NALP1 Influences Susceptibility to Human Congenital Toxoplasmosis, Proinflammatory Cytokine Response, and Fate of *Toxoplasma gondii*-Infected Monocytic Cells^{∇†}

William H. Witola,^{1‡} Ernest Mui,¹ Aubrey Hargrave,¹ Susan Liu,¹ Magali Hypolite,²
Alexandre Montpetit,³ Pierre Cavaillès,² Cordelia Bisanz,² Marie-France Cesbron-Delauw,^{2§}
Gilbert J. Fournié,^{4,5§} and Rima McLeod^{1§*}

Departments of Surgery (Ophthalmology) and Pediatrics (Infectious Disease), The University of Chicago, Chicago, Illinois 60637¹;
Laboratoire Adaptation et Pathogénie des Micro-Organismes, CNRS UMR 5163, Université Joseph Fourier Grenoble 1,
Institut Jean Roget, BP 170, 38042 Grenoble Cedex 9, France²; Centre d'Innovation, Génome Québec, Montréal,
Québec H3A 1A4, Canada³; INSERM, U563, F-31000 Toulouse, France⁴; and
University Toulouse III Paul Sabatier, F-31000 Toulouse, France⁵

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NALP1 is a member of the NOD-like receptor (NLR) family of proteins that form inflammasomes. Upon cellular infection or stress, inflammasomes are activated, triggering maturation of proinflammatory cytokines and downstream cellular signaling mediated through the MyD88 adaptor. *Toxoplasma gondii* is an obligate intracellular parasite that stimulates production of high levels of proinflammatory cytokines that are important in innate immunity. In this study, susceptibility alleles for human congenital toxoplasmosis were identified in the NALP1 gene. To investigate the role of the NALP1 inflammasome during infection with *T. gondii*, we genetically engineered a human monocytic cell line for NALP1 gene knockdown by RNA interference. NALP1 silencing attenuated progression of *T. gondii* infection, with accelerated host cell death and eventual cell disintegration. In line with this observation, upregulation of the proinflammatory cytokines interleukin-1 β (IL-1 β), IL-18, and IL-12 upon *T. gondii* infection was not observed in monocytic cells with NALP1 knockdown. These findings suggest that the NALP1 inflammasome is critical for mediating innate immune responses to *T. gondii* infection and pathogenesis. Although there have been recent advances in understanding the potent activity of inflammasomes in directing innate immune responses to disease, this is the first report, to our knowledge, on the crucial role of the NALP1 inflammasome in the pathogenesis of *T. gondii* infections in humans.

The innate immune system plays an important role in sensing pathogens and triggering biological mechanisms to control infection and eliminate pathogens, as well as marshalling the T- and B-cell responses of adaptive immunity. These functions are achieved through engaging an array of germ line-encoded pattern recognition receptors (PRRs) to detect invariant pathogen motifs (26). PRRs are expressed by cells such as monocytes, macrophages, dendritic cells, neutrophils, and epithelial cells, as well as other cells of the adaptive immune system. PRRs include Toll-like receptors (32), C-type lectins (17), RIG-like helicases (40), cytosolic DNA sensors (11), and members of the NOD-like receptor (NLR) family (21). The NLRs are a large group of cytosolic sensors whose apparent main function is to modulate the expression of the proinflammatory cytokines interleukin-1 β (IL-1 β) and IL-18. There are >23 NLRs encoded in the human genome, some of which are

important in regulation and activation of proinflammatory caspases (34).

The NLRs have a tripartite structure composed of a C-terminal leucine-rich repeat domain, a central nucleotide-binding oligomerization domain (NOD or NACHT), and a variable N-terminal protein-protein interaction domain that can be either a caspase recruitment and activation domain, a pyrin domain, or a baculovirus inhibitor of apoptosis repeat domain (13). The C-terminal leucine-rich repeat domain functions in ligand sensing and autoregulation, while the N-terminal caspase recruitment or pyrin domain mediates homotypic protein-protein interactions for downstream signaling. The NACHT domain enables activation of the signaling complex via ATP-dependent oligomerization (36). Members of the NLR family assemble into large multiprotein complexes (≥ 700 kDa), termed inflammasomes, that can be activated by cellular pathogens (bacteria, fungi, viruses, and protozoa) or stress to engage innate immune defenses (23, 35). Inflammasomes activate caspase-1, a proteolytic enzyme that cleaves and activates the secreted cytokines IL-1 β and IL-18 (13, 27). Inflammasomes are also closely linked to pyroptosis, a caspase-1-dependent highly inflammatory cell death process often observed during infection with cytosolic pathogens (36).

NALP1 is a member of the NLR family and is the only NLR protein that contains an additional C-terminal caspase activa-

* Corresponding author. Mailing address: Department of Medicine, The University of Chicago, Room AMBH S206, 5841 South Maryland Ave., Chicago, IL 60637. Phone: (773) 834-4130. Fax: (773) 834-3577. E-mail: rmcLeod@midway.uchicago.edu.

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§ M.-F.C.-D., G.J.F., and R.M. contributed equally to this study.

‡ Present address: Tuskegee University, 312 Milbank Hall, Tuskegee, AL 36088.

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tion and recruitment domain (CARD) (23). Upon stimulation, NALP1 is thought to assemble an inflammasome complex consisting of NALP1, apoptosis-associated speck-like protein containing a CARD, caspase-1, and caspase-5 (23, 37). The physiological stimulus of this activation has yet to be elucidated, although it is thought that the leucine-rich repeats of NALP1 recognize a danger signal, triggering inflammasome assembly, the activation of caspases 1 and -5, and subsequent processing of IL-1 β (19).

Toxoplasma gondii is an intracellular apicomplexan parasite that has the ability to infect virtually all warm-blooded animals, with about one-third of the human world population being seropositive for *T. gondii* (28). It can cause serious illness in immunocompromised individuals and in those infected congenitally. The hallmark of *T. gondii* is its ability to induce long-term chronic infections necessitated by specific parasite-host interactions, with conversion from the prolific tachyzoite stage to the quiescent bradyzoite stage through modulation of host cell responses associated with pathway signaling, cytokine production, and antimicrobial effector mechanisms, among others (1). Recognizing that the NALP1 gene is within a robust toxoplasmosis susceptibility/resistance region in the rat genome (Toxo1) (6, 7) that is orthologous to a region in the human genome, we hypothesized that the NALP1 gene might have susceptibility alleles for human congenital toxoplasmosis and could therefore have a significant role in the pathogenesis of *Toxoplasma gondii* infection in humans. In this study, transmission disequilibrium testing (TDT) (18) studies were performed to determine whether there is an association of alleles of the NALP1 gene with human congenital toxoplasmosis. Furthermore, in the present study, we endeavored to elucidate the role of NALP1 in the pathogenesis of *T. gondii* infection in a human monocytic cell line. We provide evidence that silencing of expression of the NALP1 gene by RNA interference (RNAi) alters the *T. gondii*-induced expression profiles of certain proinflammatory cytokines and increases the progression rate of *T. gondii* infection in a human monocytic cell line.

MATERIALS AND METHODS

Patient cohort and genotyping of NALP1. Case-parent trios were from the National Collaborative Chicago-Based Congenital Toxoplasmosis Study (NCCCTS), as previously described (18). Briefly, the NCCCTS consisted of 179 North American children (clinically diagnosed with congenital toxoplasmosis) and their parents. Of the 179 infected children, 124 (83%) had clinically confirmed brain calcifications, with or without hydrocephalus and/or retinal lesions, at birth or the time of diagnosis. DNAs were extracted successfully from 149 case-parent samples and genotyped at 23 single-nucleotide polymorphism tags (tag-SNPs) distributed throughout the NALP1 gene. The tag-SNPs were selected from the HapMap project, release 21 (<http://www.hapmap.org>), using 10 kb of flanking sequence on each side of the NALP1 gene, a minor allele frequency (MAF) cutoff of 5% in CEU, and an r^2 threshold of 0.8. The Tagger tool within Haploview (<http://www.broad.mit.edu/mpg/haploview/>) was used to select tag-SNPs. Allelic association analysis was performed for the 124 infected children in the cohort with confirmed clinical findings for the eye and/or brain, using UNPHASED (<http://www.mrc-bsu.cam.ac.uk/personal/frank/software/unphased/>).

Plasmid constructs. Short hairpin RNA (shRNA) sequences for the human NALP1 gene coding sequence (GenBank accession no. NM_001033053) and the *Salmonella enterica* tetracycline repressor (TetR) gene coding sequence (GenBank accession no. NC_006856) were designed for cloning into a Gateway-adapted entry vector (Invitrogen). For the NALP1 coding sequence, the sense and antisense shRNA sequences were 5'-caccAACAGACATGGAGCTCTTAGTGTGCACTTcgaaAAGTGCACACTAAGAGCTCCATGTCTG-3' and 5'-aaaaCAGACATGGAGCTCTTAGTGTGCACTTctcgAAGTGCACACTAAGAGCTC CATGTCTGTT-3' (the first four nucleotides in lowercase facilitate directional

cloning, the sequence in bold uppercase is the target sequence, the subsequent italicized lowercase sequence is for loop formation, and the underlined sequence is the antisense sequence for the target sequence), respectively. For the TetR coding sequence, the sense and antisense shRNA sequences were 5'-caccAAGCGCCGACGCGCAGCTTCGCTCGGCGTA-3' and 5'-aaaaTACGGCCGACGCGCAGCTTCGCTTCCTCTGtcgCAGAGGAAGCGAAGCTGCGCGTTCGCGTTC-3', respectively. For NALP1 and TetR, the target sequences were 2,238 to 2,265 bp and 3,360 to 3,389 bp of the coding sequences, respectively. To clone double-stranded shRNA sequences into the entry vector (pENTR/H1/TO), the sense and antisense shRNA sequences were annealed *in vitro* and ligated directionally into the pENTR/H1/TO entry vector, essentially as described in the manufacturer's user manual (17a). Upon propagation of the recombinant plasmids, the shRNA inserts were confirmed by sequencing. The recombinant plasmids were used in LR recombination reactions to transfer the shRNA into the expression vector pLenti4/Block-iT-DEST following the manufacturer's instructions (17b). The recombinant destination plasmids were propagated in *Escherichia coli*, extracted, and sequenced to ascertain that the shRNA inserts were correct.

Generation of NALP1- and TetR-expressing lentiviruses. To produce lentiviral stocks, the pLenti4/Block-iT-DEST vector carrying the shRNA of interest (NALP1 or TetR) and optimized ViraPowerPackaging mix (containing plasmids pLP1, pLP2, and pLP/VSVG, which facilitate viral packaging) were transfected into 293 FT cells following the manufacturer's instructions (Invitrogen). The transfected cells were cultured for 72 h in complete Dulbecco's modified Eagle's medium (DMEM; 10% fetal calf serum, 2 mM L-glutamine, 0.1 mM MEM nonessential amino acids, 1 mM sodium pyruvate, 1% penicillin-streptomycin). The virus-containing supernatants were harvested by centrifugation and stored in aliquots at -70°C. The viral yield was determined by titration following the instructions outlined in the user manual (17c).

Establishment of human monocytic cell lines for stable expression of NALP1 or TetR shRNA. To establish a human monocytic cell line stably expressing either NALP1 or TetR shRNA, *in vitro* culture-adapted human monocytes, i.e., MonoMac6 cells (41), were used. MonoMac6 cells were seeded at 4×10^5 /well in a 24-well plate in RPMI medium supplemented with 10% fetal calf serum, 2.05 mM L-glutamine, $1 \times$ nonessential amino acids (Sigma), OPI medium supplement (Hybri Max; Sigma), and 1% penicillin-streptomycin. About 250 μ l of the NALP1 or TetR shRNA-expressing lentiviral stock (3×10^6 transducing units [TU]/ml) was added to the cultures and mixed. Untransduced cell cultures were maintained as controls. The cells were maintained at 37°C with 5% CO $_2$. The following day, Zeocin (Invitrogen) was introduced into the cultures at 100 μ g/ml to select for transduced cells. The cultures were maintained by changing the medium (with Zeocin) every 2 days continuously for about 3 weeks, when cells resistant to Zeocin started propagating.

Analysis of RNAi-based knockdown of NALP1 gene transcription. To determine the effect of lentivirus-expressed NALP1 shRNA on the silencing of NALP1 gene expression via RNAi in MonoMac6 cells, triplicate sets of MonoMac6 cells engineered to express either NALP1 or TetR gene shRNA and of wild-type cells were seeded at 1×10^5 cells per well in a 12-well plate and cultured for 72 h. The cells were harvested by centrifugation, and total RNA was extracted by use of Trizol reagent. Exactly 2 μ g of RNA for each sample was treated with DNase I to remove residual genomic DNA, and reverse transcription (RT) was performed using SuperScript III first-strand synthesis supermix for quantitative RT-PCR (qRT-PCR) (Invitrogen). The primer set used for the NALP1 gene was NALP1-F and NALP1-R (Table 1). This set of primers amplified a 280-bp fragment of the NALP1 open reading frame. Primers for the human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene used for real-time PCR were GAPD-F and GAPD-R (Table 1). This set amplified a 300-bp fragment of the open reading frame of the human GAPDH gene. Both the NALP1 and GAPDH fragments were synthesized by conventional PCR from cDNA and then gel extracted, and serial 10-fold dilutions were made as templates to generate a quantification standard curve. The real-time PCR mix consisted of 1 μ l of cDNA template, 1 μ l of primer mix (500 nM [each]), and 10 μ l of SsoFast EvaGreen supermix (Bio-Rad), with the final volume made up to 20 μ l with RNase- and DNase-free water. The cycling conditions included an initial denaturation for 30 s at 95°C, 40 cycles of 95°C for 5 s, 57°C for 5 s, and 60°C for 10 s, and a final cooling step to 40°C. Cycling was performed using a CFX 96 real-time system (Bio-Rad), and transcript quantities were derived by the system software, using the generated standard curves.

Western blot analysis of NALP1 protein expression. MonoMac6 cells engineered to express either NALP1 or TetR gene shRNA and wild-type cells were seeded at 4×10^5 cells per well in a 6-well plate and cultured for 72 h. The cells were harvested by centrifugation, and the pellet was washed twice in phosphate-buffered saline (PBS) and lysed in SDS sample buffer. Equal protein amounts

TABLE 1. Primers used in this study

Primer	Sequence (5'-3')
NALP1-F	ATTCCACCACAGCTGCTACTTCTACAAAGA
NALP1-R	TCTGCAGCTGAAGTAGAAGACATGCT
GAPD-F	TTCATTGACCTCAACTACATGGTTTACA
GAPD-R	GTGATGGCATGGACTGTGGTCATGAGT
IL-18-F	TCATTGACCAAGGAAATCGGCCTCT
IL-18-R	CATGTCCTGGGACACTTCTCTGAAAGA
IL-1β-F	TGGACAAGCTGAGGAAGATGCTGGT
IL-1β-R	AGGACATGGAGAACACCACTTGTGCT
IL-12A-F	ACCACCTCAGTTTGGCCAGAAACCT
IL-12A-R	AGGCCAGGCAACTCCCATTAGTTATGA
TNF-α-F	TCTTCTCCTTCTGATCGTGGCA
TNF-α-R	ACCACCATGGTATCTCTCA
IFN-γ-F	TGGCTGTTACTGCCAGGACCCATATGT
IFN-γ-R	TGCGTTGGACATTCAAGTCAGTTACCGA
hACT-F	TATGGTCAGAAAGATTCTACGT
hACT-R	TCTTCATCAGGTATCGGTAGAT
BCL2A-F	AATGTTGTGTCCGTAGACACTGCCAGAA
BCL2A-R	AGAAAAGTCACCAGCCAGATTTAGGTT
Casp-F	AGCTATGCCACATCCTCAGGCTCAGA
Casp-R	AAATGCCTCCAGCTCTGTAGTCATGT

were loaded into wells of a 12% SDS-polyacrylamide gel and fractionated by electrophoresis. The proteins were transferred to nitrocellulose membranes, and immunoblotting was done using either rabbit anti-NALP1 (Sigma) or mouse anti-GAPDH (LifeSpan Biosciences) antibodies as primary antibodies, with conjugated goat anti-rabbit and anti-mouse antibodies as the secondary antibodies. Signal generation was performed using an ECL chemiluminescence kit (PerkinElmer Life Sciences).

Cell viability assay. To study the effect of NALP1 gene knockdown on the viability of human monocytic cell cultures during infection with *T. gondii*, wild-type MonoMac6 cells and those genetically modified for the stable knockdown of the NALP1 or TetR gene were seeded in triplicate into 96-well plates at a density of 10^4 cells/well and cultured in 200 μ l of supplemented RPMI medium in the presence or absence of *T. gondii* strain RH parasites at a multiplicity of infection (MOI) of 1:4 (parasite:monocyte) for different times (0, 1, 2, 3, and 4 days). Wells containing parasites only, without MonoMac6 cells, were also set up as controls. A colorimetric assay using the cell proliferation reagent WST-1 (Roche) for the quantification of cell viability was performed on the cultures at different time points by adding 10 μ l of the WST-1 reagent to each well. After mixing of the samples, the plates were wrapped in aluminum foil and incubated for 1 h at 37°C with 5% CO₂. Three independent experiments were performed, with triplicate samples for each experiment, and quantification of the formazan dye produced by metabolically active cells was read as the absorbance at a wavelength of 420 nm, using a scanning multiwell spectrophotometer (Spectra Max 250; Molecular Devices).

Preparation and microscopic analysis of Giemsa-stained MonoMac6 smears. To determine the effect of NALP1 gene knockdown on monocytic cell viability when cells were cultured with or without parasites, wild-type MonoMac6 cells or those genetically modified for the stable knockdown of the NALP1 or TetR gene were seeded into 24-well plates at a density of 10^5 cells/well and cultured with or without parasites at an MOI of 1:4 (parasites:monocytes) for different times (0, 1, 2, 3, and 4 days). At 0, 1, 2, 3, and 4 days postinfection, 50 μ l of the thoroughly mixed culture was used to prepare cytospin smears on glass slides. The slides were air dried and fixed in amino acridine, followed by Giemsa staining and light microscopic examination. The number of infected cells per microscopic field was counted using a $\times 20$ objective and derived as a percentage of the total number of cells per field. For each sample, a total of 50 microscopic fields per time point were counted, and the data are presented as the mean percentages of infected cells for 50 fields. Furthermore, the average number of parasites in one parasitophorous vacuole of an infected cell was determined for 20 infected cells per field, with a total of 10 fields being counted per time point.

Quantitation of expression of cytokines, BCL2A1, and caspase-1. To determine the effect of NALP1 gene knockdown in MonoMac6 cells on the expression of the cytokines IL-18, IL-1β, IL-12A, tumor necrosis factor alpha (TNF-α), and gamma interferon (IFN-γ), as well as that of the BCL2-related protein A1 (BCL2A1) and caspase-1, triplicate sets of wild-type MonoMac6 cells and MonoMac6 cells engineered to express either NALP1 or TetR shRNA were cultured with or without parasites at an MOI of 1:4 for 36 h. The cells were

harvested, and either total RNA was extracted for transcript analysis or total protein lysates were prepared for Western blotting of cytokine protein expression. Total RNA was extracted by use of Trizol reagent, followed by first-strand cDNA synthesis as described above. Quantitative real-time PCR was performed on each sample of cDNA to determine the transcript levels. Primers used were designed to amplify a fragment of approximately 300 bp for the coding sequences of IL-18, IL-1β, IL-12A, TNF-α, IFN-γ, BCL2A1, and caspase-1 (GenBank accession numbers NM_001562, NM_000576, NM_000882, NM_000594, NM_000619, BT007103, and NM_033294, respectively). Primer sets for IL-18, IL-1β, IL-12A, TNF-α, IFN-γ, BCL2A1, and caspase-1 were IL-18-F and IL-18-R, IL-1β-F and IL-1β-R, IL-12A-F and IL-12A-R, TNF-α-F and TNF-α-R, IFN-γ-F and IFN-γ-R, BCL2A-F and BCL2A-R, and Casp-F and Casp-R, respectively (Table 1). The respective gene fragments were amplified by conventional PCR from the cDNA and sequenced to confirm their identity. The real-time PCR mixture and cycling conditions were essentially as described above. The transcript levels of human GAPDH (as described above) and human actin (GenBank accession number NM_001100) were determined for each sample and used for normalization. The primer set for human actin was hACT-F and hACT-R (Table 1). Western blotting was performed on equal amounts of lysate protein, using antibodies generated against human IL-1β and GAPDH.

Statistical analysis. Statistical analyses for all *in vitro* assays were performed using two-tailed Student's *t* test. For the genetic study, allelic association analysis was performed using conventional TDT to determine the linkage disequilibrium (LD). LD is the nonrandom association of alleles at two or more loci. It is essentially an approximation of the existence of historical recombination between 2 loci. *P* values were calculated using Haploview (<http://www.broadinstitute.org/haploview>). *P* values of 0.05 or less were considered significant for association with disease.

RESULTS

Congenital toxoplasmosis associates with NALP1 in humans. To test the association of NALP1 with congenital toxoplasmosis, we genotyped a total of 23 tag-SNPs that were selected in the human NALP1 gene in a North American cohort of case-parent trios comprising 124 congenitally infected children, many with ocular and/or brain disease, using an *r*² threshold of 0.8. SNPs with a call rate of >90% and in HWE in the parents were kept for further analysis.

Among the SNPs tested, NALP1 rs8081261 and rs11652907 associated with congenital toxoplasmosis (*P* < 0.00268 and *P* < 0.02, respectively) (Fig. 1). An etiological variant of SNPs in strong LD with these markers could account for the observed association with susceptibility to this congenital disease.

Establishment of human monocytic cell lines stably expressing NALP1 and TetR gene shRNAs. To generate human monocytic cell lines that stably expressed either NALP1 or TetR gene shRNA for targeted knockdown of the respective gene by RNAi, lentiviral stocks expressing these shRNAs were engineered and titrated and found to be present at approximately 3×10^6 TU/ml, sufficient to transduce about 1×10^7 cells at an MOI of 1. The NALP1 shRNA-expressing lentivirus was used for targeted silencing of the endogenous NALP1 gene in monocytic cells, while the TetR shRNA was generated as an off-target control. The design of the promoter region that drives the expression of the target gene shRNA (Fig. 2A) facilitates tetracycline-based activation of shRNA expression in a cell line expressing a tetracycline repressor protein. However, in the absence of the tetracycline repressor protein, the transduced cell line would constitutively express the target gene shRNA. We therefore first conducted a preliminary study to determine the effect of constitutive expression of NALP1 or TetR shRNA in MonoMac6 cells lacking the tetracycline repressor expression vector. We found that this (lack of tetracycline repressor protein regulation of promoter activity) did not

FIG. 1. Analysis of NALP1 SNPs. The upper diagram shows the positions of genotyped SNPs relative to the intron-exon structure of the gene. The lower diagram shows the LD plots generated in Haploview, using NALP1 gene data from our North American patient cohort. LD values ($D' \times 100$) between markers are indicated at the intercept of the 2 markers on the matrix. Where there is no value, $D' = 1$ (i.e., 100). In outlining gene SNP associations with susceptibility to congenital toxoplasmosis, D' values between loci were calculated and displayed using Haploview. Where a high confidence for the value of D' existed (LOD score [decimal logarithm of the odds of linkage between two loci] of ≥ 2), shades of pink and bright red were used. If the confidence was low (LOD score of < 2), blue shading ($D' = 1$) or no shading ($D' < 1$) was used.

affect the morphological appearance or viability of the monocytic cells (data not shown). Based on these observations, we then generated stable monocytic cell lines (lacking the tetracycline repressor expression vector) constitutively expressing either NALP1 or TetR shRNA. These cells did not require the use of tetracycline for expression of the shRNA. Stable cell lines were achieved by taking advantage of the presence of an *Sh ble* gene in the lentivirus shRNA expression plasmid whose product confers resistance to the antibiotic Zeocin. Upon transduction with the lentivirus, monocytic cells were cultured in the presence of Zeocin at 100 $\mu\text{g/ml}$. This resulted in the majority of the cells dying in about 2 weeks, after which Zeocin-resistant cells started propagating and attained a wild-type growth rate after a further 2 weeks.

Expression of NALP1 shRNA effectively silences endogenous NALP1 gene expression in human monocytic cells. To assess the effectiveness of the lentivirus-expressed NALP1 shRNA in silencing the expression of NALP1 gene expression in monocytic cells, triplicate sets of wild-type MonoMac6 cells and those engineered to express either NALP1 gene shRNA or TetR shRNA were cultured for 72 h, after which total RNA was extracted and reverse transcribed. Cell viability was the

same in the two transduced cell lines and was compatible with the cell viability of the nontransduced cells. Quantitative real-time PCR analysis of the NALP1 gene transcripts normalized to human GAPDH transcripts showed a significant ($P < 0.001$) reduction (~ 6 -fold) in the NALP1 gene transcripts in MonoMac6 cells expressing NALP1 shRNA compared to the wild-type cells as well as those expressing TetR shRNA (Fig. 2B). To correlate this effect to NALP1 protein levels, protein extracts from cells treated as described above were analyzed by Western blotting, using anti-human NALP1 and anti-human GAPDH. While there was no notable difference in the detectable protein signals for GAPDH among all samples, there was a significant reduction in the detectable NALP1 protein signal in MonoMac6 cells expressing NALP1 shRNA (Fig. 2C). These results corroborated the quantitative real-time PCR analysis and showed that NALP1 gene silencing was achieved effectively.

NALP1 knockdown reduces viability of human monocytic cells *in vitro* during infection with *T. gondii*. To study if NALP1 gene knockdown affects the progression of *T. gondii* infection in human monocytic cells, the viability of *in vitro*-cultured wild-type or NALP1 or TetR shRNA-expressing MonoMac6 cells

FIG. 2. Analysis of the effect of NALP1 and TetR shRNAs on expression of NALP1. (A) Illustration of the promoter features of the pLenti4/Block-iT-DEST shRNA expression vector. In the absence of a Tet repressor protein, there is constitutive expression of the cloned NALP1 or TetR shRNA fragment. (B) Quantitative real-time PCR analysis of NALP1 transcript levels in wild-type MonoMac6 cells (MC) and MonoMac6 cells engineered to express either NALP1 gene shRNA (N-KO) or tetracycline repressor gene shRNA (TetRep) after 72 h of culture. The relative transcript levels of NALP1 were derived by dividing the obtained NALP1 value by the GAPDH value for each respective sample. The data shown are means for three independent experiments with standard error bars. The reduction in NALP1 transcript levels in N-KO cells was found to be significant (**, $P < 0.001$) compared to both the wild-type and TetR shRNA-expressing cells. (C) Western blotting of NALP1 protein expression in N-KO, TetRep, and MC cells was performed on cell lysates after 72 h of culture, using anti-NALP1 antibody. A similar blot was hybridized with anti-GAPDH primary antibody to check for loading. The NALP1 protein was detectable as an ~155-kDa band.

was analyzed at different time points of culture, using a colorimetric cell viability (WST-1 reagent-based) assay and a microscopic assay. The cell viability reagent WST-1 relies on the cleavage of tetrazolium salts to formazan by cellular mitochondrial dehydrogenases in the sample. The augmentation in dehydrogenase activity leads to an increase in the amount of formazan dye formed, which directly correlates with the number of metabolically active cells in the culture (3).

The absorbances obtained from the *T. gondii*-infected MonoMac6 cells were normalized using the absorbances obtained from the respective uninfected MonoMac6 cells at each time point, and these data are presented as relative cell viability values (infected A_{420} /uninfected A_{420}). At day 0 (immediately after establishing the cultures), there were no significant differences in cell viability among the infected wild-type, NALP1 knockdown, and TetR shRNA-expressing cells (Fig. 3A). Interestingly, after 1 day of culture, while the relative viability of the infected wild-type and TetR shRNA-expressing MonoMac6 cells had increased, the relative viability of the infected NALP1 knockdown cells was reduced significantly. On subsequent days of culture, this reduction in viability of the infected NALP1 knockdown cells was more pronounced, with almost no cell viability being observed by the fourth day of culture. In contrast, the numbers of infected wild-type and TetR shRNA-expressing cells started to decrease from day 2 of culture, but the relative cell viability was sustainably and significantly higher than that of the NALP1 knockdown cells at all subsequent days (Fig. 3A). The values for the assay performed on wells containing parasites only (without MonoMac6 cells) were insignificant and declined rapidly over time (data not

shown), suggesting that in the infected cultures, almost all of the dehydrogenase enzyme activity was from MonoMac6 cells.

To visualize the state of the cells in culture and to assess how the relative cell viability values correlated with the state of the cells in culture, wild-type, NALP1 knockdown, and TetR shRNA-expressing MonoMac6 cells were cultured with or without parasites under the same conditions as those described above, except that the culture volumes were increased to 1 ml in 24-well plates. Cytospin Giemsa-stained smears made from these cultures at different time points showed morphologically intact cells with similar amounts of cells (in all categories of MonoMac6 cells) for both the infected and uninfected cells at day 0 of culture (Fig. 4). At 1 day postculture, infected NALP1 knockdown cells appeared sparse, while the rest of the cell categories looked about the same (with approximately the same density). By day 2 postculture, infected NALP1 knockdown cells had become sparser and were disintegrating. On the other hand, despite the presence of intracellular parasites in some of the infected wild-type and TetR shRNA-expressing cells, the cells looked relatively denser and morphologically normal, in contrast to the infected NALP1 knockdown cells. By day 3 postculture, the infected NALP1 knockdown cell cytospin preparations contained mostly cell debris, and by day 4, there were almost no observable morphologically intact MonoMac6 cells and only a small number of sparsely distributed extracellular parasites.

Interestingly, while there was a gradual increase in the number of infected cells in the preparations of the wild-type and TetR shRNA-expressing cells at subsequent days, the cells remained mostly intact and dense, except for days 3 and 4,

FIG. 3. Effect of NALP1 knockdown on viability of human monocytic cells during infection with *T. gondii* (A), rate of host cell infection (B), and rate of parasite multiplication within a parasitophorous vacuole in an infected cell (C). Wild-type MonoMac6 cells (solid lines with circles) and those genetically modified for stable knockdown of NALP1 (dotted lines with squares) or tetracycline repressor (dashed lines with rhombuses) gene transcription were cultured with or without parasites. The relative cell viability values were derived by dividing the absorbance obtained from the infected cells by that of the uninfected cells for each respective cell line, while the percent infected cells per field was derived as an average percentage of infected cells for 50 microscopic fields. The mean number of parasites per vacuole was determined as the average number of parasites in one parasitophorous vacuole for 200 infected cells. The data shown represent means for three independent experiments with standard error bars, and levels of statistical significance are depicted by asterisks (*, $P < 0.05$; **, $P < 0.001$).

when the cell intensities decreased notably but were still denser than that of the infected NALP1 knockdown culture (Fig. 4). The uninfected cell cultures in all categories remained dense and morphologically intact at subsequent days of culture, except at day 4, when they were observed to be less dense, though the majority of the cells still appeared morphologically intact. These microscopic observations of cell culture progression provided visual evidence indicating that the decrease in cell viability (observed using the WST-1 assay) correlated with MonoMac6 cell death and that the results of both assays were consistent. Also, as can be seen in the representative images shown in Fig. 4 (from one of three independent replicate studies), there were more parasites in the NALP1 knockdown cells and cultures than in the NALP1-sufficient cells.

To assess the effect of NALP1 knockdown on the rate of progression of infection, the number of infected cells as well as the number of parasites in one parasitophorous vacuole of an infected cell at each time point was determined. Starting from day 1 postinfection, the rate of MonoMac6 cell infection with NALP1 knockdown was found to be significantly higher than those for both the wild-type and TetR shRNA-expressing cells (Fig. 3B). Furthermore, infected MonoMac6 cells with NALP1 knockdown were observed to contain a larger number of parasites per parasitophorous vacuole than the NALP1-sufficient cells (Fig. 3C).

NALP1 knockdown modulates *T. gondii*-induced proinflammatory cytokine expression in MonoMac6 cells. To assess the effect of NALP1 knockdown on the expression of proinflammatory cytokines in human monocytic cells during infection with *T. gondii*, wild-type MonoMac6 cells and MonoMac6 cells engineered to express either NALP1 gene shRNA or tetracycline repressor gene shRNA were cultured with or without parasites at an MOI of 1:4 (parasite/MonoMac6 cell ratio) for 36 h. The cells were harvested and total RNA extracted and reverse transcribed to synthesize first-strand cDNA. Quantitative real-time PCR was performed on each sample of the cDNA to determine the transcript levels of the IL-1 β , IL-18,

TNF- α , IL-12, human actin, and GAPDH genes. The transcript values obtained for IL-1 β , IL-18, TNF- α , IFN- γ , and IL-12 were normalized interchangeably with either human GAPDH or human actin transcript levels. When GAPDH was used for normalization, for all infected wild-type and TetR shRNA-expressing MonoMac6 cells, IL-1 β , IL-18, TNF- α , and IL-12 were found to be significantly higher than the levels in noninfected cells (Fig. 5A to D). The results were found to be consistent when human actin was used for normalization (see Fig. S1 in the supplemental material).

Interestingly, in both infected and uninfected MonoMac6 cells with NALP1 knockdown, IL-1 β expression was found to be lower than that in the uninfected wild-type or TetR shRNA-expressing MonoMac6 cells (Fig. 5A). Moreover, there was no notable significant difference in IL-1 β transcript levels between the infected and uninfected NALP1 knockdown MonoMac6 cells (Fig. 5A), indicating that knockdown of NALP1 by itself dampens IL-1 β transcription. In the case of IL-18 and IL-12 expression, while the transcript levels in the infected cells were about the same as those in the uninfected NALP1 knockdown cells, there was no observable dampening of the background expression (Fig. 5B and D). Specifically, IL-18 and IL-12 transcript levels in the uninfected NALP1 knockdown cells were similar to those in the uninfected wild-type and TetR shRNA-expressing cells (Fig. 5B). The expression of TNF- α was found to be consistently higher for all infected cell categories than for the noninfected cells (Fig. 5C), although transcript levels in the infected NALP1 knockdown cells were slightly lower (though the difference was not statistically significant) than those in the infected wild-type and TetR shRNA-expressing cells. This suggested that the activation of TNF- α expression during *T. gondii* infection was also affected, to some extent, by the lack of NALP1 inflammasome activity. For all cell categories, transcripts of IFN- γ could not be detected (data not shown), which was expected because the cell cultures consisted of monocytic cells only, with no T cells.

To ascertain that the observed low cytokine transcript levels

FIG. 4. Giemsa-stained preparations of monocytic cells examined by light microscopy to determine the effect of NALP1 gene knockdown on monocytic cell viability when cells were cultured with or without parasites. Preparations of monocytic cells genetically modified for stable knockdown of NALP1 (NALP1 KO) or tetracycline repressor (TetRep KO) gene transcription and of wild-type cells are shown at different days postinfection (DPI). Images are representative of three independent experiments, with red arrowheads depicting parasite-laden cells.

in infected NALP1 knockdown cells were not simply a consequence of a global reduction in transcription due to reduced cell viability, the transcript levels of the *BCL2A1* gene, whose transcription has been shown to be increased greatly by *T. gondii* infection in mammalian cells (12), were measured. For both the NALP1 knockdown and NALP1-sufficient MonoMac6 cells, *BCL2A1* transcripts were found to be significantly higher in infected cells than in uninfected cells (Fig. 6A). This result indicated that the reduction in cytokine expression was a result of NALP1 knockdown and not necessarily because of a global reduction in transcription.

IL-1 β protein accumulates as pro-IL-1 β coding transcripts during NALP1 knockdown. To determine the effect of NALP1 knockdown on the expression of mature IL-1 β , *T. gondii*-infected and uninfected wild-type, TetR shRNA-expressing, and NALP1 knockdown monocytic cells were cultured for 36 h. The cells were harvested, and Western blotting was performed on the total cell lysates. While mature IL-1 β was detectable as a strong signal of approximately 17 kDa in the *T. gondii*-infected wild-type and TetR shRNA-expressing cell lysates, it was barely detectable in the infected NALP1 knockdown cell

lysates. Interestingly, in these lysates, in addition to the weak signal of mature IL-1 β (17 kDa), there was a signal at ~35 kDa consistent with pro-IL-1 β (Fig. 7).

To assess the direct role of NALP1 knockdown on the processing of pro-IL-1 β , the transcript levels of caspase-1 (a proteolytic enzyme that is activated by the NALP1 inflammasome to cleave and activate the secretion of mature IL-1 β and IL-18) were determined. While the transcript levels of caspase-1 were found to be increased significantly in infected cells expressing NALP1, those in infected NALP1 knockdown cells remained at essentially the same level as in the uninfected cells (Fig. 6B). These results indicated that the lack of processing of pro-IL-1 β to mature IL-1 β in NALP1 knockdown cells was because of the lack of caspase-1 activation by the NALP1 inflammasome.

DISCUSSION

Mammalian monocytes, a pleomorphic and pleiotropic population of circulating mononuclear cells, contribute to microbial defense by supplying tissues with macrophages and dendritic cell precursors (25, 33). *T. gondii* is an obligate

FIG. 5. Analysis of effects of NALP1 gene knockdown on expression of proinflammatory cytokines (IL-1 β , IL-18, TNF- α , and IL-12) in a human monocytic cell line (MonoMac6). Quantitative real-time PCR was performed on cDNAs synthesized using equal amounts of total RNA from wild-type MonoMac6 cells (MC) and MonoMac6 cells engineered to express either NALP1 gene shRNA (NALP1-KO) or tetracycline repressor gene shRNA (TetR) that had been cultured with (gray columns) or without (white columns) parasites for 36 h. The transcript values for IL-1 β (A), IL-18 (B), TNF- α (C), and IL-12 (D) were normalized by dividing the observed value by the value for GAPDH for each respective sample to derive the relative gene transcript level. The significance of augmentation of cytokine expression attributed to infection is shown by asterisks (*, $P < 0.05$; **, $P < 0.001$). The data are shown as means for three independent experiments with standard error bars.

intracellular parasite that resides in a vacuole in many different nucleated cell populations. Macrophage recruitment has been shown to be essential for initial restriction of *T. gondii* growth in murine models of toxoplasmosis and in human monocytes (2, 38). This restriction of parasites in murine macrophages is

mediated through MyD88 signaling and through induction of IL-12 and IFN- γ (24, 39). NALP1 has been shown to be important in susceptibility to a variety of intracellular pathogens invading mononuclear phagocytic cells (8, 36). In the present study, we found that NALP1 has alleles associated with sus-

FIG. 6. Analysis of effects of NALP1 gene knockdown on expression of caspase-1 and BCL2A1 in MonoMac6 cells infected with *T. gondii*. Quantitative real-time PCR was performed on cDNAs synthesized from equal amounts of total RNA from wild-type MonoMac6 cells (MC) and MonoMac6 cells engineered to express either NALP1 gene shRNA (NALP1-KO) or tetracycline repressor gene shRNA (TetR) that had been cultured with (gray columns) or without (white columns) parasites for 36 h. The obtained transcript values for caspase-1 (A) and BCL2A1 (B) were normalized by dividing the observed value by the value for GAPDH for each respective sample to derive the relative gene transcript level. The significance of the levels of augmentation of caspase-1 and BCL2A1 expression attributed to infection is shown by asterisks (**, $P < 0.001$). The data are shown as means for three independent experiments with standard error bars.

FIG. 7. Western blot of the effect of NALP1 knockdown on the expression of IL-1 β in MonoMac6 cells with or without *T. gondii* infection for 36 h. Wt, wild-type MonoMac6 cells; Tet, MonoMac6 cells engineered to express tetracycline repressor gene shRNA; KO, MonoMac6 cells expressing NALP1 gene shRNA. The pro-IL-1 β protein was detectable as an ~35-kDa band, while mature IL-1 β was detectable as an ~17-kDa band. The GAPDH protein band is shown as a loading check.

ceptibility to congenital toxoplasmosis. To facilitate the elucidation of the role played by NALP1 and/or the NALP1 inflammasome during infection with *T. gondii*, we successfully genetically engineered human monocytic cells for NALP1 gene knockdown by RNAi.

Our studies to determine the outcome of *T. gondii* infection in monocytic cells showed that NALP1 knockdown attenuated the progression of the infection, with accelerated loss of cell viability and eventual disintegration of monocytic cells. Consistent with these findings, *T. gondii* infection of monocytic cells with NALP1 knockdown could not induce upregulation of the proinflammatory cytokines IL-1 β , IL-18, and IL-12. Intracellular accumulation of pro-IL-1 β has been reported to be regulated transcriptionally, presumably via NF- κ B and mitogen-activated protein (MAP) kinase signaling (15, 16). An initial pathogen stimulus generated via PRRs causes accumulation of the intracellular stores of pro-IL-1 β (5). The preceding event involves the intracellular pathogen activation of inflammasome assembly, leading to the activation of the cysteinyl aspartate protease caspase-1 and the cleavage of proforms of IL-1 β and IL-18, enabling the release of active mature IL-1 β and IL-18 (27).

In our study, we observed upregulated expression of IL-1 β transcripts in *T. gondii*-infected monocytic cells expressing NALP1, with a concomitant increase in the amount of mature IL-1 β protein, consistent with reports that intracellular pathogens activate inflammasomes (5). On the other hand, our *T. gondii*-infected monocytic cells with NALP1 knockdown did not show upregulation of IL-1 β transcript levels, nor did they show an increase in the amount of mature IL-1 β produced, suggesting that the NALP1 inflammasome plays a significant role in the maturation of IL-1 β under these circumstances. Interestingly, we found that *T. gondii* infection did not lead to increased transcription of caspase-1 in NALP1 knockdown cells, implying that the NALP1 inflammasome plays a critical

role in the processing and activation of caspase-1, which in turn leads to maturation of IL-1 β and IL-18. Upon production, mature IL-1 β activates its cognate receptor, the IL-1 receptor, which signals through the MyD88 adaptor leading to the initiation of the NF- κ B and MAP kinase pathways, with the ultimate expression of inflammatory cytokines that then fight infection (4, 9, 10). Our findings are in line with this report, in that the lack of mature IL-1 β production in infected NALP1 knockdown cells was also associated with decreased expression of IL-12, IL-18, and TNF- α (important inflammatory cytokines in combating this infection), indicating that the initiation of NF- κ B and MAP kinase pathways via IL-1 β is crucial during *T. gondii* infection.

While the lack of an increase in mature IL-1 β was expected to be due to the absence of NALP1 inflammasome activation because of NALP1 knockdown, the absence of IL-1 β transcript upregulation was striking and unexpected based on what is known about NALP1 in other systems to date. To protect against *T. gondii* infection, as stated above, an initial *T. gondii* stimulus generated via PRRs would cause an accumulation of intracellular stores of pro-IL-1 β transcripts that would then be translated into pro-IL-1 β protein. Because of the lack of NALP1 inflammasome activation of caspase-1, which in turn led to a lack of processing and secretion of mature IL-1 β (because of NALP1 knockdown), pro-IL-1 β accumulated, and this might have exerted negative feedback on the transcription of pro-IL-1 β . This could explain the low level of pro-IL-1 β transcripts in infected NALP1 knockdown cells. This diminished transcription was not due to a global cellular reduction in transcription, since equal amounts of total RNA were used to synthesize cDNA for each cell type (wild type and knockdown). Furthermore, analysis of the transcript levels of the BCL2A1 gene, whose transcription has been shown to be increased greatly by *T. gondii* infection in mammalian cells (12), showed equal augmentation of BCL2A1 transcription in both NALP1 knockdown and NALP1-sufficient cells, implying that the reduction in cytokine expression was a result of NALP1 knockdown, not a global reduction in transcription.

Transcripts for IL-18 were also found not to be upregulated in infected NALP1 knockdown cells. Just like that of pro-IL-1 β , pro-IL-18 transcription can be induced via NF- κ B, mediated by signaling through Toll-like receptors (TLRs) (22). TLR signaling then primes the assembly of caspase-1-containing inflammasomes that process the proforms of IL-1 β and IL-18, two crucial proinflammatory cytokines (9). It can be postulated that in a manner similar to that for pro-IL-1 β , the reduced inflammasome activity due to NALP1 knockdown leads to an accumulation of pro-IL-18 protein that may impart negative-feedback inhibition on the transcription of IL-18. It is noteworthy that, to date, three NLRs, namely, NALP1, NALP3, and Apaf, have been reported to form caspase-1-activating inflammasomes and therefore to be involved in the processing of the proforms of IL-1 β and IL-18 (20, 22). Our finding that the knockdown of NALP1 significantly reduces the levels of mature IL-1 β protein and the transcripts of both proforms of IL-1 β and IL-18 suggests that the NALP1 inflammasome is a major player in the processing of these cytokines during *T. gondii* infection.

We found that while infected monocytic cells with NALP1 knockdown had an accelerated loss of cell viability and cell

disintegration, the infected wild-type cells had a gradual progression of parasite growth and host cell death, suggesting that the NALP1 inflammasome is critical for mediating the inhibition and killing of *T. gondii* in human monocytic cells. As is the case with other intracellular pathogens, *T. gondii* has been shown to prominently modulate host cell apoptosis, which may be critical for the course of infection. *T. gondii* has been reported to exert opposite effects on the host cell death program by either triggering (14, 29) or inhibiting (30) apoptosis. Based on our findings and the available literature, it can be postulated that activation of the NALP1 inflammasome triggers a cascade of innate immune mechanisms, including production of proinflammatory cytokines and mechanisms leading to death of infected cells. Such mechanisms could include activation of pyroptosis and apoptosis. This would thereby limit parasite proliferation and infection of other cells. This would not take place in infected monocytic cells with NALP1 knockdown, resulting in the uncontrolled proliferation of parasites and, ultimately, in more rapid death of host cells than that of wild-type cells. The proinflammatory response could thus be beneficial. Alternatively, if the proinflammatory response is too robust, it might be harmful to the host. Such harm does not necessarily correlate with a high parasite burden.

Underlying the success of *T. gondii* infection is a delicate balance between the host's immune response and the parasite's modulation of many of the intricate mechanisms that the host uses to try to kill the parasite, resulting in the survival of both host and parasite. Initial infection with *T. gondii* provokes the innate immune mechanism of the host, leading to production of substantial amounts of IL-12, TNF- α , and IFN- γ (1). These cytokines play a crucial role in resistance to *T. gondii*, with ultimate generation of a robust Th1-biased CD4⁺ and CD8⁺ cell-mediated immune response which is also characterized by high levels of IL-12 and IFN- γ (31). In our study, unlike the infected wild-type cells, infected monocytic cells with NALP1 knockdown did not have upregulated expression of IL-12, suggesting that signaling of inflammatory cytokine expression mediated through the IL-1 β receptor contributes significantly to the upregulation of IL-12 expression.

Our findings are consistent with the NALP1 inflammasome playing a crucial role in mediating the expression during *T. gondii* infection of proinflammatory cytokines that are important in controlling pathogenesis in this infection. Understanding the mechanisms of inflammasome regulation and how their downstream pathways contribute to pathogenesis of *T. gondii* will be important for elucidating the host innate immune responses to *T. gondii* and the susceptibility to disease caused by this parasite.

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