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Toxoplasma gondii Triggers Myeloid Differentiation Factor 88-Dependent IL-12 and Chemokine Ligand 2 (Monocyte Chemoattractant Protein 1) Responses Using Distinct Parasite Molecules and Host Receptors

Laura Del Rio,* Barbara A. Butcher,* Soumaya Bennouna,* Sara Hieny,† Alan Sher,‡ and Eric Y. Denkers²*

Toll-like receptors (TLR) that signal through the common adaptor molecule myeloid differentiation factor 88 (MyD88) are essential in proinflammatory cytokine responses to many microbial pathogens. In this study we report that Toxoplasma gondii triggers neutrophil IL-12 and chemokine ligand 2 (CCL2; monocyte chemoattractant protein 1) production in strict dependence upon functional MyD88. Nevertheless, the responses are distinct. Although we identify TLR2 as the receptor triggering CCL2 production, parasite-induced IL-12 release did not involve this TLR. The production of both IL-12 and CCL2 was increased after neutrophil activation with IFN-γ. However, the synergistic effect of IFN-γ on IL-12, but not CCL2, was dependent upon Stat1 signal transduction. Although IL-10 was a potent down-regulator of Toxoplasma-triggered neutrophil IL-12 release, the cytokine had no effect on parasite-induced CCL2 production. Soluble tachyzoite Ag fractionation demonstrated that CCL2- and IL-12-inducing activities are biochemically distinct. Importantly, Toxoplasma cyclophilin-18, a molecule previously shown to induce dendritic cell IL-12, was not involved in neutrophil IL-12 production. Our results show for the first time that T. gondii possesses multiple molecules triggering distinct MyD88-dependent signaling cascades, that these pathways are independently regulated, and that they lead to distinct profiles of cytokine production. The Journal of Immunology, 2004, 172: 6954–6960.

Immunity to the intracellular protozoan Toxoplasma gondii consists of high level production of type 1 cytokines, and both IL-12 and IFN-γ are essential for resistance to this opportunistic pathogen (1–5). In recent years, innate immunity has emerged as a key element shaping the strength and character of the acquired immune response that is necessary to survive infection. Toxoplasma provides a highly potent stimulus for IL-12 production, which, in turn, is required for Th1 response ignition. Innate immune cells, such as polymorphonuclear neutrophils (PMN), dendritic cells (DC), and macrophages, are important sources of IL-12 during T. gondii infection (6–9). Molecular definition of parasite factors and host receptors, elucidation of responses triggered by such receptor-ligand interactions, and determination of how the responses are regulated remain high priority areas of investigation.

Toll-like receptors (TLR) are a family of evolutionarily conserved transmembrane molecules that recognize specific molecular patterns associated with microbes. There are 10 TLR family members that together recognize a diverse collection of pathogen-associated molecular patterns. Recognition by TLR initiates signaling pathways through the common adaptor molecule myeloid differentiation factor 88 (MyD88), leading to activation of NF-κB transcription factors and members of the mitogen-activated protein kinase family (10–12). The finding that MyD88−/− mice are acutely susceptible to T. gondii infection implicates TLR in innate immune recognition of this parasite (13). Furthermore, TLR2−/− mice have recently been shown to display higher susceptibility to T. gondii infection than wild-type (WT) animals (14). Nevertheless, increased mortality during infection of TLR2−/− mice occurs only at an extremely high parasite dose. In contrast, MyD88-deficient animals display extreme susceptibility to infection that is identical with the phenotype of IL-12−/− and IFN-γ−/− mice (13). These results indirectly implicate additional TLR and multiple TLR ligands in the innate immune response to T. gondii.

Here we focus on neutrophil responses to T. gondii. These cells produce cytokines such as IL-12 and TNF-α, and chemokines such as chemokine ligand 2 (CCL2), CCL3, CCL4, and CCL20 in response to Toxoplasma and other stimuli (15–18). Neutrophils also respond to T. gondii by producing potent DC chemoattractants and activation stimuli, suggesting a role for these cells in instructing early immune activation (16). In addition, evidence supports a role for PMN in Th1 generation and resistance to infection with Toxoplasma and other microbial pathogens (19–24).

We now report that MyD88-dependent neutrophil production of IL-12 and CCL2 (monocyte chemoattractant protein 1) is triggered...
by distinct biochemical fractions derived from the parasite. In addition, the production of CCL2, but not IL-12, is dependent upon TLR2 signaling. Importantly, neutrophil IL-12 production is not dependent upon parasite cyclophilin-18 (C-18), a protein recently shown to trigger DC IL-12 through CCR5 ligation (25). Our results are the first to show that *T. gondii* possesses multiple molecules that trigger MyD88-dependent signaling cascades. Moreover, our data reveal a hitherto unknown underlying complexity to MyD88-dependent signaling, in that multiple pathways leading to distinct outcomes can be triggered dependent upon upstream receptor-ligand interactions.

### Materials and Methods

#### Mice

C57BL/6, C3H/HeJ, and C3H/HeOuJ strain mice were obtained from The Jackson Laboratory (Bar Harbor, ME). Breeding pairs of MyD88−/− and TLR2−/− mice on a partially backcrossed 129/Ola background were provided by Dr. S. Akira (Osaka University, Osaka, Japan) via Dr. D. Golenbock (University of Massachusetts Medical School, Worcester, MA). C57Bl/6/Ola×C57BL/6 F, animals were obtained from The Jackson Laboratory. Breeding pairs of 129Sv/Ev-StatomH/2H2 strain (Stat1−/−) were purchased from Taconic Farms (Germantown, NY), and a breeding colony was established in the Transgenic Mouse Facility of Cornell University College of Veterinary Medicine. WT mice of the 129Sv/Ev strain were purchased from Taconic Farms. Female mice between 5 and 12 wk of age were used for experiments. Animals were housed under specific pathogen-free conditions at the Cornell University College of Veterinary Medicine animal facility, which accredited by the American Association for Accreditation of Laboratory Animal Care.

#### Ag preparation

Tachyzoites of the RH strain were maintained in human fibroblast monolayers by biweekly passage, as previously described (26). To prepare soluble tachyzoite Ag (STAg), tachyzoites were sonicated in the presence of a protease inhibitor mixture consisting of 0.2 mM PMPS (Sigma-Aldrich, St. Louis, MO), 0.2 μM aprotinin (Roche, Indianapolis, IN), 1 μM leupeptin (Roche), and 1 mM EDTA (Sigma-Aldrich). The resulting sonicate was dialyzed into PBS and centrifuged at 10,000 × g for 1 h, then the supernatant was collected and filtered through a 0.22-μm pore size membrane (Corning Costar, Cambridge, MA). The STAg concentration was determined using a BCA protein assay according to the manufacturer’s instructions (BD PharMingen, San Diego, CA). 0.25 mL of the diluted sonicate was added to 1 mL of DMEM supplemented with 10% FCS (HyClone, Logan, UT), 1 mM sodium pyruvate, 0.1 mM nonessential amino acids, 30 mM HEPES, 100 U/ml penicillin, 0.1 mg/ml streptomycin (Life Technologies, Grand Island, NY), and 50 μM 2-ME (Sigma-Aldrich). Neutrophils obtained in this manner were routinely 90–95% pure and >95% viable as determined by trypan blue exclusion.

#### Bone marrow-derived macrophage preparation

Bone marrow cells were flushed from femur and tibia, then macrophages were generated in the presence of growth factors as previously described (28). Briefly, macrophages were generated over period of 4 days by culture in 30% L929 cell supernatant as a source of M-CSF. On the day of analysis, cells were washed and resuspended in cDMEM.

### Cell culture

Cells were plated at 104/well in 96-well tissue culture plates, stimulated with parasite Ag and recombinant cytokines, then incubated (37°C, 5% CO2) for 18 h before collecting supernatants. Recombinant mouse IL-10 and IFN-γ were obtained from R&D Systems (Minneapolis, MN).

#### Cytokine measurement

IL-12 p40 was detected by cytokine ELISA as described previously (9). CCL2 levels were determined using a murine-specific ELISA kit, according to the manufacturer’s instructions (BD PharMingen, San Diego, CA). The ELISA detection sensitivities were 31.2 and 15.6 pg/ml for IL-12 p40 and CCL2, respectively.

#### Biochemical fractionation

STAg was fractionated by stepwise precipitation with increasing amounts of (NH4)2SO4. The fractions were dialyzed into 0.025 mM Tris buffer, pH 7, and filtered through a 0.2-μm pore size membrane, and protein content was measured by BCA assay.

For anion exchange chromatography, a UnoQ column connected to a Biologic Chromatography System apparatus (Bio-Rad, Hercules, CA) was equilibrated with 0.025 mM Tris buffer (Sigma-Aldrich), pH 7.0. After loading parasite protein extracts, the column was eluted (2 ml/min) with a 36-ml linear NaCl gradient (0–1 M) in 0.025 mM Tris buffer, pH 7.0. Fractions were desalted, and protein was concentrated by centrifugation through a nanoporous Amicon membrane (10 kDa pore; Millipore, Billerica, MA) for 15 min at 3700 × g. The protein concentration was determined by BCA assay, and samples were stored at −70°C until use.

#### Gel electrophoresis and silver staining

Proteins were resolved by SDS-PAGE under reducing conditions as described previously (26). For silver staining, gels were fixed in 6% formaldehyde and 26% ethanol for 1 h, and then washed with water overnight. After incubation in DTT (5 μg/ml, 30 min), gels were incubated for 30 min in 0.1% AgNO3. After briefly rinsing in H2O, gels were developed in 3% NaCO3 with 0.02% formaldehyde. The reaction was stopped with 0.1 M citric acid.

#### Immunoblotting

Protein fractions were separated by reducing SDS-PAGE and subsequently electrotransferred onto nitrocellulose membrane (Schleicher & Schuell, Keene, NH). Membranes were blocked in 5% nonfat dry milk (Nestle USA, Solon, OH) in TBST for 60 min at room temperature. Detection of *Toxoplasma* C-18 was accomplished using a rabbit polyclonal anti-C-18 antiserum (25). We also found up-regulation of cyclophilin-18 (C-18), in 5% nonfat dry milk (Nestle USA, Solon, OH) in TBST for 60 min at room temperature. Detection of *Toxoplasma* C-18 was accomplished using a rabbit polyclonal anti-C-18 antiserum (25). After several washes with TBST, Ab binding was detected using an HRP-conjugated anti-rabbit Ab (Cell Signaling Technology, Beverly, MA) in TBST containing 5% nonfat dry milk. After washing in TBST, bands were visualized using ECL detection, following the manufacturer’s instructions (LumiGLO; Upstate Biotechnology, Lake Placid, NY).

#### Statistical analysis

Significance of differences between groups was determined by Student’s *t* test or the F test. A value of *p* < 0.05 was considered significant.

### Results

#### T. gondii triggers neutrophil IL-12 and CCL2 production

IL-12 is well known as a central cytokine in resistance to *T. gondii* (4). As shown previously (8, 9) and in Fig. 1A, neutrophils release high levels of this cytokine during parasite Ag stimulation. The chemokine CCL2 (monocyte chemoattractant protein 1) is an important mediator of macrophage recruitment (29–31), and we now show that neutrophils produce this chemokine in response to *T. gondii* stimulation (Fig. 1B). We also found up-regulation of IL-12p40 and CCL2 mRNA within 2–4 h after STAg stimulation (data not shown).

We next compared regulation of CCL2 vs IL-12 production. Interestingly, exogenous IFN-γ displayed different effects on STAg-triggered IL-12 and CCL2 release. In the presence of suboptimal amounts of STAg (1 μg/ml), IFN-γ increased IL-12 release in a dose-dependent manner, whereas the cytokine itself was unable to induce IL-12 (Fig. 1C). In contrast, IFN-γ alone was a potent CCL2 stimulus and when added together with STAg acted synergistically to promote CCL2 production (Fig. 1D).

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The Journal of Immunology

6955

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The effects of IL-10 on STAg-induced CCL2 and IL-12 also differed. It was previously demonstrated that IL-10 down-regulates LPS-induced PMN IL-12 production (32), and we also found potent down-regulatory effects on STAg-induced IL-12 that was maintained even in IFN-γ-primed cells (Fig. 1E). In striking contrast, IL-10 was completely ineffective at down-modulating STAg-induced CCL2 regardless of whether IFN-γ/H9253 was present (Fig. 1F).

These findings show that parasite Ag simultaneously triggers an IL-10-sensitive pathway leading to IL-12 production and an IL-10-insensitive pathway that culminates in CCL2 release.

Effects of IFN-γ on IL-12 production are Stat1 dependent, but IFN-γ-driven CCL2 release does not require Stat1

Signaling by IFN-γ proceeds largely, but not completely, through intracellular signaling intermediate Stat1 (33, 34). Nevertheless, the involvement of Stat1 signaling in neutrophil cytokine responses has not previously been evaluated. In this study we show that IFN-γ fails to promote STAg-induced IL-12 production in Stat1−/− neutrophils (Fig. 2A). The result is consistent with a role for this signaling intermediate in mediating the synergistic effects of IFN-γ on PMN IL-12 release. In contrast, exogenous IFN-γ signaled CCL2 release even in the absence of a functional Stat1 molecule (Fig. 2B). Furthermore, the ability of IFN-γ to promote STAg-induced CCL2 release also did not require Stat1, although in this study Stat1 was needed for optimal CCL2 production. These results show that IFN-γ up-regulates STAg-induced IL-12 through a Stat1-dependent pathway, but IFN-γ can up-regulate CCL2 production in the absence of a functional Stat1 molecule.

IL-12 and CCL2 inducing activities in STAg are biochemically distinct

The lack of coregulation in T. gondii triggered IL-12 and CCL2 production suggested that distinct parasite molecules might trigger each cytokine. Therefore, we undertook biochemical fractionation experiments in which STAg was subjected to sequential precipitation with increasing amounts of ammonium sulfate. As predicted, IL-12 and CCL2-inducing activities did not cofractionate. Thus, most IL-12-inducing activity was present in the fraction precipitating under high salt concentration (Fig. 3A; 60–80% saturation). In contrast, CCL2-inducing activity precipitated under low salt conditions (Fig. 3B; 0–25% saturation). Titration of the fractions demonstrated that IL-12-inducing activity was enriched in the 60–80% fraction relative to STAg (p < 0.05) and in the 0–25% (p < 0.01) fraction over an extended range of protein concentrations (Fig. 3C). Conversely, CCL2 activity was clearly enriched in the 0–25% fraction over a wide range of Ag doses (Fig. 3D; p < 0.06). We confirmed that these fractions contained distinct sets of proteins by SDS-PAGE, followed by silver staining of proteins within the gel (Fig. 3E).

Distinct TLR/MyD88 signaling pathways are involved in T. gondii-triggered IL-12 and CCL2 production

TLR are vitally important transmembrane molecules for recognition and signaling in the innate immune system (10). We previously found that absence of the common adapter for TLR signaling, MyD88, resulted in defective IL-12 production in STAg-stimulated, bone
marrow-derived neutrophils (13). In this study we show that thiglycylate-elicited peritoneal PMN from MyD88−/− mice are also completely defective in IL-12 (Fig. 4A) and CCL2 (Fig. 4B) production during STAg stimulation.

A recent report indicating partial susceptibility of TLR2 knock-out (KO) mice to high dose T. gondii infection (14) prompted us to examine neutrophil cytokine responses in the absence of TLR2. Fig. 3A shows that TLR2−/− neutrophils are indistinguishable from WT counterparts in terms of IL-12 production (Fig. 4A). In Stat1 KO cells: *p < 0.01 vs medium alone; **p < 0.05 vs STAg alone. In WT cells: *p < 0.01 vs medium alone; **p < 0.01 vs STAg alone. These experiments were repeated three times with similar results.

Discussion

The TLR family in conjunction with the common adaptor MyD88 are key to innate immune recognition of bacterial ligands and are also emerging as critical components in response to protozoan pathogens. This study demonstrates that distinct T. gondii-derived molecules trigger MyD88-dependent IL-12 and CCL2 production using independent surface receptors. Downstream signaling pathways leading to IL-12 and CCL2 production are different, and each is subject to distinct cytokine regulation (Fig. 6).
Although neutrophils produce both IL-12 and CCL2 in response to soluble parasite extracts, addition of exogenous IFN-γ and IL-10 to PMN cultures had drastically different effects on the production of these cytokines. IFN-γ displayed synergistic activity on IL-12 and CCL2 production when added with STAg. However, although IFN-γ alone failed to induce IL-12 release, the cytokine itself was a potent stimulus of CCL2. The effects of IL-10 on STAg-induced IL-12 and CCL2 also differed. It has been previously demonstrated that IL-10 displays a potent down-regulatory activity on IL-12 production by LPS-stimulated PMN (32). In this study IL-10 was also a strong down-regulator of STAg-induced neutrophil IL-12 regardless of whether IFN-γ was present. In contrast, CCL2 production was completely unaffected by exogenous IL-10.

The Stat1 molecule is key to signaling through the IFN-γ receptor (34). Indeed, we found that this transducing molecule is required for IFN-γ-mediated up-regulation of STAg-induced IL-12 production. In contrast, IFN-γ signaled CCL2 production even in the absence of the Stat1 molecule. The latter results are in accord with a recent microarray analysis of IFN-γ-activated bone marrow-derived macrophages that showed Stat1-independent IFN-γ regulation of CCL2 gene expression (33). The different requirement of Stat1 signaling for IL-12 and CCL2 production by neutrophils supports the idea that the pathways triggering these soluble mediators are regulated in a nonidentical fashion. This hypothesis was confirmed by biochemical separation of Toxoplasma proteins, because IL-12- and CCL2-inducing activities fractionated independently.

Signaling through MyD88 is crucial for resistance during Toxoplasma infection, as shown in MyD88−/− mice that display an acute susceptibility indistinguishable from that of IL-12−/− or IFN-γ−/− mice (13, 14, 35). The susceptibility of MyD88-deficient mice is associated with severely impaired IL-12 production in Toxoplasma-activated DC, bone marrow-derived macrophages, and bone marrow-derived PMN, leading, in turn, to reduced IFN-γ production (13). In this study we show that CCL2 production is also impaired in the absence of MyD88. Importantly, our data demonstrate that T. gondii triggers neutrophil CCL2 production through TLR2. In dramatic contrast, MyD88-dependent IL-12 production was independent of this TLR.

It was recently shown that TLR2−/− animals display increased susceptibility to very high dose T. gondii infection (14). Nevertheless, the animals were significantly more resistant than...
FIGURE 6. Regulation of Toxoplasma-triggered neutrophil IL-12 and CCL2 production. A tachyzoite-derived factor triggers MyD88-dependent IL-12, presumably through a presently undefined TLR. The pathway is sensitive to Stat1-dependent, IFN-γ-mediated up-regulation. The anti-inflammatory cytokine IL-10 is a potent antagonist of this signaling cascade. A distinct parasite factor triggers neutrophil CCL2 production in dependence upon TLR2/MyD88. This pathway is insensitive to IL-10-mediated down-regulation. IFN-γ alone up-regulates CCL2 production in a Stat1-independent manner. In combination with parasite Ag, the effects of IFN-γ display a partial dependence on Stat1. This suggests that in addition to its direct effects on CCL2 induction, IFN-γ acts through Stat1 on the Toxoplasma gondii-triggered pathway leading to CCL2 production.

MyD88−/− or IFN-γ−/− mice, which cannot survive low dose infection (13, 35). The basis for the partial susceptibility of the TLR2−/− strain was unclear, as both MyD88−/− and TLR2−/− animals were defective in production of macrophage proinflammatory mediators.

In this study we clearly show that TLR2 KO PMN display normal IL-12 responses to Toxoplasma, although production of the cytokine was defective in macrophages. In addition, we showed that DC production of IL-12 in response to Toxoplasma is not defective in the absence of TLR2 (13). Thus, the animals may produce sufficient IL-12 to mediate partial protection that is nonetheless not enough to provide complete resistance to high dose infection. Alternatively, defective CCL2 release could confer partial susceptibility to infection. CCL2 possesses potent chemotactic activity toward monocytes, DC, lymphocytes, and NK cells (30, 31, 36, 37). Together with its receptor (CCR2), it has been found to play a role in Th1 development against Cryptococcus neoformans, and its expression is increased during Leishmania infections (31, 38). The role of CCL2 during in vivo Toxoplasma infection is currently under investigation in our laboratory.

Toxoplasma ligands that trigger the production of cytokines are not yet completely defined, but recent studies have identified some parasite-derived molecules that possess this activity. A recent report implicates Toxoplasma glycosylphosphatidylinositol (GPI) as a stimulus for TNF-α release in the RAW 264.7 macrophage cell line (39). In this paper, highly purified tachyzoite-derived GPI as well as their core glycans induced NF-κB activation and TNF-α production when added to macrophages in vitro. GPI are highly abundant protein anchors in the membranes of tachyzoites (40), and GPI derived from other protozoan parasites, such as Plasmodium falciparum or Trypanosoma brucei, are known to exhibit some immunostimulatory activities on macrophages, as measured by production of TNF-α, IL-1, or NO, and the expression of inducible NO synthase (41–43). The involvement of parasite GPI in induction of macrophage cytokines has also been reported in Trypanosoma cruzi, and interestingly, GPI-induced IL-12, TNF-α, and NO are dependent upon TLR2 (44). We have not yet identified the Toxoplasma-derived TLR2 ligand that triggers neutrophil CCL2 production, but the present study shows that it is chemically distinct from an additional MyD88-dependent Toxoplasma ligand triggering DC and neutrophil IL-12 production.

Toxoplasma tachyzoites express a secreted 18-kDa cyclophilin (C-18) that triggers splenic DC IL-12 production through binding to CCR5 (25, 45). Past and present results from our laboratories implicate another pathway leading to DC IL-12 production involving MyD88 signaling (13). In neutrophils, our results unequivocally demonstrate that C-18 is not involved in STAg-induced IL-12 production, and indeed, MyD88 is required for parasite-induced PMN IL-12 secretion. Further study is required to identify the TLR and parasite ligand involved in this pathway.

Our data show that neutrophil IL-12 and CCL2 production is triggered by distinct molecules derived from the parasite, and that the signaling pathways involved in the production of these mediators are distinct (summarized in Fig. 6). Both responses are dependent on MyD88 signaling, but, importantly, they use distinct receptors and trigger different signaling pathways subject to their own mechanisms of regulation. These data dramatically demonstrate that distinct TLR work in concert to provide optimal resistance against infection with a single microbial pathogen. Defining the molecular components of the different TLR pathways and determining how they are activated by microbial Ag will provide crucial insight into innate immune response initiation and may ultimately prove useful for the treatment of disease caused by T. gondii and other microbial pathogens.

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