Mechanism of Entry Determines the Ability of *Toxoplasma gondii* To Inhibit Macrophage Proinflammatory Cytokine Production

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Macrophages (M ϕ) infected with tachyzoites of the opportunistic protozoan *Toxoplasma gondii* are blocked in production of the proinflammatory cytokines tumor necrosis factor alpha (TNF- α) and interleukin-12 (IL-12) in response to lipopolysaccharide (LPS) triggering, and this is associated with parasite-induced inhibition of NF κ B translocation. Here, we demonstrate a requirement for active invasion in the ability of the parasite to mediate suppression. Neither soluble tachyzoite antigen nor secreted products were suppressive, and heat-inactivated, antibody-coated tachyzoites, which efficiently entered the cell through receptor-mediated uptake, failed to inhibit LPS responses. Cytochalasin D, a drug blocking tachyzoite invasion of, but not adherence to, M ϕ , severely curtailed *Toxoplasma*-induced suppression. In addition, parasite-induced nonresponsiveness, as measured by TNF- α production, was reversed by treating infected cells with the toxoplasma-static drugs pyrimethamine and 6-thioxanthine prior to LPS stimulation. A divergence in IL-12 and TNF- α responses was found during extended incubation of tachyzoites and M ϕ in that 24 h of incubation of infected M ϕ resulted in IL-12, but not TNF- α , secretion, and production of the latter cytokine remained suppressed when these cells were subjected to LPS triggering. Our results demonstrate that active invasion and survival of the parasite within the parasitophorous vacuole are required to induce and maintain M ϕ cytokine-specific nonresponsiveness to LPS. They also show that the effects of *Toxoplasma* on IL-12 and TNF- α production are nonidentical, with the parasite exerting a longer-lasting suppression of the latter.

Microbial pathogens that parasitize macrophages (M ϕ) must survive and replicate within the potentially hostile environment of the host cell. M ϕ functions such as phagocytosis and phagolysosomal degradation, nitric oxide intermediate production, and respiratory burst activity are highly effective microbicidal effector mechanisms (1, 36, 55). M ϕ may also play a role in triggering acquired immunity through release of cytokines such as interleukin-12 (IL-12) and presentation of peptide antigen (Ag) to T cells within the context of major histocompatibility complex (MHC) and costimulatory molecules (26, 29, 33). Bacterial and protozoan pathogens residing within M ϕ have adopted unique strategies for intracellular survival. Protozoan parasites such as *Leishmania major*, while generally thought to reside within an acidified phagolysosome, have been suggested to actively block phagosome-endosome fusion (15). *Trypanosoma cruzi* trypomastigotes, which enter cells by a process of induced phagocytosis, escape into the host cell cytoplasm through a mechanism involving the pore-forming toxin Tc-TOX (4, 9).

A different strategy of intracellular survival is employed by *Toxoplasma gondii* and other apicomplexan parasites. For *T. gondii*, tachyzoite invasion is an active process involving parasite actin-based motility coupled with discharge of the contents of rhoptry and dense-granule apical organelles (13, 16, 18, 23). In this manner, *T. gondii* avoids entry into the phagolysosomal pathway, instead establishing and replicating within a specialized parasitophorous vacuole composed of host cell lipid and

parasite-derived, as well as host-derived, proteins (39, 52). Indeed, the intracellular fate of *T. gondii* within the M ϕ is determined by the route through which the parasite enters the host cell. Thus, tachyzoites entering the cell through Fc receptor-mediated phagocytosis find themselves within vacuoles that undergo normal acidification and lysosomal fusion, resulting in nonproductive infection (27, 41).

Many bacterial and protozoan pathogens are capable of subverting pathways leading to inflammatory cytokine responses. For example, *L. major* prevents IL-12 synthesis in M ϕ by a mechanism that may involve intracellular pathways triggered by interaction with M ϕ Fc receptors (12, 38, 53). In contrast, *Salmonella* spp. and *Yersinia enterocolitica* can block inflammatory responses by directly disabling the NF κ B activation pathway (44, 49). Inasmuch as in vivo *Toxoplasma* infection is associated with robust proinflammatory cytokine production, the parasite has not generally been regarded as a pathogen that subverts early immunity. Nevertheless, we recently demonstrated a powerful immunosuppressive effect on the ability of infected M ϕ to produce tumor necrosis factor alpha (TNF- α) and IL-12 in response to lipopolysaccharide (LPS) stimulation in vitro (11). Although the inhibitor molecule I κ B α underwent infection-induced phosphorylation and degradation, NF κ B p50-p65 heterodimers did not translocate to the nucleus and subsequent LPS triggering was unable to promote translocation (11). Similar results were recently reported by Shapira et al. (50). Highly relevant to these data is the fact that *T. gondii* infection has also been shown to result in down-regulation of M ϕ MHC class II gene expression as a result of interference with the STAT-1 α activation pathway (34, 35).

In this study, we further examined the ability of *T. gondii* to

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inhibit M ϕ cytokine responses. We examined the role of activation stimulus, route of entry, and tachyzoite-parasitophorous vacuole persistence in the suppressive properties of the parasite. Our results demonstrate that TNF- α and IL-12 production induced by several microbial stimuli, in addition to LPS, are blocked by the parasite. It is of particular interest that heat-killed-antibody-opsonized tachyzoites entering the cell through Fc receptor-mediated endocytosis were unable to suppress M ϕ cytokine production. Cytochalasin (CytD), a drug blocking the parasite's ability to invade M ϕ but not its ability to adhere to M ϕ , severely curtailed tachyzoite-mediated suppression. In addition, in cells in which active infection was halted by pyrimethamine and 6-thioxanthine treatment, the ability to respond to LPS triggering was restored. Finally, M ϕ infected for 24 h produced IL-12 but TNF- α production remained potently suppressed. The latter cells were sensitive to LPS-triggered NF κ B translocation, unlike cells infected for 2 to 3 h. Together, these results establish that active invasion and the presence of live parasites are required to initiate pathways leading to inhibition of cytokine production. The results also demonstrate a divergence in parasite-triggered IL-12 and TNF- α responses.

MATERIALS AND METHODS

Mice. Female C57BL/6 animals (6 to 8 weeks of age) were obtained from The Jackson Laboratory (Bar Harbor, Maine). The mice were housed under specific-pathogen-free conditions in the Cornell University College of Veterinary Medicine animal facility, which is accredited by the Association for Assessment and Accreditation of Laboratory Care.

Parasites and Ag. Tachyzoites of the virulent RH strain were maintained by biweekly passage on human foreskin fibroblasts in complete medium (CM) composed of Dulbecco modified Eagle medium (Life Technologies, Gaithersburg, Md.) supplemented with 1% fetal calf serum (HyClone, Logan, Utah), penicillin (100 U/ml; Life Technologies), and streptomycin (100 μ g/ml; Life Technologies). Tachyzoites were harvested from freshly lysed cultures, washed once in endotoxin-free phosphate-buffered saline (PBS; Sigma Chemical Co., St Louis, Mo.), and employed for in vitro assays. In some cases, tachyzoites were liberated from heavily infected fibroblasts by forced passage of parasite-bearing cells through an 18-gauge needle. Tachyzoite opsonization was accomplished by heat inactivation of parasites (56°C, 25 min), followed by incubation (4°C, 25 min) with mouse anti-*Toxoplasma* serum obtained from animals chronically infected with *T. gondii* strain ME49. Unbound antibody was removed by washing prior to the use of tachyzoites in assays. Soluble tachyzoite Ag (STAg) was prepared by parasite sonication in the presence of a protease inhibitor cocktail, followed by high-speed centrifugation and supernatant collection, as described elsewhere (8).

Reagents. LPS (*Escherichia coli* strain O26:B6), staphylococcal enterotoxin A (SEA), pyrimethamine, and 6-thioxanthine were obtained from Sigma. Murine recombinant gamma interferon (IFN- γ) was purchased from R & D Systems, Inc. (Minneapolis, Minn.). Heat-killed bacillus Calmette-Guérin (BCG) was kindly provided by D. Russell (Cornell University).

M ϕ preparation. Bone marrow-derived M ϕ were prepared as described in detail elsewhere (24). Briefly, bone marrow was collected from tibia and femur bones of 6- to 8-week-old C57BL/6 mice. Cells were washed and resuspended in bone marrow culture medium, consisting of Dulbecco modified Eagle medium (Life Technologies), 10% fetal calf serum (HyClone), 5% horse serum (Life Technologies), 2 mM glutamine, penicillin (100 U/ml; Life Technologies), and streptomycin (100 μ g/ml; Life Technologies). The medium was supplemented with 30% supernatant from confluent cultures of L929 cells (American Type Culture Collection, Manassas, Va.) as a source of M ϕ colony-stimulating factor. After 4 days of culture, nonadherent cells were washed away and cells were replated in CM and subjected to infection and/or stimulation 18 h later.

Inhibition assays. M ϕ (2×10^5 per well in 96-well tissue culture plates) were infected by adding RH tachyzoites and briefly centrifuging plates ($500 \times g$, 3 min) to initiate parasite-cell contact. After 2 h (37°C, 5% CO₂), M ϕ activation stimuli were added and plates were incubated for 30 min (37°C, 5% CO₂). The cells were subsequently gently washed with 37°C CM, fresh medium was added,

and the cells were returned to the incubator for 6 to 36 h, depending upon the experiment.

To examine the ability of CytD to block suppression, tachyzoites were pretreated (10 min, 0°C) with 1 μ M drug (Sigma) and then infections were carried out in the continued presence of 1 μ M CytD. Cells were subjected to LPS triggering, and supernatant was collected 6 h later as described above. For tachyzoite inactivation, pyrimethamine (final concentration, 10 μ M) and 6-thioxanthine (final concentration, 40 μ g/ml) were added 2 h after infection, cells were subjected to LPS stimulation 24 h later, and supernatants were collected 6 h after LPS treatment.

Transwell experiments were conducted by plating 10^6 bone marrow-derived M ϕ on a 24-well plate containing a transwell insert (0.1 μ M polycarbonate membrane; Costar, Cambridge, Mass.). Tachyzoites were placed in transwells or directly on cells, and 2 h later, M ϕ were subjected to LPS triggering for cytokine analysis as described above.

Cytokine ELISA. IL-12(p40) was measured by two-site enzyme-linked immunosorbent assay (ELISA) as previously described (8), and TNF- α was measured with a commercially available kit (B-D Pharmingen, San Diego, Calif.).

Fluorescence microscopy. Visualization of NF κ B p65 (RelA) nuclear translocation was achieved by confocal fluorescence microscopy as described in detail elsewhere (11). Briefly, M ϕ were plated onto 12-mm glass coverslips and subjected to infection and LPS stimulation as described above. Cells were fixed in 3% formaldehyde, washed, and incubated with goat anti-p65 serum (Santa Cruz Biotechnology, Santa Cruz, Calif.) and rabbit anti-*Toxoplasma* serum diluted in PBS with 0.075% saponin (Sigma). After washing, cells were incubated with fluorescein isothiocyanate-conjugated anti-goat and Texas Red-conjugated anti-rabbit antibody (both reagents from Jackson ImmunoResearch, West Grove, Pa.) diluted in PBS with 0.075% saponin and 5% normal mouse serum. Cells were washed and mounted with Pro-Long Antifade (Molecular Probes, Eugene, Oreg.). Fluorescence images were acquired with a Zeiss Axioskop equipped with an AxioCam and analyzed with Axiovision software (Carl Zeiss, Inc. Thornwood, N.Y.).

RESULTS

***T. gondii* inhibits M ϕ cytokine production driven by multiple stimuli.** Our previous results demonstrated that tachyzoite infection blocked LPS-triggered M ϕ IL-12 and TNF- α production. To determine whether suppressed cytokine production was restricted to M ϕ activated with LPS, we infected cells and then, 2 h later, stimulated them with BCG and SEA, in addition to LPS. While LPS signals M ϕ exclusively through Toll-like receptor 4 (TLR-4), BCG likely signals through multiple receptors, including TLR-2 and TLR-4 (25, 54). In contrast, SEA, a bacterial superantigen, activates M ϕ by binding to MHC class II molecules (17). Six hours after stimulation, supernatants were collected for ELISA. As shown in Fig. 1, LPS was a strikingly more potent stimulus for M ϕ inflammatory cytokine production than was either BCG or SEA. Nevertheless, parasite infection clearly inhibited cytokine production in response to both of these microbial stimuli, in addition to LPS.

Live infection, but not STAg, inhibits LPS-driven M ϕ TNF- α and IL-12 production. We previously showed that tachyzoite infection results in suppressed proinflammatory cytokine production in infected, but not bystander noninfected, M ϕ (11). This result suggested that infection per se, and not release of soluble factors by extracellular parasites or infected cells, is required to mediate suppression. This issue was further addressed by comparing the abilities of live parasites and STAg, a soluble fraction from total parasite sonicate, to mediate cytokine suppression in M ϕ . As shown in Fig 2, while tachyzoites blocked LPS-induced M ϕ IL-12(p40) and TNF- α production, at no STAg dose tested was suppression evident. Importantly, we estimate that the largest dose examined was approximately equivalent to 10^7 tachyzoites, while the highest

FIG. 1. Multiple NF κ B activation stimuli are blocked by tachyzoite (TZ) preinfection. Bone marrow-derived M ϕ (Ø) were infected with *T. gondii* (4:1 ratio of parasites to cells) and then, 24 h later, subjected to stimulation with LPS (100 ng/ml), heat-killed BCG (5×10^8 /ml), SEA (50 μ g/ml), or medium. After 30 min, stimuli were removed and fresh medium was added. A cytokine ELISA was performed on culture supernatants 6 h after addition of microbial activation stimuli. This experiment was repeated twice with essentially identical results.

multiplicity of infection tested corresponded to 1.6×10^6 parasites.

Tachyzoite release of soluble factors fails to account for parasite-mediated inhibition. Although STAg failed to suppress M ϕ responses, it was possible that live tachyzoites release a subset of inhibitory molecules not represented in our STAg preparation. Alternatively, it was possible that sonication during the course of STAg preparation results in loss of the biological activity of suppressive molecules. To address these issues, a transwell experiment was performed in which tachyzoites were separated from M ϕ by a membrane that is impermeable to parasites but nonetheless allows free diffusion of macromolecules. As shown in Fig. 3A, when parasite-host cell contact was prevented, M ϕ remained fully responsive to subsequent LPS triggering, unlike the situation in which parasites were placed directly into contact with host cells.

These results suggested that the parasite exerted its effects from within the host cell. To examine this further, infection and subsequent LPS triggering were performed in the presence of CytD. The latter drug blocks both gliding motility and entry into host cells by disrupting the parasite actin cytoskeleton while leaving intact the parasite's ability to adhere to the cell surface membrane (16) (Fig. 3C). Treatment with CytD almost completely blocked suppression of M ϕ cytokine production by *T. gondii* (Fig. 3B). Nevertheless, a small amount of suppression was evident relative to M ϕ incubated with heat-inactivated tachyzoites, and this became more pronounced at higher tachyzoite/cell ratios (data not shown). While it is possible that the latter phenomenon results from rhoptry discharge and formation of empty vacuoles (evacuoles) (23), we cannot exclude the possibility of low-level host cell invasion in the presence of the drug.

FIG. 2. Live parasites, but not STAg, block LPS-triggered M ϕ IL-12(p40) and TNF- α production. Bone marrow-derived M ϕ were infected with RH strain tachyzoites (TZ) or incubated with STAg. Two hours later, extracellular parasites and STAg were removed and LPS (100 ng/ml) was added. After 30 min, LPS was washed away, fresh medium was added, and 6 h later, supernatants were collected for cytokine ELISA. Symbols: ●, cells incubated in the presence of LPS; ○, cells incubated in the absence of LPS. This experiment was repeated three times with equivalent results.

Heat-inactivated tachyzoites internalized by receptor-mediated endocytosis fail to block LPS-triggered cytokine production. We then asked whether M ϕ that had internalized antibody-coated tachyzoites by receptor-mediated endocytosis showed suppressed responses to LPS, as was the case for cells harboring live parasites. These experiments were complicated by the fact that live tachyzoites coated with antibodies may enter M ϕ by both receptor-mediated endocytosis and active invasion. Therefore, we examined suppression mediated by live, heat-inactivated, opsonized live, and opsonized and heat-inactivated tachyzoites. In this set of experiments, we also examined whether M ϕ preactivated with IFN- γ are subjected to parasite-induced suppression.

As shown in Fig. 4, tachyzoites subjected to either heat inactivation or heat inactivation followed by opsonization were unable to suppress LPS-triggered TNF- α production (H/L and HO/L) relative to live parasites (T/L) in M ϕ that were not subjected to overnight IFN- γ activation. Live, antibody-coated tachyzoites maintained the ability to prevent LPS-driven M ϕ TNF- α (O/L), most likely as a result of continued active invasion by the parasite. Similar results were obtained when IL-12 production in M ϕ not preactivated with IFN- γ was examined. Nevertheless, in this case, we found that opsonized, heat-treated parasites displayed a partial (approximately 50%) ability to suppress IL-12 production (HO/L). In addition, live

FIG. 3. Direct contact between the parasite and host cell is necessary, but not sufficient, to mediate suppression of LPS-triggered TNF- α production. (A) M ϕ were stimulated with LPS (100 ng/ml), preinfected for 2 h with a 1:1 ratio of parasites to host cells and then stimulated with LPS, or incubated for 2 h with the same number of tachyzoites (TZ) in a transwell and then stimulated with LPS. Cell supernatants were collected 6 h later for cytokine assay. M, medium. (B) Live, CytD (1 μ M)- and heat-treated tachyzoites were cultured for 2 h with M ϕ , LPS (100 ng/ml) was added, and supernatants were collected 6 h later for cytokine ELISA. For CytD, M ϕ were maintained in the drug for the duration of the experiment. Cultures with live and heat-killed parasites were carried out in an percentage of the solvent dimethyl sulfoxide equivalent to that used for the CytD experiment. (C) Diff-Quik staining of M ϕ infected with live and CytD-treated tachyzoites. Cells were fixed and stained 6 h after parasite exposure. The proportions of cells containing internalized parasites were determined to be 99 and 5% in live and CytD experiments, respectively. This experiment was repeated three times with the same result.

opsonized parasites were more effective at inhibiting LPS-induced IL-12 than were live parasites (O/L and T/L, respectively). The basis for this effect may be the reported ability of Fc receptor cross-linking to specifically suppress IL-12 production in M ϕ (22).

IFN- γ -preactivated M ϕ were also sensitive to live and opsonized tachyzoite-induced blockade of LPS-induced cytokine responses (T/L and O/L in Fig. 4). Interestingly, while live tachyzoites (T in Fig. 4) alone failed to induce cytokine production by IFN- γ -activated M ϕ , parasites subjected to heat inactivation (H in Fig. 4) became capable of inducing both IL-12 and TNF- α production by M ϕ .

Drug-induced tachyzoite inactivation following infection reverses inhibition of LPS-triggered TNF- α production. To determine whether live infection and parasite replication are required to maintain M ϕ LPS nonresponsiveness, cells were infected and then, 2 h later, pyrimethamine and 6-thioxanthine were added to the cultures. The latter drugs interfere with nucleotide metabolic pathways required for parasite replication and long-term survival within the parasitophorous vacuole (45, 48). Treatment of infected cells with pyrimethamine results in parasite stasis and dissolution of the parasitophorous vacuole (27). As shown in Fig. 5A, in the absence of drug inhibitors, replicating parasites are clearly visible within a distinct parasitophorous vacuole. After 24 h in the presence of pyrimethamine and 6-thioxanthine, intracellular parasites were difficult to distinguish. In those cells where they could be identified, tachyzoites displayed a rounded-up morphology and, in

most cases, a distinct parasitophorous vacuole was inapparent (Fig. 5B). At this time point, cells were pulsed for 30 min with LPS and supernatants were collected 6 h later for cytokine ELISA.

Interestingly, we found that the TNF- α and IL-12 responses diverged at this late time point following infection. As shown in Fig. 5C, cells in which tachyzoites were subjected to 24-h drug inactivation exhibited a reversal in TNF- α suppression. In contrast, cells infected for 24 h showed dose-dependent IL-12(p40) production in the absence of further LPS stimulation (Fig. 5D), unlike the TNF- α response at this time point (Fig. 5C) and unlike IL-12 responses 6 h after infection (Fig. 2 and 4). Indirect immunofluorescence of IL-12 production confirmed the ELISA results and further showed that both infected and noninfected cells produced the cytokine (data not shown).

IL-12 production during late infection is accompanied by release of inhibition of NF κ B activation. We further examined the kinetics of IL-12 production during infection with *T. gondii* relative to stimulation with LPS. Figure 6 shows that M ϕ IL-12 production induced by tachyzoites was first detectable 24 h postinfection and that the response was maximal 36 h after initiation of infection. This differed from the response to LPS, which was strong at 6 h and peaked at 8 h after stimulation. At no time point did live parasites induce TNF- α production (Fig. 6). It is notable that in 30-h cultures, no TNF- α was produced and, indeed, LPS-induced TNF- α production remained strongly suppressed (Fig. 5C).

FIG. 4. Effects of heat inactivation and antibody coating on the ability of tachyzoites to suppress M ϕ proinflammatory cytokine production. Cells were cultured with live, opsonized, heat-inactivated, or heat-inactivated and opsonized tachyzoites (3:1 ratio of parasites to cells) in the presence or absence of recombinant IFN- γ . Six hours after LPS stimulation, supernatants were collected for cytokine assay. Cells were cultured with medium alone (C), 100 ng of LPS per ml (L), live tachyzoites (T), live tachyzoites followed by LPS (T/L), heat-killed tachyzoites (H), heat-killed parasites followed by LPS (H/L), opsonized parasites (O), opsonized parasites followed by LPS (O/L), heat-killed and opsonized tachyzoites (HO), and heat-killed and opsonized tachyzoites followed by LPS (HO/L). In this experiment, the percentages of cells with internalized tachyzoites were $61\% \pm 15\%$ (live), $25\% \pm 3\%$ (heat killed), $75\% \pm 2\%$ (opsonized), and $68\% \pm 14\%$ (heat killed, opsonized). The data shown are representative of three experiments.

Previous results from our laboratory and others demonstrated inhibition of NF κ B nuclear translocation during *Toxoplasma* infection of M ϕ (11, 50). The observation that infected M ϕ initiate IL-12 production after a delay of approximately 24 h led us to ask whether such cells maintain a block in NF κ B translocation. To answer this question, M ϕ were infected with parasites, triggered with LPS either 2 or 24 h later, and then examined for RelA nuclear translocation 60 min after endotoxin addition. As shown in Fig. 7, cells cultured in medium alone for 2 (panel A) or 24 (panel B) h displayed cytoplasmic NF κ B localization, shown by green fluorescence. Similarly, M ϕ infected with tachyzoites (shown in red) for 2 (panel C) or 24 (panel D) h displayed little evidence of RelA nuclear translocation. Triggering with LPS resulted in NF κ B relocation to the nucleus (panels E and F). As previously demonstrated, cells preinfected for 2 h displayed a block in NF κ B nuclear translocation during LPS stimulation, unlike surrounding non-infected cells, in which NF κ B was translocated to the nucleus (panel G). A different pattern was apparent in the case of M ϕ infected 24 h prior to LPS triggering. Here, cells harboring tachyzoites were capable of NF κ B translocation in response to LPS, as shown by strong green fluorescence in the nuclei of infected cells (Fig. 7H).

DISCUSSION

T. gondii may enter M ϕ through either active invasion or receptor-mediated uptake. Active invasion, completed within approximately 15 to 20 s (42), is a complex, parasite-initiated process involving sequential discharge of microneme, rhoptry, and dense-granule proteins. Microneme protein discharge is associated with initial attachment of the parasite to a host cell (10, 19). Rhoptry proteins are released concurrent with formation of a moving junction during cell penetration (23). Dense-

granule exocytosis occurs in the nascent vacuole and is likely involved in modification of the intracellular compartment occupied by the tachyzoite (13). Phagocytosis and receptor-mediated endocytosis are host cell-mediated processes that may involve clathrin-based mechanisms (1). Phagocytosis typically leads to vacuole acidification and fusion of the vacuole with lysosomes. The latter processes are evaded during *T. gondii* invasion (27, 40, 51).

FIG. 5. Inactivation of intracellular parasites with pyrimethamine (Pyr) and 6-thioxanthine (Thio) fails to reverse LPS-triggered TNF- α production. Bone marrow-derived M ϕ were infected with live RH strain tachyzoites. Two hours later, a combination of pyrimethamine (10 μ M) and 6-thioxanthine (40 μ g/ml) or an equivalent dilution of carrier solvent alone was added. After 24 h, control and drug-treated cells (panels A and B; Diff-Quik staining) were pulsed for 30 min with LPS, fresh medium was added, and 6 h later, supernatants were collected for TNF- α and IL-12 ELISA (panels C and D, respectively). At the time of LPS addition, parallel cultures on glass coverslips were fixed and stained with Diff-Quik and examined by light microscopy (original magnification, $\times 100$). The arrows in panels A and B point to parasites. The experiment was repeated three times with similar results.

FIG. 6. Kinetics of M ϕ IL-12 production during *T. gondii* infection or LPS stimulation. Cultures with increasing amounts of tachyzoites or LPS were incubated for the indicated times, and supernatants were collected for cytokine ELISA. This experiment was repeated twice with essentially the same results.

Our results suggest that active invasion and survival of the parasite within the parasitophorous vacuole are required to induce and maintain M ϕ cytokine-specific nonresponsiveness to LPS. STAg and molecules secreted by live parasites failed to affect LPS-driven cytokine production, arguing against a receptor-mediated event at the cell surface. Additionally, in the presence of CytD, which allows parasite attachment but not invasion, tachyzoites were almost completely ineffective at suppressing responses to LPS. Nevertheless, a small amount of suppression was apparent in the presence of the drug. Indeed, at high parasite/cell ratios (approximately 20-fold relative to those of control cultures in the absence of CytD), levels of cytokine suppression were similar. Inasmuch as rhoptry discharge and vacuole formation may occur in the absence of tachyzoite invasion (23), it is possible that vacuole formation in the presence of CytD accounts for M ϕ nonresponsiveness when high numbers of tachyzoites are present.

Heat-killed parasites entering host cells through phagocytosis or receptor-mediated endocytosis following opsonization failed to prevent LPS-triggered cytokine production. In addition, parasite-induced nonresponsiveness, as measured by TNF- α production, was reversed by drug inactivation of the parasites. Combined with our previous study showing that LPS nonresponsiveness was established within 30 min of infection (11), these results reveal that tachyzoite-mediated suppression occurs rapidly after infection and that its continued maintenance requires active parasites and/or an intact parasitophorous vacuole. A hypothesis we suggest is that M ϕ nonresponsiveness is initiated during parasite entry, possibly involving discharge of apical organelles. Several tachyzoite proteins, including rhoptry-derived ROP-2 and dense-granule-derived GRA5, undergo transmembrane insertion into the parasitophorous vacuole (5, 30). It is possible that such proteins interfere with intracellular M ϕ functions, leading to a block in NF κ B activation.

M ϕ nonresponsiveness to tachyzoite infection is not restricted to cells activated by LPS. Thus, BCG-driven TNF- α and IL-12(p40) production was also inhibited by preinfection. Components of BCG signal through both TLR-4 and TLR-2 (54). Since both receptors lie upstream of the I κ B kinase ac-

tivation pathway required for NF κ B translocation (28), it is not surprising that the BCG response is blocked. Similarly, superantigen-induced MHC class II multimerization leads to IL-12(p40) production through an NF κ B-dependent pathway (17) and, as predicted, tachyzoite infection also blocked this response.

The results presented here suggest that *Toxoplasma* possesses at least two mechanisms that result in suppressed proinflammatory cytokine production. Early during infection, production of IL-12 and TNF- α triggered by LPS is prevented, and this is associated with a blockade in NF κ B nuclear import. However, after 24 h of infection, tachyzoites themselves elicit M ϕ IL-12, yet the cells remain actively suppressed in the ability to produce TNF- α . Importantly, at this late time point, LPS-driven RelA translocation is no longer prevented in infected cells. These results suggest nonidentical control of IL-12 and TNF- α expression. While control of IL-12(p40) and TNF- α gene promoters is not fully understood, both contain binding sites for AP-1 and CCAAT enhancer-binding protein, in addition to NF κ B (32, 43). Nevertheless, functional promoter analysis suggests that these genes are unlikely to be regulated identically (32, 46). Determination of how *T. gondii* targets different aspects of M ϕ proinflammatory signaling cascades is an area of ongoing research.

Our experiments show that at 24 h post-*Toxoplasma* infection, when IL-12 is being produced, there is no evidence of NF κ B translocation in infected cells. In this regard, it is of interest that production of IL-12 during *T. gondii* infection also occurs in the absence of NF κ B family member c-Rel (37). Together, these results suggest that parasite-driven IL-12 production may occur independently of NF κ B signaling pathways. Nevertheless, with regard to the present results, it is possible that parasite-driven nuclear translocation and re-export of RelA to the cytoplasm were events that preceded the 24-h time point at which cells were examined, a possibility that we are now examining.

Our findings and similar reports by others (50) suggesting that *Toxoplasma* disables the NF κ B activation pathway parallel other studies demonstrating parasite interference with the Janus kinase/signal transducer and activator of transcription signaling pathway in M ϕ . Thus, the parasite also down-regulates M ϕ expression of IFN- γ -induced MHC class II molecules, a process dependent upon signaling by the Janus kinase/signal transducer and activator of transcription (34). The suppressive effect results from parasite interference with nuclear translocation of STAT1 α , despite normal phosphorylation of the latter transcription factor (35). It is tempting to speculate that the inhibitory effects of *Toxoplasma* on NF κ B and STAT1 α stem from a common mechanism of action. A hypothesis consistent with these data is that *T. gondii* infection results in a nuclear protein import blockade.

Toxoplasma infection is well known to result in robust proinflammatory mediator production in vivo (2, 14, 56). Therefore, the parasite's suppressive effects on M ϕ cannot be globally applicable to all cell types. Indeed, the present study shows that M ϕ themselves produce IL-12(p40), but not TNF- α , during extended periods (i.e., >24 h) of contact with *T. gondii*. Both dendritic cells and neutrophils serve as early cytokine sources during infection (3, 6–8, 47). We are currently examining the role of NF κ B activation during infection of these cell types

FIG. 7. LPS-triggered RelA translocation is blocked 2 h post-*Toxoplasma* invasion but restored 24 h after infection. M ϕ were infected (1:1 ratio of tachyzoites to cells), and then, either 2 (panels A, C, E, and G) or 24 (panels B, D, F, and H) h later, cells were triggered with LPS (100 ng/ml). Sixty minutes later, cells were fixed, permeabilized, and stained with antibodies to RelA (p65) (shown in green) and *Toxoplasma* (shown in red). Panels: A and B, medium; C and D, *Toxoplasma*; E and F, LPS; G and H, *Toxoplasma* preinfection plus LPS. Scale bar = 20 μ m. This experiment was repeated twice with similar results.

with *T. gondii*. The present results showing M ϕ IL-12 production during extended culture also reconcile our results with previous studies showing M ϕ IL-12 production in response to *T. gondii* (20).

It is not clear why *Toxoplasma* possesses mechanisms to down-regulate M ϕ proinflammatory responses. Control of infection depends upon balanced production of type 1 cytokines such as IL-12 and IFN- γ (2, 14, 21, 31, 56). Therefore, it is possible that the suppressive activity of the parasite represents an immune evasion strategy or a means by which to avoid overinduction of proinflammatory cytokines. Molecular identification of the parasite-inhibitory factor is required to understand its role in the host-parasite interaction and may lead to development of anti-inflammatory agents of broad clinical utility.

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