**Toxoplasma gondii** Inhibits Toll-Like Receptor 4 Ligand-Induced Mobilization of Intracellular Tumor Necrosis Factor Alpha to the Surface of Mouse Peritoneal Neutrophils

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Neutrophils are well-known to rapidly respond to infection through chemotactic infiltration at sites of inflammation, followed by rapid release of microbicidal molecules, chemokines, and proinflammatory cytokines. For tumor necrosis factor alpha (TNF-α), we recently found that neutrophils contain intracellular pools of the cytokine and display the capacity to upregulate transcriptional activity of the gene during lipopolysaccharide (LPS) stimulation. We now show that triggering of mouse peritoneal neutrophils with Toll-like receptor 2 (TLR2), TLR4, and TLR9 ligands, but not ligands of TLR3, induces upregulation of surface membrane TNF-α. However, neutrophils infected with the protozoan *Toxoplasma gondii* displayed an inability to respond fully in terms of TLR ligand-induced increases in membrane TNF-α expression. Infected neutrophils failed to display decreased levels of intracellular TNF-α upon LPS exposure. In contrast to intermediate inhibitory effects in nontreated neutrophils, *T. gondii* induced a complete blockade in LPS-induced surface TNF-α expression in the presence of the protein synthesis inhibitor cycloheximide. Despite these inhibitory effects, the parasite did not affect LPS-induced upregulation of TNF-α gene transcription. Collectively, the results show that *Toxoplasma* prevents TLR ligand-triggered mobilization of TNF-α to the neutrophil surface, revealing a novel immunosuppressive activity of the parasite.

*Toxoplasma gondii* is an intracellular protozoan parasite with global distribution among humans and animals (17, 20). The parasite is a major opportunistic pathogen in immunocompromised patients and can cause severe birth defects during congenital infection (24, 27). Nevertheless, in immunocompetent individuals, infection with *Toxoplasma* is asymptomatic under most circumstances. An ongoing strong type 1 cytokine response and robust cell-mediated immunity are necessary to survive both acute and chronic phases of infection, but in some situations overproduction of these mediators has pathological consequences (1, 15, 39).

Our work focuses on the role of neutrophils in *Toxoplasma* infection. Polymorphonuclear leukocytes (PMN) are known to be the first immune cell type to arrive at foci of infection where they exert their effector potential through phagocytosis and release of granules rich in antimicrobial and tissue-degrading enzymes (32). These cells have been considered terminally differentiated antimicrobial immune effectors but more recently have emerged as immunoregulatory cells that are capable of producing proinflammatory mediators and that express cytokines such as interleukin 12 (IL-12) and tumor necrosis factor alpha (TNF-α) as preformed intracellular pools (3, 6, 11, 14, 28, 36).

The expression of TNF-α itself involves transport to the cell surface where it is subsequently subjected to cleavage and release by the metalloproteinase TNF-α-converting enzyme (4, 5). Expression of a membrane form of TNF-α (mTNF-α) suggests that direct cell-to-cell contact may be important in mediating the effects of this proinflammatory cytokine.

When neutrophils are subjected to Toll-like receptor 4 (TLR4) triggering, intracellular TNF-α is rapidly expressed at the neutrophil surface membrane (3). As a result of increased membrane TNF-α expression, PMN acquire the ability to induce high-level dendritic cell TNF-α expression in a cell contact-dependent manner. Further complexity is added by the finding that lipopolysaccharide (LPS)-triggered neutrophils also induce dendritic cell IL-12 production, but in this case the response involves neutrophil release of presently undefined soluble factors unrelated to TNF-α. Because polymorphonuclear leukocytes also produce several chemokines important in dendritic cell recruitment, we hypothesize that neutrophils may play an important role in dendritic cell activation during infection (2). Such findings are reinforced by recent data indicating near-identical effects using human PMN and dendritic cells (37).

In other studies, we found that *Toxoplasma* infection results in potent long-lasting suppression of TNF-α release in LPS-triggered macrophages (9, 16). We previously reported that neutrophils release low levels of TNF-α in response to *T. gondii*, but these studies employed soluble tachyzoite lysate as the stimulus (7, 8). Here, we asked whether parasite infection affected the ability of PMN to upregulate membrane TNF-α in response to LPS. We show that while noninfected peritoneal neutrophils upregulate surface TNF-α expression induced by TLR ligands, this response is downregulated in infected populations. For triggering through TLR4, mobilization of intracellular TNF-α is blocked without affecting increased transcription of the cytokine. As such, this represents a novel mechanism that the parasite employs to manipulate cells of innate immunity.

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MATERIALS AND METHODS

Mice. C57BL/6 strain mice were obtained from The Jackson Laboratory (Bar Harbor, ME). Female mice between 6 and 8 weeks of age were used throughout. Animals were housed in filter-covered isolator cages in the animal facility of the College of Veterinary Medicine at Cornell University, which is accredited by the American Association for Accreditation of Laboratory Care.

Parasites. Transgenic yellow fluorescent protein (YFP)-expressing parasites of the virulent T. gondii strain RH (kindly provided by D. Roos, University of Pennsylvania) and green fluorescent protein (GFP)-expressing tachyzoites of the virulent T. gondii strain PTG (American Type Tissue Culture Collection, Manassas, VA) were maintained by biweekly passage on human foreskin fibroblasts in medium composed of Dulbecco modified Eagle medium (DMEM) (Life Technologies, Gaithersburg, MD) supplemented with 1% fetal calf serum (HyClone Laboratories, Logan, UT), 100 U/ml penicillin, and 100 µg/ml streptomycin (Life Technologies). YFP-expressing RH cultures were supplemented with 1 mM pyrithiamine (Sigma-Aldrich).

Reagents and Ab. Ultrapure LPS (Escherichia coli strain O111:B4) was purchased from List Biological Laboratories (Campbell, CA), Polyclonal anti-TNF-α (clone M5.114; American Type Culture Collection), and then washed twice with Dulbecco’s PBS (Life Technology) containing 0.5% bovine serum albumin and 0.075% sodium azide. For intracellular cytokine detection, permeabilization buffer, unlabeled hamster anti-TNF-α (MP6-XT22), and hamster anti-mouse TNF-α (M6-4630; clone M6-4630) were obtained from BD Pharmingen (San Diego, CA). Normal mouse IgG and normal mouse serum (NMS) were purchased from Jackson ImmunoResearch (West Grove, PA). Cycloheximide (CHX) was obtained from Calbiochem (La Jolla, CA). 

Peritoneal neutrophils. Mice were injected with 1 ml of 10% thioglycolate (Difco Laboratories, Detroit, MI) intraperitoneally, and 18 h later, peritoneal exudate cells were obtained by lavage with ice-cold phosphate-buffered saline (PBS). Cells were washed in PBS and resuspended through a 70-µm nylon cell strainer, and erythrocytes in the suspension were lysed using red cell lysis buffer (Sigma). Cells were then washed, resuspended at 2 × 10⁶ cells/ml in buffer composed of Dulbecco’s PBS (Life Technology) containing 0.5% bovine serum albumin (Sigma) and 1 mM EDTA (Fisher Scientific), incubated for 15 min at 4°C with rat anti-mouse major histocompatibility complex class II monoclonal antibody (clone M5.114; American Type Culture Collection), and then washed twice with ice-cold PBS. Cells were then incubated at 4°C with goat anti-rat IgG coupled to magnetic beads under gentle mixing conditions. After 30 min, cells were placed in a magnet apparatus (MPC-2; Dynal, Oslo, Norway) to remove major histocompatibility complex class II-positive cells. The procedure was repeated twice, and then the cells were washed in cold PBS and resuspended in DMEM supplemented with 10% horse serum (HyClone, Logan, UT), 100 mM/ml penicillin, 100 µg/ml streptomycin (Life Technologies, Grand Island, NY), 5 × 10⁻⁶ M 2-mercaptoethanol, 10 mM HEPES (Invitrogen Life Technologies), 100 µM nonessential amino acids (Invitrogen Life Technologies), 1 mM sodium pyruvate (Invitrogen Life Technologies) alone or in the presence of different stimuli at 37°C in 5% CO₂. Neutrophils obtained in this manner were routinely 90 to 95% viable as determined by trypan blue exclusion.

In some experiments, purified PMN were infected with RH strain tachyzoites and stimulated with LPS 2 h later, and 4 h later, the supernatants were collected for TNF-α enzyme-linked immunosorbent assay.

Flow cytometry. To analyze surface markers on PMN, Fc receptors were blocked in fluorescence-activated cell sorting buffer (PBS, 1% bovine serum albumin, and 0.1% sodium azide) with 5 µg/ml anti-mouse CD16/CD32 (BD Pharmingen) and 10% NMS for 15 min at 0°C, and then the cells were stained with optimal concentrations of PerCP-Cy5.5-conjugated anti-Gr-1 (Ly-6G) in combination with PE-conjugated antibodies specific for TLR9, and allophycocyanin-conjugated anti-F4/80 Ab, and unlabeled anti-TNF-α Ab, and then the cells were fixed in 3% paraformaldehyde (Sigma), 0.1 mM CaCl₂, and 0.1 mM MgCl₂, for 30 min at 0°C. For intracellular cytokine detection, peritoneal cells were blocked as described above, stained with PerCP-Cy5.5-conjugated anti-Gr-1 Ab, allophycocyanin-conjugated anti-F4/80 Ab, and unlabeled anti-TNF-α Ab, and then the cells were fixed in 3% paraformaldehyde (Sigma), 0.1 mM CaCl₂, and 0.1 mM MgCl₂, for 30 min at 0°C. Cells were subsequently washed in permeabilization buffer (PBS with 0.075% saponin) and incubated for 15 min at 0°C in permeabilization buffer containing 10% NMS, 5% normal goat serum, and 5 µg/ml anti-mouse CD16/CD32. After two washes in permeabilization buffer, unlabeled hamster anti-TNF-α was added, and the cells were incubated for 30 min at 0°C. After the cells were washed in permeabilization buffer, PE-conjugated anti-hamster Ab was added, and the cells were incubated for 30 min at 0°C and subsequently washed for flow cytometric analysis. Expression of TLR was examined on purified neutrophils by fixation (3% paraformaldehyde) followed by blocking in 10% normal mouse serum and 10% normal donkey serum. Samples were permeabilized with 0.075% saponin and subjected to intracellular blocking with 10% normal donkey serum. Anti- TLR3 (eBioscience) Ab, anti-TLR4 (Santa Cruz Biotech) Ab, and rat isotype control Ab were added, followed by PE-conjugated donkey anti-rat IgG. Samples were subsequently washed and subjected to flow cytometric analysis. Data were acquired on a FACS Calibur system (10,000 events per sample) and analyzed with CellQuest software (Becton Dickinson Immunocytometry Systems, San Jose, CA).

RESULTS

TLR ligands trigger rapid upregulation of transmembrane TNF-α on the surface of peritoneal PMN. We previously established that TLR4 ligand LPS stimulates rapid mobilization of intracellular TNF-α to the surface of neutrophils (3). In Fig. 1A, we examined the influence of other TLR ligands on upregulation of neutrophil surface membrane TNF-α expression. Flow cytometric analysis revealed virtually identical kinetics of increased mTNF-α in response to LPS and TLR2 ligand Pam3Cys. Our Pam3Cys preparation contained insignificant amounts of contaminating LPS, because it was equally active on cells from C3H/HeJ (LPS nonresponder) mice (data not shown). Transmembrane TNF-α expression was also triggered by TLR9 ligand CpG, but in this case expression kinetics were delayed (Fig. 1A). This may reflect the fact that TLR9 is expressed within the endoplasmic reticulum and must be recruited to lysosome-like compartments during CpG recognition, whereas TLR2 and TLR4 ligand binding occurs at the cell surface (22). In contrast to LPS, Pam3Cys, and CpG, the TLR3 ligand poly(I:C) failed to increase mTNF-α, despite the fact that it is active on macrophages (Fig. 1A and data not shown). This was true at the dose shown in Fig. 1 and at higher doses and extended incubation times (data not shown). Although neutrophils did not upregulate surface TNF-α in response to poly(I:C), the cells expressed TLR3 at levels similar to those of TLR4 (Fig. 1B). This is of interest, because whereas TLR2, -4, and -9 signaling involves intracellular MyD88, TLR3 signal transduction is independent of this adaptor molecule (35).

T. gondii interferes with TLR ligand-induced upregulation of mTNF-α on peritoneal neutrophils. We next tested the influence of live Toxoplasma on the ability of neutrophils to respond to TLR ligand stimulation. In this set of experiments, we in vitro infected cells isolated from the peritoneal cavities of thioglycollate-injected mice and then 2 h later reisolated cells for flow cytometric analysis. As shown in Fig. 2, using transgenic YFP-expressing tachyzoites, it was possible to unambiguously identify and select for analysis infected versus noninfected Gr-1⁺ F4/80⁺ myeloid neutrophils.

To determine whether infection with Toxoplasma tachyzoites altered the ability of neutrophils to upregulate mTNF-α, we in-
cubated peritoneal exudate cells with tachyzoites from the highly virulent type I parasite strain RH expressing YFP (Fig. 3A) or from the less virulent type II strain PTG expressing GFP (Fig. 3B). Two hours later, we stimulated the cells with LPS or Pam3Cys for 1 h or with CpG for 4 h and then stained for flow cytometric analysis. The data show surface TNF-α expression profiles gated on Gr-1<sup>high</sup> F4/80<sup>low</sup> cells. (B) Expression levels of thioglycolate-elicited neutrophil TLR3 and TLR4 in nonstimulated PMN. Neutrophils were isolated (85% purity) and examined for TLR expression by flow cytometry. Broken line, isotype control; solid line, anti-TLR staining.

FIG. 1. Surface expression of transmembrane TNF-α on neutrophils following stimulation with different microbial factors. (A) Thioglycolate-elicited peritoneal exudate cells were cultured with medium alone (thin broken lines) or in the presence of 10 μg/ml Pam3Cys, 100 μg/ml poly(I:C), 1 μg/ml LPS, or 5 μM CpG (thick lines). Cells were collected at the indicated times and stained for flow cytometric analysis. The data show surface TNF-α expression profiles gated on Gr-1<sup>high</sup> F4/80<sup>low</sup> cells. (B) Expression levels of thioglycolate-elicited neutrophil TLR3 and TLR4 in nonstimulated PMN. Neutrophils were isolated (85% purity) and examined for TLR expression by flow cytometry. Broken line, isotype control; solid line, anti-TLR staining.

FIG. 2. Toxoplasma-infected and noninfected neutrophils can be distinguished by flow cytometric analysis. Thioglycolate-elicited peritoneal exudate cells were subjected to in vitro infection with transgenic YFP-expressing RH strain tachyzoites (3:1 ratio of parasites to cells). After 2 h, cells were labeled with PerCP-Cy5.5-conjugated anti-Gr-1 Ab and allophycocyanin-conjugated anti-F4/80 Ab. Cells were gated on the Gr-1<sup>high</sup> F4/80<sup>low</sup> population, then among this population T. gondii-infected and noninfected (NI) cells were examined. This gating strategy was employed to determine TNF-α expression profiles shown in Fig. 3, 4, and 6.
determine whether this was a unique property of the RH parasite strain, the same experiment was performed with GFP-labeled PTG tachyzoites (Fig. 3B). The results are similar to those obtained employing the RH strain, but the suppressive effects of PTG appeared to be less severe than those mediated by the virulent parasite strain.

We also examined accumulation of TNF-α in supernatants of infected PMN subjected to LPS triggering. As shown in Fig. 4, parasites blocked LPS-induced release of neutrophil TNF-α. Nevertheless, the inhibitory effect of Toxoplasma was partial (approximately 55%). This was true even at the very high ratios of parasites to cells where the vast majority of PMN were infected.

**FIG. 3. T. gondii infected PMN are defective in their ability to upregulate surface expression of TNF-α in response to TLR triggering.** Thioglycolate-elicited peritoneal cells were incubated in the presence of medium alone (thin black lines) or exposed to fluorochrome-labeled *T. gondii* parasites of (A) RH strain and (B) PTG strains (green lines). A 3:1 ratio of parasite:cells was used. After 2 h of exposure to parasites, cells were stimulated with LPS (1 h), Pam3Cys (1 h), or CpG (4 h) (blue lines). Cells incubated in the presence of TLR ligands only are represented by red histograms. The profiles shown are gated on Gr-1<sup>hi</sup>F4/80<sup>lo</sup> cells. Noninfected (NI) cells are shown for comparison. This experiment was repeated twice with essentially identical results.

*T. gondii* blocks neutrophil surface membrane expression of intracellular TNF-α but does not prevent LPS-induced increases in mRNA levels. We previously identified pools of intracellular TNF-α and newly synthesized cytokine as two LPS-inducible sources of mTNF-α (3). Therefore, the parasite-induced block in TLR-induced mTNF-α could result from interference with one or both of these TNF-α sources. To address this issue, we incubated cells in the presence of the protein synthesis inhibitor cycloheximide prior to culture with LPS. Under these conditions, any increase in mTNF-α must result from mobilization of preformed cytokine rather than from its de novo synthesis.

As shown in Fig. 5, in the absence of CHX, RH tachyzoites...
partially blocked surface expression of TNF-α in infected neutrophils (Fig. 5A) but had no effect on noninfected (Fig. 5B) neutrophils (red line, LPS; blue line, RH plus LPS). However, *T. gondii* infection completely blocked the ability of LPS to upregulate mTNF-α in the presence of CHX (Fig. 5C). As expected, in noninfected neutrophils, the parasite did not affect the levels of LPS-induced mTNF-α (Fig. 5D).

The findings in Fig. 5 point to preformed intracellular pools of TNF-α as a target of manipulation by *T. gondii*. To support this result, we measured TNF-α mRNA expression levels in noninfected and infected neutrophils subjected to LPS triggering. In this experiment purified PMN were preinfected with RH strain tachyzoites and then 1, 2, and 4 h later cells were subjected to 60 min of stimulation with LPS. Using conditions where >85% of neutrophils were infected, RH alone failed to elicit increases in TNF-α mRNA at any time point (Fig. 6A). In contrast, stimulation with LPS alone resulted in increased transcripts for TNF-α (Fig. 6A, lane LPS). Most importantly, pre-infection with *T. gondii* for 1 to 4 h did not affect the ability to upregulate TNF-α mRNA levels in response to LPS. This result provides independent evidence that the parasite interferes with mobilization of intracellular pools of the cytokine, rather than blocking de novo TNF-α synthesis. We also examined LPS-induced CD11b mRNA upregulation and found that it, too, was unaffected by *T. gondii* infection (Fig. 6B).

**TNF-α is retained in intracellular form in infected cells.** We next stimulated PMN with LPS and then assessed intracellular TNF-α levels in infected versus noninfected cells. We previously reported that within 10 min of LPS stimulation, the intracellular pool of this cytokine decreased, and we confirm
here that LPS stimulation decreases the mean fluorescence intensity of cells stained for intracellular TNF-α (Fig. 7A, LPS). When intracellular TNF-α was assessed in parasite-infected neutrophils, there was no change in intracellular TNF-α levels (Fig. 7A, RH). Furthermore, LPS stimulation of RH-infected PMN failed to result in decreased intracellular TNF-α (Fig. 7A, RH+LPS). In contrast, when noninfected PMN in the same population were assessed for intracellular TNF-α, the presence of parasites did not alter the ability of LPS to decrease the level of intracellular cytokine (Fig. 7B, LPS versus RH+LPS). Taken together, the data argue that neutrophils infected with *T. gondii* cannot mobilize intracellular pools of TNF-α to the surface membrane when the cells are activated by TLR4 ligand LPS. In contrast, the ability of LPS to upregulate transcription of TNF-α is unaffected by the parasite.

**DISCUSSION**

The cytokine TNF-α is an important pleiotropic mediator that displays protective effects through its ability to activate innate immunity, as well as pathological effects when its production is dysregulated (19). Gene knockout studies in mice have demonstrated that TNF-α is required for long-term survival during *Toxoplasma* infection, although its expression is dispensable during acute infection (31, 38). In the context of oral *T. gondii* infection, TNF-α mediates immunopathology in the small intestine that underlies tissue necrosis and death in susceptible mouse strains (23, 34).

The results of the present study demonstrate that PMN infected with the protozoan *T. gondii* are unable to mobilize intracellular TNF-α pools to the cell surface during TLR4 triggering. Direct infection was required for this inhibitory effect, because noninfected neutrophils upregulated TNF-α in a mixed population of infected and noninfected cells. Nevertheless, the parasite did not prevent LPS-induced upregulation of TNF-α gene transcription. Furthermore, when de novo protein synthesis was blocked with cycloheximide, tachyzoites completely blocked TLR-induced TNF-α surface expression in contrast to less complete effects seen in the absence of the drug. The combined results argue that TLR4-stimulated neutrophils increase mTNF-α through mechanisms involving both increased gene transcription and surface mobilization of intracellular cytokine and that *Toxoplasma* targets the latter pathway for disassembly. The data in Fig. 3 suggest that *T. gondii* infection has similar effects on triggering and signaling through TLR2 and TLR9. Whether this is the case and whether signaling through other neutrophil TLRs is similarly affected are currently under investigation.

TNF-α is expressed in the surface membrane-bound form prior to enzymatic cleavage and release, suggesting that the cytokine and its receptors, TNF receptor I (TNFRI) and TNFRII, may be important in directing cell-to-cell interactions. In this regard, we recently showed that triggering mouse neutrophils with TLR4 ligand LPS rapidly mobilizes intracellular stores of TNF-α to the cell surface (3). Neutrophil expression of mTNF-α induces strong cell contact-dependent dendritic cell activation, suggesting that such interactions may be important during initiation of innate immunity.

The TNF-α expressed in membrane-associated form on the cell surface is functionally distinct from the cytokine released from the cell. For example, while soluble TNF-α is the predominant form driving autoimmune inflammation, the membrane-bound cytokine is sufficient to support secondary lymphoid organ ultrastructure (29). Transmembrane TNF-α is the primary ligand for TNFRII (18). Because mTNF-α also signals through TNFRI, cooperativity between the receptors leads to cellular responses stronger than those induced by the soluble cytokine. In this regard, we recently reported that neutrophil mTNF-α was an extremely potent activator of dendritic cells, displaying an activity superior to that of soluble TNF-α (2, 3). Recently, it was found that membrane and soluble TNF display disparate effects during *Mycobacterium tuberculosis* infection in mice in that mTNF-α alone could induce cell migration and granuloma formation and early resistance but that long-term control of this pathogen required soluble TNF (30). The effects of membrane versus soluble TNF-α during *Toxoplasma* infection remain to be investigated.

**FIG. 7.** *T. gondii* prevents intracellular TNF-α from translocating to the cell surface of peritoneal PMN. Intracellular TNF-α was examined in thioglycolate-elicited cells by blocking surface TNF-α using an unlabeled rat anti-mouse TNF-α Ab, then fixing and permeabilizing cells, followed by staining with a hamster anti-mouse TNF-α Ab to reveal intracellular expression of TNF-α. The bar graphs show the differences in the mean fluorescence intensity (ΔmFI) of parasite- and LPS-exposed cells relative to unstimulated cells. The profiles shown are gated on (A) infected Gr1<sup>high</sup> F4/80<sup>low</sup> and (B) noninfected Gr1 high F4/80<sup>low</sup> cells. The cells were exposed to different treatments as follows: LPS, cells were exposed to LPS for 10 min; RH, cells were cocultured with parasites for 2 h; RH+LPS, cells were exposed to RH for 2 h followed by 10 min of stimulation with LPS. Noninfected (NI) cells are shown for comparison. This experiment was repeated twice with essentially identical results.
We and others have reported the ability of T. gondii to suppress responses in bone marrow-derived macrophages and dendritic cells (9, 13, 25). Release of LPS triggered TNF-α, and IL-12 is prevented in both cell types. Infection of immature dendritic cells also renders the cells unable to attain a maturation status required for T-cell activation (25). For macrophages, the suppressive effects of Toxoplasma involve cytokine-independent activation of signal transducer and activator of transcription 3 (STAT3), possibly acting in part through inhibition of NF-κB and p38 mitogen-activated protein kinase (MAPK) activity (9, 10, 21, 33). The results reported here show for the first time parasite-induced suppression of PMN responsiveness. Nonetheless, unlike the effects of T. gondii on macrophages, the parasite does not block de novo TNF-α synthesis in neutrophils, instead targeting mobilization of intracellular cytokine.

At present, we cannot distinguish whether Toxoplasma specifically blocks mobilization of preformed TNF-α or whether the parasite blocks exocytosis of both preformed and newly synthesized cytokine. Nevertheless, the data in Fig. 5 suggest that surface expression of preformed TNF-α may be specifically targeted. This is because the parasite completely blocks LPS-triggered mTNF-α expression under conditions where de novo protein synthesis is blocked. In addition, the inability to completely block LPS-induced TNF-α release is also consistent with a mechanism of PMN TNF-α secretion that is not affected by the parasite. Further experiments are required to definitively address this issue.

Although we find that T. gondii blocks mobilization of preformed TNF-α to the neutrophil surface, our previous studies demonstrated neutrophil release of TNF-α in response to the parasite (2, 8). However, our previous studies were performed on supernatants from overnight cultures of parasites and cells. Therefore, TNF-α secretion could result from an alleviation in the block in release of newly synthesized cytokine in long-term culture. Additionally, many of our previous studies employed soluble tachyzoite lysates (8, 12), a situation clearly distinct from the live tachyzoites that were employed in the present study. We also found that TNF-α-dependent PMN activation of dendritic cells was consistently more efficient when neutrophils were subjected to triggering with low tachyzoite numbers (2). The latter suggests that uninfected PMN respond to secreted parasite molecules or products of infected cells and that parasite-infected cells per se are targeted for suppression.

Why Toxoplasma would inhibit TLR signaling leading to neutrophil TNF-α mobilization is currently a matter for speculation. This may be a means by which the parasite avoids TNF-α induction mediated by its own TLR ligands that it is known to express (40). Alternatively, it is possible that infection-induced exposure to bacterial TLR ligands requires the parasite to block responses to endotoxins and other inflammatory bacterial products.

The finding that Toxoplasma-infected neutrophils cannot mobilize preformed pools of intracellular TNF-α to the cell surface raises the possibility that the parasite has the capacity to generally block neutrophil exocytosis of preformed mediators. In this regard, PMN are well-known to harbor granules rich in potent microbialic molecules, such as myeloperoxidase, cathepsin G, and neutrophil elastase (26, 32). These mediators are rapidly released into the extracellular milieu or fuse with phagocytic vacuoles upon microbial stimulation. Inhibition of surface expression of preformed TNF-α may reflect a general parasite-induced blockade in mobilization of neutrophil granules and as such could represent a major defensive strategy of the parasite.

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