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Production of IL-12 by Macrophages Infected with *Toxoplasma gondii* Depends on the Parasite Genotype¹

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Three clonal strain types (I, II, and III) of *Toxoplasma gondii* predominate worldwide. The outcome of infection in mice is highly dependent on the parasite genotype with type I strains being uniformly virulent, while types II and III are nonvirulent. Interactions with the innate immune response play a major role in determining the outcome of infection in the murine model. To identify key early differences in the innate immune response that contribute to pathogenesis, we examined the cytokine production of macrophages after in vitro infection with parasites of virulent type I and nonvirulent type II genotypes. Infection with type II strain parasites stimulated the production of proinflammatory cytokines, and particularly high levels of the Th1-polarizing cytokine, IL-12. Infection with type II strain parasites stimulated NF- κ B nuclear translocation at early time points and led to the up-regulation of mRNA levels of IL-12 and other proinflammatory cytokines that was dependent on the myeloid differentiation factor 88 signaling pathway. Induction of IL-12 required active invasion by live parasites and was not blocked by infection with virulent type I strain parasites, arguing against an active inhibition of signaling. Our findings suggest that early induction of high levels of IL-12 by macrophages infected with type II strain parasites may contribute to more effective control. *The Journal of Immunology*, 2004, 172: 3686–3694.

Toxoplasma *gondii* is an important food and waterborne pathogen that can be acquired postnatally in two ways (1). Infection can occur by ingestion of a spore-like stage called an oocyst that is released in the feces of the definitive host, the cat. The alternative is consumption of undercooked, cyst-containing meat from a chronically infected intermediate host. *T. gondii* can infect virtually any warm-blooded vertebrate. Acute infection is marked by the proliferation of fast-growing tachyzoites that can encyst as slow-growing bradyzoites and persist for the lifetime of the host, leading to a chronic state of infection. *T. gondii* is an exceptionally successful parasite with approximately one-quarter of the U.S. population (2), and as many as 1 billion humans worldwide are thought to be chronically infected (3).

Acute toxoplasmosis may pass asymptotically in immunocompetent individuals, and usually results in a mild flu-like syndrome when symptomatic (4). Severe manifestations can occur during acute infections, however, and include retinochoroiditis, pneumonitis, and encephalitis (3). Reactivation of chronic infections is a common feature of toxoplasmosis in immunocompromised individuals, resulting in an encephalitis that is uniformly fatal if untreated (5, 6).

T. gondii has an unusual clonal population structure in which there exists limited genetic diversity, and three closely related strain types predominate (7). Despite the limited genetic diversity, different strain types display markedly different levels of virulence toward mice. A tight correlation exists between strain type and acute virulence toward mice, with type I strains possessing an LD₁₀₀ = 1, while type II and III parasites display LD₅₀ values at least two orders of magnitude greater (8). Although infections with type I strains are uniformly fatal in mice, strain types II and III result in controlled infections that persist chronically. Parasite strain type may also play a role in determining the severity of human infections. In several studies, type I strains also lead to more severe sequelae in a human host (9, 10). Despite this, the majority of human toxoplasmosis is due to infection by type II strains, which are the most prevalent in nature (7, 11).

The immune response to *T. gondii* is characterized by the induction of a vigorous Th1 cell-mediated response, and high levels of IFN- γ are necessary for control of both acute and chronic stages of infection in the murine system (12, 13). IL-12 is required for resistance to acute and chronic toxoplasmosis due to its essential role in stimulating production of IFN- γ (14, 15). Early production of IL-12 by dendritic cells (DCs)⁴ (16), neutrophils (17), and macrophages (15) occurs in response to Ags from *T. gondii*. IL-12 induction in response to Ags is dependent on myeloid differentiation factor 88 (MyD88) (18), a crucial adaptor protein in the Toll-like receptor (TLR) signaling pathway (19), suggesting an important role for innate immunity in control of infection.

Strain types of *T. gondii* display different levels of virulence toward mice (20, 21). A conspicuous feature of lethal infections by type I strains is the rapid establishment of a high parasite burden in the host, suggesting that events early in the infection may set the

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⁴ Abbreviations used in this paper: DC, dendritic cell; BMM, bone marrow-derived macrophage; C-18, cyclophilin-18; CytoD, cytochalasin D; ESA, *Toxoplasma* excreted-secreted Ag; LOD, low density; MOI, multiplicity of infection; MyD88, myeloid differentiation factor 88; RT, room temperature; STAg, soluble tachyzoite Ag; TLR, Toll-like receptor; TTA_g, total tachyzoite Ag.

stage for the eventual outcome. To examine the initial interaction that occurs between naive APC and live *T. gondii*, we determined whether virulent (type I) and nonvirulent (type II) strain types stimulate different patterns of cytokine production from APC. Our results show that infection of macrophages with nonvirulent (type II) strain parasites stimulates very high levels of IL-12, suggesting that an efficient early innate response results in effective control.

Materials and Methods

Experimental animals

CD1 mice were obtained from Charles River Breeding Laboratories (Wilmington, MA). C57BL/6J, C3H/HeJ, and C3H/HeOJ mice were obtained from The Jackson Laboratory (Bar Harbor, ME). MyD88^{-/-} and TLR2^{-/-} mice (22, 23) on a partially backcrossed 129/Ola × C57BL/6 background were produced in the laboratory of S. Akira (Osaka University, Osaka, Japan). Mice of both sexes between 8 and 15 wk of age were used. Animals were housed under specific pathogen-free conditions at Washington University School of Medicine (St. Louis, MO).

Host cell culture

Low density (LOD) splenocytes were harvested by injecting spleens from CD1 mice with Liberase CI (400 μg/ml; Roche Biochemicals, Indianapolis, IN) and incubating for 30 min at 37°C. Following incubation, cells were released by mincing spleens and passing cells through a 100-μm cell strainer (Falcon, Franklin Lakes, NJ). Cells were washed, then suspended in a 30% BSA solution (Sigma-Aldrich, St. Louis, MO) and centrifuged at 15,000 × *g* for 15 min. The resulting interface cells were washed and then plated 2 × 10⁵ per well in 96-well plates, in DMEM supplemented with 10% FBS, L-glutamine (2 mM), 2-ME (50 μM), and 20 μg/ml gentamicin (referred to as complete DMEM).

Macrophages were obtained from CD1 mice that had been inoculated i.p. with 1.5 ml of 5% proteose peptone (Sigma-Aldrich) or 3% thioglycolate (Edge Biologicals, Memphis, TN), respectively, 3 and 4 days prior. Cells were plated in 96-well plates, 2 × 10⁵ per well, in DMEM supplemented with 2% FBS. After incubating 2 h at 37°C, nonadherent cells were removed by washing wells three times and replacing medium with complete DMEM.

Bone marrow-derived macrophages (BMM) were obtained, as previously described (24). Briefly, bone marrow cells were obtained by flushing marrow from hind tibias and femurs. Cells were suspended in DMEM supplemented with 10% FBS, 5% horse serum, L-glutamine (2 mM), 20 μg/ml gentamicin, and 20% L929 cell-conditioned medium, and incubated at 37°C in humidified 5% CO₂ in air. Nonadherent cells were transferred the following day to plastic 10-cm bacteriological petri dishes (Kord-Valmark, Brampton, Ontario, Canada). BMM were harvested by gently rinsing cells from dishes after replacing the medium with cold PBS and incubating cells on ice for 10 min. Cells were resuspended in DMEM supplemented with 10% FBS, L-glutamine (1 mM), and 20 μg/ml gentamicin (referred to as D10). BMM (5 × 10⁵ cells/ml) were seeded at 0.5 ml/well in 24-well plates. Macrophages were allowed to rest 8–12 h, then either cultured overnight in control medium or primed overnight by replacing medium with D10 containing 10 U/ml murine rIFN-γ (BD PharMingen, San Diego, CA). Cells were rinsed gently three times with D10 before inoculation of wells with parasites or parasite products suspended in 0.5 ml of D10.

Parasite culture and preparations

T. gondii were maintained by serial 2-day passage of tachyzoites in human foreskin fibroblast monolayers, as described previously (25). The number of viable parasites in inocula was determined by plaque formation on fibroblast monolayers. RH (American Type Culture Collection, Manassas, VA; 50174) and GT1 (ATCC 50853) served as representative type I strains. PTG (ATCC 50841) and BEV (ATCC 50854) served as representative type II strains. CTG (ATCC 50842) and VEG (ATCC 50861) served as representative type III strains. All parasite strains were tested for *Mycoplasma* contamination using the GenProbe kit (Fischer Scientific, Pittsburgh, PA) and remained negative throughout the experiments.

Total tachyzoite Ag (TTag) was prepared by harvesting tachyzoites, as previously described (25), and then subjecting them to four cycles of rapid freezing and thawing, and storing at -20°C until use. *Toxoplasma* excreted-secreted Ags (ESA) were prepared by incubating purified, washed tachyzoites for 30 min at 37°C in HBSS supplemented with 10 mM HEPES and 1 mM EGTA containing 1% ethanol, removing parasites via centrifugation, and then concentrating ESA-containing supernatants using centrifugal filter devices (Centricon YM-10; Millipore, Billerica, MA), and storing at -80°C until use. Cytochalasin D was obtained from Calbiochem

(La Jolla, CA). LPS (*Escherichia coli* O55:B5) was obtained from Sigma-Aldrich.

ELISA measurement of cytokine levels

Following in vitro culture, samples of supernatant medium were removed at specific time points and frozen at -20°C. IL-12p40, IL-12p70, TNF-α, IL-1β, IL-18, and IL-10 protein levels of the samples were determined using OptEIA kits (BD PharMingen), following the manufacturer's directions.

RNase protection assay of cytokine mRNAs

Macrophages (1.5 × 10⁶ cells/ml) were seeded at 2.0 ml/well in six-well plates. After allowing macrophages to rest overnight, cells were inoculated at a macrophage-parasite ratio of 3:1, or treated with LPS. Total RNA was isolated using TRIzol (Invitrogen, Carlsbad, CA). RNase protection assays (BD Biosciences, San Diego, CA) were performed using 5'-[α-³²P]UTP (Amersham Pharmacia Biotech, Piscataway, NJ), according to the kit manufacturer's directions, using 10 μg of total RNA per sample and mCK-2b probe kit supplied by the manufacturer. Phosphor imaging and data analysis of the resulting gels were accomplished using a FLA-5000 phosphor imager and software supplied by the manufacturer (Fujifilm, Stamford, CT). For quantification, signal intensity values for individual cytokines were normalized against the value of a housekeeping gene for the respective treatment group.

Western blotting

T. gondii (10⁷) was lysed in SDS-PAGE reducing sample buffer, resolved under reducing conditions on Bio-Rad 4–15% Gradient, Ready Gels (Bio-Rad, Hercules, CA), and then electroblotted onto nylon membranes. Immunoblots were probed with rabbit anti-cyclophilin-18 (C-18) antiserum (obtained from A. Sher, National Institutes of Health) and rabbit anti-*T. gondii* β-tubulin antiserum (26) and developed by chemiluminescence (ECL⁺; Amersham Pharmacia Biotech). Band intensities were quantified by direct detection of light output using a Model FLA-5000 imaging system (Fujifilm).

NF-κB detection and quantification

BMM (6.5 × 10⁵/ml) were plated onto 12-mm glass coverslips in 24-well plates. After allowing macrophages to rest overnight, cells were inoculated at multiplicity of infection (MOI) 0.5 with RH (type I) or PTG (type II) strain parasites, or mock infected for 6 or 12 h. Noninfected BMM were treated with LPS (100 ng/ml) 30 min before the 6- or 12-h time point, and then processed in parallel with infected cells. Cells were washed gently and then fixed in 3% paraformaldehyde for 10 min at room temperature (RT). Following fixation, coverslips were washed and cells were permeabilized and blocked (30 min, RT) in PBS supplemented with 5% FBS, 5% normal goat serum, and 0.5% Triton X-100. After rinsing, cells were incubated (1 h, RT) with mouse anti-NF-κB p65 mAb (Santa Cruz Biotechnology, Santa Cruz, CA) and rabbit anti-tachyzoite antiserum diluted in 1% FBS in PBS. After washing, coverslips were incubated (1 h, RT) with secondary Abs (Alexa Fluor 488-conjugated goat anti-rabbit IgG and Alexa Fluor 594-conjugated goat anti-mouse IgG) diluted in 1% FBS in PBS. Coverslips were subsequently washed and mounted with Vectashield mounting medium with 4',6'-diamidino-2-phenylindole (Vector Laboratories, Burlingame, CA). Immunofluorescence images were collected on a Zeiss Axioskop using a Zeiss AxioCam camera and Axiovision 3.1 software (Carl Zeiss, Thornwood, NY). To calculate the nuclear-cytoplasmic NF-κB ratio, three infected cells were selected from each of three randomly selected fields per coverslip in each experiment. Representative nuclear, cytoplasmic, and background regions for each infected cell were selected, and the mean red channel (NF-κB staining) fluorescence of each region was measured using the Axiovision 3.1 Quantitative Analysis Package. Background values were subtracted from nuclear and cytoplasmic measurements, and ratios were calculated. The mean values for the cells within an experiment were averaged between three experiments, and the SEM was calculated.

Statistics

Experiments were performed three or more times using triplicate samples, unless otherwise noted. Data are expressed as mean ± SE where experiments are combined and mean ± SD where a single representative experiment is shown. Comparison of means was performed using a one-way ANOVA (F-statistic) to establish whether significant differences were present. Pairwise comparisons between means were performed using the least significant difference test (based on Student's *t* statistic), allowing for estimation of *p* values, when the F-statistic indicated that significant differences existed (27).

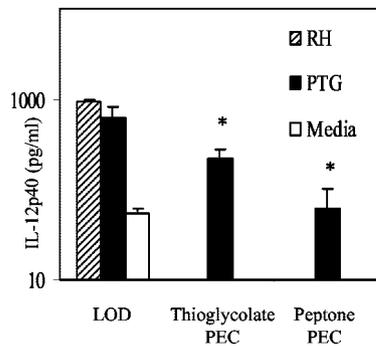


FIGURE 1. IL-12 production following *T. gondii* infection of LOD splenocytes and macrophages. LOD splenocytes (enriched in DCs and macrophages), and adherent thioglycolate- or proteose peptone-elicited peritoneal exudate cells (2×10^5) from CD1 mice were infected in vitro with either RH (type I) or PTG (type II) strain *T. gondii*. Following overnight infection, resulting IL-12p40 levels were measured by ELISA. Data shown are means \pm SD of triplicate cultures from a representative of two or more experiments performed that had similar outcomes (*, type II significantly different from type I, $p < 0.01$).

Results

Strain-specific induction of IL-12 following parasite infection

To examine the production of cytokines that might contribute to or suppress the induction of the robust Th1-polarized immune response essential for resistance to *T. gondii*, we challenged APCs with tachyzoites of *T. gondii*. A representative type I strain (RH) and representative type II strain (PTG) were used to compare the response with virulent and nonvirulent parasites, respectively. We compared the response of LOD splenocytes (enriched in CD8 α^+ DC cells and macrophages) and peritoneal macrophages elicited with peptone or thioglycolate. Following overnight infection, supernatants were collected and analyzed via ELISA for production of IL-12 (Fig. 1). The different strain types were found to induce similar levels of IL-12 from splenocytes (Fig. 1). In contrast, infection of both thioglycolate-elicited and proteose peptone-elicited macrophages by PTG (type II) strain parasites led to consistently higher levels of IL-12 production than infection by RH (type I) strain *T. gondii*.

To examine whether strain-specific induction of IL-12 was also a feature of naive macrophages, we examined the production of cytokines following parasite challenge of BMM. Levels of IL-12p40, TNF- α , IL-1 β , IL-18, and IL-10 (Table I) produced in response to infection (MOI = 1) were determined by ELISA on samples collected after 24 h of infection. Importantly, PTG (type II) strain parasites were found to induce nearly 200-fold higher levels ($p < 0.001$) of IL-12 than RH (type I) strain parasites. Pairwise comparisons (PTG vs RH) of mean values revealed no other difference in cytokine production as statistically significant at

the $p < 0.05$ level. These data are corrected for viable MOI; hence, the difference observed between parasite strains is not simply a consequence of the efficiency of infection.

Strain-specific IL-12 induction reflects infectious dose

In light of the striking difference (>175-fold) in levels of IL-12 induced by infection of BMM with different strains, we focused our efforts on analyzing this phenomenon in more detail. Both IFN- γ -primed and unprimed (± 10 U/ml) BMM were infected in vitro over a range of MOIs with either RH (type I) or PTG (type II) strain *T. gondii*. Importantly, PTG (type II) strain parasites were found to induce substantially higher levels of both IL-12p40 (Fig. 2A) and IL-12p70 (Fig. 2B) after 24 h of infection than RH (type I) strain parasites. The level of induction was found to reflect the MOI with higher inputs of parasites resulting in higher levels of IL-12 induction. Furthermore, the substantial strain type difference in IL-12 induction was observed regardless of the prior IFN- γ -priming status of the BMM.

Induction of IL-12 by parasite lysate is not strain specific

In earlier studies, soluble tachyzoite Ag (STAg), generally from RH strain, was found to induce IL-12 in several different cell types (14, 28–30). Conceivably, the method of preparation might result in the removal of some immunogenic constituents of parasite lysate. For this reason, we initially tested both STAg and TTAG for their ability to induce IL-12. Although patterns of IL-12 induction were similar for both STAg (a soluble fraction) and TTAG, STAg was found to be a less potent stimulus of IL-12 induction (data not shown). Both IFN- γ -primed and unprimed (± 10 U/ml) BMM were challenged in vitro with various concentrations of TTAG. Unlike the situation of infection by live parasites (above), TTAG from the PTG (type II) strain *T. gondii* was no more potent a stimulus for IL-12 induction than that prepared from RH (type I) strain (Fig. 3). In this instance as well, the IFN- γ -priming status of the BMM did not significantly alter the pattern of IL-12 induction. Importantly, total levels of IL-12 measured were much lower than those observed following tachyzoite challenge (Fig. 2) even at the highest concentration of TTAG (100 μ g/ml). For example, at an MOI 1 for PTG (type II) strain, ~ 30 – 40 ng/ml IL-12p40 is produced; by comparison, the equivalent amount of TTAG (≤ 0.5 μ g of protein) only induced levels of ≤ 1 ng/ml IL-12p40.

IL-12 induction is a property of type II strain *T. gondii*

To examine the association between strain type and the induction of IL-12, we infected BMM in vitro with several different parasite strains representing the three clonal genotypes of *T. gondii*. After 24 h, levels of IL-12p70 were measured via ELISA (Table II). Although the strains were originally isolated from a variety of different host organisms and geographic locales, only type II strains of *T. gondii* were found to induce significant levels of IL-12.

Table I. Levels of cytokines produced by BMM following challenge with different stimuli^a

Challenge	IL-12p40	TNF- α	IL-1 β	IL-18	IL-10
Type I	181 \pm 54 ^{b,c}	15 \pm 13	56 \pm 41	19 \pm 20	28 \pm 33
Type II	32,271 \pm 6,394 ^{d,e}	80 \pm 31 ^c	269 \pm 179	49 \pm 17	61 \pm 13 ^d
LPS	22,844 \pm 8,138	3,074 \pm 1,120	307 \pm 356	18 \pm 13	48 \pm 9
Medium	1 \pm 2	23 \pm 10	54 \pm 27	23 \pm 22	4 \pm 4

^a BMM were infected with tachyzoites for 24 h at MOI 1 or treated with LPS (100 ng/ml).

^b Data are means \pm SE from two or three experiments expressed in picograms per milliliter.

^c Significantly different from medium (background) control ($p < 0.05$).

^d Significantly different from medium (background) control ($p < 0.001$).

^e Significantly different from type I infection ($p < 0.001$).

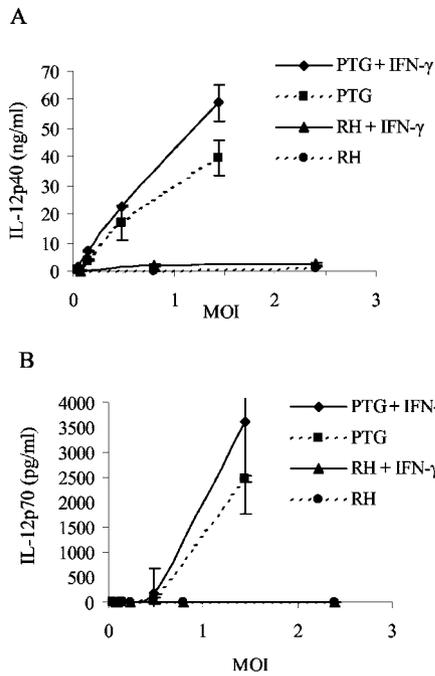


FIGURE 2. Induction of IL-12p40 or IL-12p70 following infection by different strains of *T. gondii*. Primed and unprimed (± 10 U/ml IFN- γ) BMM from CD1 mice were infected in vitro with either RH (type I) or PTG (type II) strain *T. gondii* at various MOIs. The data points on x-axis are adjusted to represent viable parasites based on plaquing assays. After 24 h, IL-12p40 (A) and IL-12p70 (B) levels were measured by ELISA. Data shown are means \pm SD from a representative of three experiments that had similar outcomes.

Requirements for IL-12 induction

One possible explanation for the strain-specific induction of IL-12 would be if different strains invaded and/or survived differently in macrophages. To discern the requirement for active invasion on IL-12 induction, we infected BMM in vitro for 24 h with PTG (type II) strain parasites at MOI 0.5, or heat-treated and opsonized parasites at MOI 3, in the presence or absence of cytochalasin D (CytoD).

When BMM were challenged with live PTG (type II) strain parasites in the presence of 1 μ M CytoD to block entry, induction of IL-12p70 was significantly diminished ($p < 0.001$) (Fig. 4A). Abrogation of IL-12 induction by CytoD treatment suggests that host cell invasion is required for the potent induction of IL-12p70 by live parasites. Challenge of BMM for 24 h with heat-treated and mAb-opsonized parasites resulted in no induction of IL-12p70, irrespective of the presence or absence of CytoD. The failure of phagocytosed PTG (type II) strain parasites to induce IL-12p70 in this experiment indicates that potent IL-12 induction only follows active invasion by parasites. Induction of IL-12p70 following treatment of BMM with LPS was unaffected by the presence of CytoD, demonstrating that CytoD does not impair the production or secretion of IL-12p70.

The more efficient induction of IL-12 by infection vs TTAG suggested that invasion represented a highly efficient Ag delivery mechanism, possibly due to release of organellar contents during host cell invasion. We tested this possibility by inoculating BMM with PTG (type II) strain tachyzoite ESA or TTAG at either 10 or 100 μ g/ml. Exposure of BMM to ESA resulted in IL-12p70 induction to a similar extent to that produced in response to TTAG (Fig. 4B). However, ESA represents only a fraction of the total protein content of a tachyzoite. When potency of induction was

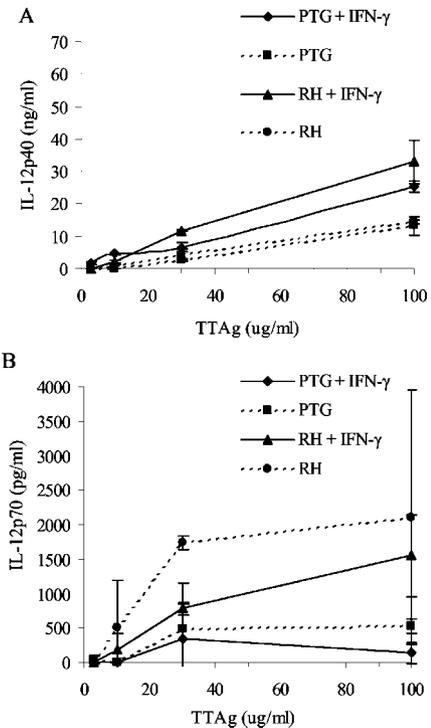


FIGURE 3. IL-12 induction by total Ag prepared from different strains of *T. gondii*. Primed and unprimed (± 10 U/ml IFN- γ) BMM from CD1 mice were challenged in vitro with either RH (type I) or PTG (type II) strain TTAG at various concentrations. After 24 h, IL-12p40 (A) and IL-12p70 (B) levels were measured by ELISA. Data shown are means \pm SD from a representative of three experiments performed that had similar outcomes.

considered after normalizing for the approximate number of tachyzoites that the challenge represents, ESA was no more potent than TTAG at stimulating IL-12 induction in BMM. The findings of this experiment corroborate our finding that infection of BMM with live parasites is required for potent stimulation of IL-12 induction.

Kinetics of IL-12 induction indicates T. gondii infection results in nascent synthesis

To more closely analyze the strain-specific induction of IL-12 by *T. gondii* parasites and determine whether the IL-12p70 observed was being nascently synthesized, we studied the kinetics of IL-12 induction at both the mRNA and protein level following infection with *T. gondii* or exposure to LPS.

For analysis of mRNA levels, total RNA harvested from BMM infected in vitro with RH (type I) or PTG (type II) strain parasites or treated with LPS was used in an RNase protection assay. BMM exposed to LPS up-regulated both IL-12p35 and IL-12p40 mRNA levels greater than 80- and 70-fold, respectively, relative to a mock-infected control by 12 h (Fig. 5, A and B). PTG (type II)

Table II. Levels of IL-12p70 produced by BMM following challenge with different strains of *T. gondii*^a

		Type I	Type II	Type III	
RH	– ^b	PTG	2832 \pm 1382 ^c	CTG	–
GT1	–	BEV	5930 \pm 23	VEG	–

^a BMM were infected with tachyzoites for 24 h at MOI 3.
^b \leq Lower limit of detection (62.5 pg/ml).
^c Data are means \pm SE from two experiments expressed in picograms per milliliter.

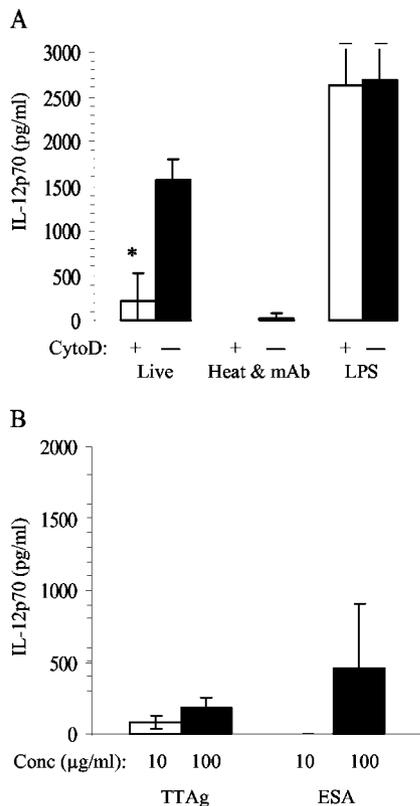


FIGURE 4. Invasion is required for induction of IL-12. Bone marrow macrophages from CD1 mice were exposed to PTG (type II) strain *T. gondii* tachyzoites (A) or their products (B) under various conditions in vitro to determine the minimal requirements for IL-12 induction by *T. gondii*. After 24 h, IL-12p70 levels were measured by ELISA. Data shown are means \pm SE from three experiments performed (*, significantly different from untreated parasites, $p < 0.001$). The uptake of heat-killed and Ab-opsonized parasites (Heat & mAb) was comparable to infection.

strain parasites resulted in less substantial increases in IL-12 subunit mRNA levels (Fig. 5A). At 12 h postinfection, IL-12p35 and IL-12p40 subunit mRNA levels were found to be increased 25- and nearly 40-fold above background levels, respectively (Fig. 5B). In line with their failure to induce significant levels of IL-12, RH (type I) strain parasites stimulated a less than 3-fold increase in IL-12p35 subunit mRNA, and no discernible increase in the level of IL-12p40 subunit mRNA levels. Consonant with the results presented in Table I, infection with PTG (type II) strain parasites resulted in elevation of mRNA levels for several proinflammatory cytokines, while infection with RH (type I) strain parasites failed to produce discernible elevations in the mRNA levels of any other cytokines analyzed.

Analysis of IL-12 levels in medium supernatants at various time points revealed that LPS stimulation resulted in early production of IL-12p70, with levels above background being produced within 6 h of exposure (Fig. 5C). Robust release of IL-12 in response to LPS was observed between 6 and 12 h after initial exposure, with peak levels measured at 12 h, and levels subsequently plateauing. In contrast, robust IL-12p70 production in response to tachyzoite infection was not observed until 12–18 h postinfection. Taken together, these results demonstrate that PTG (type II) strain infection stimulates nascent synthesis of IL-12p70, but with delayed kinetics relative to when LPS is used as the trigger.

Type I strains fail to inhibit IL-12 induction

The failure of infection with virulent RH (type I) strain parasites to result in potent IL-12 induction could result from either of two

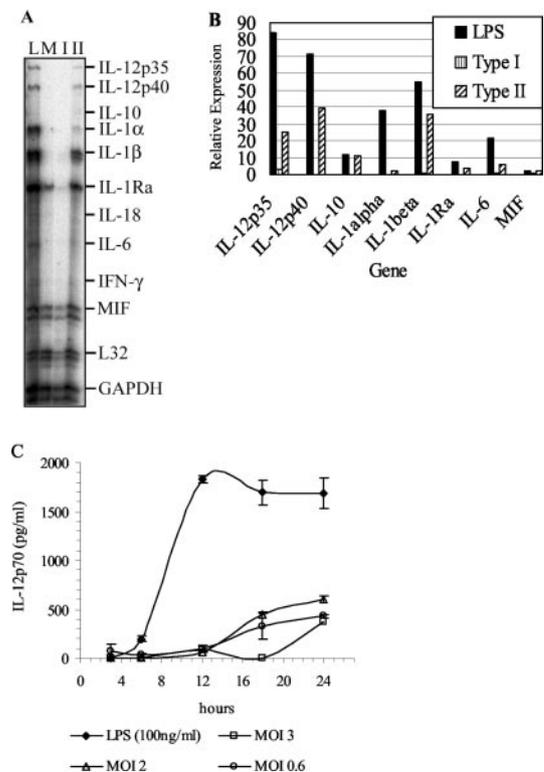


FIGURE 5. IL-12 is nascently synthesized in response to infection. A, Bone marrow macrophages were treated with 10 ng/ml LPS (L), mock infected (M) or infected at MOI 3 with type I or II strain parasites for 12 h. Total RNA was isolated and subjected to RNase protection assay analysis for the indicated genes. Relative levels of expression for those cytokine genes undergoing changes when compared with a mock-infected medium control were determined by phosphor image analysis and are shown in B. The experiment was performed three times with similar results. A and B, Representative data from one experiment. C, BMM were infected at various MOIs or treated with LPS (100 ng/ml), and IL-12p70 levels were determined by ELISA from collected supernatants. Data shown are means \pm SE from three experiments performed.

possible scenarios: 1) type I strains possess the stimulus for IL-12 induction, but actively inhibit the process, or 2) type I strains simply lack the stimulus possessed by nonvirulent type II strains and fail to trigger IL-12 induction. We discriminated between these two possibilities by performing mixed infection experiments. To establish mixed infections, BMM were infected in vitro with RH (type I) strain parasites at MOI 3. After 2 h, infected cells were washed gently and then infected with the PTG (type II) strain parasites at MOI 3.

Two-hour preinfection with RH (type I) strain parasites failed to significantly alter the level of IL-12p70 induction observed (Fig. 6) following infection with PTG (type II) strain parasites ($p > 0.1$), arguing that type I strains do not actively inhibit the IL-12 induction stimulated by infection with type II strain parasites. Coinfection experiments, in which RH (type I) and PTG (type II) strain parasites were inoculated simultaneously in various proportions, revealed that IL-12 induction was proportional to the level of type II parasites input (data not shown). These results further demonstrate that type I strains do not inhibit the IL-12 induction triggered by infection with type II strain parasites.

IL-12 induction is MyD88 dependent

MyD88 serves as an adapter protein for all murine TLRs, and is necessary for proinflammatory cytokine production in response to

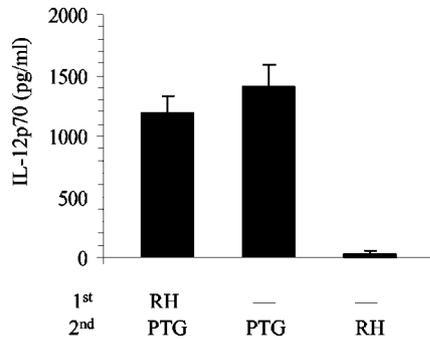


FIGURE 6. Type I strain infection cannot block induction of IL-12 due to infection with type II strain. Bone marrow macrophages from CD1 mice were preinfected with RH (type I) strain parasites at MOI 3. Two hours later, preinfected cells were washed gently and infected with PTG (type II) strain at MOI 3 to establish mixed infections. After 24 h total infection time, IL-12p70 levels were measured by ELISA. Data shown are means \pm SE from two experiments performed.

TLR binding (19). Furthermore, MyD88 is involved in the induction of IL-12 by various cell types in response to STAg (18). We used cells from MyD88^{-/-} strain mice to test for possible TLR involvement in the IL-12 induction stimulated by infection with type II parasites. BMM from MyD88^{-/-} and C57BL/6J background control mice were infected in vitro with RH (type I) or PTG (type II) strain parasites at MOI 1. Induction of IL-12p70 by PTG (type II) strain parasites was abrogated in MyD88^{-/-} strain BMM >90% ($p < 0.05$) relative to levels observed in BMM from C57BL/6J background control mice (Fig. 7A). The results of this experiment indicate that type II strain parasites induce IL-12 in a MyD88-dependent manner.

Two TLRs that recognize microbial ligands are TLR4 and TLR2, the latter of which has been implicated in response to protozoal ligands (31). BMM from TLR2^{-/-} and wild-type control C57BL/6J, as well as C3H/HeJ (functionally *tlr4*^{-/-}) and wild-type control C3H/HeOuJ mice, were infected in vitro with PTG (type II) strain parasites at MOI 1. The ability of PTG (type II) strain parasites to induce similar levels of IL-12 from BMM from both experimental and background control strains of mice (Fig. 7B) argues against either TLR2 or TLR4 being independently necessary for the observed IL-12 induction. The lack of TLR2 and TLR4 dependence argues strongly against a role for contamination with LPS or *Mycoplasma* lipoproteins in the IL-12 induction observed in response to parasite infection.

C-18 was recently identified as the ligand predominantly responsible for the CCR5-mediated IL-12 induction from DCs exposed to *T. gondii* Ag (32). To determine whether the amount of C-18 produced by different strain types plays a role in the strain-specific IL-12 induction we observe, we compared the amounts of C-18 produced by RH (type I) and PTG (type II) strain parasites by immunoblotting parasite lysates for C-18 (Fig. 7C), and quantifying chemiluminescent signals via phosphor imaging. Type I strain parasites were found to produce ~2-fold more C-18 than type II strain parasites, when immunoblot C-18 signals were normalized against a loading control (*T. gondii* β -tubulin). The slightly higher level of C-18 found in type I strain parasites argues against a significant role for C-18 in the IL-12 induction observed in response to parasite infection.

Type II strain *Toxoplasma* induces nuclear localization of NF- κ B

Production of IL-12p70 is thought to require the activation of NF- κ B, and MyD88-dependent TLR signaling is known to result in

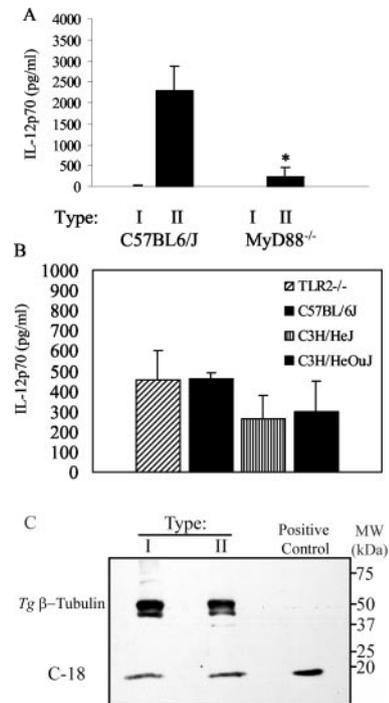


FIGURE 7. MyD88- vs CCR5-dependent IL-12 induction. *A*, IL-12 induction by type II strain infection is MyD88 dependent. *B*, IL-12 induction does not require TLR2 or TLR4. *C*, Comparison of C-18 in type I vs type II strains by Western blot. BMM from MyD88^{-/-} and wild-type control C57BL/6J (*A*) and TLR2^{-/-}, wild-type control C57BL/6J, C3H/HeJ (functionally TLR4^{-/-}), and wild-type control C3H/HeOuJ (*B*) mice were infected in vitro with RH (type I) (*A*) or PTG (type II) (*A* and *B*) strain *T. gondii*. After 24 h, supernatants were collected and IL-12p70 levels were measured via ELISA. Data shown are means \pm SE from two experiments (*A*) and three experiments (*B*) (*, significantly different from background strain control, $p < 0.05$). *C*, RH (type I) or PTG (type II) strain parasites were lysed in SDS-PAGE reducing sample buffer, subjected to SDS-PAGE alongside 100 ng of recombinant C-18, and blotted with C-18 or *Toxoplasma* β -tubulin antisera. The blot shown is representative of the results of three separate experiments with similar outcomes (band of ~45 kDa is most likely β -tubulin breakdown product).

activation of NF- κ B (19). We hypothesized that infection with type II strain parasites might activate nuclear translocation of NF- κ B more efficiently than infection with type I strain parasites.

To test this hypothesis, we examined by indirect immunofluorescence microscopy the cellular localization of NF- κ B p65 in BMM infected with either RH (type I) or PTG (type II) strain parasites 6 h (data not shown) and 12 h postinfection (Fig. 8, *A* and *B*). We quantified the fluorescence intensity of representative nuclear and cytoplasmic regions of infected cells (Fig. 8, *C* and *D*), and calculated the nuclear-cytoplasmic ratio of NF- κ B immunostaining (Fig. 8*E*). Infection with type I strain parasites led to no significant increase in NF- κ B p65 nuclear localization, even at 12 h postinfection (nuclear-cytoplasmic ratio \pm SE = 1.07 ± 0.13 cf medium control = 1.00 ± 0.05 ; $p > 0.2$). Infection with type II strain parasites, however, led to highly significant increases in the nuclear-cytoplasmic ratio (1.86 ± 0.11 ; $p < 0.005$ vs RH infection or medium) by 12 h postinfection. Collectively, these results demonstrate that NF- κ B activation occurs more efficiently in response to infection with type II strains.

Discussion

T. gondii has a unique population genetic pattern consisting of three predominant clonal lineages. In the mouse model, all members of the type I lineage are acutely virulent in the mouse, while

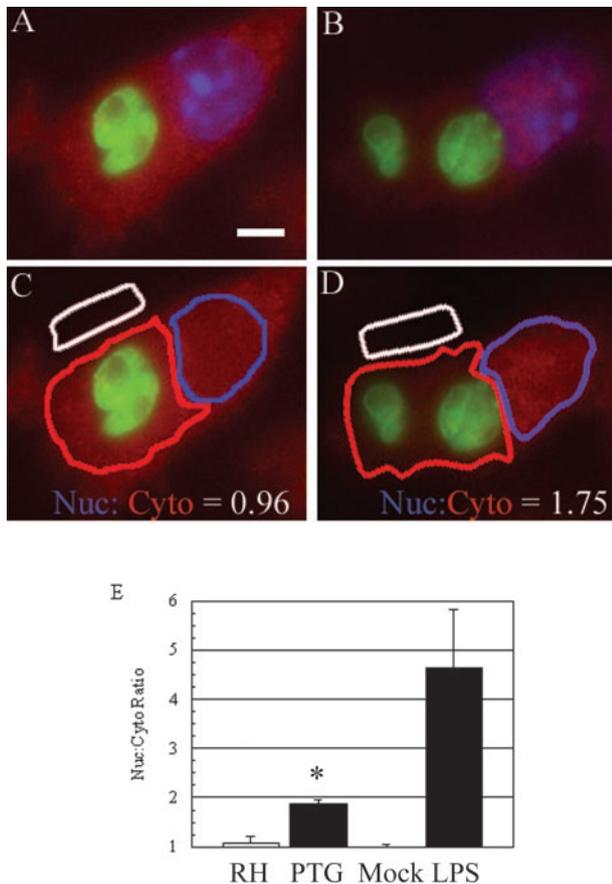


FIGURE 8. NF- κ B is activated during infection with type II strain *Toxoplasma*. Representative staining for NF- κ B (red) in type I strain-infected cells (A and C) vs type II strain-infected cells (B and D) (*Toxoplasma* in green, nuclei in blue). Mean intensity of NF- κ B staining was measured for nuclear and cytoplasmic portions of infected cells and nearby background regions (representative sampling and ratios are shown in C and D). E, The mean ratio of nuclear to cytoplasmic NF- κ B (after subtraction of background signal) in infected, mock-infected, or LPS-treated cells was calculated from randomly selected fields from separate coverslips (see *Materials and Methods* for details) (*, significantly different from RH (type I) strain parasites and medium control, $p < 0.005$; scale bar = 5 μ m).

types II and III are nonvirulent. We have examined the release of proinflammatory cytokines from APC following infection to determine to what extent parasite lineage contributes to the induction of immune responses. Although infection of LOD splenocytes enriched in DC cells did not respond differentially, both naive and inflammatory macrophages responded in a strain-specific manner. Infection of naive BMM in vitro with nonvirulent type II strain parasites induces high levels of the Th1-polarizing cytokine, IL-12, as well as more modest levels of other inflammatory cytokines. In contrast, infections with virulent type I strain parasites are characterized by the induction of very low levels of proinflammatory cytokines. Consistent with this, infection by type II strain parasites was found to result in more effective activation of NF- κ B than infection with type I strain parasites.

Several different cell types have been shown to be potent producers of IL-12 following stimulation by parasite infection of Ags, including neutrophils (17), DCs (16), and macrophages (15). In the spleen, the primary producers of IL-12 are CD8 α^+ DC cells that respond strongly to soluble parasite Ag (STAg) (16, 18). Induction of IL-12 by these DC cells involves both a MyD88-dependent pathway and a separate CCR5-dependent pathway (29, 32). Previous studies characterizing the response of splenic DC cells have

used the RH strain. In this study, we demonstrate that LOD splenocytes, which are enriched in CD8 α^+ DC cells, produce IL-12 equally well when stimulated with type I (RH) or II (PTG) strains. The response of splenic DC may be important for mounting an early immune response, in particular following stimulation by circulating Ags. However, given the rapid advance of the parasite during the acute phase, compartmentalization of interactions between the parasite and immune response is also likely to be important for determining the outcome of infection. In this regard, it may be significant that both naive (BMM) and inflammatory (peptone- or thioglycolate-elicited) macrophages respond differentially and produced IL-12 in appreciable amounts only in response to type II strain parasites. It has recently been shown that inflammatory macrophages home to sites of infection and are important for local control of parasites in the peritoneum (33). Consequently, the differential response of macrophages may be important in peripheral tissues during dissemination of toxoplasmosis. The failure of type I strains to induce appreciable levels of Th1 cytokines from naive or inflammatory macrophages may hamper the host from mounting an appropriate local immune response during acute infections. Conversely, the early induction of IL-12 by type II strains may contribute to their effective control and diminished virulence.

Infections of mice by type I strains of *T. gondii* are universally lethal (8), and marked by the rapid establishment of high parasite burdens, followed by toxic serum levels of Th1 cytokines later in infection (21). During nonlethal infections, type II strains take longer to establish high parasite numbers that are accompanied by modest serum levels of Th1 cytokines (20, 21). We were surprised, therefore, to uncover the extremely potent induction of IL-12 in vitro by type II strain parasites, which are not lethal in mice unless inoculated in high doses. In contrast, type I strains failed to induce appreciable levels of cytokines in vitro. The failure of type I strains to induce IL-12 may result in a window of opportunity for the parasite to replicate unchecked, thereby enhancing the virulence of these infections. Such early activation of innate effectors by type II strains may lead to better control of parasite replication. Consistent with this hypothesis, higher levels of IFN- γ are produced by peritoneal cells 2 days following i.p. inoculation of type II strain parasites vs type I strain parasites (33). A similar association between disease severity and the ability of different parasite genotypes to induce IL-12 has been observed in studies of the protozoan pathogen, *Leishmania*. Infection by *Leishmania tropica* and *Leishmania donovani* results in nonhealing cutaneous and severe visceral forms of leishmaniasis, respectively. These two species are deficient in the ability to prime DCs for CD40L-dependent IL-12 production relative to *Leishmania major*, which causes a less severe form of cutaneous disease (34).

The IL-12 p40 subunit associates with the IL-12 p35 subunit to form the heterodimeric IL-12p70, as well as another protein, p19, to form IL-23. Moreover, both agonistic and antagonistic activities have been ascribed to (p40) $_2$ homodimers (35). In agreement with literature reports, we found IL-12p40 to be produced in large excess over IL-12p70 following infection with type II strain parasites (36). Stimuli that can induce IL-12 production can often stimulate production of IL-23 as well (36). Whether IL-23 is also induced in a strain-specific fashion will require further study. Previous reports have documented that administration of rIL-12p70 is sufficient for the restoration of wild-type levels of survival in IL-12p40 $^{-/-}$ mice following a *T. gondii* challenge (37), arguing against an essential role for IL-23 in resistance to acute infection.

Virulence is a multigenic trait controlled in part by a major locus on *T. gondii* chromosome VII. This locus is unique to the type I lineage (38), and is necessary for the acute virulence phenotype. This factor presumably acts independent of the induction of IL-12,

because the failure to induce IL-12 is clearly not alone sufficient to result in a virulent phenotype. Type III strains were also unable to elicit strong responses in vitro and yet they are relatively nonvirulent in vivo (39). The differences in induction of IL-12 between type II and III lineages, however, may have direct bearing on the development of pathology during infection with nonvirulent strains in two previously characterized murine models of toxoplasmosis. First, peroral infection of C57BL/6 mice with type II strains (i.e., ME49) results in severe pathology in the gut during early infection (40). Second, reactivation of chronic infections with the type II strain ME49 also results in severe pathology in the CNS (41). The pathology of both acute and chronic infection is mediated in part by high levels of inflammatory cytokines (42–44). Although it is not known whether the pattern of strain-specific, proinflammatory cytokine induction extends to human cells, it is notable that type II strains are the most frequently isolated from CNS and congenital disease in humans (7, 11).

Studies on the induction of IL-12 both in vivo and in vitro by *T. gondii* Ags have revealed the importance of both a MyD88-dependent pathway and a separate pathway that signals through CCR5 (14, 18, 28, 29). The parasite ligand that triggers signaling via CCR5 has recently been identified as a cyclophilin homologue (C-18) that is secreted from dense granules (32). We found that type I parasites produce slightly higher amounts of C-18 (~2-fold more), excluding greater production of this protein by type II strain parasites as the explanation for their induction of IL-12.

High level IL-12 production following infection by type II strains was MyD88 dependent, suggesting the possible involvement of a TLR. Glycophosphatidylinositol anchors and glycoinositolphospholipids from the protozoan parasite *Trypanosoma cruzi* (31), as well as lipopeptides/glycolipids from *Mycoplasma* spp (45) and *Mycobacteria tuberculosis* (46), have been shown to trigger inflammatory cytokine production by signaling via TLR2. Moreover, a role for TLR2 has been implicated in resistance to in vivo challenge with high numbers of oocysts from an avirulent strain of *T. gondii* (47). In addition to signaling in response to LPS, TLR4 has been implicated in the binding of ligands that are produced in response to cellular injury, such as murine heat shock protein 70 and components of the extracellular matrix (19). Our results, however, indicate that neither of these TLRs is solely responsible for the IL-12 induction we observe, suggesting the involvement of a different TLR (perhaps a previously unrecognized TLR), or redundant recognition of the responsible ligand(s).

Invasive *L. donovani* have previously been shown to lead to the release of membrane-bound stores of IL-12 from DCs (48). The extremely high levels and delayed kinetics of IL-12 release following infection with type II strains of *T. gondii*, though, argue against a similar model and instead indicate that new synthesis is involved. Moreover, production of proinflammatory cytokines as a result of MyD88-dependent signaling occurs via activation of c-Jun N-terminal kinase and NF- κ B (19), supporting a model of nascent transcription. Other studies have emphasized that following infection of BMM with *T. gondii*, there is a block in nuclear translocation of NF- κ B (49, 50), a result that might be expected to result in inhibition of IL-12 and proinflammatory cytokine synthesis. In agreement with these studies, we observed no activation of NF- κ B in response to infection with type I strain parasites. In contrast, significant NF- κ B nuclear translocation occurred 6–12 h postinfection with type II strain parasites. This early activation of NF- κ B is consistent with increases in the mRNA levels observed for both IL-12 subunits. Collectively, our results suggest that signaling through MyD88 results in activation and nuclear translocation of NF- κ B, leading to induction of proinflammatory cytokines. The resulting high level production of IL-12 may result in better

control of parasite replication at early time points. Such interactions are likely to be important in the periphery, where naive and inflammatory macrophages represent an important line of defense and may explain in part the reduced virulence of type II strain parasites.

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