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Impact of IFN-γ and CD40 signalling on *Toxoplasma gondii* cyst formation in differentiated Neuro-2a neuroblastoma cells

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Abstract

Signalling by IFN-γ and CD40 is known to trigger anti-microbial activity in macrophages infected with *Toxoplasma gondii*, but their effects on infected neurons are less well known. Here, we compared how stimulation with IFN-γ and an agonistic anti-CD40 mAb impacts infection and cyst formation in the mouse neuroblastoma cell line Neuro-2a relative to bone marrow-derived macrophages. Both IFN-γ and CD40 mAb decreased cyst emergence in Neuro-2a cells. In macrophages, these stimuli decreased infection, but had no impact on infection in the neuroblastoma cell line. Resistance to killing in Neuro-2a cells may explain why neurons preferentially harbour parasites during chronic infection in the brain.

Keywords

cyst; macrophage; neuroblastoma; *Toxoplasma gondii*

1 | INTRODUCTION

A globally prevalent parasite, *Toxoplasma gondii* is an intracellular protozoan that owes its success largely to a remarkable ability to manipulate host immunity.[1,2] Infection with *T. gondii* begins with an acute phase characterized by actively invading tachyzoites that disseminate widely throughout host tissues.[3,4] After 10–14 days, parasites become preferentially localized in tissues of the central nervous system. Once in neuronal tissue, *T. gondii* establishes latency, which is characterized by slow-growing bradyzoites contained within tissue cysts. While healthy individuals are normally asymptomatic during latent *T. gondii* infection, immunocompromised individuals are at serious risk of parasite recrudescence that may result in lethal toxoplasmic encephalitis.[5] In the brain, two

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AUTHOR CONTRIBUTIONS

CMD, ADR and EYD designed the studies, CMD and ADR performed the experiments and CMD and EYD wrote the manuscript.

CONFLICT OF INTEREST

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critical immune mediators that are involved in preventing parasite reactivation include the proinflammatory cytokine IFN-y and the costimulatory molecule CD40.[6,7]

Production of IFN-y has long been known to play a critical role in host immune defence during *Toxoplasma* infection.[8,9] In mice, this is in large part due to the induction of immune-related GTPases (IRGs) that mediate disassembly of the parasitophorous vacuole membrane.[3,10–12] In humans, IFN-y contributes to host immunity through tryptophan degradation, production of reactive oxygen species and triggering of cell death.[13–15] Ultimately, the role of IFN-y in host immunity is to facilitate host control of both acute and latent *T. gondii* infection by controlling parasite expansion. Signalling through CD40 also plays a role in macrophage control of infection. In this case, CD40 stimulates autophagy-dependent vacuole-lysosomal fusion and parasite killing dependent upon host proteins such as ATG5, ATG7 and Beclin-1.[16,17] CD40 signalling appears to trigger multiple pathways leading to autophagic elimination of *Toxoplasma*. These pathways include AMPK-mediated ULK1 phosphorylation, autocrine TNF-α secretion leading to Beclin-1 activation, regulation of Beclin-1 protein and activation of PKR.[16,18–21]

Although the effects of IFN-y and CD40 signalling in macrophages are well known, less is known about how these mediators impact *Toxoplasma* infection in neurons. This is critical because neurons are thought to preferentially harbour *T. gondii* during chronic infection.[22–25] To gain insight into this issue, we used the mouse neuroblastoma cell line Neuro-2a (N2a) to examine how infection and cyst formation are impacted by signalling through IFN-y and CD40 in comparison with infection in mouse macrophages.

2 | MATERIALS AND METHODS

2.1 | Ethics statement

All experiments were performed in strict accordance with the recommendations set forth by the National Institutes of Health *Guide for the Care and Use of Laboratory Animals* (8th Edition). Protocols were approved by the Institutional Animal Care and Use Committee at the University of New Mexico (Animal Welfare Assurance Number A4023-01). All efforts were made to minimize animal suffering and distress over the course of the studies.

2.2 | Mice

Swiss Webster and C57BL/6 mouse strains were obtained from The Jackson Laboratory. Both male and female mice (6–12 weeks of age) were used in these studies.

2.3 | Parasites and infections

Type II (PTG) parasites were purchased from the American Type Culture Collection (ATCC) and were maintained *in vitro* by passage on confluent monolayers of human foreskin fibroblasts (ATCC). ME49 cysts were originally provided by C. A. Hunter (University of Pennsylvania). A colony of chronically infected Swiss Webster mice was used to maintain ME49 cysts. Infection was established by intraperitoneal injection of 20 cysts. After 4–8 weeks, brains were isolated and homogenized to obtain cysts for passage in mice and for experimental studies.

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2.4 | Cell culture

Bone marrow from C57BL/6 mice was collected using a 10-mL syringe and a 27-Ga needle to flush cells from tibia. Cells were differentiated into bone marrow-derived macrophages (BMDM) using media composed of Dulbecco's modified Eagle media (DMEM; Cat#10-017-CV), 10% bovine growth serum (BGS; HyClone Laboratories, Cat# SH30541.03), nonessential amino acids (ThermoFisher Scientific, Cat#15630-080), 100 U/mL penicillin (ThermoFisher Scientific, Cat#15140-122), 0.1 mg/mL streptomycin (ThermoFisher Scientific, Cat #15140-122) and L929 culture supernatant as previously described.[26] The BMDM were used 5 days after culture initiation. N2a cells (ATCC, Cat#CCL-131) were maintained in media composed of DMEM containing 10% BGS, 100 U/mL penicillin and 0.1 mg/mL streptomycin. At 24 h prior to infection with PTG, N2a cells were differentiated with the addition of 10 μM al-trans retinoic acid (Sigma-Aldrich, Cat#302-79-4). At time of infection, cells were treated with either 100 U/mL recombinant IFN-y (Peprotec, Cat#315-05), 50 mg/mL agonistic anti-CD40 mAb (BioXCell, Cat#BE0016-2) or left untreated.

2.5 | Western blot analysis

Differentiated N2a cells and BMDM were plated on 24-well tissue culture plates (Corning, Cat#3524) and allowed to become confluent by overnight incubation at 37°C in 5% CO_2. For the case of BMDM, cells were switched to medium composed of DMEM supplemented with 1% BGS 18 h prior to infection. The following day, cells were infected with PTG tachyzoites and stimulated with either recombinant IFN-y, anti-CD40 mAb or left untreated. Lysates were prepared with the addition of reducing SDS lysis buffer and sample DNA was sheared by 3× passage through a 27-Ga needle. The samples were boiled for 3 min and then loaded onto a 10% acrylamide protein gel (Bio-Rad Laboratories, Cat#4561033). After electrophoresis, proteins were electrotransferred onto a nitrocellulose membrane (Bio-Rad, Cat#1620115). The membranes were blocked using 5% non-fat dry milk (Bio-Rad, Cat#1706404) in 20 mM Tris-buffered 150 mM NaCl (pH 7.4) with 0.05% Tween 20 (TBST) for 2 h at 25°C. Primary antibodies diluted in TBST were added and membranes were incubated overnight at 4°C on a rotating platform. Primary antibodies included anti-phospho-ERK1/2 (Cell Signaling Technologies, Cat#4511), total-ERK1/2 (Cell Signaling, Cat#4695), anti-phospho-STAT1 (Cell Signaling, Cat#9167), anti-total-STAT1 (Cell Signaling, Cat#14994), anti-phospho-STAT3 (Cell Signaling, Cat#9145) and anti-total-STAT3 (Cell Signaling, Cat#9139). After washing membranes in TBST, secondary horseradish peroxidase-linked anti-rabbit antibody (Invitrogen, Cat #A16035) diluted in TBST was added and blots were incubated on a rotating platform (2 h, room temperature). Membranes were washed in TBST, and then developed using an enhanced chemiluminescent substrate system (ThermoFisher Scientific, Cat#34580).

2.6 | Immunofluorescence microscopy

Differentiated N2a cells and BMDM were plated in DMEM containing 10% BGS, 100 U/mL penicillin and 0.1 mg/mL streptomycin on glass coverslips and allowed to become confluent by overnight culture (37°C, 5% CO_2). Following a 2:1 infection with PTG, coverslips were collected and processed for microscopy. After rinsing with PBS, cells on

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coverslips were fixed in 3.7% formaldehyde (MilliporeSigma, Cat#FX0410-5) for 20 min at room temperature. Following fixation, coverslips were blocked for 1 h at room temperature using 5% normal mouse serum (Invitrogen, Cat#10410) in permeabilization buffer (0.1% saponin in phosphate-buffered saline). Staining was accomplished in permeabilization buffer using fluorescein isothiocyanate (FITC)-conjugated anti-*Toxoplasma* polyclonal antibody (Invitrogen, Cat#PA17253) and rhodamine-conjugated *Dolichos biflorus* agglutinin (DBA; Vector Laboratories, Cat#RL-1032). Coverslips were mounted onto microscope slides using permount solution containing 4′,6-diamidino-2-phenylindole (DAPI; Invitrogen, Cat#P36962). Imaging was performed using a BX53 fluorescence microscope (Olympus America, Inc) and DP manager software (Olympus). Each slide was imaged over 5 different fields each containing approximately 100 DAPI-positive cells.

2.7 | Flow cytometry

Single cell suspensions of differentiated N2a cells and BMDM were stained with allophycocyanin-conjugated anti-CD40 Ab (BioLegend, San Diego, CA, Cat #124611) for 20 min at 4°C in FACS buffer composed of 1% bovine growth serum, 0.01% sodium azide (VWR, Cat#0639) in phosphate-buffered saline. Controls remained un-stained. Cells were then fixed in 3.7% formaldehyde. Samples were interrogated on a four laser (violet, blue, yellow, red) Attune NxT flow cytometer (ThermoFisher Scientific) and the data were processed using FlowJo v.10 software (FlowJo).

2.8 | Statistical analyses

Statistical analyses were performed using GraphPad Prism v.9 (GraphPad). Two-tailed paired t-tests were used to compare the normally distributed data of the two groups. A confidence interval of 95% (a = 0.05) was used as the cut-off to denote significant changes between groups.

3 | RESULTS AND DISCUSSION

3.1 | Retinoic acid-differentiated N2a cells support generation of *Toxoplasma* cysts

We used PTG tachyzoites, a low-virulence *Toxoplasma* strain capable of establishing chronic infection in mice, to examine *in vitro* cyst formation in N2a mouse neuroblastoma cells relative to mouse bone marrow-derived macrophages (BMDM). Cysts were visualized by staining with rhodamine-conjugated DBA, a lectin that binds carbohydrate moieties present in the cyst wall.[27] Tachyzoites were counter-stained with a FITC-labelled anti-*Toxoplasma* polyclonal antibody. As shown in Figure 1A, cysts spontaneously formed in both retinoic acid (RA)-differentiated N2a cells and BMDM. The cysts generated *in vitro* were overall approximately three-fold decreased in diameter relative to cysts isolated from latently infected mouse brain (Figure 1A). This is likely not surprising given that the in vivo generated cysts were isolated 30–60 days following infection, whereas cysts generated *in vitro* were visualized 72 h after infection. Overall, there was no significant difference in diameter of cysts generated in N2a cells vs. BMDM (data not shown). In contrast, cyst formation in human fibroblast monolayers was extremely rare (Figure 1B). Next, we determined if non-differentiated vs. RA-differentiated N2a cells differed in ability to support cyst formation. While the percent infection in differentiated vs. nondifferentiated N2a cells

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did not differ over multiple experiments (data not shown), N2a were significantly more permissive to cyst generation following RA differentiation (Figure 1C,D). We also examined the kinetics of cyst formation over 72 h in BMDM compared with RA-differentiated N2a cells. While there was a significant increase in cyst formation in BMDM compared with N2a cells at early time points, by 72 h post-infection, the percentage of infected cells harbouring cysts was equivalent in both cell types (Figure 1E). Finally, we examined percent infection in BMDM and RA-differentiated N2a cells over a range of MOI. While percent infection was significantly higher in N2A cells at intermediate MOI, at high MOI (10), infection rates converged at 100% (Figure 1F).

3.2 | N2a cells and BMDM respond strongly but differently to agonistic anti-CD40 mAb

N2a cells are known to express CD40, a costimulatory molecule important in control of chronic *T. gondii* infection.[28,29] Signalling through CD40 is also known to be capable of promoting parasite destruction in an autophagy-related manner.[16] The cytokine IFN-y and its widely expressed receptor are well known to be involved in *Toxoplasma* killing through multiple mechanisms including the IRG system of GTPases.[30] Therefore, we sought to determine how CD40 and IFN-y influenced cell signalling in RA-differentiated N2a cells compared with BMDM. As shown in Figure 2A, stimulation of N2a cells with exogenous IFN-y triggered rapid signal transducer and activator of transcription (STAT)-1 activation followed by deactivation in a manner kinetically similar to that occurring in BMDM. We then examined the ability of an agonistic CD40 mAb to trigger signalling in N2a cells compared with BMDM. Signalling through CD40 has previously been shown to activate a p42/p44 mitogen-activated protein kinase (MAPK) signalling cascade.[28] Indeed, in RA-differentiated N2a cells, p42/p44 MAPK was strongly activated by CD40 mAb (Figure 2B). In contrast to STAT-1 activation, the p42/p44 MAPK phosphorylation response was both delayed and sustained (Figure 2A vs. B) in the RA-differentiated N2a cells. In parallel BMDM cultures, we did not detect activation of p42/p44 MAPK in response to agonistic anti-CD40 mAb. Previous studies have reported STAT3 activation in response to CD40 signalling.[31] Interestingly, we found that this STAT molecule was phosphorylated in anti-CD40 stimulated BMDM, but not N2a cells (Figure 2C). In Figure 2D, we compared expression of CD40 RA-differentiated N2a cells and BMDM. Levels of expression were overall similar in both cell types (Figure 2D). While expression of CD40 was relatively low, this is in line with previous studies that examined CD40 expression in BMDM.[32,33]

3.3 | rIFN-y and agonistic anti-CD40 mAb differentially impact infection and cyst formation in N2a cells and BMDM

We then investigated how infection and cyst formation were influenced by IFN-y added at the time of infection. The presence of IFN-y had no impact on infection at 72 h in RA-differentiated N2a cells (Figure 3A). However, stimulation with IFN-y impeded emergence of cysts in N2a cells (Figure 3B). In BMDM, and as expected, IFN-y triggering at the time of infection decreased the percent of parasite-positive cells at 72 h (Figure 3C). Emergence of cysts in BMDM was not significantly impacted by the presence of IFN-y (Figure 3D).

We next wanted to determine if signalling through CD40 affected persistence of infection or cyst emergence in N2a cells compared with BMDM. Accordingly, agonistic anti-CD40

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mAb was added to RA-differentiated N2a cells concurrently with parasite inoculation, and then 72 h later, percent infection and percent of infected cells harbouring cysts were evaluated. Similar to the stimulation with IFN-y, signalling through CD40 had no significant effect on the degree of infection in N2a cells (Figure 4A). However, emergence of cysts was significantly hindered in the presence of anti-CD40 mAb (Figure 4B). In BMDM, signalling through CD40 decreased the percentage of infected cells at 72 h (Figure 4C). Signalling through CD40 appeared to promote emergence of cysts, although in this case the results did not attain statistical significance (Figure 4D; $p = .19$). Overall, neither CD40 nor IFN-y signalling impacted short-term parasite persistence, but both pathways impeded cyst formation in differentiated N2a cells. In contrast, both pathways decreased infection in BMDM, but the effects of IFN-y and CD40 signalling may diverge in influencing cyst emergence in this cell type. Divergent activation of p42/44 MAPK and STAT3 may underpin the differential effects of CD40 triggering in BMDM compared with N2a cells.

In Figures 3A and 4A, we observed that activation of IFN-y and CD40 signalling did not impact infection in RA-differentiated N2a cells. This may be of relevance to *in vivo* infection insofar as neurons in the central nervous system are preferentially used as host cells during chronic *T. gondii* infection.[22] Lack of inherent killing mechanisms may make these cells attractive targets of infection when the parasite reaches the brain. In contrast, other brain-resident cells such as astrocytes and microglia are capable of toxoplasmacidal activity in humans and mice.[34–38] An alternative view is that neurons are hypersusceptible to infection.[22] Indeed, inoculation of parasites in our experiments routinely led to greater infection in N2a cells relative to BMDM (Figure 3A vs. C; Figure 4A vs. C).

While IFN-y or CD40 signalling did not affect persistence of infection in N2a cells, we observed an inhibitory effect of both on cyst differentiation over 72 h (Figures 3B and 4B). This may have relevance to *in vivo* infection, since it was long ago established that both IFN-y and CD40/CD40L are required to prevent emergence of toxoplasmic encephalitis (TE) and concomitant uncontrolled parasite growth in mouse models of infection.[11,29,39–41] $CD4^+$ and $CD8^+$ T lymphocytes are also required to avoid TE.[39] Therefore, a plausible model is that T cell CD40L-neuronal CD40 interactions are required to prevent establishment of high numbers of cysts that would otherwise lead to inflammatory pathology.

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DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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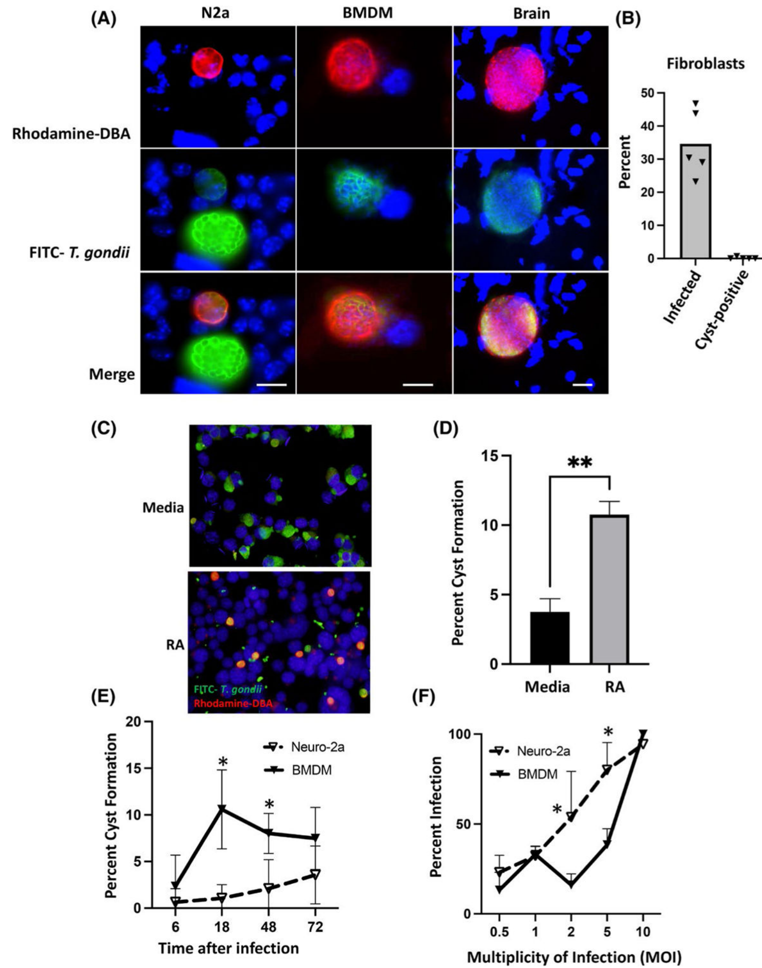

FIGURE 1.

RA-differentiated N2a cells and BMDM support rapid *T. gondii* cyst formation. A, Immunofluorescence staining of RA-differentiated N2a and BMDM 72 h post-infection with PTG strain tachyzoites. Staining of an ME49 cyst in mouse brain homogenate 30 days post-infection is shown for comparison. Parasite cyst walls were stained using rhodamine-conjugated DBA (red), and individual parasites were labelled with FITC-conjugated anti-*T. gondii* Ab (green). Nuclei are stained with DAPI (blue). The scale bars in merged images of N2a cells and BMDM indicate 10 mm. Scale bar in merged image of the brain cyst indicates 20 mm. B, percent infection and percent cyst formation in human foreskin fibroblasts. C, Cyst formation in non-differentiated vs RA-differentiated N2a cells. Cysts and individual parasites were imaged as in A. Scale bar indicates 10 mm. D, Quantification of percent cyst formation in differentiated vs non-differentiated N2a cells. Percent Cyst Formation, percent of infected cells staining with rhodamine-DBA. E, Kinetics of cyst formation in RA-differentiated N2a cells and BMDM. F, Comparison of infection over a range of MOI in N2a vs. BMDM. In D, an unpaired t test with a Welch's correction was employed to determine statistical significance where ** indicates $p < .01$. In E and F, a Multiple Mann-Whitney test was employed to determine statistical significance where * indicates $p < .05$. Each experiment was repeated three times with the same essential result

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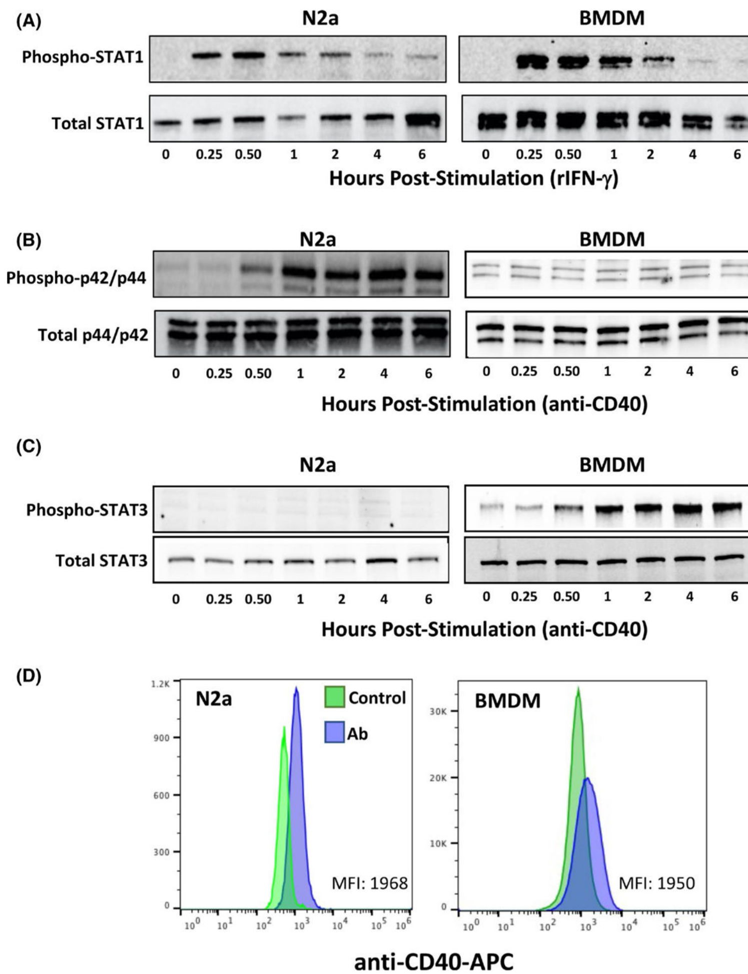

FIGURE 2.

Effect of recombinant IFN-y and agonistic anti-CD40 mAb on signalling in N2a cells and BMDM. A, STAT-1 tyrosine phosphorylation in response to rIFN-y in RA-differentiated N2a cells and BMDM. B, Ser-Thr phosphorylation of p42/p44 MAPK in RA-differentiated N2a cells and BMDM after stimulation with agonistic anti-CD40 antibody. C, Tyr phosphorylation of STAT3 in responses to anti-CD40 antibody treatment. D, Flow cytometric analysis of cell surface CD40 expression on differentiated N2a cells and BMDM. Control, unstained cells. Ab, anti-CD40 Ab stained. These experiments were repeated three times with the same result

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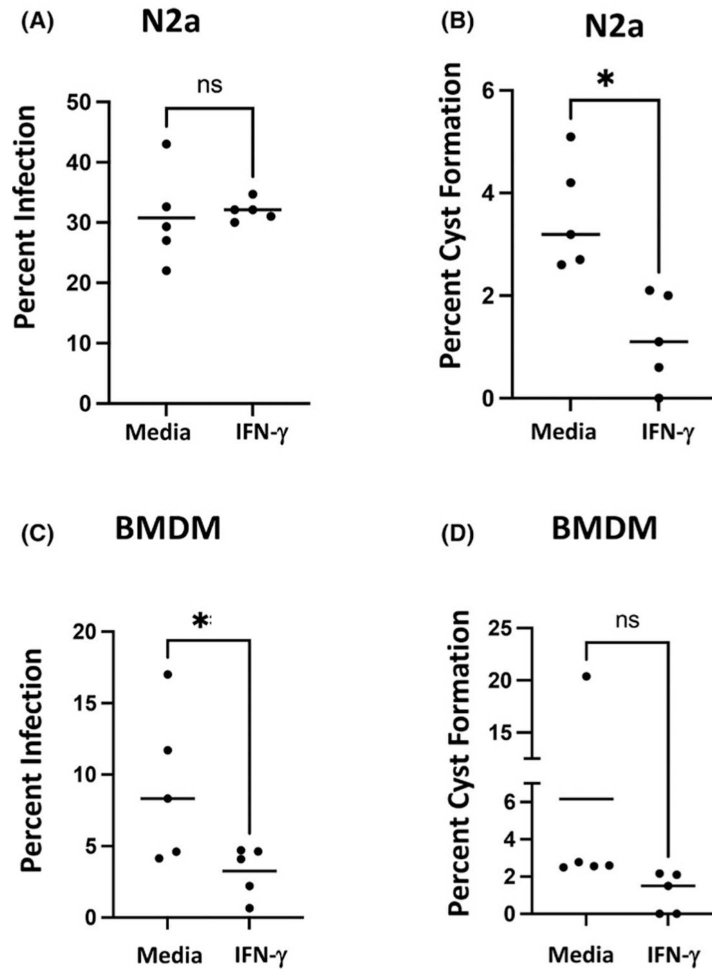

FIGURE 3.

Disparate effects of rIFN-y on percent infection and cyst formation in BMDM and RA-differentiated N2a cells. RA-differentiated N2a cells (A and B) and BMDM (C and D) were infected with PTG tachyzoites simultaneously with the addition of rIFN-y at a 2:1 MOI. At 72 h post-infection, cells were fixed and stained with FITC-labelled anti-*Toxoplasma* Ab and rhodamine-conjugated DBA as described in Figure 1 legend. Percent infection (A and C) and percent cyst formation in infected cells (B and D) are shown. Each symbol represents an independent experiment ($n = 5$) quantifying approximately 500 cells over 5 imaging fields. *$p < .05$ (unpaired t test). ns, not significant

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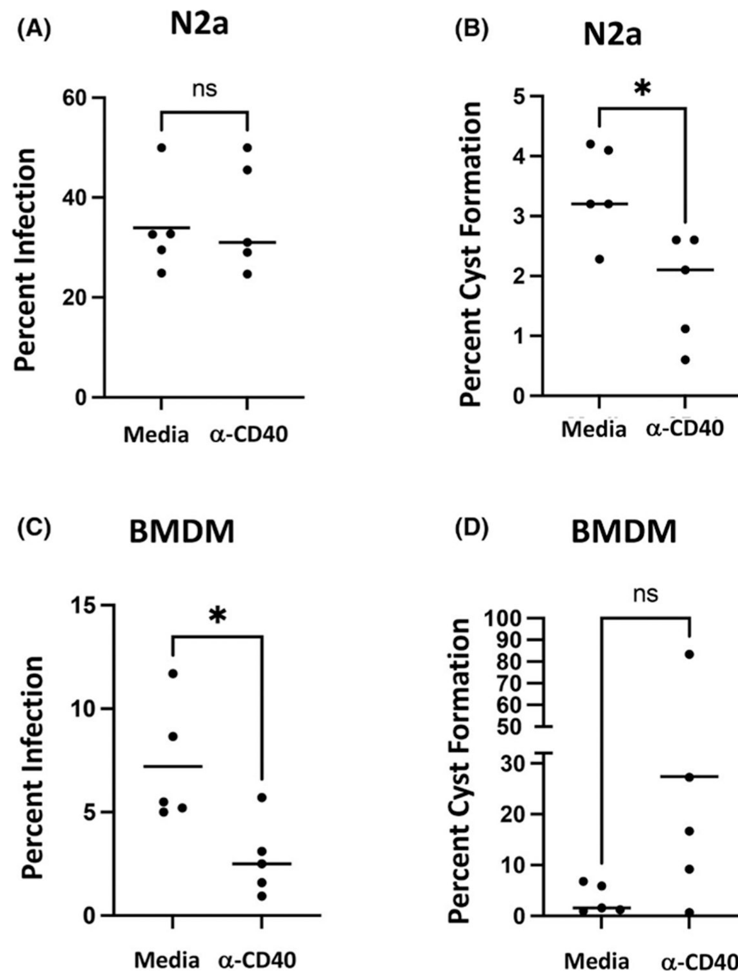

FIGURE 4.

Signalling through CD40 decreases infection in BMDM and decreases cysts formation in N2a cells. Cells were infected with PTG tachyzoites and agonistic anti-CD40 mAb was added at Day 0. PTG tachyzoites were added in a 2:1 MOI to host cells. On Day 3, cells were fixed and stained, and then, percent infection and percent cyst formation were determined as in Figure 3. $*p < .05$; ns, not significant