for protection is an important feature of acute toxoplasmosis. ***The Journal of Immunology,* 2001, 167: 4574–4584.**

Toxoplasma gondii is an intracellular parasite that infects a wide variety of hosts, including humans (1). Human infection occurs through two main routes, ingestion of undercooked meat containing cysts of the parasite and ingestion of oocysts passed into the environment by cats (2). During acute infection, rapid growth of the parasite is contained by a vigorous IFN-γ-dependent, cell-mediated immune response. Chronic infection is a consequence of parasite differentiation into slow-growing cysts that are capable of persisting for the life of the host (3). In immunocompromised individuals, *Toxoplasma* is an important opportunistic pathogen due to fulminate re-emergence of chronic infections (4). Acute infections acquired during pregnancy are also capable of causing severe birth defects, including hydrocephaly, calcification, neurological defects, and chorioretinitis, which may be recurrent (5).

The outcome of toxoplasmosis in the mouse model is strongly dependent on the strain of *T. gondii* and can be predicted based on the parasite genotype (6, 7). Although *T. gondii* strains are genetically very similar, they comprise three distinct clonal lineages (6, 8). Strains of the type I genotype are highly virulent in mice, and regardless of the genetic background of the mouse host, they have a lethal dose of a single viable parasite (6, 7). In contrast, type II and III strains have a 50% lethal dose of $\geq 10^3$ parasites, and the outcome of infection is dependent on the genotype of the host (9–12).

Type II strains are the most prevalent in animals and are most commonly associated with human toxoplasmosis (8, 13). Despite an overall lower prevalence, type I strains are often associated with severe congenital toxoplasmosis (14, 15) and ocular toxoplasmosis (16), suggesting that they are also more pathogenic in humans. *T. gondii* is capable of infecting sites of immune privilege, including the retina, CNS, and placenta. In these tissues, cellular immunity is important for control of infection; however, the extent of tissue inflammation is often disproportionate to the presence of parasites, suggesting that the resulting pathology is partially immune-mediated (2, 17–20). Furthermore, in cases of disseminated toxoplasmosis, which occur primarily in immunocompromised patients, systemic infections are characterized by a septic shock-like syndrome leading to death (21).

Control of toxoplasmosis during nonlethal infection with nonvirulent strains has been extensively studied in the mouse model where a vigorous IFN-γ-dependent, Th1 cytokine response controls infection. IL-12 is released early in infection and potentiates the production of IFN-γ by NK cells and T cells (22–24). IFN-γ is absolutely essential to the control of both acute and chronic infections (25–27). The innate immune response is implicated in the early induction of proinflammatory cytokines during infection of naive animals, and studies have shown that dendritic cells (28), neutrophils (29), and macrophages (30) respond directly to parasite Ags by producing IL-12 and TNF-α. TNF-α is also important for controlling resistance to acute (31, 32) and chronic (33, 34) infections by *T. gondii*.

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While nonvirulent strains are generally well controlled by the immune response, type I strains are uniformly lethal in mice, suggesting that they induce inadequate immune control or are endowed with destructive properties that directly cause pathology. To determine the basis of the remarkable difference in virulence of *T. gondii* strains, we examined the response of immunocompetent outbred mice to infection by type I and type II strains of *T. gondii*. Surprisingly, our findings indicate that lethal infections are associated with massive overstimulation of Th1 cytokines, which probably contribute to pathology.

Materials and Methods

Mice

CD1 mice were obtained from Charles River Laboratories (Wilmington, MA). Double-knockout mice lacking both TNFR I (p55) and TNFR II (p75; strain name, B6;129S-*Tnfrsf1a*^{tm1Imx}*Tnfrsf1b*^{m1Imx}), referred to as TNFR knockout (KO)[4] mice, and control mice representing a cross between C57BL/6J and 129 (strain name, B6129SF2/J), referred to as B6/129 controls, were obtained from The Jackson Laboratory (Bar Harbor, ME). Mice with normal responsiveness to LPS (strain name, C3H/HeOuJ MMTV$^-$) and LPS-hyporesponsive mice (strain name, C3H/HeJ MMTV$^-$) were obtained from The Jackson Laboratory. Fas-deficient mice (B6.MRL-*Tnfrs6lpr),* referred to as B6-*lpr*, and control C57BL/6J mice were obtained from The Jackson Laboratory. Animals were housed under specific pathogen-free conditions at Washington University School of Medicine (St. Louis, MO). Female mice between 8 and 12 wk of age were used for experiments.

Parasite growth

T. gondii strains were maintained by serial 2-day passage of tachyzoite stages in human foreskin fibroblast monolayers as described previously (35). The RH strain (36) was used as a representative of the type I lineage. A cloned line of the ME49 strain, called PTG (37), was used as a representative of the type II lineage. All parasite strains were tested for *Mycoplasma* contamination using the GenProbe kit (Fisher Scientific, Pittsburgh, PA) and remained negative throughout the experiments.

Parasite numbers in tissue and blood were determined every other day starting on day 2 throughout the acute stage of infection. Mice were sacrificed, and lungs, hearts, livers, spleens, mesenteric lymph nodes, peripheral lymph nodes, ileums, kidneys, peritoneal fluid, and brains were isolated. Tissue samples were homogenized using a saw-tooth generator at low speed (PowerGen 125, Fisher Scientific) under conditions that did not affect parasite viability (data not shown). The number of viable parasites in tissues was determined by plaque formation on fibroblast monolayers (duplicate 12-well cultures per sample) (38).

Histopathology

Tissues were fixed in 4% buffered formaldehyde and embedded in paraffin wax, and 5-μm serial sections were stained with H&E or periodic acid-Schiff (PAS) according to standard procedures. Serial sections were deparaffinized, rehydrated, and stained with specific primary Abs followed by use of the Vectastain Elite ABC HRP kit and Vector DAB or Vector Red substrates (Vector Laboratories, Burlingame, CA). Liver sections were screened for lipid accumulation by staining frozen sections with Oil-Red-O. H&E-stained liver sections were blindly scored for pathology.

Demonstration of apoptosis in situ

Apoptosis was detected by TUNEL labeling using the In Situ Cell Death Detection Kit AP (Roche, Indianapolis, IN). Formalin-fixed, paraffin-embedded sections of spleen or liver from control and infected mice were labeled by TUNEL reaction and visualized using Vector Fast Red, which provides colorimetric and fluorescence signals (Vector Laboratories). Apoptosis was quantified by counting the number of apoptotic cells per total nucleated cells within lymphoid follicles. Five random fields at $\times 20$, consisting of ≥ 400 cells/field, were counted from three or four mice per group, and the values are reported as the mean $\pm$ SE.

For FACS-based analysis of apoptosis, cells were stained with Alexa 488-conjugated annexin V, and syntaxin green was used to identify dead cells (Molecular Probes, Eugene, OR). Stained cells were analyzed on a FACScan and evaluated using CellQuest software (Becton Dickinson, Cockeysville, MD).

Serum liver enzymes and cytokines

Blood was collected by cardiac puncture, and serum was isolated using separator tubes (Sarstedt, Numbrecht, Germany). Serum enzyme levels of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were quantified using a commercial kit (Sigma, St. Louis, MO) that was adapted for use in 96-well plates. Serum levels of IFN-γ, IL-18, TNF-α, IL-12 p40, IL-10, and IL-1β were assayed using OptEIA, two-site ELISA sets from BD PharMingen (San Diego, CA). The sensitivities of detection in the ELISAs were 31.3 pg/ml for IFN-γ and IL-12 p40, and 62.5 pg/ml for IL-18, TNF-α, IL-10, and IL-1β.

Antibodies

The following cell markers were detected using biotin-conjugated Abs obtained from BD PharMingen (La Jolla, CA): Ly-6G (Gr-1) was detected with mAb RB6-8C5, NK cells were detected using mAb DX5, CD8α cells were detected with mAb 53-6.7, CD4-positive T cells were detected with mAb GK1.5, B cells were detected using mAb RA3-6B2 specific for CD45R/B220, and TCRβ was detected with mAb H57-597. PE-conjugated mAb GL3 to $\gamma\delta$TCR was obtained from BD PharMingen. Unlabeled rabbit polyclonal antiserum against murine inducible NO synthase (iNOS) was obtained from BD Transduction Laboratories (Franklin Lakes, NJ). mAb FA-11 to CD68 was obtained from Serotec (Oxford, U.K.). The F4/80 hybridoma was obtained from American Type Culture Collection (Manassas, VA).

Ab neutralization of cytokines

For IL-18 neutralization, female CD1 outbred mice were given 200 μl i.p. of rabbit anti-IL-18 antiserum (39), rabbit anti-GST (40) as an irrelevant control antisera, or sterile pyrogen-free PBS. A hamster mAb H22, (41) against murine IFN-γ was used to neutralize IFN-γ. A hamster mAb TN3-19.12 against murine TNF-α was used to neutralize TNF-α. Purified hamster IgG (Jackson ImmunoResearch Laboratories, West Grove, PA) and sterile pyrogen-free PBS were used as controls for neutralization with hamster Abs. Five mice per group were used for each time of death study and three to five additional mice per group were sacrificed on day 9 postinfection for determination of pathology and serum concentrations of cytokines and liver transaminases.

Results

Establishment of a model for acute toxoplasmosis

Previous studies have established that a single viable type I strain parasite is sufficient to cause death in the mouse (7, 8). To establish a model for examining acute virulence, mice were challenged i.p. with low doses of the type I RH or type II PTG strain of *T. gondii*. Challenge with 100 organisms of the RH strain resulted in 100% mortality within 8–9 days. In contrast, 80% of mice given a similar dose of the PTG strain survived beyond 20 days (Fig. 1*A*). Challenge with a high dose of PTG (10^5 parasites) resulted in 80% mortality (Fig. 1*A*). Differences in virulence in vivo were not a result of inherent differences in viability, as shown by the similar capability of the strains to cause plaques on host cell monolayers in vitro (data not shown). This model, which discriminates between strains that are virulent regardless of dose (type I) from those which show a dose-dependent lethality (type II), was used to evaluate the acute virulence of toxoplasmosis.

Growth and dissemination of virulent and nonvirulent T. gondii *strains in vivo*

We reasoned that the acute virulence of type I *T. gondii* strains might be due to a greater ability to disseminate and/or replicate in vivo relative to type II strains. To examine this possibility, mice were challenged with a low dose (100) of RH or PTG strain parasites, and the number of viable parasites in blood and tissues was determined at intervals postinfection by plaque formation in vitro.

[4] Abbreviations used in this paper: KO, knockout; ALT, alanine aminotransferase; AST, aspartate aminotransferase; iNOS, inducible NO synthase; MLN, mesenteric lymph nodes; PAS, periodic acid-Schiff; PLN, peripheral lymph nodes.

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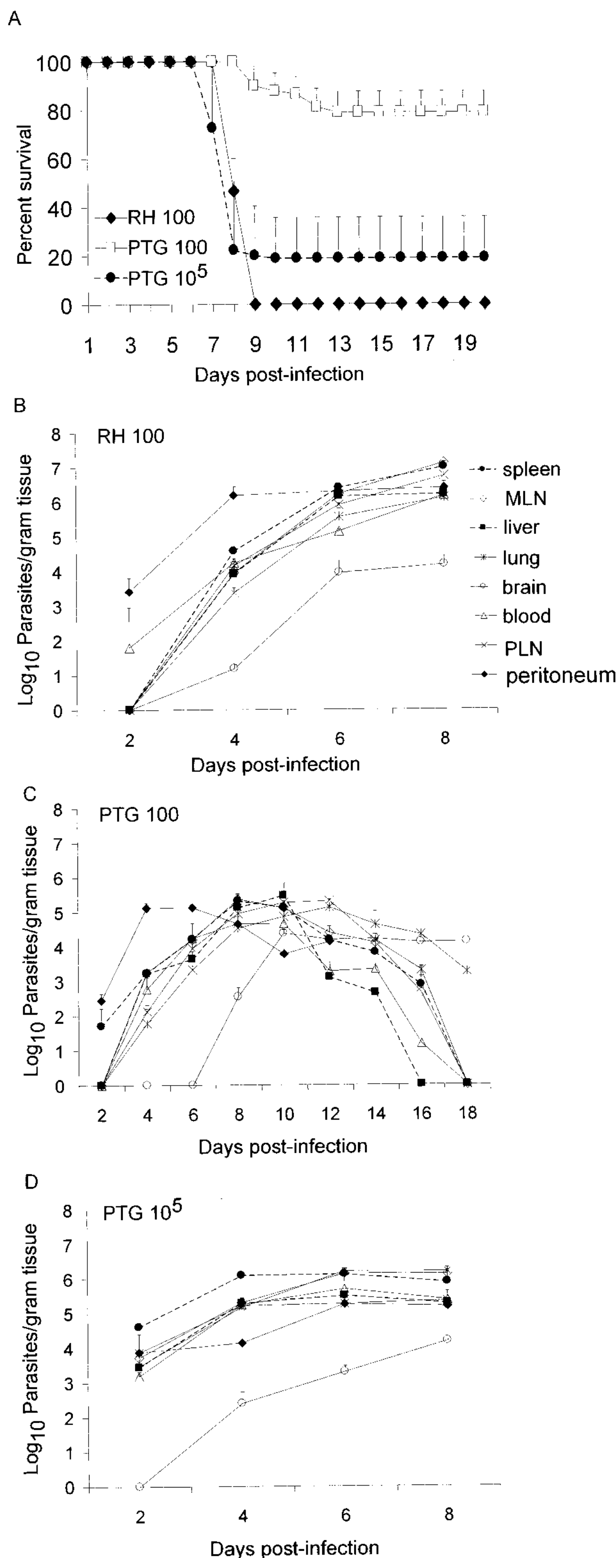

FIGURE 1. Comparison of type I (RH) vs type II (PTG) strains of *T. gondii* in the mouse model for virulence. *A*, Effects of parasite genotype and challenge dose on mortality in CD1 mice. The results shown are the mean ± SE from two or three experiments consisting of 4–15 mice/experiment. *B–D*, Tissue parasite burdens during infection with type I (RH strain) vs type II (PTG strain) *T. gondii*. Lethal infections with a low dose of RH (RH 100) or a high dose of PTG (PTG 10^5) parasites resulted in parasite levels ~1 log higher than nonlethal infections caused by a low dose of PTG (PTG 100). The results shown are the mean ± SE of three experiments (except for peritoneum, which represents a single experiment) consisting of three to five mice per time point per experiment.

RH strain (Fig. 1*B*) and PTG strain (Fig. 1*C*) parasites were equally capable of dissemination in vivo and did not show tissue tropism despite a slight delay in dissemination to the brain compared with other tissues. RH strain parasites did have a slight replication/survival advantage in vivo, and peak parasite numbers were achieved in 6 days compared with 8 days with PTG infection. Importantly, the initial growth rate was similar, and peak numbers of viable parasites achieved by the two strains differed by only approximately 10- to 20-fold.

To determine whether lethal infection with PTG strain parasites induced significantly higher tissue burdens than nonlethal infections, mice were challenged with a low dose (100) or a high dose (10^5) of PTG strain parasites, and parasite burdens were determined (Fig. 1, *C* and *D*). Peak parasite numbers were achieved in 4 days after high dose challenge with PTG strain parasites, although the maximum levels were less than a log greater than with low dose challenge (Fig. 1, *C* and *D*). Collectively, these results indicate that a threshold number of parasites is associated with mortality and that the timing in reaching this tissue burden is critical to survival.

Lethal toxoplasmosis is associated with liver and lymphoid damage

To explore the pathology induced during acute toxoplasmosis, tissues were examined at intervals postinfection. Significant pathology during lethal RH strain infections was restricted to the liver and lymphoid tissue and was accompanied by peritonitis and accumulation of plural fluid. Lethal infections with a high dose of PTG strain parasites generally induced similar pathology as that with RH strain parasites, although to a lesser extent. In contrast, low dose challenge with PTG strain parasites induced little tissue pathology during acute infection.

To quantify liver damage during infection, the levels of hepatocyte cytoplasmic enzymes released into the serum were determined. Levels of AST were elevated 10–15 times over control levels during lethal infections with either RH or PTG strain parasites (Fig. 2*B* and Table I). In contrast, AST levels were only modestly elevated during nonlethal infections with a low dose of PTG strain parasites (Fig. 2*B* and Table I). The level of the liver-associated enzyme ALT was 10–12 times higher in RH-infected mice compared with control mice and 5 times higher than that in mice infected with either high or low doses of PTG strain parasites when sampled on days 8–9 (Fig. 2*A* and Table I).

To evaluate whether the liver damage induced during infection with RH strain parasites was indicative of multisystem organ failure, serum levels of enzymes associated with renal failure were measured. Blood urea nitrogen and creatinine were not elevated during lethal infection with RH strain parasites (Fig. 2*C*). Bilirubin and γ-glutamyl transpeptidase levels were also unaltered relative to control values, indicating that liver damage was not a consequence of bile duct obstruction or hepatobiliary damage, respectively (Fig. 2*C*).

Liver damage is independent of hepatocyte apoptosis and intracellular infection

Histopathological examination of the liver in mice infected with RH strain parasites revealed generalized hepatocyte enlargement, cytoplasmic vacuolization, and loss of sinusoid architecture along with regions of cellular infiltration and foci of coagulative necrosis (Fig. 3*A*, Table I, and data not shown). Parasites were restricted to regions of cellular infiltration, but were seldom detected within hepatocytes, indicating that liver pathology was independent of

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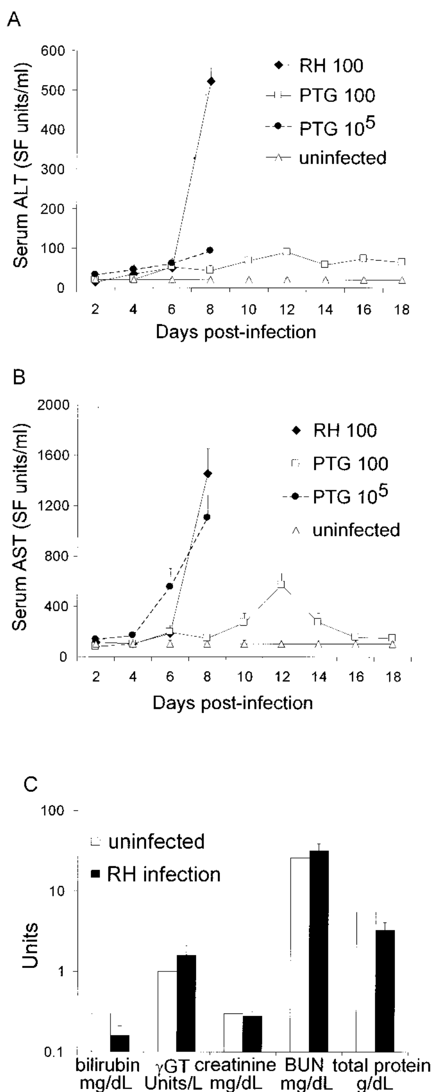

FIGURE 2. Release of liver enzymes into the serum accompanies lethal infection with *T. gondii*. The release of ALT (alanine aminotransferase) (*A*) and AST (*B*) into serum correlated with time of death during infection with a low dose of RH strain parasites (RH 100). Lethal infection with a high dose of PTG strain parasites (PTG 10^5) induced high levels of AST, but not ALT. Values represent the mean ± SE from three experiments consisting of three to five mice per time point. *C*, Lethal infection with RH strain parasites did not affect serum levels of total bilirubin, γ-glutamyl transpeptidase (γGT), creatinine, blood urea nitrogen (BUN), or total protein. Values represent the mean ± SD from one experiment consisting of five infected mice and two controls analyzed at 8 days postinfection. SF units, Sigma-Frankel units.

cellular infection (Fig. 3*A*). Immunohistochemical staining identified the cellular infiltrate to consist largely of F4/80$^+$ macrophages; however, only a subset of the macrophage infiltrate appeared fully activated, as evidenced by low levels of iNOS expression (Fig. 3*A*).

In contrast to infection with RH strain parasites, mice infected with either a high or a low dose of PTG strain parasites developed less pronounced pathology (Fig. 3*A* and Table I). Macrophage infiltration and iNOS production in the liver occurred to a similar extent and with a similar pattern regardless of the strain or dose of infecting parasite (Fig. 3*A*). Collectively, these findings indicate that the liver damage associated with lethal infections by the RH strain was widespread and probably due to a soluble mediator.

To evaluate whether hepatocyte apoptosis contributed to liver damage during lethal infection, liver sections from RH strain-infected mice were labeled by TUNEL staining. Very few TUNEL-positive cells were detected, indicating that liver damage was not a consequence of hepatocyte apoptosis (Fig. 3*B*).

To identify the mechanism of hepatocyte vacuolization during lethal infection, liver sections from RH strain-infected mice were stained histochemically to distinguish glycogen deposition (PAS positive) from fatty liver degeneration (Oil Red O positive), or hydropic degeneration (PAS and Oil Red O negative). The staining pattern shown in Fig. 3*B* indicated that cytoplasmic vacuolization was due to a combination of fatty liver degeneration and hydropic degeneration and was not a consequence of glycogen deposition.

Destruction of lymphoid follicles accompanies lethal infection

Lethal *T. gondii* infections led to a marked acellularity in lymphoid compartments and loss of tissue architecture in the spleen, peripheral lymph nodes, mesenteric lymph nodes, and Peyer's patches (Fig. 4*A* and data not shown). Following lethal infection with either a low dose of RH strain or a high dose of PTG strain parasites, loss of cells in the spleen occurred in clusters that were concentrated in follicle regions (Fig. 4*A* and data not shown). TUNEL staining of spleen sections indicated that cell loss was due to extensive apoptosis or late stage necrosis (Fig. 4*A*). Staining serial spleen sections with TUNEL vs anti-*T. gondii* Ab revealed that cell death did not occur in infected cells but, rather, occurred primarily at sites distal from parasite replication. To identify the cell types that were deleted during RH strain infection, spleen cells were doubly stained with annexin V and cell type-specific Abs and were analyzed by FACS. Increased levels of cell death in the spleen were largely confined to αβ T cells and NK cells (Fig. 4*B*). In contrast, infection with a low dose (100) of PTG strain parasites induced considerably less destruction and stimulated germinal center formation in the spleen (Fig. 4*A* and data not shown).

To determine the mechanism of cell death during lethal toxoplasmosis, splenocyte apoptosis was assessed in mice deficient in Fas (B6-*lpr*) or mice lacking TNFR I and TNFR II (TNFR KO). The level of apoptosis in uninfected control B6/129 mice was approximately 1–2%, but rose dramatically to 15–20% following challenge with RH strain parasites (Fig. 4*C*). Significantly less apoptosis was observed in RH strain-infected TNFR KO mice compared with B6/129 controls (Fig. 4*C*); however, there was no change in survival (Fig. 4*D*) or serum cytokine levels (data not shown). Apoptosis in control C57BL/6J mice infected with RH strain parasites was elevated to approximately 14% vs 1–2% in noninfected mice. The level of apoptosis was not appreciably changed in Fas-deficient mice (B6-*lpr*; 11%) infected with RH strain parasites (Fig. 4*C*), and there was no difference in survival (Fig. 4*D*). Therefore, the apoptosis of lymphocytes associated with infection by RH strain parasites was predominantly mediated through TNFRs and was independent of Fas.

High serum levels of IL-18 and IFN-γ correlate with lethality

The systemic nature of the liver and lymphoid pathology during lethal infections with RH strain suggested that tissue damage might be a consequence of overinduction of inflammatory cytokines. To determine whether lethal infections with *T. gondii* induced greater systemic levels of inflammatory cytokines than nonlethal infections, serum levels of IL-12, IFN-γ, IL-18, IL-1β, and TNF-α were determined throughout acute infection. Extremely high serum levels of IL-18, IFN-γ, TNF-α, and IL-12 were induced during lethal infections caused by a low dose (100) of RH

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Table I. *Effect of parasite genotype, challenge dose, and cytokine neutralization on liver pathology*[a]

Mouse Strain	Infection and Treatment	Hydropic Degeneration	Cellular Infiltration	Coagulative Necrosis	Serum ALT	Serum AST
CD1	Uninfected	0 (0)	0 (0)	0 (0)	20 (4)	105 (27)
CD1	RH100	4 (0)	1.25 (0.5)	1.5 (0.6)	1115 (219)*	1820 (266)*
CD1	PTG 100	1.5 (1.3)	1.5 (0.6)	0.5 (0.6)	68.7 (10)	272 (76)
CD1	PTG 10^5	1.4 (1.5)	1.6 (0.5) 0	1.0 (0)	93 (20)†	1104 (181)*
CD1	RH + PBS	3.5 (0.58)	1 (0)	1.8 (0.5)	680 (152)	1780 (447)
CD1	RH + IgG (0.5 mg)	2.2 (1.0)	1 (0)	1.7 (0.8)	716 (109)	1575 (276)
CD1	RH + anti-IFN-γ (0.5 mg)	2.2 (1.3)	1.7 (0.5)	1.8 (0.7)	956 (8)	1692 (108)
CD1	RH + PBS	4 (0)	1 (0)	1 (0)	713 (129)	1848 (92)
CD1	RH + IgG (1 mg)	4 (0)	1 (0)	2 (1)	532 (128)	1014 (117)
CD1	RH + anti-IFN-γ (1 mg)	1.3 (0.6)*	1.3 (0.6)	1.7 (0.6)	576 (60)	849 (76)
CD1	RH + PBS	4 (0)	1 (0)	1.3 (0.5)	623 (179)	1500 (207)
CD1	RH + anti-GST	3.3 (1)	1 (0)	1 (0)	513 (163)	1423 (356)
CD1	RH + anti-IL-18	3.3 (1)	1 (0)	1 (0)	509 (207)	1166 (245)
B6/129 or TNFR KO	Uninfected	0 (0)	0 (0)	0 (0)	2 (2)	52 (7)
B6/129	RH 100	3 (1.4)	1 (0)	1 (0)	325 (103)‡	1315 (115)†
TNFR KO	RH 100	2 (1.2)	1 (0)	1 (0)	661 (226)‡	1351 (258)†

[a] Hydropic degeneration, cellular infiltration, and coagulative necrosis were evaluated on a scale from 0 to 4: 0 = no pathology; 1 = 1–20% of the tissue section; 2 = 21–50%; 3 = 50–75%; and 4 = >75% of the tissue section affected. Liver sections and serum were collected between 8 and 10 days postinfection. Values for histopathology are the mean (SD) from three to six mice per group except for uninfected control mice which consisted of two mice per group. Serum transaminase values are the mean (SE) from at least four mice per group except for uninfected B6/129 and TNFR KO mice which consisted of two mice per group (‡, $p < 0.01$; †, $p < 0.005$; *, $p < 0.0005$ compared with values for uninfected mice).

strain or a high dose (10^5) of PTG strain parasites (Fig. 5, *A–D*). During lethal infections, serum levels of IL-18, IFN-γ, and TNF-α increased until death, whereas IL-12 levels peaked and then decreased (Fig. 5, *A–D*). During RH infection, IL-1β increased until death, whereas during high dose PTG infection, levels of IL-1β declined following an initial rise (Fig. 5*E*). In contrast, nonlethal

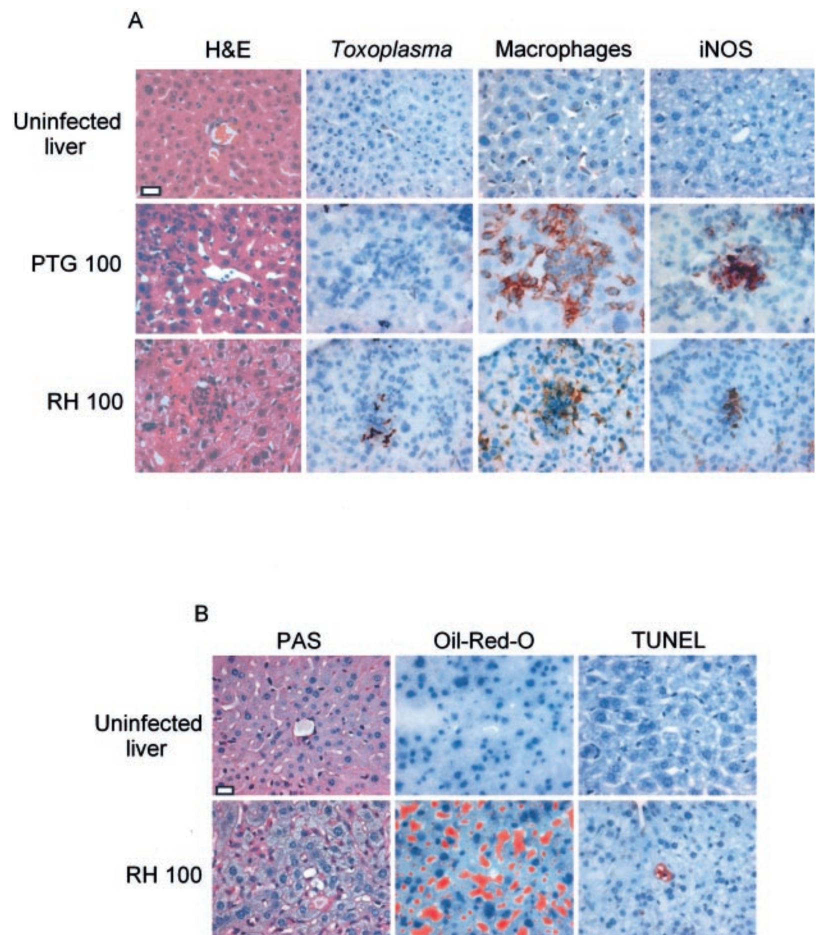

FIGURE 3. Systemic liver damage occurs during lethal infection by RH strain *T. gondii*. *A*, Liver damage was induced by lethal infection with RH strain parasites at sites distal from parasite replication (day 8 postinfection for RH and day 10 postinfection for PTG). Recruitment of F4/80$^+$ macrophages and expression of iNOS in the liver were common to infection with both RH and PTG strain parasites. H&E-stained liver sections from control mice or infected mice and serial sections stained with Ab specific for the parasite (*T. gondii*), macrophages (F4/80), or iNOS are shown. Scale bar = 20 μm. *B*, Liver damage during lethal infection with RH strain parasites resulted in fatty liver degeneration and hydropic vacuolization, but minimal apoptosis (TUNEL straining). Liver sections from control mice or mice infected with RH strain parasites (9 days postinoculation) stained with PAS, Oil Red O, and TUNEL. Scale bar = 20 μm.

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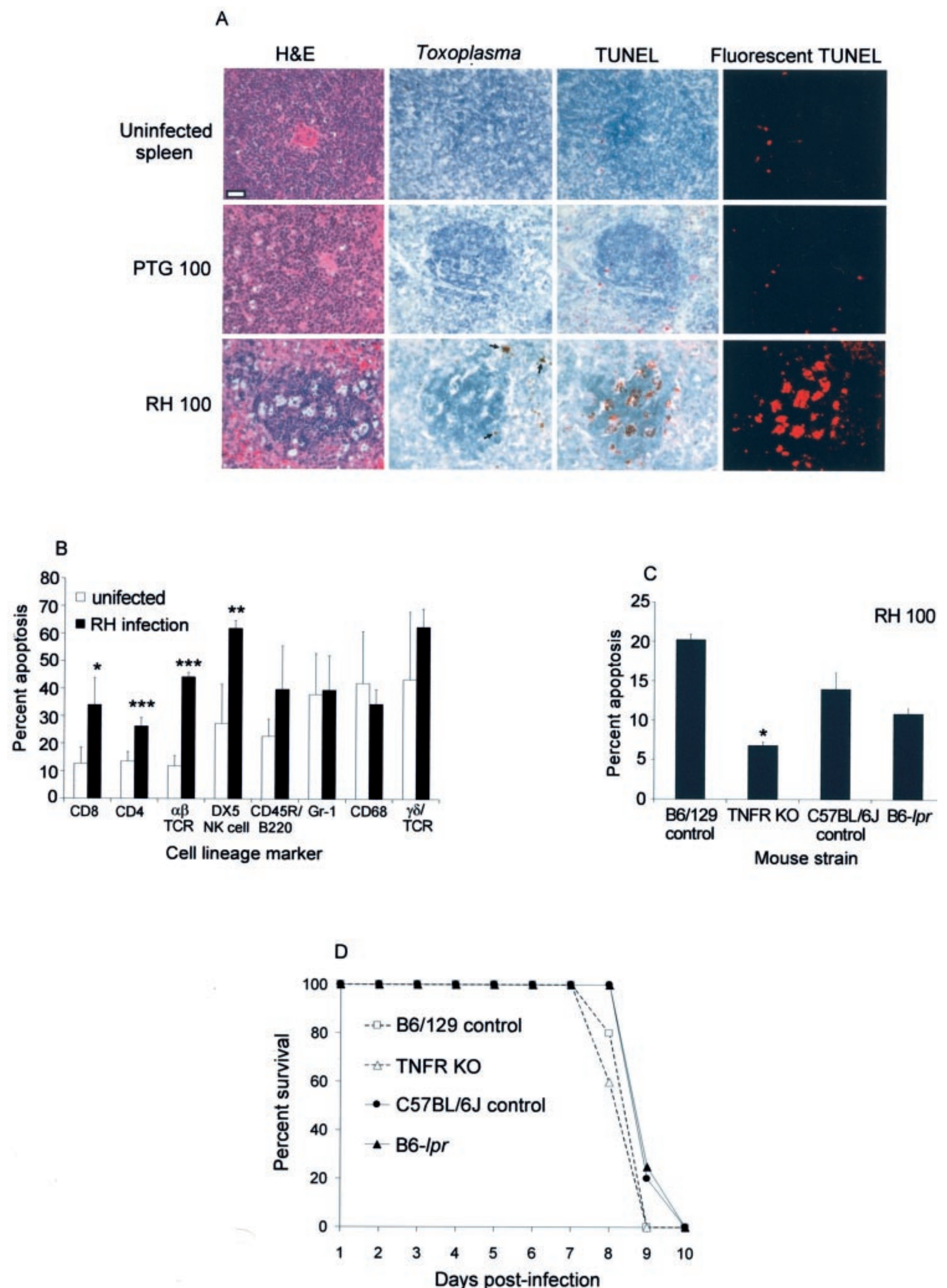

FIGURE 4. Apoptosis occurs in the spleen during lethal infection with RH strain *T. gondii*. *A*, Infection with RH strain parasites induced cell loss in spleen follicles that occurred through apoptosis in the absence of detectable parasite Ag. Parasites were detected with a polyclonal rabbit serum against *T. gondii* (arrows), and TUNEL staining was used to detect apoptotic cells at 8 or 10 days postinfection. Scale bar = 20 μm. *B*, Apoptosis in the spleen of mice infected with RH strain parasites was largely selective for $\alpha\beta$ T cells and NK cells. Apoptosis was detected at 8 days postinfection using annexin V staining. Data shown are the mean ± SE of two or three experiments consisting of two or three mice each. *C*, RH infection induced significantly less apoptosis in TNFR KO mice (*) compared with B6/129 controls ($p \leq 0.005$). In contrast, apoptosis levels in infected B6-*lpr* mice did not differ from those in C57BL/6J controls ($p > 0.05$). Data shown are the mean ± SD of three or four mice per group. Statistical comparisons were performed using Student's *t* test: *, $p \leq 0.05$; **, $p \leq 0.01$; ***, $p \leq 0.005$. *D*, Survival after infection with 100 RH strain parasites. Mice deficient in TNFR (TNFR KO) or Fas (B6-*lpr*) died with the same kinetics as B6/129 or C57BL/6 controls. Results are from a single experiment with five animals per group.

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infection with a low dose of PTG strain parasites induced almost no IL-18 or IL-1β and substantially less IFN-γ (100 ng/ml; Fig. 5, *A–E*). TNF-α and IL-12 were elevated to similar levels during both lethal and nonlethal infections, although the increases occurred later in nonlethal infections (Fig. 5, *C* and *D*).

The high serum levels of inflammatory cytokines induced during lethal toxoplasmosis could be due to inadequate stimulation of down-regulatory cytokines such as IL-10. Therefore, we determined whether lethal *T. gondii* infections induced similar levels of IL-10 compared with nonlethal infections. Lethal infections with a low dose (100) of RH strain or a high dose (10^5) of PTG strain parasites induced IL-10 to a greater extent than nonlethal infections with a low dose of PTG strain parasites (Fig. 5*F*). Therefore, overproduction of IL-18 and IFN-γ during toxoplasmosis is unlikely to be a consequence of inadequate regulation by IL-10.

Disease progression is independent of LPS or bacterial sepsis

Although there was minimal pathology in the small intestine during lethal toxoplasmosis, mice did develop pronounced peritonitis. Thus, a possible trivial explanation for death of mice during late stage toxoplasmosis could be a breach in the intestinal barrier resulting in bacterial sepsis. Consequently, we examined whether LPS contributed to the virulence of RH strain parasites by infecting mice that were genetically unresponsive (C3H/HeJ) or fully responsive (C3H/HeOuJ) to LPS. C3H/HeJ mice displayed no difference in survival, serum liver enzymes, or serum cytokine levels compared with LPS-responsive C3H/HeOuJ mice (Fig. 6*A* and data not shown). Furthermore, when mice were challenged with RH strain parasites by the s.c. route, they developed similar liver and lymphoid pathology that was associated with elevated

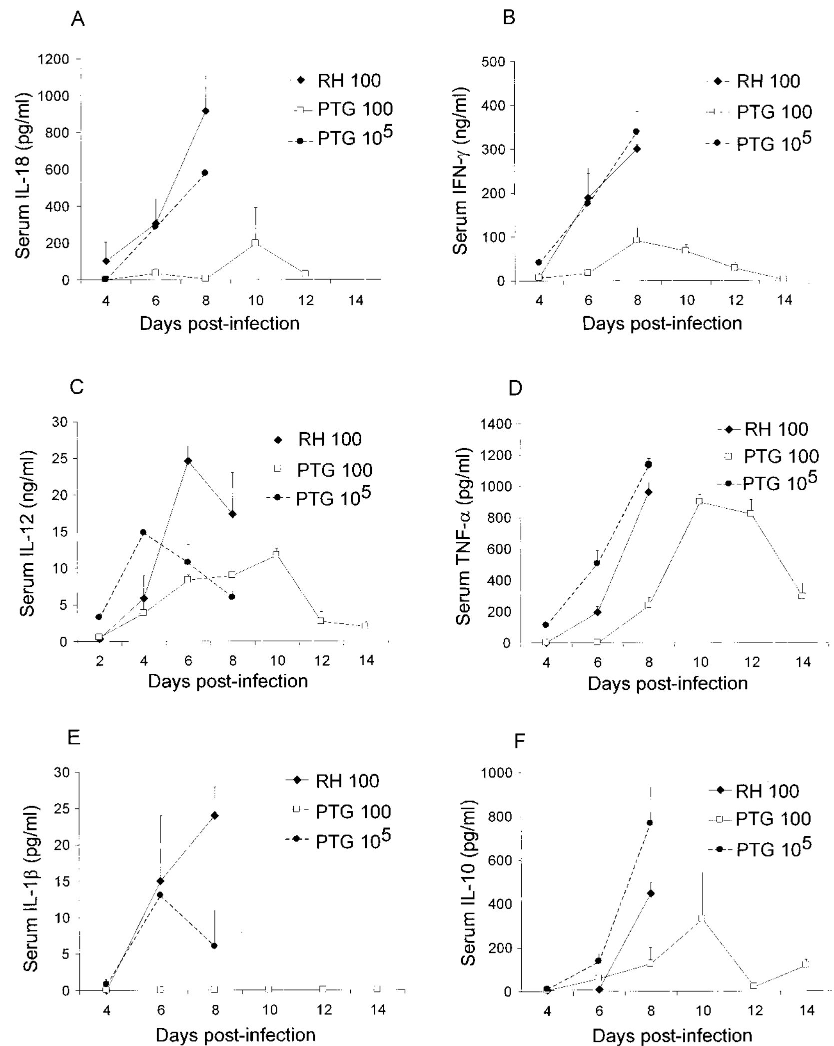

FIGURE 5. Serum cytokine levels during infection with type I (RH) vs type II (PTG) strain *T. gondii*. Lethal infections with a low dose of RH (RH 100) or a high dose of PTG (PTG 10^5) strain parasites induced high serum levels of IL-18 (*A*) and IFN-γ (*B*) compared with moderate levels produced during nonlethal infections with a low dose of PTG (PTG 100) strain parasites. The results shown represent the mean ± SE from two (PTG) or three (RH) experiments consisting of three to five mice per time point. Serum IFN-γ and IL-12p40 are expressed as nanograms per milliliter, and the other cytokines are expressed as picograms per milliliter. Serum cytokine levels of IFN-γ, IL-18, IL-1β, IL-10, and TNF-α in control mice were below the level of detection, and the IL-12 level was 0.7 ng/ml.

cytokines and liver damage (data not shown). Collectively, these results indicate that liver damage, proinflammatory cytokine induction, and lethal infections caused by RH strain were not due to endotoxemia or bacterial sepsis.

Neutralization of IL-18 in vivo prolongs survival

The high levels of Th1 cytokines produced during lethal infection suggest that cytokine-induced shock was responsible for death. Therefore, we attempted to mitigate the severity of disease by administering neutralizing Abs to IFN-γ, TNF-α, or IL-18 during infection with RH strain parasites. Neutralization of IL-18, but not control antiserum (anti-GST) or PBS, enhanced the survival of infected mice by up to 2 days, a result that was observed consistently in three separate experiments ($p \leq 0.01$, by Student's *t* test; Fig. 6*B*). Prolonged survival was not accompanied by decreased serum levels of IFN-γ and TNF-α (data not shown) or a decrease in liver or lymphoid pathology (Table I). Moreover, treatment with anti-IL-18 did not alter parasite tissue burdens (data not shown). These results suggest that the beneficial effects of neutralizing IL-18 are largely independent of high levels of TNF-α or IFN-γ.

To directly test the role of elevated IFN-γ, RH strain-infected mice were given 0.5 mg of a neutralizing Ab against IFN-γ (mAb H22) or control hamster IgG on day 5 postinfection. Ab neutralization was successful in causing more than a 10-fold reduction in serum IFN-γ levels, resulting in a lower level (~5 ng/ml) in treated mice than that induced by low dose, nonlethal PTG strain infection (~70 ng/ml). However, no change was evident in the numbers of parasites in the tissues or in survival or liver damage in mice receiving anti-IFN-γ Ab relative to controls (Fig. 6*C* and Table I). When a higher dose of mAb H22 was used (1 mg given on day 5 postinfection), a 5-fold increase in parasite numbers in the tissues was observed, and there was no change in survival (data not shown). While treatment with the lower dose of mAb H22 did not affect liver pathology, there was a significant decrease in hepatocyte vacuolization with the higher dose of neutralizing Ab (Table I). Neutralization of TNF-α during RH strain infection using mAb TN3-19.12 (0.25 mg given on day 5 postinfection), also had no effect on survival relative to that of mice receiving control hamster IgG (Fig. 6*C*). Furthermore, in TNFR KO mice, similar liver damage occurred, indicating that pathology during lethal infection was not dependent on signaling through TNFRs (Table I). Collectively these results indicate that IL-18 contributes to pathology during toxoplasmosis and suggest that its detrimental effects are independent of IFN-γ or TNF-α.

Discussion

T. gondii strains are remarkably clonal, and virulence in the mouse model is strongly influenced by the genotype of the parasite. Our studies demonstrate that the ability of type I strains to uniformly

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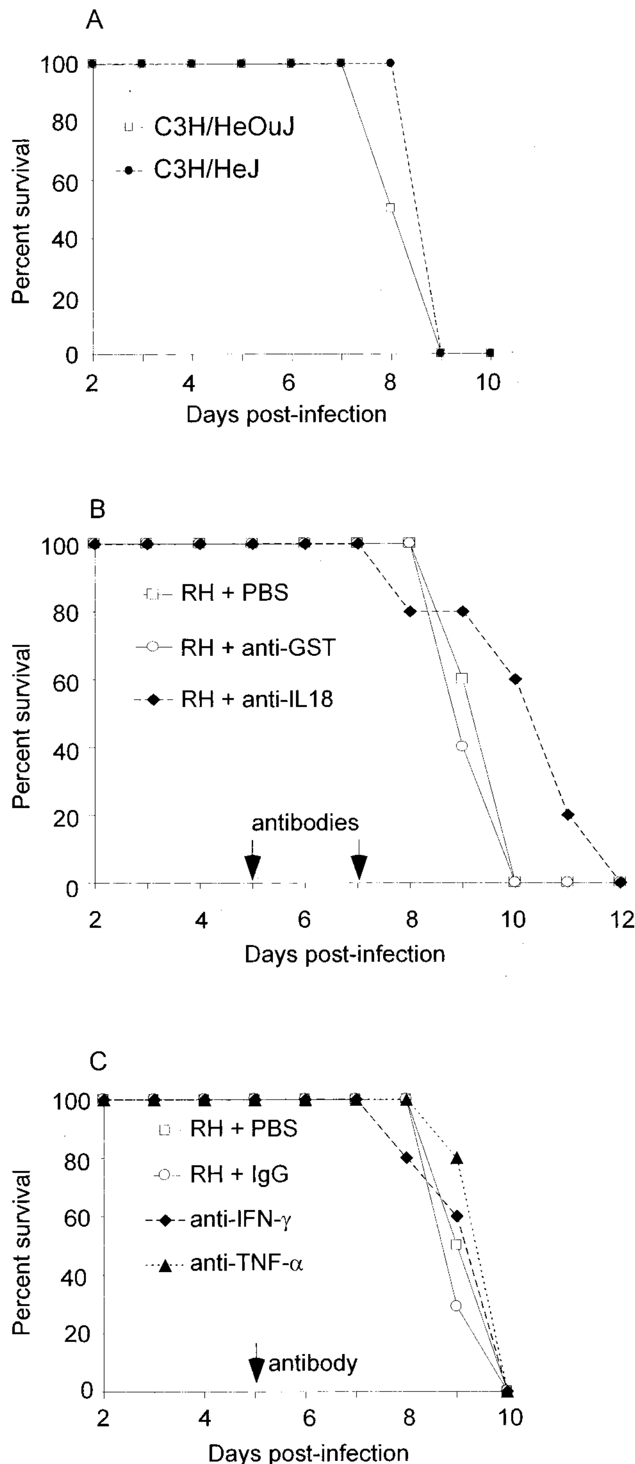

FIGURE 6. Extended survival after lethal challenge by abrogation of IL-18, but not IFN-γ or TNF-α. *A*, Response to lethal infection with the RH strain is not altered in LPS-nonresponsive mice (C3H/HeJ). Results shown are from a single experiment consisting of four mice per group. *B*, Neutralization of IL-18 partially protects against lethal infection. Anti-IL-18 prolonged time until death in mice challenged with lethal RH infection compared with PBS or an irrelevant control rabbit antiserum (anti-GST; $p \leq 0.01$, by Student's t test; $n = 3$ experiments). *C*, Neutralization of IFN-γ or TNF-α did not protect against lethal infection. Treatment with Ab against IFN-γ (H22) or TNF-α (TN3-19.12) on day 5 postinfection with RH did not affect survival compared with that after treatment with PBS or control hamster IgG.

cause lethal infections in mice was not due to direct destruction by the parasite. Instead, infections with the type I RH strain were characterized by their rapid ability to reach tissue burdens associated with lethality ($\sim 10^6$ parasites/g tissue). In contrast, low dose infections with the type II PTG strain were delayed in reaching this threshold, and lethality was only observed with a higher initial inoculum. Unexpectedly, lethal infections were associated with overinduction of inflammatory cytokines rather than an insufficient immune response. Lethality was associated with excessive levels of Th1 cytokines, particularly IL-18 and IFN-γ, in the serum. Our findings indicate that acute virulence in *T. gondii* is associated with overstimulation of Th1 cytokines, which paradoxically are also required for protection.

Following primary infection, type I and type II strains of *T. gondii* did not differ substantially in their ability to disseminate in vivo or to reach similar tissues. However, a key property of the RH strain of *T. gondii* is the dramatic increase in serum cytokine levels after low dose challenge, whereas the PTG strain required a much higher challenge dose (10^5 parasites). This difference may be due to the ability of a low dose of RH strain parasites to rapidly reach tissue levels of $\sim 10^6$ parasites/g tissue (within 4 days), whereas PTG strain parasites only reached this level at 6–8 days postinfection. This difference was overcome by administering a high initial dose of PTG strain parasite, resulting in a faster increase in tissue burdens and a lethal outcome. Collectively, these results suggest that the early interaction of the parasite with the innate immune response is critical to triggering the lethal cascade of cytokines. The basis for the dramatic difference in virulence between type I (RH) and II (PTG) strains of *T. gondii* is presently unknown, but is probably due to the 1–2% underlying genetic differences between these strain types (6, 8).

The major site of tissue pathology during lethal toxoplasmosis was the liver. Damage was not directly attributable to intracellular parasite replication or apoptosis. Instead, lethal infections with RH strain induced widespread alterations in hepatocytes, including enlargement, cytoplasmic vacuolization, and release of liver enzymes. These results suggest that liver damage is mediated through a soluble parasite-derived factor(s) or an induced host factor(s) that reaches toxic levels during lethal infections. Liver pathology is probably due in part to elevated cytokine levels; however, additional factors may also contribute, as mice infected with lethal doses of RH strain vs PTG strain showed similar elevated cytokines, but developed different levels of tissue damage.

Lethal toxoplasmosis also induced extensive necrosis/apoptosis of noninfected cells within lymphoid tissues. Apoptosis was selective for $\alpha\beta$ T cells and NK cells, and was predominantly mediated through TNF-α, as shown by the significant reduction in apoptotic cells in TNFR KO mice challenged with RH strain *T. gondii*. It has previously been reported that direct infection by *T. gondii* prevents activation of apoptosis within the parasitized host cell (42). Nonetheless, splenic $CD4^+$ T cells isolated from mice challenged with high doses of ME49 (PTG) strain parasites undergo apoptosis in vitro, resulting in diminished immune responsiveness (43). Collectively, these studies indicate that infection by *T. gondii* protects the resident cell from apoptosis, but cell death is efficiently activated in noninfected cells. Bacterial sepsis is also frequently accompanied by profound depletion of lymphocytes associated with apoptosis in the white pulp of the spleen, and this response is detrimental to host survival (44, 45). In contrast, during acute toxoplasmosis, apoptosis occurred only late in infection. Combined with the similar outcome of infection in TNFR KO

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mice, which succumb at the same rate despite exhibiting less apoptosis, these findings indicate that apoptosis is probably a secondary consequence of pathogenesis during toxoplasmosis rather than causal.

Previous studies have shown that infection with *T. gondii* drives a potent Th1 response that is necessary for control of infection in the mouse. Induction of IL-12 (22–24), which drives production of IFN-γ (26, 30), is essential to control parasite replication and prevent death due to toxoplasmosis in the murine system. Consistent with this, infection of mice with a low dose of type II strain (PTG) parasites resulted in an immune response characterized by moderate levels of IFN-γ, IL-12, and TNF-α and leading to eventual control of parasite replication and minimal tissue pathology. In marked contrast, lethal infections were associated with systemic induction of extremely high levels of IL-18, IFN-γ, and IL-12.

The finding that pathology during acute toxoplasmosis was associated with an overstimulation of the immune system was surprising, as previous models have suggested that lethality of type I strains was due to the inherent virulence of the parasite. However, this finding does have precedent in previous studies using C57BL/6 mice, which are uniquely susceptible to infection by normally nonlethal doses of the type II strain ME49 when administered orally (46, 47). Importantly, pathology in this previously described model differs substantially from the results of studies reported here using the RH strain, where 1) damage is systemic rather than localized to the gut, 2) expression is independent of host genotype rather than highly specific, 3) and disease is independent of route of challenge. Thus, our findings indicate that rather than being a limited case, overinduction of Th1 cytokines may be an important general mediator of pathology in toxoplasmosis, especially during infection by type I strains.

Although Th1 cytokines are essential for parasite control, this response must be tightly regulated to prevent lethal immunopathology. Evidence for this is provided by IL-10 knockout mice, in which liver damage and death occur when mice are challenged with normally nonlethal type II strain parasites as a consequence of overinduction of IFN-γ, IL-12, and TNF-α (48). In the present study high serum levels of Th1 cytokines during lethal toxoplasmosis in wild-type mice was not due to an absence of down-regulatory IL-10, since this cytokine was also substantially elevated in serum during lethal infection. Recent studies indicate IL-10 can be proinflammatory during human endotoxemia (49), suggesting that during lethal toxoplasmosis in mice, IL-10 could act to further enhance the induction of inflammatory cytokines.

In contrast to other Th1 cytokines, high levels of IL-18 were only observed in mice destined to succumb to infection, and in the case of mice infected with RH strain parasites, neutralization of this cytokine prolonged survival. This effect, while only transiently protective, is nonetheless highly significant given the extreme virulence of the RH strain, which causes death with a lethal dose of a single viable organism. Despite prolonging survival, anti-IL-18 was not able to protect mice against liver damage, and these mice ultimately died, suggesting that the cumulative effects of high levels of other inflammatory mediators also contributed to death. Indeed, the high levels of IFN-γ and IL-12 would be expected to result in considerable pathology, and previous studies have shown that coadministration of IL-12 and IL-18 is capable of causing death in mice (50, 51).

IL-18 is related to IL-1 and shares biological properties with IL-12, including induction of IFN-γ and enhancement of Th1 responses (52, 53). IL-18 and IL-12 synergize to promote high levels of IFN-γ production, and this leads to a more effective immune response against certain intracellular pathogens (54, 55). However, overproduction of IL-18, as occurs in murine models of endotoxemia, results in lethal liver damage (56) and splenocyte apoptosis (50, 56). IL-18 exerts its toxic activity through overproduction of IFN-γ and through IFN-γ-independent mechanisms (50, 56). In the present study neutralization of toxic levels of IL-18 that occurred during lethal toxoplasmosis did not result in decreased IFN-γ or reduction of hepatic damage, suggesting that the detrimental effects of IL-18 in toxoplasmosis are independent of IFN-γ.

The elevated levels of cytokines preceding death due to acute toxoplasmosis resemble systemic infections with Gram-negative bacterial pathogens in that both stimulate high levels of IL-1β, TNF-α, and IL-18. However, *T. gondii* differs from these bacterial pathogens in several important traits. First, it does not contain LPS, and the innate immune response to parasites such as *T. gondii* is not mediated by Toll-like receptor 4, the classical pattern recognition receptor for bacterial LPS. This conclusion is supported by the similar outcome of *T. gondii* infection in C3H/HEJ mice, which are genetically unresponsive to LPS due to a mutant allele of the Toll-like receptor 4 (57). Second, the production of IFN-γ that is necessary for control of infection by *T. gondii* relies primarily on IL-12, but only minimally on IL-18 (58). In contrast, IL-18 is required for induction of an inflammatory pathway that results in control of shigellosis (59). Third, endotoxemia leads to liver damage that is typically associated with apoptosis mediated by Fas and TNFR (60), while the hepatic damage in toxoplasmosis was independent of these mediators.

The potent induction of Th1 cytokines during toxoplasmosis suggests that *T. gondii* contains a substance that is a potent inflammatory mediator. Importantly, our results cannot be ascribed to contamination with *Mycoplasma* lipopeptide, which has potent immunostimulatory properties (61), as we took extreme care to avoid contamination throughout this study. There are two possible models to explain the massive inflammatory response that occurs during toxoplasmosis: 1) the host responds to a substance produced by the parasite, analogous to the inflammatory response generated to bacterial LPS (yet clearly distinct); or 2) pathology is a secondary effect due to release of endogenous triggers from infected and/or damaged host cells, as has been suggested previously (62). Further studies to elucidate the mechanism of immune damage during acute toxoplasmosis could have important implications for the treatment of parasitic infections.

Note added in proof. Independently, another group has recently reported that RH strain infection induces splenocyte apoptosis and high levels of IFN-γ (63).

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