Ocular toxoplasmosis is the most common clinical manifestation of an infection with *Toxoplasma gondii*.\(^1\) This Apicomplexan parasite occurs in all regions of the world and infects the full range of mammals, as well as humans, most commonly through oral ingestion followed by dissemination via blood or the lymphatic stream.\(^2\) The eye disease typically presents as necrotizing retinitis, which often spreads to involve the adjacent choroid and vitreous, and also may cause anterior uveitis.\(^3,4\) Microscopic examinations of human eye samples demonstrate extensive destruction of the retina, with characteristic disruption of the retinal pigment epithelium.\(^5-8\) In addition to parasites, which may be present as replicating tachyzoites or dormant bradyzoites, ocular tissues contain a mixed leukocytic infiltrate of T lymphocytes, B lymphocytes and plasma cells, granulocytes including neutrophils and eosinophils, and mononuclear phagocytes.

The activities of the different leukocyte subsets that enter the human eye during ocular toxoplasmosis have not been fully elucidated. Previous study by our group has explored the role of one leukocyte population: using Boyden transwell migration assays, we demonstrated that infected monocyte-derived dendritic cells develop a hypermotile phenotype to transport *T. gondii* tachyzoites across the human retinal vascular endothelium from blood stream to retina.\(^9\) Research using mouse models of ocular toxoplasmosis indicates that CD8-positive and CD4-positive T and B lymphocytes are likely to play a lead role in controlling an eye infection.\(^10\) Although to our knowledge, these investigations have not been carried over to human retina-based experimental systems. The involvements of neutrophils in human ocular toxoplasmosis have not been addressed to date.

Neutrophils are a central pillar of the human innate immune system, and the most prevalent leukocyte in circulating blood.\(^11\) Studies in the mouse have shown that following ingestion, *T. gondii* infects and uses neutrophils as taxis to facilitate its dissemination within the intestine.\(^12\) These observations suggest neutrophils have the potential to transport *T. gondii* from the circulation into the retina. During a microbial infection, neutrophils swarm to the area, where they release granules containing antimicrobial molecules and other

**RESULTS.** Infection with *T. gondii* arrested neutrophil migration across retinal endothelium regardless of the presence of CXCL8. Migration to CXCL1, CXCL2, and CXCL8 also was significantly inhibited in infected neutrophils. Neutrophils generated more ROS when cocultured with infected versus uninfected ARPE-19 cells and three of four primary retinal pigment epithelial cell isolates. Infected ARPE-19 cells augmented the synthesis of 12 neutrophil-activating proteins also expressed by primary retinal pigment epithelial cells. Antibody blockade of granulocyte-macrophage colony-stimulating factor, interleukin-6 (IL-6) and IL-18 significantly reduced ROS production by neutrophils cocultured with *T. gondii*-infected ARPE-19 cells.

**CONCLUSIONS.** Our findings support involvement of neutrophils in retinal inflammation, but not parasite transport, in the setting of ocular toxoplasmosis.

Keywords: *Toxoplasma gondii*, toxoplasmosis, retina, neutrophil, retinal pigment epithelium
proteins that promote an immune response, including reactive oxygen species (ROS) and inflammatory cytokines. In the posterior eye, the retinal pigment epithelium is a key target cell for *T. gondii*, and has capacity to promote or suppress neutrophil activity, by synthesis of diverse inflammatory mediators or immuno-modulatory products, respectively. We explored the potential activities of neutrophils in human ocular toxoplasmosis, using neutrophils isolated from human peripheral blood in Bowden transwell migration assays, and in activation assays that involved coculture with *T. gondii*-infected human retinal pigment epithelial cells.

**Materials and Methods**

**Cytokines and Antibodies**

Human recombinant cytokines were purchased from PeproTech (Rocky Hill, NJ, USA); melanoma growth stimulating activity-z/CXCL1, used at a working concentration of 50 ng/ml; macrophage inflammatory protein 2-z/CXCL2, used at a working concentration of 100 ng/ml; and interleukin (IL)-8/CXCL8, used at a working concentration of 50 ng/ml. Antibodies were purchased from R&D Systems (Minneapolis, MN, USA): mouse anti-human granulocyte-macrophage colony-stimulating factor (GM-CSF) antibody (clone 3209, isotype IgG1), used at a working concentration of 0.5 to 1.5 μg/ml; mouse anti-human CXCL8 antibody (clone 6217, isotype IgG1), used at a working concentration of 0.5 to 1.5 μg/ml; anti-human IL-18 antibody (clone 125-2H, isotype IgG1), used at a working concentration of 0 to 0.5 μg/ml; mouse anti-human IL-6 antibody (clone 1956, isotype IgG2b), used at a working concentration of 0.15 to 0.45 μg/ml; and mouse anti-human CXCL1 antibody (clone 20326, isotype IgG2b), used at a working concentration of 7.5 μg/ml. Purified mouse IgG1 (clone MOPC-21) and Mouse IgG2b (clone MPC-11; BD Pharmingen, San Diego, CA, USA) were used as negative control antibodies at the same concentrations as the specific blocking antibodies.

**Cells**

Neutrophils were obtained from peripheral blood of healthy adult human volunteers. Cells were isolated by density gradient centrifugation using Ficoll-Paque PLUS (GE Healthcare, Uppsala, Sweden), followed by lysis of red blood cells in 3% wt/vol Dextran T500 (GE Healthcare). Across the experiments described in this study, neutrophil purity was at least 95% as estimated by counts made from hematoxylin and eosin-stained smears on glass slides. Following isolation, cells were routinely suspended in Roswell Park Memorial Institute medium (RPMI)-1640 medium (Thermo Fisher Scientific-GIBCO, Waltham, MA, USA) with 10% heat-inactivated fetal bovine serum (FBS; Bovogen Biologicals, Keilor East, Australia; reduced to 5% for parasite infections) and required to be at least 15%, which is the expected viability of freshly egressed GT-1 strain tachyzoites. Parasite viability was evaluated by plaque assay in fibroblast monolayers and required to be at least 15%, which is the expected viability of freshly egressed GT-1 strain tachyzoites.

**Neutrophil Migration Assays**

Neutrophil suspensions (1.0 × 10^6 cells) were incubated in isolation or with tachyzoites (multiplicity of infection, 5) at 37°C and 5% CO₂ in air. After a 20-hour incubation, the cell suspensions were added to upper chambers of transwells (Merck Sigma-Aldrich [Corning]; catalogue number: CLS5472; 0.33 cm² growth area, 3 micron pores). Lower chambers were filled with medium alone or supplemented with chemokine (one of CXCL1, CXCL2, and CXCL8). For some experiments, transwell perforated membranes were prepopulated 3 to 4 days before the assay with retinal endothelial cells, seeded at 70,000 cells per transwell onto bovine type I collagen (50 μg/ml; Santa Cruz Biotechnology, Santa Cruz, CA, USA) as described previously. The transwells were incubated for 2 (perforated membrane alone) or 5 (perforated membrane seeded with retinal endothelial cells) hours at 37°C and 5% CO₂ in air. Subsequently, migrated neutrophils were recovered from lower chambers and counted on a hemocytometer. For experiments with endothelial cell-seeded membranes, integrity of endothelial monolayers was confirmed by measuring diffusive flux of 1 mg/ml Texas Red-conjugated dextran (molecular weight 70,000; Thermo Fisher Scientific-Molecular Probes, Eugene, OR, USA), per the method of Harhaj et al.

**Neutrophil-Retinal Pigment Epithelial Cells Coculture**

Neutrophils were incubated with confluent monolayers of retinal pigment epithelial cells in 12-well plates (3.6 cm² growth area) at 37°C and 5% CO₂ in air, at a neutrophil:epi-
the epithelial cell ratio of 10:1, and in the presence of blocking antibodies or negative control antibodies in some experiments. Epithelial cells were infected 4 hours before assay with *T. gondii* tachyzoites (multiplicity of infection, 5), followed by washing to remove extracellular parasites, or not infected and treated with fresh medium only. Following an 18-hour incubation, plates were agitated to permit removal of neutrophils from epithelial cell monolayers, which were collected by aspiration and pelleted. Neutrophils were resuspended in RPMI-1640 medium with 10% FBS, and divided for: (1) immediate measurement of ROS or (2) transfer to RLT buffer (Qiagen, Hilden, Germany) and storage at −80°C for later extraction of total RNA using the RNeasy Mini Kit (Qiagen).

**Assay for Detection of ROS**

Neutrophils, suspended in RPMI without phenol (Thermo Fisher Scientific-GIBCO) were aliquoted in black 96-well plates (1 × 10^6 cells/well), with technical duplicate, and stained with 5 μM dihydroethidium (Thermo Fisher Scientific-Molecular Probes) for 15 minutes at 37°C in the dark. Following the incubation, the center of each well was photographed on the EVOS FL Cell Imaging System (Thermo Fisher Scientific-Invotegen, Carlsbad, CA, USA) at ×10 magnification. Relative fluorescence of the stained neutrophils was measured on black-and-white photomicrographs using ImageJ software (version 1.48; National Institutes of Health, Bethesda, MD, USA), with a threshold that was set from readings taken at the time of assay from wells of the plates that contained reagent only.

**Reverse Transcription of RNA and Quantitative Real-Time PCR (RT-qPCR)**

RNA was reverse-transcribed using the iScript Reverse Transcription Supermix for RT-qPCR (Bio-Rad, Hercules, CA, USA) with up to 50 ng of RNA template. qPCR was performed on the CFX Connect Real-Time PCR Detection System (Bio-Rad) with iQ SYBRGreen Supermix (Bio-Rad), using up to 0.75 ng of cDNA per reaction. Primer sequences and product sizes are presented in Supplementary Table S1. The standard amplification consisted of: a precycling hold at 95°C for 5 minutes; 40 cycles of denaturation for 30 seconds at 95°C; annealing for 30 seconds at 60°C; extension for 30 seconds at 72°C; and a postextension hold at 75°C for 1 second. A melting curve, representing a 1-second hold at every 0.5°C between 70°C and 95°C, was generated to confirm that a single peak was produced for each primer set. Size of qPCR products were checked by agarose gel electrophoresis. Standard curves, produced for each primer set. Size of qPCR products were measured using CFX Manager software, normalized to two stable reference genes: expression of transcripts was calculated using CFX Manager version 3.1 software, normalized to two stable reference genes: expression of transcripts was calculated using CFX Manager version 3.1 software, normalized to two stable reference genes.

**Protein Immunoarray**

Confluent retinal pigment epithelial cell monolayers in 6-well plates (9.5 cm² growth area) were infected with tachyzoites (multiplicity of infection, 5) or retained in medium alone. At 4 hours, monolayers were washed with Dulbecco’s phosphate-buffered saline (Thermo Fisher Scientific-GIBCO) to remove extracellular tachyzoites. The medium was refreshed, and cell monolayers were incubated for a further 20 hours, at which time culture supernatant was collected. The levels of 36 human cytokines in the supernatant were measured using the Proteome Profiler Human Cytokine Array (R&D Systems), which is a membrane-based immunoarray, following the manufacturer’s instructions. Immediately after addition of the chemiluminescence substrate, immunoblots were photographed on the LAS-4000 luminescence analyzer (Fujifilm Imaging Systems, Tokyo, Japan). Level of cytokine expression in each sample was determined by spot densitometry using AlphaEaseFC software (version 4.0.0; Alpha Innotech, San Leandro, CA, USA). Correction for background signal was performed by subtracting mean optical density of negative control spots on each immunoblot. Mean optical density of three reference spots was used to generate an adjustment factor that was applied to correct for any differences in development time between immunoblots.

**Statistical Analysis**

Data were analyzed using GraphPad Prism v6.04 (GraphPad Software, La Jolla, CA, USA). Two-tailed Student’s *t*-test was used to make comparisons between two groups, and 1-way ANOVA test was used to make comparisons across multiple groups. Statistical significance was defined as *P* < 0.05.

**Research Compliance**

The use of human cadaver donor eyes and blood from adult volunteers in research was approved by the Southern Adelaide clinical human research ethics committee (protocol numbers: 175.13 and 350.13). In vitro research with *T. gondii* was approved by the Flinders University Institutional Biosafety Committee (Microbiological Dealing protocol number: 2013-08, and Notifiable Low Risk Dealing protocol number: 2013-09). This research followed the tenets of the Declaration of Helsinki.

**RESULTS**

**Human Neutrophils Infected With *T. gondii* Do Not Transmigrate Retinal Endothelium or Respond to Chemokines Expressed Within the Eye in Ocular Toxoplasmosis**

Leukocytes traffic *T. gondii* tachyzoites in the blood stream from the intestine to target organs, and within these organs. To examine a potential role for neutrophils in moving tachyzoites into the retina, we examined CXCL8-induced migration of human neutrophils in Boyden transwells divided by perforated membranes that were populated with human retinal endothelial cells on collagen. Neutrophils infected with GT-1 natural isolate strain tachyzoites were significantly and substantially retarded in moving through the transwells toward CXCL8 (*P* ≤ 0.001; Fig. 1A). To address the potential for infected neutrophils to transport tachyzoites within the retina, we studied chemokine-induced neutrophil migration in Boyden transwells divided by perforated membranes alone. In contrast to uninfected neutrophils, which were actively motile, GT-1 strain *T. gondii*-infected neutrophils did not migrate toward CXCL1, CXCL2, or CXCL8 (*P* ≤ 0.002; Fig. 1B). Taken together, these results indicate that neutrophils are unlikely to participate in the movement of *T. gondii* tachyzoites into and within the human retina; instead, it is likely that neutrophils are uninfected as they enter the retina, which they may access to participate in the immune response against the parasite.
**Human Neutrophils Produce ROS in the Presence of T. gondii-Infected Retinal Pigment Epithelial Cells**

The retinal pigment epithelium is a principal target for *T. gondii* tachyzoites in ocular toxoplasmosis.\(^{14}\) We examined the activities of neutrophils in response to *T. gondii* in a coculture system with retinal pigment epithelial cells that had been infected with GF1 strain *T. gondii* tachyzoites 24 hours previously. In the first series of experiments, the ARPE-19 human retinal pigment epithelial cell line was used (Figs. 2A-C, left column). Human neutrophils were cultured with infected or uninfected ARPE-19 cells, and subsequently isolated and tested for their production of ROS and inflammatory cytokines. When neutrophils were exposed to ARPE-19 cells that were infected with tachyzoites, the leukocytes produced significantly more ROS, as well as TNF-α and IL-1β transcripts, compared to uninfected ARPE-19 cells.

In a second series of experiments, similar assays were performed using retinal pigment epithelial cells separately isolated from four human eye pairs, in place of ARPE-19 cells (Figs. 2A-C, right column). Human neutrophils increased their production of ROS when cocultured with three of four primary epithelial cell isolates infected with *T. gondii* tachyzoites compared to uninfected epithelial cells (\(P < 0.003\) for two isolates and \(P = 0.056\) for a third isolate). In contrast, neutrophils did not upregulate TNF-α transcript when exposed to any *T. gondii*-infected primary epithelial cell isolates (\(P > 0.05\)), and the change in expression of IL-1β transcript varied (increased for two isolates, \(P \leq 0.003\), and decreased for one isolate, \(P = 0.04\)). These results suggested that human neutrophils are involved in the ocular immune response to *T. gondii* infection, being activated by *T. gondii*-infected retinal pigment epithelial cells to produce ROS. Although IL-1β expression also may be impacted by the infection, this effect appears to vary between individuals.

**Human Neutrophils Are Activated by Inflammatory Products of Retinal Pigment Epithelium that Include GM-CSF, IL-6, and IL-18**

To identify the molecular basis of the activation of human neutrophils by *T. gondii*-infected retinal pigment epithelial cells, we performed a 36-protein immunoarray (in quadruplicate) to screen for retinal pigment epithelial proteins. Results were cross-referenced against the immunologic literature to identify proteins known to activate neutrophils, as well as the transcriptome of primary human retinal pigment epithelial cells that we recently reported to confirm production by primary cells.\(^{14}\) ARPE-19 cells were infected with GF1 strain *T. gondii* tachyzoites or incubated with medium alone for 24 hours, after which time, culture supernatant was analyzed by the R&D Systems Proteome Profiler Human Cytokine Array. Twelve proteins expressed by primary human retinal epithelial cells and known to activate neutrophils, as well as the transcriptome of primary human retinal pigment epithelial cells that we recently reported to confirm production by primary cells, were cross-referenced against the immunologic literature to identify proteins known to activate neutrophils, as well as the transcriptome of primary human retinal pigment epithelial cells that we recently reported to confirm production by primary cells.\(^{14}\) ARPE-19 cells were infected with GF1 strain *T. gondii* tachyzoites or incubated with medium alone for 24 hours, after which time, culture supernatant was analyzed by the R&D Systems Proteome Profiler Human Cytokine Array. Twelve proteins expressed by primary human retinal epithelial cells and known to activate neutrophils were significantly differentially expressed between *T. gondii*-infected and uninfected ARPE-19 cells (\(P \leq 0.05\): CCL5 (3.4-fold), CXCL1 (20.8-fold), CXCL8 (9.4-fold), G-CSF (2.6-fold), GM-CSF (10.3-fold), intercellular adhesion molecule 1 (ICAM-1; 19.4-fold), IL-1α (2.6-fold), IL-6 (20.4-fold), IL-13 (4.2-fold), IL-18 (14.1-fold), and IL-1β (19.4-fold).

**Figure 1.** Migration of human neutrophils in Boyden transwells 20 hours after infection with GT-1 strain *T. gondii* tachyzoites (1 × 10⁶ neutrophils, multiplicity of infection = 5) or parallel incubation in medium alone. **(A)** 5 hours after migration across human retinal endothelial cell monolayers seeded on the perforated transwell membrane in presence or absence of CXCL8, and **(B)** 2 hours after migration across the transwell perforated membrane in the presence or absence of one of three chemokines: CXCL1, CXCL2, or CXCL8. All graphs are representative of two independent experiments. Data were analyzed by 1-way ANOVA with post hoc Tukey procedure. \(n = 4–6\) transwells/condition. **Status bars:** mean number. **Error bars:** SEM.
FIGURE 2. Synthesis of ROS and expression of inflammatory cytokines by human neutrophils exposed to *T. gondii*-infected human retinal pigment epithelial cell monolayers. ARPE-19 cells and primary retinal pigment epithelial cell isolates were infected with GT-1 strain *T. gondii* tachyzoites (multiplicity of infection = 5) or incubated with medium alone for 4 hours. Neutrophils were cocultured with retinal pigment epithelial cells at a ratio of 10:1, or incubated with medium alone, for 20 hours, and subsequently isolated and assayed for production of (A) ROS with dihydroethidium, and (B) TNF-α and (C) IL-1β transcripts by RT-qPCR. Graphs of ARPE-19 cells are representative of two independent experiments, and graphs of primary retinal pigment epithelial cells are from experiments with four different isolates. Data were analyzed by 1-way ANOVA with post hoc Tukey procedure. \( n = 4–6 \) cultures/condition. *Status bars*: mean fluorescence or relative expression. *Error bars*: SEM.
32 (2.8-fold), and macrophage migration inhibitory factor (MIF; 2.1-fold; Fig. 3).

To identify proteins that were responsible for neutrophil activation induced by T. gondii-infected human retinal pigment epithelial cells, we prioritized cytokines with >5-fold significant increase for evaluation in antibody blockade studies: CXCL1, CXCL8, GM-CSF, IL-6, and IL-18. Significant reduction in the production of ROS by neutrophils cultured with GT-1 strain T. gondii tachyzoite-infected ARPE-19 cells was observed in the presence of antibody directed against GM-CSF, IL-6, or IL-18, when blockade was applied at the higher end of the neutralization range (Fig. 4). Taken together, these results demonstrate that T. gondii-infected human retinal pigment epithelial cells produce multiple cytokines that might promote neutrophil activation, and implicate GM-CSF, IL-6, and IL-18 in particular, in this process.

DISCUSSION

Neutrophils are the most prevalent innate immune leukocyte, and the critical first responder cell in many infections,\(^1\) including those that are based in the eye.\(^2\) Human neutrophils are well known to be permissive to infection with T. gondii tachyzoites, and to respond to the infection by synthesis of immune mediators that may vary according to tissue location.\(^3\) Neutrophils are present in the retina-based inflammatory infiltrate in persons with active ocular toxoplasmosis,\(^4\) but their role(s) in the pathogenesis of the disease have not been considered to date to our knowledge. Our experiments suggest that neutrophils are not involved in transporting T. gondii tachyzoites into and within the retina. Instead, this work indicates that neutrophils are likely to participate actively in an inflammatory response to T. gondii, which often is aggressive and may contribute to retinal damage,\(^5\) through their interaction with the retinal pigment epithelium, which is an intraocular target for the parasite.\(^6\)

Monocytes and dendritic cells have been identified as leukocytes with capacity to carry T. gondii tachyzoites from the gut via the circulation into target tissues.\(^7\) Using a transwell system, we have shown that dendritic cells infected with tachyzoites use Ig superfamily adhesion molecules to move the parasite across human retinal endothelial cell monolayers, simulating movement from the bloodstream into the retina.\(^8\) Neutrophils are relatively less susceptible to infection with T. gondii than these mononuclear phagocytes,\(^9\) but in the mouse gut, they account for a high proportion of T. gondii-infected leukocytes, and represent the primary mechanism for parasite transport in that organ.\(^10\) Although this suggests neutrophils might contribute to moving tachyzoites into and within the retina, neutrophil arrest at encounter with a pathogen is an established immunologic concept that has been linked to oxidant sensing.\(^11\) Consistently, we observed
Neutrophils exhibit considerable phenotypic and functional heterogeneity.32

One important observation about our findings is the capacity for the *T. gondii*-infected human retina pigment epithelium to activate neutrophils. In general, the retinal pigment epithelium is considered immunoregulatory, being the major contributor to ocular immune privilege in the posterior segment of the eye.33 The retinal pigment epithelium downregulates activation of CD4+ T helper cells and B cells, and promotes the differentiation of regulatory T cells.15 However, in the context of infection, this role may change. Through independent research, we have observed dysregulation of the expression of immunoregulatory molecules by human retinal pigment epithelium in the context of Zaire ebolavirus infection, which causes uveitis in Ebola survivors.34 Indeed, we observed that, compared to uninfected retinal pigment epithelial cells, *T. gondii*-infected epithelial cells augmented ROS production by neutrophils exposed to *T. gondii* lysate (data not shown). Our experiments associate the activation of neutrophils to production of several inflammatory cytokines by *T. gondii*-infected human retinal pigment epithelial cells: GM-CSF, IL-6, and IL-18.

The main limitation of our research is that all experiments have been conducted in vitro. However, there is no other option for addressing the activities of neutrophils in the human eye. It also is highly relevant to investigate a human-based system: the mouse is used frequently for in vivo research on ocular toxoplasmosis, but exhibits significant differences in reacting to the parasite.35 We worked with the ARPE-19 human retinal pigment epithelial cell line and primary retinal pigment epithelial cells that we isolated from human donor eyes. Recently we compared gene expression responses of ARPE-19 cells and primary epithelial cells to infection with GT-1 strain *T. gondii*.
Neutrophil Activities in Human Ocular Toxoplasmosis

**gondii** tachyzoites, and observed that although both mounted strong immunologic responses to the parasite, these responses showed some variance at the molecular level. Thus, in this study, we conducted key experiments on neutrophil activation by *T. gondii*-infected retinal pigment epithelium with both cell types. Acknowledging this limitation, to our knowledge, our work is the first study of neutrophil activities in human ocular toxoplasmosisis, and it highlights the role of the cell in the ocular immune response to the parasite. Production of ROS by neutrophils that contact *T. gondii*-infected retinal pigment epithelium is expected to be highly inflammatory, and justifies the use of corticosteroid in conjunction with antimicrobial drugs for treatment of ocular toxoplasmosis, to limit tissue damage.

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