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Human neutrophil-like cells demonstrate antimicrobial responses to the chronic cyst form of *Toxoplasma gondii*

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Abstract

The protozoan parasite *Toxoplasma gondii* infects approximately 2.5 billion people worldwide. Infection induces a rapid dissemination of parasites throughout the body followed by the formation of lifelong cysts within neurons of the host brain. Both stages require a dynamic immune response comprised of both innate and adaptive cells. Neutrophils are a primary responding cell to acute infection and have been observed in the brain during murine chronic infection. Previous studies investigating human neutrophils found that invasion by Toxoplasma tachyzoites inhibits apoptosis of neutrophils, prolonging their survival under inflammatory conditions. Here, we demonstrate the differentiation of two distinct subsets following exposure of human neutrophil-like-cells (HNLC) to Toxoplasma cysts. *In vitro* stimulation and imaging studies show cyst-specific induction of cytokines and cyst clearance by HNLCs. Further testing demonstrates that aged HNLCs perform less phagocytosis of cysts compared to non-aged HNLCs. In conclusion, this study identifies a novel response of HNLCs to Toxoplasma cysts and may indicate a role for neutrophils in the clearance of cysts during human infection with Toxoplasma.

Keywords

neutrophils; human; *Toxoplasma gondii* ; chronic cysts; immune response

INTRODUCTION

Toxoplasma gondii is estimated to infect 1 in 3 people around the world[1], and infection in the immunocompromised can have devastating results[2]. The immune response to the acute stage of infection is characterized by early recruitment of innate immune cells including neutrophils to sites of infection and innate cytokine production[3,4]. Neutrophils produce some vital early cytokines and chemokines that encourage additional cell recruitment and are

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Authors Contributions

KVB, ADR, and EHW designed and conducted experiments; FM provided guidance and feedback on project design and results; KVB, BK, and EHW analyzed data; KVB, ADR, and EHW wrote the manuscript.

Conflict of Interest Statement

The authors declare that they have no conflicts of interest.

able to directly kill the parasite through mechanisms like phagocytosis[5], reactive oxygen species (ROS) production[6], and the process of NETosis to ensnare and kill free-floating pathogens[7,8].

While neutrophils are a well-known first line of defense against Toxoplasma infection, recent work demonstrates the ability of Toxoplasma to extend the lifespan of human neutrophils[9]. Additional work describes a small but defined population of neutrophils in the brain during chronic Toxoplasma infection[10]. During this chronic stage, the parasite transitions to a slow replicating bradyzoite that forms lifelong cysts inside brain and muscular neurons[11]. Although there are drugs available to kill tachyzoites, there are no clinically approved drugs that remove cysts. While these dormant cysts are less immunogenic than tachyzoites, effector mechanisms employed by infiltrating immune cells have been described in murine studies that suggest the potential for innate control of cyst burden in the CNS[12,13].

Extensive work has been conducted in murine models of Toxoplasmosis, but much less is known about the responses of human immune cells during chronic Toxoplasma infection. Peripheral blood provides a source of human neutrophils, and studies have demonstrated the ability of Toxoplasma tachyzoites to inhibit apoptosis in these cells and prolong their lifespan during inflammatory stimulation[9]. However, due to the inaccessibility and asymptomatic nature of chronic infection, human studies are restricted to those of antibody detection[14,15] or post-mortem studies to observe tissue cysts in histological brain sections[16–18]. Recent work utilizing primary human neutrophils has shown that Toxoplasma infection leads to NET-dependent amplification of innate and adaptive immune responses to tachyzoites[19,20]. While this work supports neutrophil response to the acute stage of Toxoplasma, the presence of neutrophils in the brain during chronic murine Toxoplasma infection suggests neutrophils may participate in immune responses against the cyst form of infection. However, whether and how neutrophils respond to the chronic cyst form of the parasite remains unknown.

In this study, we demonstrate phagocytic uptake of cyst material by a human neutrophil-like cell (HNLC) line. Cyst-specific cytokine production is found in the production of IL-1α and IFNγ compared to tachyzoite exposure. Furthermore, the response of neutrophils to cysts is dependent on the maturation and age of the neutrophil, with less phagocytosis occurring in aged-HNLCs compared to non-aged HNLCs. These studies characterize responses of human neutrophils to the cyst stage of Toxoplasma and may lead to improved understanding of immune mechanisms during chronic infection.

MATERIALS AND METHODS

Human Neutrophil Cell (HNLC) Culturing and Aging

Culturing.—HL-60 cells were provided by the Collins lab at the University of California Davis. Cells were cultured between 0.2–1.5million cells/ml in complete RPMI media at 37°C with 5% CO_2. Differentiation media (complete RPMI with 1.3% DMSO and 100ng/ml of G-CSF) was used to differentiate HL-60 cells into HNLCs, and cells were differentiated

at a concentration of 0.2million cells/ml. Successful differentiation was confirmed via neutrophil morphology after 7 days[21,22].

Aging.—To create aged HNLCs, cells were kept in culture for 7 days following completion of differentiation. Differentiation media was replaced after maturation, and experiments were optimized for induction of CXCR4.

Parasite Culturing and Antigen Preparation

Parasite and Cyst Maintenance.—Toxoplasma tachyzoites of the Me49 (B7) strain were maintained in HFF cultures, and Me49 cysts were maintained using alternating infections of Swiss Webster and CBA mice[23]. Purification of parasites was conducted by needle passage of fibroblasts to release parasites followed by filtration through a 3μm filter to remove cell debris and dead parasites as previously described[24]. Parasites in media were spun down at 1200rpm for 10min at room temperature, supernatant was decanted, and parasite pellet was resuspended in 1mL of parasite media. Parasites were counted using a hemacytometer, PBS was added, and parasites were spun down to rinse residual media. Cysts were harvested by needle passage of brain homogenate and spun down in 1x PBS followed by a crude 30% Percoll spin to remove myelin. After Percoll, pellet was incubated in RBC lysis buffer for 10min on ice and spun down in 1x PBS to rinse. Cysts were resuspended in 1mL of 1x PBS and counted manually using a bright field microscope.

Tachyzoite and Cyst Antigen Preparation.—Parasite antigen from cultured Me49 tachyzoites or purified Me49 cysts was generated. Following purification, approximately 3.0×10^6 live tachyzoites were placed into a cryovial with 200μl sterile 1x PBS. For cyst antigen between $3–5\times10^3$ intact cysts were used. Cryovials were sealed and placed in liquid Nitrogen until frozen and then allowed to thaw completely at room temperature. This freeze-thaw process was repeated a total of five times, and total concentration of parasite antigen was determined via BCA Assay (ThermoFisher). For experiment dilution, appropriate volume of solution based on total antigen concentration was added to HNLC differentiation media and then added to wells.

Stimulation Experiments

In vitro HNLCs.—For infections, fully differentiated HNLCs plated at 0.2 million cells/ml in 200μl of neutrophil differentiation media were infected with Me49 tachyzoites at an MOI of 5; 100 Me49 cysts/well; or stimulated with either antigen at a final concentration of 25μg/ml according to previously published protocols[13]. Uninfected/unstimulated cells and cells stimulated with LPS (125 ng/mL) or PMA (100μg/ml) were used as positive controls in respective experiments. Cultures were conducted in quadruplicate. Cells were incubated as above for 6 hours (for cytokine production) or 4 hours (for NET production and imaging studies). Cells were spun down at 100×g for 10min at room temperature, supernatant was collected for cytokine or NET production analysis, and coverslips were prepared for immunofluorescence.

Brain mononuclear cells.—Brain mononuclear cells (BMNCs) from 4 week-infected mice were harvested according to previously published protocols[26]. BMNCs were plated at a

concentration of 0.2million cells/mL in complete RPMI. Cells were infected and stimulated as described above.

Immunofluorescence Studies

Coverslips were washed with 1x PBS, fixed in 4% PFA for 15min, washed again with 1x PBS and permeabilized in 0.5% TritonX 100 for 15min. Incubation with donkey serum (5%) for 30min was followed by primary antibodies to CD11b (ThermoFisher), CD15 (ThermoFisher), and *T. gondii* (Abcam) for 1 hour at room temperature (1% Donkey Serum/ 0.5% Tween20/1x PBS). Coverslips were washed and incubated in secondary fluorescent antibodies (488, 568, and 647, ThermoFisher) for 1 hour at room temperature in same buffer. Coverslips were washed and mounted on microscope slides using Vectashield with DAPI. HNLCs were imaged on a Leica inverted DMI6000 B microscope using 40x magnification with Leica LASX software. For quantification of CD15+ cells, total numbers of CD15+ cells (minimum of 50) and total cells (minimum of 100) were counted from a minimum of 7 regions of interest (ROIs) in each condition. For quantification of parasite (tachyzoite stage) positive cells, total numbers of CD15+ cells containing parasites (minimum 50), total numbers of CD15− cells containing parasites (minimum 50), and total cells (minimum 200) were counted from a minimum of 10 randomized ROIs.

LEGENDplex Cytokine Analysis

LEGENDplex cytokine assay for Human Inflammation Panel 1 (13-plex) (BioLegend) was performed on undiluted cell supernatants according to LEGENDplex kit instructions for V-bottom plate (Manual for Cat. No. 740809). Samples were acquired on a BD FACS Canto II flow cytometer, and analysis was conducted using recommended BioLegend software.

PicoGreen Assay

For quantification of extracellular DNA indicative of NETosis, preparation of cells for PicoGreen assay (ThermoFisher) protocol was performed according to previously published protocols[8]. PicoGreen assay kit instructions (Manual Reference: MP 07581) were modified for 96-well plate format. Supernatant was incubated for 5 minutes protected from light and immediately read on a fluorimeter at an excitation of 450nm and emission of 520nm.

HNLC Time Lapse Experiments

HNLCs were cultured and differentiated as above. HNLCs were pre-activated using human serum (Innovative, Lot: ISERAB, 33930) to mimic biological conditions during infection. Me49 cysts were purified as above and stained with CMFDA Green Cell Tracker dye for 30 minutes protected from light at room temperature. Control 20μm diameter YG-fluorescent beads were coated in cyst antigen (25μg/mL). A total of 100 cysts or beads were added to wells in a glass-bottom 24-well plate in neutrophil differentiation media and imaged using a Keyence BZ-X710 microscope with Tokai-Hit Climate Control attachment (37°C, 5% CO_2). Designated areas of cysts or beads were identified for imaging based on positive fluorescent staining, and HNLCs were added to one well at a time at a concentration of 2.0 × 10^5 cells/ml. Cells were allowed to settle for 5 minutes, and images were taken every 60 seconds for 3 hours. Cysts and beads were visualized using the fluorescent GFP channel,

and cells were visualized using Bright Field imaging. Live imaging was performed at 40x magnification. Cysts alone were also imaged for the same amount of time as a control.

Flow Cytometry Experiments

Phagocytosis Inhibition.—HNLCs were pre-activated using human serum (Innovative, Lot: ISERAB, 33930) as above. Prior to cyst exposure, a cohort of HNLCs received CytochalasinD treatment (2.5μg/mL) for 1 hour to inhibit phagocytosis. Me49 cysts were purified and added to unstained HNLCs for 60 minutes as described above. Following exposure, all cells were incubated with fixable Live/Dead stain (ThermoFisher) for 30min in 1x PBS and rinsed in FACS buffer. Cells were incubated with APC-conjugated CD11b (Invitrogen) for 30 minutes and fixed in 4% PFA for 15 minutes prior to acquisition.

HNLC Aging Characterization.—HNLCs were pre-activated as above. Exposure, Live/Dead staining, and analysis of non-aged and aged HNLCs was conducted as described above. The primary and secondary antibodies (30min incubation each) used were: PE-conjugated CXCR4 (Invitrogen), Biotinylated CD15 (Invitrogen), APC-conjugated CD11b (Invitrogen), and Streptavidin-conjugated PerCPCy5.5 (Invitrogen). As a control, cyst antigen-coated fluorescent beads used in live imaging studies were also added to a cohort of cells during exposure period.

Flow Cytometer and Analysis.—All samples for all experiments were acquired using a BD FACS Canto II flow cytometer, and analysis was conducted using FlowJo software.

Ethical Use of Animal Subjects

All research using animals was conducted in accordance with the Animal Welfare Act, and all efforts were made to minimize suffering. All protocols were approved by the Institutional Animal Care and Use Committee (IACUC) of the University of California, Riverside. Female 6–8 weeks old WT C57BL/6J and CBA mice were obtained from Jackson Laboratories and were maintained in a pathogen-free environment in accordance with IACUC protocols at the University of California Riverside.

Statistical Analyses

All experiments were repeated a minimum of 3 times to confirm consistency of results, and all experiments were conducted with a minimum of n = 3. Statistical significance for all experiments was determined by either 2- tailed, unpaired Student’s t-test or One-Way ANOVA with multiple comparisons. The type of statistical test run for each experimental result is indicated in the corresponding figure legends.

RESULTS

Two different types of human neutrophil-like cells (HNLCs) are present following exposure to T. gondii.

Previous work investigating the function of neutrophils has demonstrated differentiation into distinct subsets depending on location, age, and host immune status[27,28]. The subset type and environment influence activation of “classical” antimicrobial vs. “alternative” regulatory

functions. Expression of the integrin CD15 is one way to define “classical” vs. “alternative” neutrophil phenotypes[29].

We hypothesized that HNLC differentiation into subsets would be observed in response to different stages of Toxoplasma. To test this, differentiated HNLCs were exposed to purified cysts or tachyzoites for 4 hours (Figure 1). Immunofluorescence analysis demonstrated a proportion of CD15+ cells that represented up to 20% of the neutrophil population without stimulation (Fig. 1A, **top panels**). Following exposure to either tachyzoites or cysts, the CD15+ population increased to 60% of all cells (Fig. 1A–B). CD15+ cells also demonstrated a trend towards more association with tachyzoites, but this was not significant (Fig. 1C). These results demonstrate the differentiation of HNLCs into 2 subsets (CD15+ and CD15-) that is enhanced following exposure to parasites but is independent of infection stage.

Cyst-induced responses of human neutrophils.

Inflammatory and antimicrobial responses of neutrophils to Toxoplasma have been investigated in the past[8,13], but neutrophil response to the chronic cyst form of the parasite remains less understood. To determine cytokine responses by HNLCs to Toxoplasma cysts, cells were exposed to cysts, cyst antigen (CAg), tachyzoites, and tachyzoite antigen (TAg) for 6 hours. LEGENDplex cytokine analysis demonstrated a 3-fold increase in the amount of secreted IL-1α by HNLCs following exposure to CAg compared to all other experimental and control conditions (Fig. 2A **left**). IL-1α production was specific to CAg exposure but was not significantly induced by exposure to whole cysts. Previous work has discovered a population of IFNγ-expressing murine neutrophils during chronic Toxoplasma and Mycobacterium infection[10,30]. HNLCs significantly increased IFNγ secretion in response to cysts (>2-fold increase) and CAg (>4-fold increase) but not tachyzoites or tachyzoite antigen (Fig. 2A, **right**). These results reveal previously undemonstrated inflammatory responses of HNLCs following exposure to the chronic form of Toxoplasma.

To investigate NETosis by HNLCs in response to cysts, cells were stimulated with different stages of the parasite and stage-specific antigens. Cells were stimulated for 4 hours, a time period sufficient for NET production but not cell death by parasite replication, and extracellular DNA was quantified[8]. Control murine BMNCs demonstrated a significant increase in extracellular DNA upon exposure to all stimuli (Fig. 2B). As previously described[8], exposure to PMA and tachyzoite antigen (Tag) led to a significant NET response and increased extracellular DNA in HNLC cultures (Fig. 2C). However, unlike cytokine production, stimulation of HNLC’s with cysts did not lead to NET production (Fig. 2C). Taken together, these results demonstrate inflammatory cytokine secretion but not NET production by HNLCs in response to the chronic cyst form of Toxoplasma.

HNLCs attack and phagocytose cyst material.

Our previous imaging studies revealed not only the presence of CD15+ HNLCs following cyst exposure but also a notable loss of cyst numbers and small cyst size (~10μm) after 4 hours (Fig. 1A and data not shown). Based on these results, we hypothesized that HNLCs

are capable of ingesting and degrading cyst material. To test this, we performed live time lapse imaging and flow cytometry to visualize and quantify any uptake of cyst material.

Upon addition of neutrophils to wells containing fluorescently labelled cysts, there was noticeable migration of neutrophils towards cysts (green) (Fig. 3A, **arrows**); Supplemental Video. 1, **right arrow**). Adherence of neutrophils to cysts could be observed throughout imaging (Supplemental Video. 1, **left and right arrows, respectively**). Over the course of 3 hours, the number of observable cysts decreased, and increased migrating green cells could be seen suggesting engulfment of cyst material by neutrophils (Fig. 3A). Contrary to the fewer numbers and smaller size of cysts observed after HNLC exposure, images of cysts alone after 3 hours showed no loss of numbers and ranged from 10–50μm in size (Suppl. Fig. 1). Use of CAg-coated fluorescent beads as a control condition yielded no attachment of neutrophils or uptake of fluorescent material (Supplemental Video 2). These results demonstrate that HNLCs begin responding within 5 minutes following exposure to Toxoplasma cysts, and peak cyst uptake occurs within 60 minutes of exposure followed by gradual degradation and clearance of ingested cyst material.

To quantify our imaging results, flow cytometry was conducted at the peak cyst ingestion time point of 60 minutes post-exposure. After 60minutes of FITC+ cyst incubation with CD11b+ HNLCs, more than half of the culture were CD11b+ FITC+ cells, the equivalent of 80% of neutrophils (Fig 3B, **middle panel**). In contrast, cells that had no cysts added did not show any proportion of FITC+ cells (Fig. 3B, **left panel and** Suppl. Fig. 2). As a separate control experiment, cells exposed to CAg-coated fluorescent beads for 60 minutes showed no uptake of fluorescent material (Suppl. Fig. 3).

Phagocytosis is one of the primary antimicrobial responses of neutrophils to Toxoplasma and other pathogens[5,31]. To test if phagocytosis plays a role in HNLC clearance of cysts, cells were treated with CytochalasinD to inhibit phagocytic activity prior to cyst exposure. Inhibition of phagocytosis in HNLCs resulted in a significant decrease in the uptake of cyst material compared to non-inhibited cells (Fig. 3B–C). Representative flow plots showed a drop in the percentage of CD11b+FITC+ cells from 55% in non-inhibited cells to 15% in cells pre-treated with CytochalasinD (Fig. 3B). This corresponds to a decrease from 80% of total neutrophils (CD11b+ cells) to 20%. The majority of inhibited cells expressed CD11b but not FITC (63% CD11b+FITC-) confirming successful inhibition of phagocytosis without induction of cell death. Quantification of this flow data showed a significant decrease in CD11b+FITC+ cells (Fig. 3C) and a significant increase in the percentage of CD11b+FITC− cells (Fig. 3D) following CytochalasinD treatment. These results suggest that cyst degradation and uptake is an active process most likely involving phagocytosis of cyst components. Taken together, these data indicate that neutrophils interact with Toxoplasma cysts and are capable of cyst degradation.

Characterization of HNLCs demonstrates age-dependent differences in response to cyst exposure.

Our results demonstrate the ability of human neutrophils to clear cysts in a canonical antimicrobial manner, but initial imaging identified two cell subsets (CD15+ and CD15-) following exposure to Toxoplasma (Figure 1). These findings prompted us to investigate

CD15 expression by HNLCs containing cyst material and if cyst clearance capability changes following aging. To test this, we aged neutrophils over the course of two weeks and exposed aged HNLCs to cysts for 60 minutes (Figure 4). Aging was confirmed by increased expression of CXCR4, an indicator of an aged neutrophil population (Fig. 4A).

After cyst exposure, non-aged and aged HNLCs displayed differences in their ability to consume cyst material. More than half (55%) of non-aged HNLCs were FITC+, indicating cyst engulfment, compared with only 20% of aged HNLCs (Fig. 4B). Examination of CXCR4 and CD15 expression by FITC+ cells revealed no significant difference, however FITC− cells retained the phenotype of aged cells by increased CXCR4 expression (Fig 4C). Total CD15 expression remained consistent after aging indicating the persistent expression of a “classical effector” phenotype. Taken together, these results demonstrate that cyst clearance capability of HNLCs decreases following aging.

DISCUSSION

While neutrophil responses to acute Toxoplasma infection have been extensively characterized, there is a gap in our understanding of human neutrophil responses to chronic infection. This study presents a novel response of human neutrophil-like cells (HNLCs) to the cyst stage of Toxoplasma and indicates a previously unknown role of human neutrophils in the control of chronic infection.

Increased CD15+ HNLCs were seen following exposure to acute-stage tachyzoites and chronic-stage cysts compared to media controls. Although this increased differentiation was induced by parasite exposure, it was independent of parasite stage. While neutrophil subsets are commonly identified using CD15, enhanced phenotyping using additional markers including CD66b[19] or transcriptional analysis may reveal cyst-dependent subsets[10,27,28]. CD15+ cells demonstrated a trend towards increased association with parasites that was maintained across two independent experiments, but it cannot be distinguished whether the cells were actively infected or had phagocytosed these parasites. Due to the inability to observe parasite replication, indicating active infection, after only 4 hours, future experiments could utilize a later timepoint such as 24 hours to determine preferential infection of CD15+ cells by parasites. Additional studies could investigate the production of by-products involved in phagocytosis or activation of the phagosome[32] to determine successful phagocytosis of parasites by CD15+ cells.

Functional analyses investigating HNLC responses to chronic cysts demonstrated production of inflammatory cytokines. IL-1α production was observed following exposure to chronic CAg. While human PBMCs produce IL1-β in response to acute Toxoplasma infection[33], IL-1α is generally more associated with tolerance to infection[34]. However, early work demonstrated that IL-1α production by host cells is involved in the recruitment of neutrophils via IL-8 signaling[35]. IL-1α secretion in response to CAg and not whole cysts suggests that neutrophil production of this cytokine may be dependent on receptor mediated PAMP recognition or genes involved in latent cyst recrudesence rather than contact-dependent or phagocytic stimulation[36,37], perhaps suggesting that the sugars present in the cyst wall are not a stimulus for IL-1a. The other striking cytokine response was the

secretion of IFNγ by HNLCs following exposure to both cysts and CAg. IFNγ is critical to the control of acute and chronic Toxoplasma infection. Dunay and colleagues previously identified a population of IFNγ-expressing neutrophils in the brain during chronic murine infection[10] which is supported by our own murine studies on chronic neutrophils[38]. While human neutrophils can also produce IFNγ in response to Toxoplasma infection[39], a lack of T cell-expressed IFNγ leads to fatal pathology[40]. While expression of IFNγ by neutrophils suggests a direct role for them in control of chronic infection, the dominant and well-characterized role of T cell-induced IFNγ may affect the impact of IFNγ production by neutrophils *in vivo*. Future studies could investigate the timing of IFNγ production by neutrophils and T cells to determine the complex interplay between innate vs. adaptive infection control mechanisms.

The stronger immune response to CAg compared to cysts may be expected because the primary site of recognition for an intact cyst will be a homogenous cyst wall structure dominated by glycosylated sugars in contrast to the range of soluble antigens from cyst wall and the protected bradyzoite found in cyst antigen. This suggests cytokine-inducible factors are sequestered inside cysts. Previous work has demonstrated induced immune responses after exposure to bradyzoitespecific antigens[41–44]. Intact cyst walls provide a protective barrier for these bradyzoites, which are released upon cyst reactivation in the brain. Future studies could investigate neutrophil responses to specific cyst antigens to determine potentially important immune activation targets during *in vivo* parasite reactivation.

Functional studies showed no NET production by HNLCs after exposure to cysts. Previous studies reviewed by Blanter and colleagues have identified lower NET production by HNLCs as one of the limitations of this neutrophil model[45]. Alternatively, this finding may support the concept that NET production is an innate effector mechanism to clear tachyzoites specifically or in response to the cell lysis signals seen during acute infection[19]. However, circulating neutrophils during chronic infection are less likely to encounter parasites, and their effector mechanisms within the tissue parenchyma may be dependent on highly localized signals that would still require investigation.

While our results demonstrated unexpected cytokine expression by HNLCs in response to chronic Toxoplasma cysts, these cytokines did not explain the phenomenon of fewer and smaller cysts observed in our imaging studies. Targeted experiments demonstrated striking activity of HNLCs including uptake of cyst material within 60 minutes of exposure. The uptake of cyst material by cells confirms our hypothesis that HNLCs are capable of ingesting and degrading cyst material, revealing a previously unknown antimicrobial role of neutrophils against the chronic cyst form of Toxoplasma. Although neutrophil uptake and direct killing of pathogens including Toxoplasma tachyzoites via direct phagocytosis and autophagy is well known, their ability to target cyst structures that are frequently larger than their normal size is unreported in Toxoplasma infection[31,46,47]. Inhibition of phagocytosis in HNLCs led to a significant decrease in the uptake of cyst material suggesting a possible mechanism of cyst control. However, phagocytosis can be limited to small pathogens due to the inherently small size of neutrophils and could explain the presence of leftover cyst material in both inhibited and non-inhibited cells after exposure.

To compensate for this, neutrophils have additional mechanisms for clearing larger pathogens such as trogocytosis. This process enables neutrophils to kill larger pathogens by trogocytosing or "nibbling" leading to eventual pathogen killing[48–50]. Given the broad range of Toxoplasma cyst sizes (10–100μm), it is possible that HNLCs could employ both phagocytosis and trogocytosis during chronic Toxoplasma infection. Our current imaging experiments did not provide evidence to support neutrophil trogocytosis of cysts[48]. However, previous studies have demonstrated that trogocytosis occurs very quickly after exposure to large pathogens[48]. Based on the presence of larger cysts (~50μm) seen after 3 hours in the absence of HNLCs (Suppl. Fig. 1), it is possible that trogocytosis and breakdown of larger Toxoplasma cysts by neutrophils does actually occur, but due to the rapidity of the response, is not observed because of technical limitations.

Finally, the ability of neutrophils to degrade cysts was dependent on their age and phenotype. Neutrophils can differentiate into multiple subsets, some of which do not possess the canonical antimicrobial qualities of "classical" neutrophils[28]. There is a new appreciation for these "non-classical" neutrophils as they can be beneficial and even protective[51,52]. Aged neutrophils demonstrate decreased effector functions like phagocytosis and instead adopt a more regulatory phenotype as reviewed by Peiseler and Kubes[53]. Our results reveal a decrease in the ability of aged HNLCs to consume cyst material, supporting these previous studies. It also suggests that such cells, if found in the brain, would be less pro-inflammatory, less antimicrobial, and more supportive of chronic infection regulation. As reviewed earlier by Kolaczkowska and Kubes, certain aged neutrophils become senescent and less immunoreactive as they prepare to travel back to the bone marrow for recycling[54]. This suggests that aged neutrophils may be less responsive overall and not just less phagocytic compared to non-aged cells during cyst response. During Toxoplasma infection, however, neutrophils are found during chronic infection in the brain, and our ongoing studies suggest that these cells are immunomodulatory and regulatory rather than non-functioning[38]. Transcriptomic and protein analysis of these cells show that aged neutrophils in the brain possess signatures involved in angiogenesis and lack factors related to apoptosis and canonical neutrophil functions. Future studies could investigate the relationship between these functions seen in murine neutrophils during *in vivo* chronic infection and those seen in human neutrophils in this study.

In conclusion, this study demonstrates Toxoplasma cyst-specific antimicrobial responses of HNLCs. Cysts induce the differentiation of CD15+ HNLCs and cyst-dependent cytokine production. These cells ingest and degrade cyst material which is halted upon the inhibition of phagocytosis. Aging of HNLCs leads to decreased cyst degradation and suggests that non-aged cells are the primary responders to cyst exposure. In conclusion, this study identifies a novel response of HNLCs to Toxoplasma cysts and may point to a role for neutrophils in the clearance of cysts during human infection with Toxoplasma.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Data Availability Statement

The data presented in this study are available from the corresponding author upon reasonable request.

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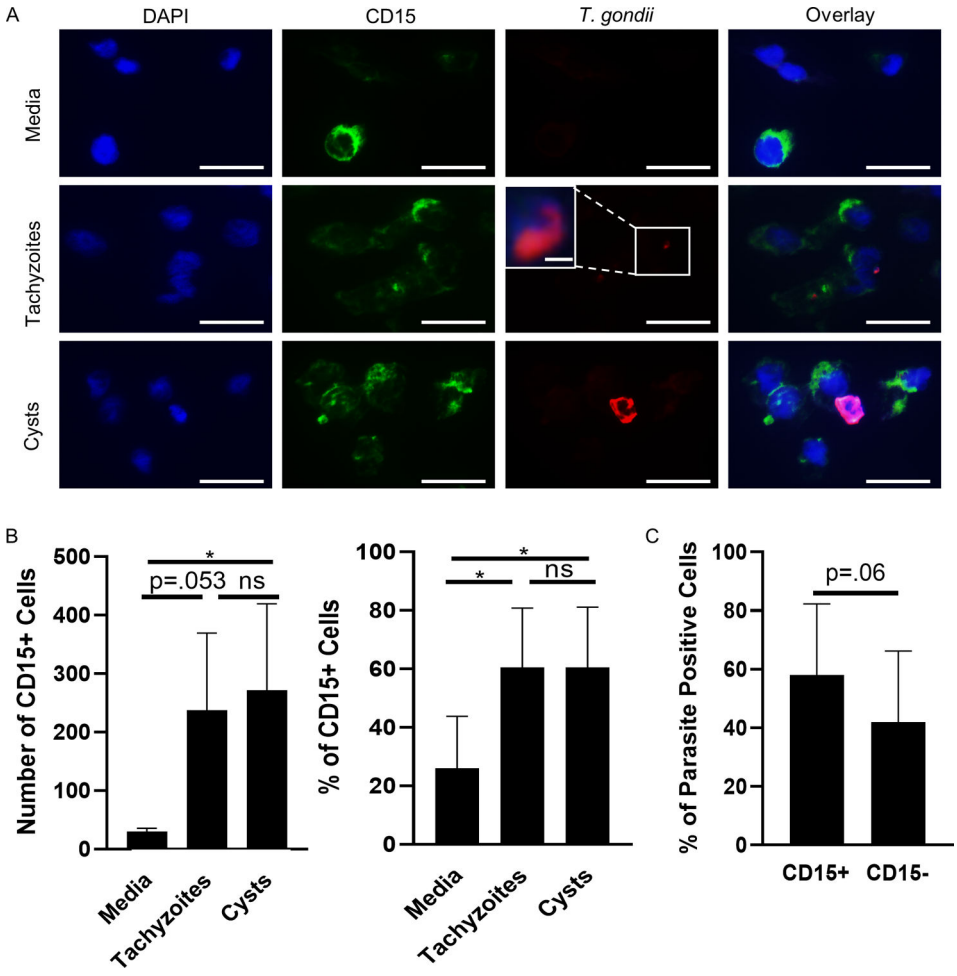

Figure 1. Two different types of human neutrophil-like cells (HNLCs) are present following exposure to T. gondii.

HNLCs were exposed to either *T. gondii* tachyzoites or chronic-stage cysts for 4 hours. Coverslips were stained with immunofluorescent antibodies, and cells were imaged at 40x magnification. A) Immunofluorescence staining of HNLCs following addition of tachyzoites and cysts (blue = DAPI, green = CD15, red = *T. gondii*, scale bar = 25μm, scale bar of zoomed tachyzoite image = 10 μm). B) Total number of CD15+ cells and percent of CD15+ cells from 7 randomized regions of interest (ROIs) per sample (n=3/group). C) Percent of parasite positive cells from 15 randomized ROIs in a total of 6 “Tachyzoite” wells.

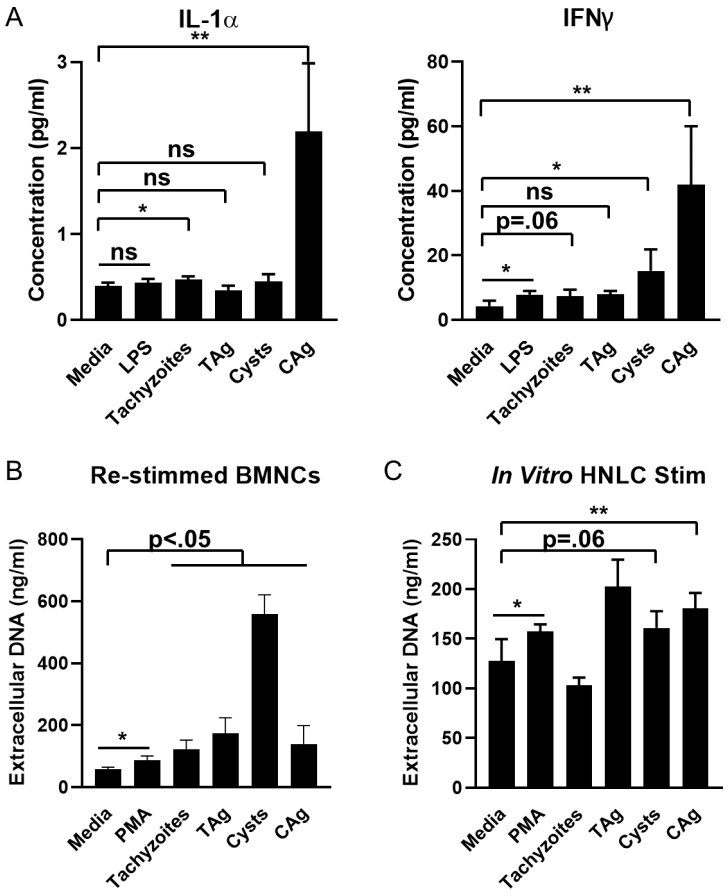

Figure 2. Cyst-induced responses of human neutrophils.

HNLCs were stimulated with media alone, LPS (or PMA for NET production), *T. gondii* Me49 tachyzoites, tachyzoite antigen, *T. gondii* Me49 cysts, or cyst antigen. Following stimulation, cell supernatants were tested for cytokine and chemokine production via LEGENDplex assay and NET production via PicoGreen assay. A) Quantification of cytokine production by HNLCs via LEGENDplex assay. B-C) Quantification of extracellular DNA from re-stimulated brain mononuclear cells (BMNCs) (B) and HNLCs (C). For all significance measures, unpaired Student T-test was performed and p-values are as follows: * = $p<0.05$, ** = $p<0.01$.

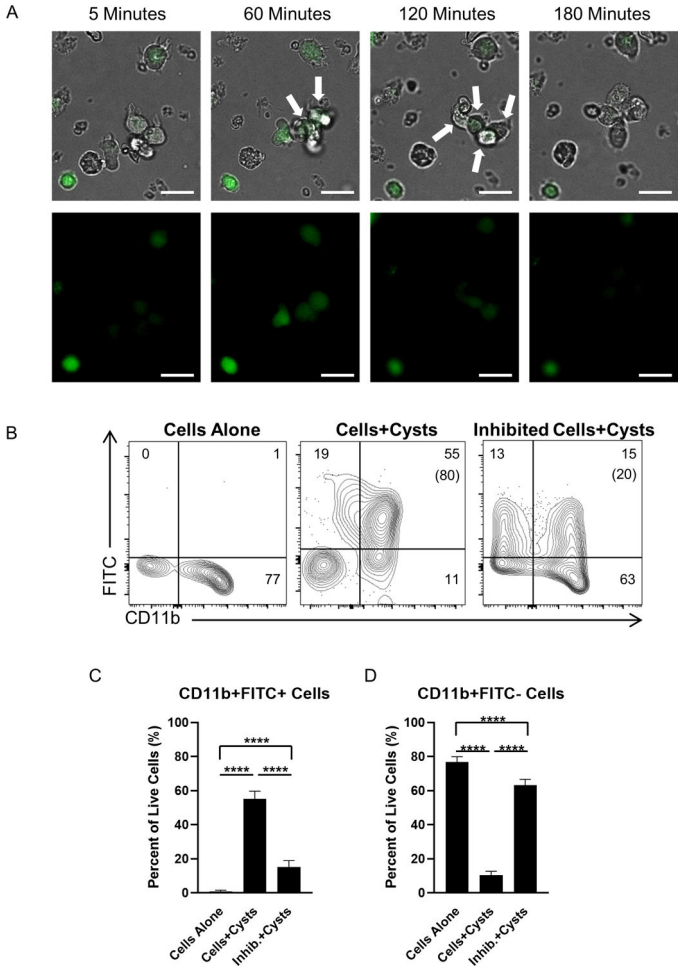

Figure 3. HNLCs attack and phagocytose cyst material.

A) Live time-lapse imaging after exposure of differentiated HNLCs to *T. gondii* cysts for 3 hours (40x magnification). Figure shows time-lapse images of brightfield cells overlayed with fluorescent cysts (green) at 5min, 60min, 120min, and 180min post-cyst exposure. Scale bar = 15μm. Arrows indicate HNLC migration towards cysts. B-D) HNLCs were exposed to cysts for 60 minutes with or without inhibition of phagocytosis using Cytochalasin D. Representative flow cytometry plots (B) of un-exposed cells, normal cells, and inhibited cells following 60min cyst exposure. Flow plots were analyzed after gating on live cells, and numbers on flow plots represent the average percentage of expression (n=4). Numbers in parentheses of upper right quadrants equal average percentages of expression of all CD11b+ cells. Quantification of CD11b+FITC+ (showing cell uptake of cyst material) (C) and CD11b+FITC− (cells without cyst material) (D) frequencies for each exposure condition. Statistical significance was determined by One-Way ANOVA (n=4 per group), **** = p<.0001.

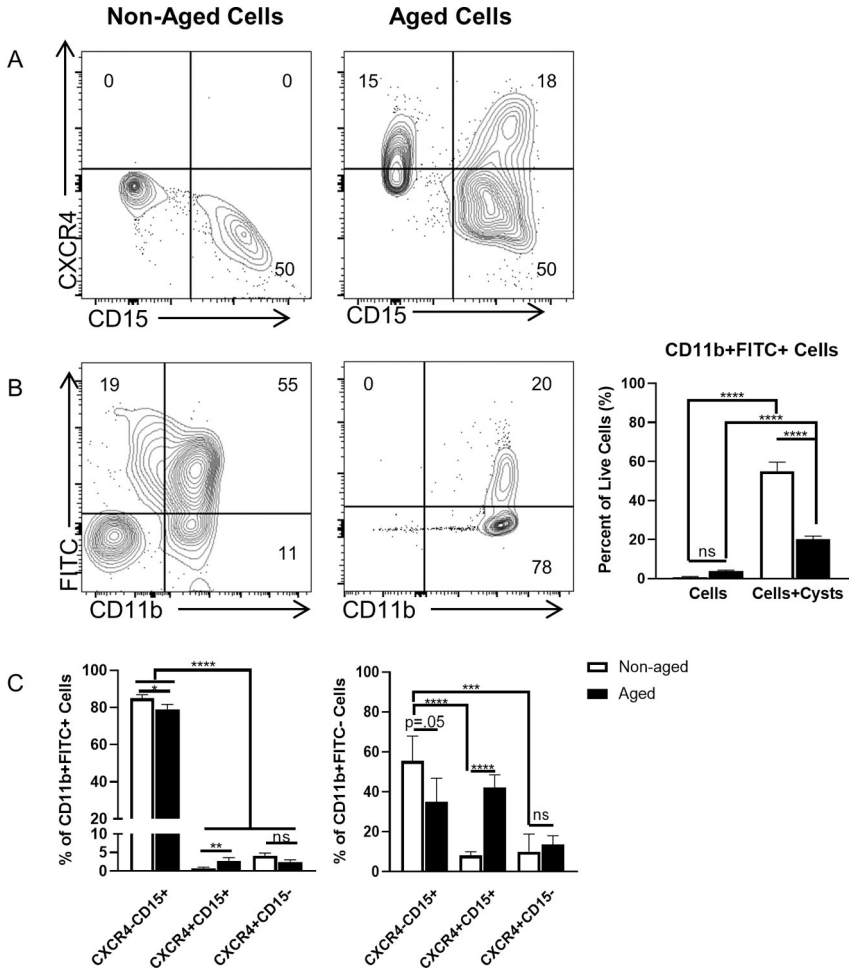

Figure 4. Characterization of HNLCs demonstrates age-dependent differences in response to cyst exposure.

Differentiated HNLCs were either exposed to cysts for 60min following completion of differentiation or were exposed to cysts after 2 weeks of aging following complete differentiation. A) Representative flow cytometry plots of CXCR4 and CD15 expression by unexposed Non-Aged (left) and Aged (right) cells. B) Representative flow cytometry plots (left) and quantification (right) of CD11b+FITC+ (showing cell uptake of cyst material) and CD11b+FITC− (cells without cyst material) frequencies in Non-aged and Aged cells after cyst exposure. Flow plots were analyzed after gating on live cells, and numbers on flow plots represent the average percentage of expression (n=4). C) Quantification of CXCR4 and CD15 frequencies in Non-aged and Aged CD11b+FITC+ (left) and CD11b+FITC− (right) populations after cyst exposure. For all quantification, statistical significance was determined by One-Way ANOVA (n=4 per group), * = $p<0.05$, ** = $p<0.01$, *** = $p<0.001$ and **** = $p<0.0001$.