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Dysregulation of Macrophage Signal Transduction by *Toxoplasma gondii*: Past Progress and Recent Advances

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SUMMARY

The opportunistic protozoan parasite *Toxoplasma gondii* is well known as a strong inducer of cell-mediated immunity, largely as a result of proinflammatory cytokine induction during in vivo infection. Yet, during intracellular infection the parasite suppresses signal transduction pathways leading to these proinflammatory responses. The opposing responses are likely to reflect the parasite's need to stimulate immunity allowing host survival and parasite persistence, and at the same time avoiding excessive responses that could result in parasite elimination and host immunopathology. This Review summarizes past and present investigations into the effects of *Toxoplasma* on host cell signal transduction. These studies reveal insight into the profound suppression of proinflammatory cytokine responses that occurs when the parasite infects macrophages and other cells of innate immunity.

Keywords

Parasite-protozoan; macrophage; cytokine; signal transduction

INTRODUCTION

The apicomplexan protozoan *Toxoplasma gondii*, estimated to infect 20−50% of the human population, is a phenominally successful parasite that normally establishes lifelong latent infection in the central nervous system and skeletal muscle tissue of the host. However, under conditions of immunodeficiency, *T. gondii* can become a pathogenic microbe that may cause serious disease or death in the host [1]. Recent advances in the immunology and cell biology of *Toxoplasma* infection are revealing in molecular detail the sophisticated ways that this parasite interacts with the host to determine the outcome of infection [2]. As such, experimental approaches to the study of intracellular *T. gondii* infection serve as a model to understand the host-parasite interaction, revealing insight into infection with this and other protozoan parasites.

Under normal conditions, *Toxoplasma* infection is controlled by a strong IL-12-dependent, IFN-γ-mediated response in which Th1 cells and $CD8^+$ T lymphocytes play predominant roles [3,4]. These responses are likely triggered in part through the activity of Toll-like receptor (TLR) ligands expressed by the parasite. For example, tachyzoite glycosylphosphatidylinositol lipid anchors activate TLR2 and 4 [5]. Also, *T. gondii* profilin induces high level TLR11-dependent IL-12 production in mouse dendritic cells, although

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this TLR molecule is not expressed in humans [6]. There is in addition a significant TLR/MyD88-independent component to innate recognition of the parasite [7,8]. In mice, IFN-γ mediates protection in large part through activation of macrophages. The latter cells display microbicidal activity through production of nitric oxide and expression of immunity-related GTPase (IRG) proteins that mediate intracellular parasite destruction [9-13].

Toxoplasma is well known for its ability to infect virtually any kind of host cell in vitro. However, during in vivo infection the parasite displays a more restricted host range, and there is evidence that *T. gondii* uses cells of innate immunity, in particular macrophages and dendritic cells, as reservoirs of infection and early dissemination [14-17]. Along similar lines, other intracellular protozoa such as *Leishmania* target dendritic cells and neutrophils for early infection [18,19]. This seems paradoxical given the potent antimicrobial effector functions of macrophages [20], and the pivotal role of dendritic cells in triggering lymphocyte activation [21]. Surviving in these potentially hostile cells would seem to present intracellular parasites such as *Toxoplasma* with special challenges to successful infection.

In recent years, considerable progress has been achieved with regard to the effects of *Toxoplasma* on host signaling pathways during intracellular infection of macrophages and other cell types. During in vivo infection, it is well known that the parasite must elicit a protective cell-mediated immune response to enable host survival and establishment of latent infection [3,4]. At the same time, the parasite must avoid inducing an overly strong proinflammatory response that can lead to strong anti-microbial activity and in some cases lethal host immunopathology [22-24]. These opposing forces are played out during intracellular infection of macrophages, where the parasite activates certain signal transduction pathways and suppresses others. For example, *T. gondii* infection leads to induction of IL-12 involving both MyD88-dependent and MyD88-independent signaling cascades [5-7,25]. *Toxoplasma* also activates anti-apoptotic pathways, thereby rendering infected cells resistant to programmed cell death [26,27]. At the same time, the parasite can suppress activation pathways triggered through TLR signaling and IFN-γ receptor/signal transducer and activator of transcription (Stat)-1 signal transduction [28,29]. Excitingly, parasite molecules that influence host cell signaling are now being identified. Therefore, a molecular picture of how *T. gondii* targets the signal transduction machinery of the host cell is beginning to emerge. In turn, this may help us understand how other intracellular protozoans dysregulate host cell signaling. This Review summarizes recent progress in our understanding of how *Toxoplasma* de-activates proinflammatory signaling pathways in macophages and other cell types, a phenomenon that appears to convert these cells from enemy combatants into safe havens for infection and dissemination.

VIEW FROM INSIDE THE PARASITOPHOROUS VACUOLE

During infection, *Toxoplasma* directs an orchestrated series of events that enable entry into the cell and creation of a parasitophorous vacuole (PV) [30-33]. A key occurrence is discharge of rhoptries during invasion. These are apical organelles that contain large numbers of proteins, some of which are now known to be kinases involved in communication with the host cell [34]. Several rhoptry proteins are directly injected into the cytoplasm within membrane-bound vesicles and new data show that a subset of these molecules targets the host nucleus [35-37]. The PV becomes closely juxtaposed with the host nucleus, recruiting both mitochondria and endoplasmic reticulum of the host cell [38,39]. This close positioning of the PV and organellar membranes thus, in principle, provides a system of molecular exchange for both metabolism and communication.

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Definitive identification of parasite proteins that target host signaling pathways has until recently remained elusive, but now *Toxoplasma* rhoptry proteins are emerging as important players in this area [36,37]. Identification of the activity these molecules was facilitated by the unusual population structure of *Toxoplasma*. In Europe and North America, individual isolates of the parasite predominantly belong to one of three clonal lineages, designated Type I, II and III [40]. These strain types differ in mouse virulence, in that Type I strains uniformly cause lethal infection prior to encystment, whereas strain Types II and III cause nonlethal infection and are therefore able to establish latent infection. The involvement of ROP16 in defense against host immunity was discovered by quantitative trait locus (QTL) mapping of virulence traits associated with progeny of *T. gondii* Type II and Type III strain crosses, and in particular with parasite strain-specific IL-12 production [41]. The polymorphic ROP16 molecule seems to be involved in tyrosine phosphorylation-dependent activation of host cell Stat3, an event that was linked to lack of IL-12 production. This is important because we had previously shown that parasite-induced Stat3 activation was involved in down-modulation of IL-12 and TNF-α production during TLR4 stimulation of infected macrophages [42]. The Stat3 molecule is also required for the anti-inflammatory effects of IL-10 [43]. Therefore, it appears that *Toxoplasma* may directly hijack the IL-10 signaling pathway to mediate down-regulation of proinflammatory signaling.

Because ROP16 is a putative serine/threonine kinase that localizes to the host nucleus after injection into the cytoplasm [41], it is unlikely to directly tyrosine phosphorylate Stat3, which is normally mediated by host Janus kinase (Jak) molecules associated with cytokine receptor signaling. Whether ROP16 phosphorylates Stat3 serine residues, a nuclear event required for maximal transcriptional activity [44], was not tested. Whatever the effect of ROP16 on the Jak-Stat pathway in *Toxoplasma* infected cells, the demonstration of nuclear targeting of a parasite kinase and its apparent association with immune response traits provides for the first time a molecular link between the parasite and the signal transduction apparatus of the host cell.

A second rhoptry kinase, ROP18, was similarly identified through QTL mapping [36,37]. Shortly after invasion, this protein is found in vesicles within the cytoplasm and associated with the parasitophorous vacuole membrane. ROP18 is an active kinase and phosphorylates at least two parasite proteins in vitro [33]. Most interestingly, when parasites were engineered to over-express ROP18, proliferation increased, and this increase was lost when parasites over-expressed a kinase-dead transgene [33]. Endogenous substrates for ROP18, whether at the parasitophorous vacuole membrane or within the host cytoplasm, are unknown at this point. It is tempting to speculate that ROP18 plays a role in remodeling and maintenance of the parasitophorous vacuole membrane, as do kinases within the endosomal contiuum [45].

Toxoplasma rhoptries also contain a metal-dependent serine-threonine phosphatase that is released upon invasion. This protein exhibits characteristics of thea PP2C family of phosphatases and has been designated PP2C-hn due to its appearance in the host nucleus shortly after invasion [46]. Endogenous substrates have not been determined for this enzyme, and while its disruption leads to a minor proliferative defect in vitro, PP2C-hn deficient parasites do not show attenuated virulence in vivo.

There is also evidence that the Type I *T. gondii* strain RH expresses a kinase activity at the parasitophorous vacuole membrane that phosphorylates host IκBα, thereby activating the NFκB cascade [47,48]. In turn, it is hypothesized that NFκB activation is involved in induction of anti-apoptotic genes that induce resistance to programmed cell death in infected cells [49]. However, we and others have found that infection with RH strain parasites does not result in NFκB activation [50-52]. The reasons for the discrepancy are unclear, but could

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possibly relate to host cell type, or possibly emergence of parasite substrains with altered in vitro activity during culture over long periods. Regardless, these results bear an interesting parallel to the apicomplexan *Theileria parva*. This parasite, which replicates in the cytoplasm of lymphocytes and induces a state of uncontrolled proliferation, has been shown to recruit the host IκB kinase complex to the parasite surface, resulting in constitutive NFκB activation [53,54]. The activation of NFκB, in turn, is associated with lymphoproliferation of infected cells [55].

The identification of parasite proteins that target host cell signaling is an exciting area that is still in its infancy. *Toxoplasma* and probably other protozoans are likely to use kinases and phosphatases to interfere with host signal transduction. Many bacterial species possess Type III secretion systems to inject virulence factors that interfere with host cell signaling [56]. *T. gondii* and protozoans in general have no such system. Thus, how these parasites subvert host signal transduction may reveal unique insights into the host-microbe interaction.

NEUTRALIZATION OF IFN-γ-INDUCED RESPONSES

Although there is not yet a mechanical model of how *Toxoplasma* proteins affect host kinase molecules, there are numerous studies that document abnormal signal transduction in infected cells. Disruption of IFN-γ/Stat1 signaling is prominent in this regard. It has long been recognized that IFN-γ is a major mediator of resistance during mouse infection with *Toxoplasma* [57-60]. The cytokine, produced by T lymphocytes and natural killer cells, is believed to act primarily through its ability to activate macrophage microbicidal activity [10,13,58,61-64]. However, the requirement for IFN-γ in protection is counter-balanced by the need to avoid inflammation-associated pathology. The uniform lethality of virulent Type I parasite strains has been associated with overinduction of proinflammatory cytokines [65,66], and mice lacking the down-regulatory cytokine IL-10 succumb to infection-induced proinflammatory cytokine overproduction [22,24]. Therefore, it is clear that production of IFN-γ and other proinflammatory cytokines must be closely regulated to provide the appropriate balance between protection and immunopathology. *Toxoplasma* induces IFN-γ, an event necessary for host survival and establishment of latency. However, in many cell types, including macrophages, the parasite interferes with signaling initiated by IFN-γ, rendering host cells nonresponsive to this cytokine.

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In the canonical IFN-γ signaling pathway (Fig. 1), the cytokine binds to a two-subunit receptor (IFNGR1 and IFNGR2) that is widely expressed on diverse cell types [67,68]. IFN-γ-induced receptor oligomerization results in tyrosine phosphorylation of receptor-associated Jak1 and Jak2. In turn, the Jak molecules phosphorylate the receptor itself, creating a docking site for Stat1. Jak-mediated phosphorylation on Tyr^{701} results in dimerization and nuclear translocation of Stat1. The Stat1 molecule binds to γ-activated sequence (GAS) elements in the promoter regions of IFN-γ-inducible genes, resulting in transcriptional activation. Maximal Stat1 activity also requires Ser^{727} phosphorylation that involves phosphatidylinositol 3-kinase and protein kinase B (Akt). The Jak-Stat pathway is subject to negative regulation at several points [69]. The nuclear and cytoplasmic phosphatases including SHP1, SHP2 and PTP1B can de-activate Jak and Stat molecules. The suppressors of cytokine synthesis (SOCS) protein family also plays a role in negative regulation of Stat signaling by binding to tyrosine-phosphorylated Jak molecules. Signaling through Jak and possibly Stat molecules can be regulated by ubiquitination-dependent proteasome-mediated degradation.

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The Stat1 signaling cascade is clearly important in host resistance to *T. gondii* [70,71], a predictable result based upon early studies on the importance of IFN-γ in immunity to the parasite. Yet, several studies make it clear that cells pre-infected with *T. gondii* are resistant

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to IFN-γ-mediated activation. The parasite down-regulates IFN-γ-induced MHC class II expression in mouse macrophages as well as in human glioblastoma cells and rat primary astrocytes and microglia [72,73]. These effects are associated with negative regulation of the MHC class II transactivator CIITA, a downstream target of Stat1 signaling [27,74]. In turn, this results in decreased antigen-presentation function in infected bone marrow-derived macrophages [75]. *Toxoplasma* also down-modulates expression of IFN-γ inducible nitric oxide synthase, the enzyme responsible for high-level production of the toxoplasmacidal molecule nitric oxide [76-78]. Recently, a genome-wide microarray analysis of infected fibroblasts revealed that over 100 IFN-γ responsive genes were down-modulated following IFN-γ treatment [79]. Thus, *T. gondii* seems to extremely effectively silence the IFN-γ/Stat1 signaling cascade. Although *Toxoplasma* strain type is now known to influence responses in the innate immune system, down regulation of Stat1 signaling appears to occur in a parasite strain-independent manner [79]. The ability to shut down Stat1 signal transduction, a phenomenon that has been shown to occur in multiple cell types and across several host species, may be an important factor in the parasite's ability to persist in the presence of a vigorous Th1 response in vivo.

While it is apparent that *T. gondii* inhibits IFN-γ-initiated signaling, how the parasite achieves this effect is less clear (Fig. 1). Early studies using mouse bone marrow-derived macrophages suggested that the parasite blocks nuclear accumulation of Stat1, even though tyrosine phosphorylation was normal during IFN-γ stimulation [75]. Circumstantial support for a block in nuclear translocation came from other data suggesting similar effects on NFκB translocation (described below) [50,80]. However, more recent studies have found normal translocation of Stat1 during IFN-γ activation of the monocyte/macrophage RAW264.7 cell line [74]. Likewise, Stat1 activation and translocation were found to be largely normal during IFN-γ stimulation of infected human fibroblasts, although there may be partial effects on dephosphorylation of nuclear Stat1 [79]. Another recent study provided evidence that *Toxoplasma* inhibits Stat1 signaling in RAW264.7 cells through induction of the negative regulator SOCS-1 [78]. In this study, infection resulted in a decrease in both tyrosine-phosphorylated and total Stat1 levels. Therefore, there is currently no consensus view on whether Stat1 signaling is blocked by proteolytic Stat1 cleavage, phosphatase dependent Stat1 deactivation or prevention of nuclear translocation, or possibly a combination of factors that might depend upon parasite strain and cell type (Fig. 1).

Other protozoa also target IFN-γ-triggered signaling. For example, infection of macrophages and dendritic cells with *Leishmania* blocks the Stat1 signaling pathway, and exposure to *Trypanosoma brucei rhodesiense* glycosylinositolphosphate soluble variant surface glycoprotein blocks IFN-γ-mediated Stat1 phosphorylation [81,82]. For *Leishmania donovani*, there is evidence that promastigotes dysregulate IFN-γ signaling by interfering with Jak1, Jak2 and Stat1 phosphorylation [83]. This appears to be in part due to induction of the host protein phosphatase SHP-1 [84]. Infection of dendritic cells with *L. amazonensis* amastigotes also causes decreases in Stat1 phosphorylation [85]. In addition, there is evidence that *L. donovani* infection stimulates host proteosome-mediated Stat1 degradation [86]. Other data suggest that *L. major* and *L. mexicana* modulate surface expression of IFNGR1 and IFNGR2, and that *L. mexicana* preferentially activates Stat1β, a dominant negative regulator of Stat1 signaling [87]. Thus, as a general principal, protozoan survival within macrophages and related cells may require that the host cell be nonresponsive to proinflammatory effects of IFN-γ.

INHIBITION OF TLR-TRIGGERED MACROPHAGE ACTIVATION

Toll-Like Receptors (TLR) play an essential role in sensing and responding to pathogens by the innate immune system [88,89]. Signaling through TLR leads to induction of

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proinflammatory cytokines and chemokines, as well as anti-microbial molecules such as inducible nitric oxide synthase. To date, 11−13 TLR molecules have been identified in mammals. Most are expressed on the cell surface, but some are expressed in intracellular compartments (TLR3, 7, 8 and 9). One of the best characterized is TLR4, which, along with MD2, serves as a receptor for Gram-negative bacterial lipopolysaccharide (LPS). With the exception of TLR3, activation through these receptors requires recruitment of adaptor molecule MyD88, in turn resulting in recruitment of IL-1 receptor-associated kinases (IRAK) 1 and 4 (Fig. 2). These molecules form a complex with TNF receptor-associated factor (TRAF)6, resulting in interaction with Uva1 and Ubc13, leading in turn to ubiquitination of TRAF6. Then, ubiquitinated TRAF6 activates transforming growth factor-β-activated kinase (TAK)-1. Interacting with TAK1-binding proteins (TAB)-1 and -2, TAK1 serves as a mitogen-activated kinase (MAPK) kinase kinase, triggering the MAPK cascade (Fig. 2). The TAK1 molecule also activates the IκB kinase complex. This results in phosphorylation-dependent ubiquitination and degradation of IκBα, enabling nuclear translocation of NFκB.

After invasion of macrophages, the *Toxoplasma* Type 1 strain RH actively down-regulates a large panel of proinflammatory cytokines and chemokines that are normally induced by TLR signaling [50,90]. We observed in particular that LPS-triggered IL-12p40 and TNF-α are suppressed in infected cells. While the parasite itself eventually initiates IL-12 synthesis, TLR4-triggered production of TNF-α remains potently suppressed. The ability of *T. gondii* to block LPS triggered responses requires active invasion. Thus, heat inactivated tachyzoites do not display suppressive activity, and when parasite entry is prevented by cytochalasin D blockade of actin polymerization, suppressive activity is also lost [91]. The suppressive activity of *Toxoplasma* on LPS-induced TNF-α requires parasite survival within the host cell. This is because drug-induced tachyzoite inactivation after invasion restores the ability of cells to respond to TLR4 triggering [91]. We also recently found that signaling through other TLR is blocked during infection [92]. Importantly, this includes TLR3, an intracellular receptor for double-stranded RNA that, unlike other TLR, signals in a manner independent of MyD88 (Fig. 3). Suppression of TLR signaling does not appear to be restricted to RH strain tachyzoites because other Type 1, as well as Type 2, strain parasites also blocks LPS induction of TNF-α [92].

The block in TLR signaling has also been reported to occur in bone marrow-derived dendritic cells [93]. In this case, infected immature dendritic cells fail to mature in response to LPS triggering, and the cells were deficient in their ability to activate T cells. During in vivo infection, we also obtained evidence that *Toxoplasma* blocks cytokine production in infected cells. Infected macrophages collected from the peritoneal cavity following i. p. parasite inoculation are suppressed in their ability to produce TNF-α, and infected dendritic cells in the spleen are defective in IL-12 production [14,92]. Thus, suppression of TLR signaling by *T. gondii* appears to be a general phenomenon that is not parasite strain restricted and that occurs in several cell types.

Deactivation of TLR signaling by *Toxoplasma* may indicate the need for the parasite to avoid triggering these pathways by the parasite's own TLR ligands. Thus, the *Toxoplasma* profilin molecule TGPRF activates TLR11 and parasite glycosylphosphoinositol (GPI) moieties associated with tachyzoite surface proteins possess the ability to activate TLR2 and TLR4 [5,6]. Since both profilin and GPI synthesis are essential for survival [94,95], the parasite may be under evolutionary pressure to block TLR signaling during intracellular infection. In this regard, the immunodominant $CD4^+$ T cell response to TGPRF characterized by Yarovinsky and colleagues [96] might result from recognition of this TLR11 ligand by noninfected antigen presenting cells. It is also possible that blocking TLR signaling is a means to prevent proinflammatory responses that would otherwise be

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triggered by exposure to gut flora now known to occur during oral *T. gondii* infection [97,98]. Another possibility is that inability to respond to TLR ligands reflects a general nonresponsiveness of cells to proinflammatory signals no matter what the initiating stimulus, rather than being specific for TLR pathways.

There is evidence that *T. gondii* inhibits TLR signaling through both NFκB and MAPK pathways. During early infection of macrophages, the parasite prevents accumulation of nuclear NFκB in response to LPS [50,80]. This may be a consequence of failure to retain this transcription factor in the nucleus, rather than a block in nuclear import [52]. The defect in NFκB nuclear accumulation is not permanent, because cells translocate this transcription factor in response to LPS when stimulation is performed 6 hr or more after infection [99] (Fig. 2). Although we and others do not see RH-induced NFκB nuclear translocation, others have observed activation of this transcription factor during infection. There is also evidence that Type 2 *T. gondii* strains themselves induce NFκB activation, and indeed, IL-12 production induced by the Type 2 ME49 strain is partially dependent upon MyD88 [7,51]. Thus, while there appears to be defects in NFκB activity during early infection, the extent to which this contributes to suppression of cytokines such as IL-12 and TNF-α is unclear.

Toxoplasma mediates a rapid but transient activation of MAPK pathways including SAPK/JNK, p38 and ERK1/2 during macrophage infection [100]. However, subsequent stimulation with LPS fails to result in robust activation normally associated with TLR stimulation [99] (Fig. 2). Whether *Toxoplasma* blocks activation of MAPK through the activity of host or parasite phosphatases, or by other phosphatase-independent mechanisms is not clear. Failure of infected macrophages to respond to LPS restimulation in some ways resembles LPS tolerance. This raises the possibility that *T. gondii* induced nonresponsiveness is an endotoxin tolerance phenomenon. Yet, based on several criteria the processes appear to be distinct. Parasite infection induces sustained activation of the MAPK kinase MKK3/6, but, during LPS stimulation of LPS tolerized cells, phosphorylation of MKK3/6 is defective [99]. In addition, IκBα is resistant to TLR4-induced degradation in LPS tolerized macrophages, whereas in *T. gondii* infected cells this molecule undergoes degradation following LPS exposure.

There is evidence that other protozoans target NFκB and MAPK pathways. For example, data suggest that *Leishmania* downregulates proinflammatory signaling through induction of SHP-1, a phosphatase that plays a role in deactivation key components of both Jak/Stat and TLR pathways [101,102]. Recently, it has been reported that *Leishmania* proteases cleave kinases involved in p38 MAPK and NFκB activation [103,104]. Infection with *T. cruzi* is also reported to induce macrophage TLR nonresponsiveness through induction of host cell phosphatase activity [105]. Taken together, while it is clear that infection with *Toxoplasma* and other protozoans interferes with the ability to respond to TLR ligands, there is not yet a definitive picture of how this is accomplished in any case.

TARGETTING CHROMATIN: A POSSIBLE UNIFYING MECHANISM OF SUPPRESSION BY TOXOPLASMA

Inducible gene expression is now understood to involve two types of regulatory cascades. One type of transduction cascade leads to transcription factor activation, most often involving kinase signaling, to enable binding to target DNA sites on gene promoters. The particular pattern of transcription factors activated plays a role in determining the specificity of genes induced. At the same time, it is now appreciated that chromatin structure itself is subject to regulation, inasmuch as signaling leading to covalent modification of histones plays a role in determining the activity of transcription factors [106].

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T. gondii suppresses TNF-α production in bone marrow-derived macrophages, and this is associated with decreased recruitment of RNA polymerase II to the promoter [92]. This finding links suppression of TNF-α release to decreased transcription rather than downstream effects such as diminished mRNA stability, altered RNA splicing or decreased translation. In a close examination of the TNF promoter during TLR4 stimulation of *T. gondii* infected macrophages, we concluded that the parasite targets chromatin modification rather than transcription factor activation [92]. In 12-hr infected macrophages, subsequent LPS-triggered NFκB nuclear translocation was unaffected by the parasite. In addition, activation and nuclear translocation of c-Jun and CREB, two other TNF-associated transcription factors, appeared largely normal in infected cells. However, NFκB as well as c-Jun and CREB were unable to bind to the TNF promoter. This is explained by the ability of the parasite to inhibit Ser^{10} phosphorylation and $Lys^{9/14}$ acetylation of histone H3 at the TNF promoter, modifications associated with increased transcriptional activity [107]. Thus, evidence indicates that chromatin structure surrounding the TNF promoter remains in a closed state in infected cells, and the transcription factors therefore cannot gain access to their binding sites. In turn, RNA polymerase II is not recruited to the TNF promoter and transcription is not initiated [92]. Because *Toxoplasma* does not affect low level IL-10 production triggered by TLR4, we examined histone H3 modication at the promoter for the gene encoding IL-10. In this case, LPS itself did not induce histone H3 modification, and the parasite therefore had no effect at the promoter for IL-10. Yet, when IL-10 was super-induced with the combination of immune complex and LPS [108,109], histone H3 phosphorylation and acetylation occurred, and *Toxoplasma* simultaneously blocked chromatin modification and high level IL-10 production (Leng and Denkers, manuscript in preparation).

Given these results, it is tempting to speculate that targeting chromatin modification in the host cell provides an explanation for the ability of *Toxoplasma* to simultaneously downregulate a large panel of proinflammatory genes [90]. Rather than targeting specific transcription factors (which conceptually would seem to be a relatively inefficient way to shut down multiple genes), the parasite may achieve the same effect by blocking inducible modification of H3 and possibly other histone molecules (Fig. 4). Such an inhibitory mechanism would also account for the ability of *Toxoplasma* to down-modulate signaling though all TLR, and possibly also through the IFN-γ receptor.

How *Toxoplasma* influences host histone modification is unclear, but as related above, the host cell nucleus is emerging as a target for parasite effector proteins. Whether the putative kinase ROP16 or the phosphatase PPC2-hn, parasite proteins that traffick to the host cell nucleus [35], influence histone modification either directly or indirectly remains to be determined (Fig. 4). It is also possible that other parasite kinases or phosphatases may be delivered into the host nucleus to actively interfere with host gene transcription by targeting chromatin remodeling.

While *Toxoplasma* is the only intracellular protozoan identified to date that is capable of interfering with host cell chromatin modification, it is possible that other parasitic protozoans possess similar properties. In this regard, a growing number of bacterial pathogens have recently been reported to interfere with host chromatin remodeling required for gene expression [110]. *Shigella flexneri* injects OspF, a dually specific serine-threonine phosphatase, into epithelial cells followed by relocalization of the protein to the host nucleus [111]. Here, OspF blocks histone H3 Ser^{10} phosphorylation, thereby preventing induction of a subset of NFκB-responsive genes including IL-8. The OspF molecule appears to act by dephosphorylating ERK and p38, MAPK that lie upstream of histone H3 phosphorylation. Studies with *OspF* null *Shigella* suggest that lack of this protein results in early IL-8-dependent neutrophil recruitment in vivo, resulting in attenuation of infection [111].

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Listeria monocytogenes, another well-known bacterial pathogen, was also recently found to dephosphorylate histone H3 on Ser^{10} and deacetylate histone H4 during early infection [112]. This relates to a decreased level of transcription of a subset of host genes, including several key immune-related genes. The *Listeria* virulence factor, listeriolysin O (LLO), was identified as the major protein required for dephosphorylation of histone H3 and deacetylation of histone H4. Interestingly, other toxins of the same family such as *Clostridium perfringens* perfringolysin and *Streptococcus pneumoniae* pneumolysin have also been found to dephosphorylate histone H3 at Ser^{10} [112].

Other evidence for pathogen interference with chromatin structure comes from studies on *Mycobacterium tuberculosis*. This opportunistic pathogen inhibits IFN-γ-induced MHC class II gene expression by upregulating histone deacetylation at the HLA-DR promoter region in human THP-1 monocyte/macrophage cells. The activity is mediated by increased expression of Sin3A, a corepressor required for MHC class II repression by histone deacetylase [113]. A related study showed that a 19-kDa lipoprotein from mycobacteria, LpaH, inhibited acetylation of histone H3 and H4 at the CIITA promoter. LpqH also blocked recruitment of Brahma-related gene 1, a chromatin remodeling protein, to the promoter [114]. The effect of LpqH is believed to be mediated through TLR2-induced p38 or ERK MAPK signaling.

More recently, a virus protein encoded by white spot syndrome virus, ICP11, has been identified as a histone-binding DNA mimic that disrupts host cell nucleosome assembly [115]. ICP11 is believed to compete with host DNA for histone proteins, in particular the H2A variant H2A.x, which functions in repairing DNA double-strand breaks. Host cell chromatin is therefore vulnerable to DNA damage, leading to disruption of the nuclear transcription machinery. This may facilitate the host cell death caused by infection, promoting transmission of the virus.

Based upon these examples and our findings in *Toxoplasma*, there are strong suggestions that the host cell chromatin modification machinery represents an Achille's heel for pathogen exploitation. Interfering with inducible histone modification potentially allows the pathogen to simultaneously down-modulate a large panel of host genes using a common mechanism. We do not yet know precisely how *Toxoplasma* - and possibly other intracellular protozoans - achieve this aim. However, studies with bacterial and viral pathogens may provide us with clues as to how *T. gondii* interferes with host chromatin modification during intracellular infection.

CONCLUSIONS AND FUTURE PERSPECTIVES

Toxoplasma was once regarded as a purely as a prototypic proinflammatory pathogen. We now have a more nuanced view of this pathogen's interaction with its host. In addition to expression of molecules involved in triggering immunity, it is clear that *T. gondii* produces molecules that actively target proinflammatory signaling pathways for down-modulation. While studies to date have focused on specific signal transduction cascades and individual transcription factors, there is now evidence that the parasite may achieve down-modulation of proinflammatory cytokines by targeting a common mechanisms of gene regulation, namely histone modification. It is not yet clear whether ROP16-Stat3 signaling may impact histone modification, or whether interference with MAPK signaling might alter histone modification. Thus, precisely how such parasite effector molecules interfere with host signal transduction is an important area for future discovery. The ability to perform forward and reverse genetics on both the murine host and the parasite itself provides the valuable opportunity to dissect this host-parasite interaction and to determine its relevance to the biology of infection.

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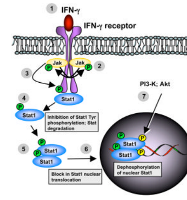

Figure 1.
The Stat1 signaling pathway and modulation by *T. gondii*. 1, IFN-γ binding causes receptor dimerization. 2, This causes receptor associated Jak molecules to tyrosine phosphorylate each other, leading in turn to recruitment and tyrosine phosphorylation of Stat1 (step 3). Activated Stat1 dissociates from the receptor complex and dimerizes in the cytosol (step 5). Stat1 dimers translocate to the nucleus (step 6) where they bind to IFN-γ responsive promoter elements. Full Stat1 transcriptional activity requires serine phosphorylation dependent upon phosphatidylinositiol 3-kinase (PI3-K) and protein kinase B/Akt (step 7). Multiple studies suggest that *Toxoplasma* interferes with Stat1 signaling, but the control points are less clear. There is evidence that *T. gondii* prevents Stat1 phosphorylation and causes its degradation. Other data suggest that the parasite prevents Stat1 nuclear translocation. Finally, some studies suggest that Stat1 is subject to dephosphorylation following nuclear translocation.

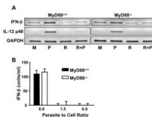

Figure 2.

The Toll-like receptor signaling cascade: Impact of *Toxoplasma* infection. In a generalized pathway, TLR binding to its ligand (step 1) results in recruitment of the MyD88 adaptor molecule (step 2). In turn, this mediates recruitment of IRAK1 and IRAK4, which form a complex with TRAF6 (step 3). In step 4, the TRAF6 molecule interacts with Uva1 and Ubc13, triggering ubiquitination of TRAF6. This stimulates activation of TAK1 which is associated with TAB1 and TAB2 (step 5). Activated TAK1 possesses MAPK kinase kinase activity, triggering MAPK activation (step 6). The TAK1 molecule also activates the IKK complex (step 7) leading in turn to degradation of IκB and nuclear translocation of NFκB p50:p65. *Toxplasma* blocks most, but not all TLR-induced cytokine responses. There is evidence that the parasite blocks nuclear NFκB translocation, although this is a temporary effect. There are also data to suggest that the parasite interferes with TLR-induced MAPK activation.

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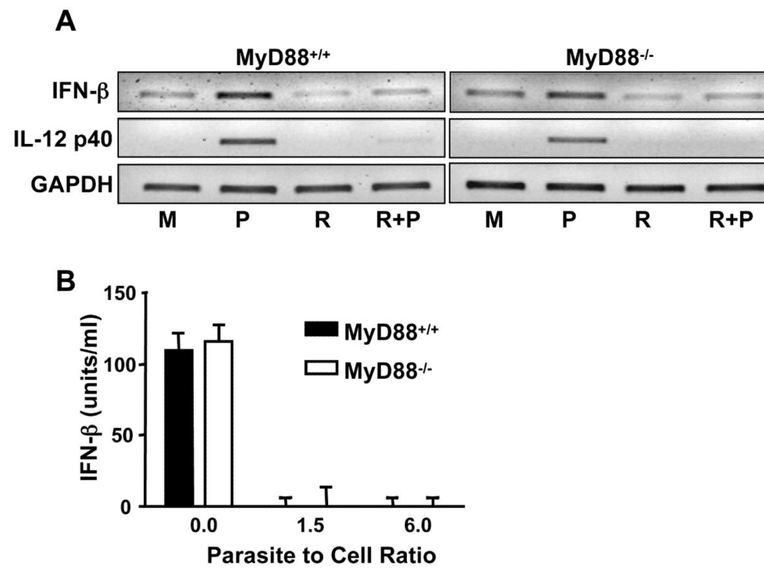

Figure 3.

T. gondii down-regulates MyD88-independent signaling mediated by poly I:C/TLR3. A, bone marrow-derived MyD88$^{+/+}$ and MyD88$^{-/-}$ macrophages were infected, subjected to 2 hr poly I:C stimulation, then cells were harvested and RNA prepared for RT-PCR analysis of IFN-γ, IL-12p40 and GAPDH. M, cells cultured in medium for the entire course of the experiment; P, uninfected cells stimulated with poly I:C; R, cells infected with RH strain tachyzoites, no subsequent poly I:C stimulation; R+P, cells infected with *Toxoplasma* RH strain, then 6 hr later subjected to poly I:C stimulation (2 hr). B, wild-type and MyD88$^{-/-}$ bone marrow-derived macrophages were infected with RH strain tachyzoites, then 6 hr later subjected to poly I:C stimulation. Supernatants were collected for IFN-γ ELISA 6 hr later.

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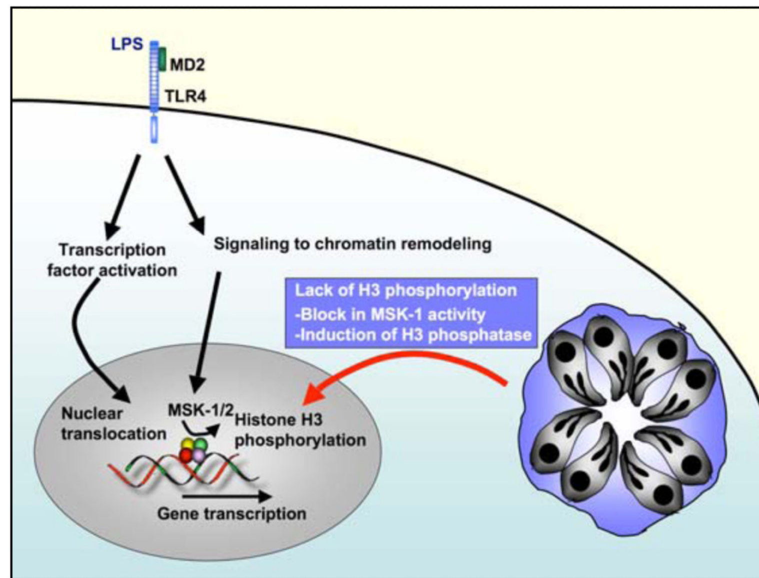

Figure 4.

Targetting histone modification as a possible unifying mechanism of suppression. Macrophage activation through receptors such as TLR4 induces production of multiple proinflammatory cytokines, but two general information pathways are needed. Activation of transcription factors and binding to promoter elements is required to activate specific genes. However, prior to binding, signal-induced histone modification is needed in many cases to re-model chromatin into an open configuration that enables transcription factors to act at target promoter regions. There is evidence that *T. gondii* targets the chromatin remodeling pathway, offering an explanation for the parasite's ability to simultaneously down-regulate a large panel of proinflammatory cytokines and chemokines. In particular, there is a block in TLR4-induced histone H3 phosphorylation. Whether this is because upstream histone H3 kinases such as MSK-1 are deactivated, or whether the parasite induces histone H3-directed phosphatase activity is not known at present.