

## CD40 Signaling in Macrophages Induces Activity against an Intracellular Pathogen Independently of Gamma Interferon and Reactive Nitrogen Intermediates

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**Gamma interferon (IFN- $\gamma$ ) is the major inducer of classical activation of macrophages. Classically activated mouse macrophages acquire antimicrobial activity that is largely dependent on the production of reactive nitrogen intermediates. However, protection against important intracellular pathogens can take place in the absence of IFN- $\gamma$  and nitric oxide synthase 2 (NOS2). Using *Toxoplasma gondii* as a model, we investigated if CD40 signaling generates mouse macrophages with effector function against an intracellular pathogen despite the absence of priming with IFN- $\gamma$  and lack of production of reactive nitrogen intermediates. CD40-stimulated macrophages acquired anti-*T. gondii* activity that was not inhibited by a neutralizing anti-IFN- $\gamma$  monoclonal antibody but was ablated by the neutralization of tumor necrosis factor alpha (TNF- $\alpha$ ). Moreover, while the induction of anti-*T. gondii* activity in response to CD40 stimulation was unimpaired in macrophages from IFN- $\gamma$ <sup>-/-</sup> mice, macrophages from TNF receptor 1/2<sup>-/-</sup> mice failed to respond to CD40 engagement. In contrast to IFN- $\gamma$ -lipopolysaccharide, CD40 stimulation did not induce NOS2 expression and did not trigger production of reactive nitrogen intermediates. Neither N<sup>G</sup>-monomethyl-L-arginine nor diphenylethionium chloride affected the induction of anti-*T. gondii* activity in response to CD40. Finally, macrophages from NOS2<sup>-/-</sup> mice acquired anti-*T. gondii* activity in response to CD40 stimulation that was similar to that of macrophages from wild-type mice. These results demonstrate that CD40 induces the antimicrobial activity of macrophages against an intracellular pathogen despite the lack of two central features of classically activated macrophages: priming with IFN- $\gamma$  and production of reactive nitrogen intermediates.**

Macrophages play key regulatory and effector functions for many aspects of the immune response. Type 1 cytokine response results in generation of the so-called “classically” activated macrophages that act as pivotal effectors of the control of intracellular pathogens (9, 19). Despite the evidence that macrophages are diverse in regards to the mechanisms that lead to their activation and effector functions (9, 19), gamma interferon (IFN- $\gamma$ ) is still considered central for the generation of macrophages with antimicrobial activity against intracellular pathogens (9, 19). However, patients with congenital defects in IFN- $\gamma$  signaling control infection with the obligate intracellular protozoan *Toxoplasma gondii* (13) and do not appear to be susceptible to *Listeria monocytogenes* and *Legionella pneumophila* (24). Similarly, IFN- $\gamma$ <sup>-/-</sup> and IFN- $\gamma$  receptor<sup>-/-</sup> (IFN- $\gamma$ R<sup>-/-</sup>) mice develop mechanisms of control of pathogens such as *L. monocytogenes*, *Leishmania donovani*, and *Histoplasma capsulatum* (36, 38, 45). These findings suggest that there is heterogeneity in regard to the mechanisms of induction of macrophages with antimicrobial activities against intracellular pathogens. Indeed, using a model of *T. gondii* infection, we recently demonstrated that CD40-CD154 interaction alone, a signaling pathway crucial for host protection against a variety of intracellular pathogens, induces human monocyte-derived

macrophages to acquire anti-*T. gondii* activity independently of IFN- $\gamma$  (2).

Reactive nitrogen intermediates (RNI) are important mediators of antimicrobial activity in mouse macrophages. Production of RNI is a hallmark of classically activated macrophages in mice (9, 19). Inhibition of RNI production blocks the antimicrobial activity of IFN- $\gamma$ -stimulated mouse macrophages (1, 4, 11, 16). In vivo data reveal that RNI are necessary for host protection against intracellular pathogens, including *Leishmania major*, *Mycobacterium tuberculosis*, *L. monocytogenes*, and *Trypanosoma cruzi* (4, 6, 17, 28, 40, 42). In the model of *T. gondii* infection, NOS2 is essential for control of the chronic phase of infection (26). Interestingly, an analogous picture has been reported for CD154 since CD154<sup>-/-</sup> mice are also susceptible to toxoplasmic encephalitis (25).

The present studies were conducted to determine if CD40 signaling alone generates mouse macrophages with antimicrobial activity against an intracellular pathogen despite the absence of two central features of classically activated macrophages: not only lack of priming with IFN- $\gamma$  but also lack of production of RNI. We considered this question important because it addresses heterogeneity in the biology of macrophages with an effector function against intracellular pathogens.

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### MATERIALS AND METHODS

**Animals.** Specific-pathogen-free female BALB/c and C57BL/6 (B6) mice were obtained from the National Cancer Institute (Frederick, MD). Female IFN- $\gamma$ <sup>-/-</sup>

(BALB/c background), tumor necrosis factor receptor 1/2<sup>-/-</sup> (TNFR1/2<sup>-/-</sup>) (B6/129 background), and nitric oxide synthase 2 knockout (NOS2<sup>-/-</sup>) (B6/129 background) mice and appropriate wild-type controls were purchased from Jackson Laboratories (Bar Harbor, ME). Animals were 8 to 10 weeks old when used.

**Macrophages.** Resident peritoneal macrophages were collected by lavage with 2 ml of ice-cold phosphate-buffered saline. Peritoneal cells were cultured on eight-chamber tissue culture glass slides (Falcon; Becton Dickinson Labware, Franklin Lakes, NJ) at  $5 \times 10^5$  cells/ml in complete medium (CM) consisting of Dulbecco's modified Eagle medium plus 10% fetal bovine serum (HyClone, Logan, UT). Nonadherent cells were removed after 6 h. Bone marrow-derived macrophages were obtained by culturing bone marrow cells for 7 days in Teflon jars containing Dulbecco's modified Eagle medium plus 30% L-cell-conditioned medium, 10% fetal bovine serum, and 5% horse serum (HyClone). Bone marrow-derived macrophages were also plated on eight-well chamber culture slides. Prior to infection with *T. gondii*, macrophages were incubated in culture slides for 24 h with either stimulatory rat anti-CD40 (1C10) or control (B39-4) monoclonal antibodies (MAbs, both at 10  $\mu$ g/ml), recombinant mouse CD154 (3  $\mu$ g/ml; Immunex Corporation, Seattle, WA), or recombinant mouse IFN- $\gamma$  (100 U/ml; Peprotech, Rocky Hill, NJ) plus lipopolysaccharide (LPS, 100 ng/ml; Sigma Chemical, St. Louis, MO). When indicated, macrophages were incubated with N<sup>G</sup>-monomethyl-L-arginine (NMA, 100  $\mu$ M; Calbiochem, San Diego, CA), anti-IFN- $\gamma$  (XMG1.2), anti-TNF- $\alpha$  (G281-2626), or control (R3-34) MAbs (all MAbs at 10  $\mu$ g/ml; BD Biosciences, San Jose, CA) for 24 h prior to infection, and reagents were added back after challenge with *T. gondii*. Macrophages were also treated with diphenyleneiodonium chloride (DPI, 5  $\mu$ M; Sigma Chemical) during the first hour after addition of IFN- $\gamma$ -LPS.

***T. gondii* infection and parasite growth.** Monolayers of macrophages were washed prior to the addition of *T. gondii*. Tachyzoites of the RH strain of *T. gondii* obtained as described previously (31) were used to infect monolayers at a ratio of 1.5 parasites per macrophage. Parasite replication was assessed by light microscopy (2). Briefly, monolayers were washed 1 h after the addition of *T. gondii* to remove extracellular parasites. Thereafter, monolayers were fixed and stained with Diff-Quick (Dade Diagnostics, Aguada, Puerto Rico) or monolayers were reincubated in fresh CM alone (except when using NMA or using anti-TNF- $\alpha$  and IFN- $\gamma$  MAbs), followed by fixation and staining 18 h after the addition of *T. gondii*. The percentage of infected macrophages, the number of tachyzoites per infected macrophage, and the number of parasites per 100 macrophages in duplicate monolayers were determined by light microscopy by counting at least 200 macrophages per monolayer. There was no difference in cell loss between monolayers of control and CD40-stimulated macrophages. The percentages of cells that detached from monolayers during washing steps prior to staining were similar in macrophage preparations from the different strains of mice used. Cell densities were determined using an eyepiece grid (1 mm by 1 mm). Three randomly selected fields were examined per monolayer.

In certain experiments, splenic T cells from uninfected BALB/c mice were obtained as described previously (32) and incubated at  $10^6$ /ml in either CM or CM plus phorbol myristate acetate (PMA, 10 ng/ml) and ionomycin (1  $\mu$ g/ml) (Sigma Chemical). After 6 h of incubation, T cells were extensively washed, resuspended in CM at  $2.5 \times 10^5$ /ml, and added to syngeneic macrophage monolayers after the removal of extracellular *T. gondii*. T cells were incubated with infected macrophages in the presence of anti-IFN- $\gamma$ , anti-CD154 (MR1; BD Biosciences), or isotype control MAbs (R3-34 and A19-3, respectively; BD Biosciences), all at 10  $\mu$ g/ml. Monolayers were examined 18 h postinfection. In parallel experiments, PMA and ionomycin added to CM alone (without T cells) and handled similarly to T-cell preparations failed to affect parasite growth in macrophages. For immunofluorescence and confocal microscopy, transgenic RH *T. gondii* that expresses yellow fluorescent protein (YFP; gift from David Roos) was used to infect resident peritoneal macrophages at a ratio of three parasites per macrophage.

**Immunoblotting.** Macrophages cultured in CM alone or CM plus either anti-CD40 or control MAb or in LPS plus IFN- $\gamma$  were lysed in buffer containing 20 mM Tris HCl (pH 7.4), 100 mM NaCl, 10% glycerol, 1% Triton X-100, 100 mM NaF, 1 mM NaVO<sub>4</sub>, 1 mM benzamide, 20  $\mu$ g/ml leupeptin, 5 mM phenylmethylsulfonyl fluoride, and 20  $\mu$ g/ml aprotinin (all from Sigma Chemical). Proteins were resolved by sodium dodecyl sulfate-10% polyacrylamide gel electrophoresis and transferred onto a polyvinylidene difluoride membrane. Membranes were probed with either a mouse MAb anti-mouse NOS2 (BD Biosciences) or polyclonal rabbit antibody to actin (Santa Cruz Biotechnologies, Santa Cruz, CA), followed by incubation with either donkey anti-mouse immunoglobulin G (IgG) or goat anti-rabbit conjugated to horseradish peroxidase (Santa Cruz Biotechnologies). Bands were developed using enhanced chemiluminescence following manufacturer's instructions (Pierce, Rockford, IL).

**PCR.** RNA was obtained from macrophage monolayers with RNeasy (QIAGEN, Valencia, CA), and 1  $\mu$ g was reverse transcribed to cDNA with SuperScript III reverse transcriptase (Invitrogen, Carlsbad, CA) and oligo(dT)<sub>12-18</sub> primers. cDNA was amplified using previously described primers specific for NOS2 and hypoxanthine phosphoribosyltransferase (HPRT) (29, 35). HPRT mRNA was transcribed to verify that equal amounts of RNA were added in all experimental conditions. After an initial incubation at 95°C for 3 min, temperature cycling was initiated using the GeneAmp PCR System 9700 (Perkin-Elmer, Wellesley, MA) as follows: 95°C for 1 min, 54°C for 1 min, and 72°C for 2 min. Then, 25 and 27 cycles were used for HPRT and NOS2 mRNA amplification, respectively. The number of cycles produced band densities that were directly proportional to the amount of cDNA amplified. The amplified products were analyzed on 1% agarose gels containing 0.05  $\mu$ g/ml ethidium bromide.

**Flow cytometry.** Peritoneal cells were incubated with Fc block reagent (BD Biosciences) plus mouse IgG (10  $\mu$ g/ml; Sigma Chemical) for 10 min at room temperature, followed by the addition of anti-F4/80 fluorescein isothiocyanate (Serotec, Oxford, United Kingdom) and anti-CD40-PE (BD Biosciences) or isotype control MAbs. After a 30-min incubation on ice, cells were washed and fixed with 1% paraformaldehyde. Expression of CD40 on F4/80<sup>+</sup> cells was analyzed using a FACScalibur instrument (BD Biosciences). In certain experiments, bone marrow-derived macrophages were sorted as CD40<sup>-</sup> and CD40<sup>+</sup> using FACSvantage (BD Biosciences). T cells were stained with anti-CD154 (BD Biosciences) or isotype control MAbs after incubation in CM with or without PMA-ionomycin.

**Immunofluorescence and confocal microscopy.** Resident peritoneal macrophages were incubated with or without *T. gondii*-YFP. At 1 and 18 h after challenge, monolayers were incubated with blocking buffer (5% bovine serum albumin plus 10% goat serum in phosphate-buffered saline), followed by incubation with biotinylated anti-CD40 MAb (HM40-3; eBioscience, San Diego, CA) or hamster IgM for 30 min at 4°C. After being washed with blocking buffer, monolayers were incubated with streptavidin-Alexa 568 (Molecular Probes, Eugene, OR) for 30 min on ice. Slides were washed, fixed with 3.75% paraformaldehyde, and mounted using Fluormount G (Southern Biotechnology, Birmingham, AL). Monolayers were analyzed by confocal microscopy using a model 510 laser scanning confocal microscope (Carl Zeiss, Thornwood, NY). Percentages of infected CD40<sup>-</sup> and CD40<sup>+</sup> macrophages were determined by counting 100 CD40<sup>-</sup> and CD40<sup>+</sup> macrophages per group. Preincubation of macrophages with stimulatory anti-CD40 MAb (1C10) did not affect the detection of CD40 by HM40-3 as assessed by flow cytometry.

**Measurement of nitrite production.** Culture supernatants of macrophages were collected 24 h after incubation with stimulatory anti-CD40 or control MAb or with IFN- $\gamma$  plus LPS and 24 h after the addition of *T. gondii*. The amount of nitrite released was calculated colorimetrically using the Griess reaction (Sigma Chemical). Data are expressed as  $\mu$ M of nitrite.

**Enzyme-linked immunosorbent assay.** Resting and polyclonally activated T cells were washed extensively and resuspended in CM and were cultured in 96-well plates at  $5 \times 10^5$ /ml. Supernatants were collected after 6 and 18 h and were used to measure the concentration of IFN- $\gamma$  by enzyme-linked immunosorbent assay (Endogen, Cambridge, MA). The lower limit of detection of the assay was 39 pg/ml.

**Statistical analysis.** Statistical significance was assessed by Student's *t* test and analysis of variance.

## RESULTS

**CD40 induces anti-*T. gondii* activity in mouse macrophages independently of IFN- $\gamma$  through a mechanism that requires TNF- $\alpha$ .** To determine if CD40 induces the anti-*T. gondii* activity in mouse macrophages, resident peritoneal macrophages from BALB/c mice were incubated with either a stimulatory anti-CD40 or control MAbs. Figure 1 shows that both groups of macrophages had similar percentages of infected macrophages and numbers of parasites per infected macrophage and per 100 macrophages at 1 h postinfection ( $P > 0.4$ ,  $n = 3$ ). Compared to that in control monolayers, a lower percentage of infected cells and parasites per 100 macrophages was noted in CD40-stimulated monolayers at 12 h postinfection ( $P < 0.003$ ,  $n = 3$ ), as was the presence of macrophages with intracellular tachyzoites that appeared degraded. The differences between

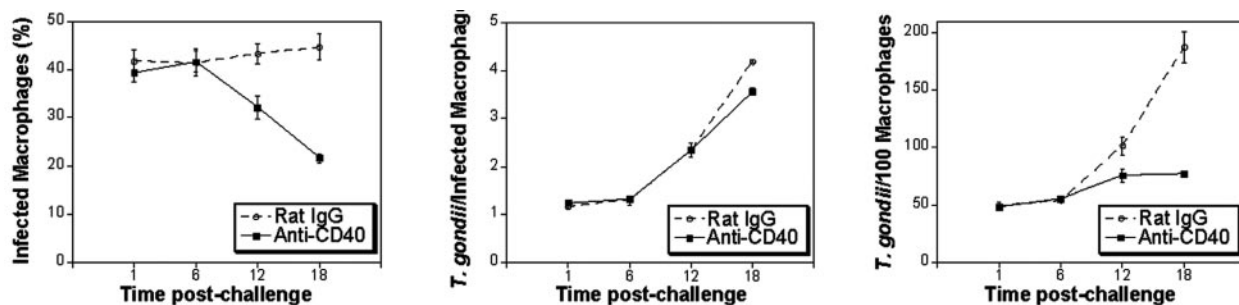


FIG. 1. CD40 enhances the anti-*T. gondii* activity of mouse macrophages. Resident peritoneal macrophages from BALB/c mice were incubated with either isotype control or stimulatory anti-CD40 MAb as described in Materials and Methods. Macrophages were washed and challenged with *T. gondii* for 1 h. Monolayers were examined by light microscopy 1, 6, 12, and 18 h after addition of *T. gondii*. Results shown are representative of one out of four independent experiments.

control and CD40-stimulated monolayers became more pronounced at 18 h postinfection. At this time point, the number of tachyzoites per 100 macrophages was on average  $47.2\% \pm 2.4\%$  lower in CD40-activated monolayers than that in controls ( $P = 0.001, n = 3$ ). This was accompanied by a  $45.9\% \pm 1.2\%$  decrease in the infection rate at 18 h compared to that at 1 h postinfection in CD40-stimulated monolayers ( $P = 0.001, n = 3$ ). Similar results were obtained with peritoneal macrophages from C57BL/6 mice ( $43.8\% \pm 1.9\%$  inhibition;  $P < 0.001, n = 7$ ) (data not shown). The decrease in the percentage of infected cells was not caused by selective cell loss in CD40-activated monolayers. Cell densities in control and CD40-activated monolayers were similar at 18 h after infection (control,  $453.5 \pm 36.1$  cells/mm<sup>2</sup>; CD40 activated,  $455.4 \pm 40.9$  cells/mm<sup>2</sup>;  $n = 4, P = 0.9$ ). Taken together, CD40 signaling alone stimulates anti-*T. gondii* activity in mouse macrophages.

We determined whether activated T cells induce macrophage anti-*T. gondii* activity through CD40-CD154 interaction. In addition, we examined whether such a response could take place when CD40 is engaged after infection with the parasite. Resting or activated T cells were added to macrophages 1 h after challenge with *T. gondii* and removal of extracellular tachyzoites. As shown in Fig. 2A, activated T cells caused a significant reduction in the number of tachyzoites per 100

macrophages ( $45.9\% \pm 1.8\%$  reduction;  $P = 0.01, n = 4$ ). This effect was not mediated by IFN- $\gamma$  because neutralization of this cytokine did not affect antimicrobial activity. At the concentration used, anti-IFN- $\gamma$  neutralized (>95% inhibition) the effect of 1 ng/ml of IFN- $\gamma$  and activated T cells secreted  $\leq 420$  pg/ml of IFN- $\gamma$ . Flow cytometric analysis confirmed that only activated T cells expressed CD154 (data not shown). Figure 2B shows that a neutralizing anti-CD154 MAb significantly inhibited the effect of activated T cells on parasite load ( $82.4\% \pm 7.0\%$  inhibition;  $P = 0.001, n = 3$ ). Taken together, activated T cells induce anti-*T. gondii* activity in macrophages through CD40-CD154 interaction.

Next, we determined if induction of anti-*T. gondii* activity by CD40 signaling alone is dependent on IFN- $\gamma$ . Peritoneal macrophages from BALB/c mice were incubated with anti-CD40 MAb in the presence of either a neutralizing anti-IFN- $\gamma$  or control MABs. As shown in Fig. 3A, addition of anti-IFN- $\gamma$  MAB did not affect the induction of anti-*T. gondii* activity in macrophages incubated with stimulatory CD40 MAB (Fig. 3A) ( $P = 0.3, n = 3$ ). In addition, macrophages from IFN- $\gamma^{-/-}$  mice acquired anti-*T. gondii* activity in response to anti-CD40 MAB and exhibited a reduction in the number of tachyzoites per 100 macrophages that was similar to those observed in macrophages from wild-type mice (Fig. 3B) ( $P > 0.5, n = 3$ ). Thus, CD40 stimulates macrophage anti-*T. gondii* activity independently of IFN- $\gamma$ .

TNF- $\alpha$  becomes central for the control of several intracellular pathogens when IFN- $\gamma$  signaling is impaired (13, 36, 38, 45). Therefore, we determined if CD40 stimulation acts through TNF- $\alpha$  signaling to induce anti-*T. gondii* activity. Control and CD40-activated peritoneal macrophages from BALB/c mice were incubated with either neutralizing anti-TNF- $\alpha$  or control MABs. Figure 3C shows that while the addition of an anti-TNF- $\alpha$  MAB to unstimulated macrophages did not affect *T. gondii* load, this MAB increased the number of tachyzoites per 100 CD40-activated macrophages. Anti-TNF- $\alpha$  caused a  $76.5\% \pm 12.9\%$  inhibition of the effect of CD40 stimulation on *T. gondii* growth ( $n = 3, P = 0.001$ ). To confirm that TNF- $\alpha$  signaling is necessary for the effect of CD40 on macrophage anti-*T. gondii* activity, peritoneal macrophages from TNFR1/2<sup>-/-</sup> and wild-type control mice were incubated with anti-CD40 or control MABs. As shown in Fig. 3D, while macrophages from control mice exhibited a significant de-

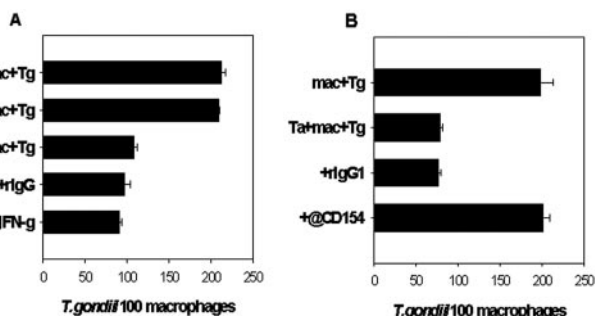


FIG. 2. Activated T cells induce macrophage anti-*T. gondii* activity through CD40-CD154 interaction. Peritoneal macrophages were infected with *T. gondii* (Tg) for 1 h, followed by removal of extracellular parasites and addition of either resting (Tr) or activated (Ta) T cells. Macrophages and T cells were cultured in the presence of anti-IFN- $\gamma$  (A), anti-CD154 (B), or control MABs. Results of one representative experiment out of four are shown.

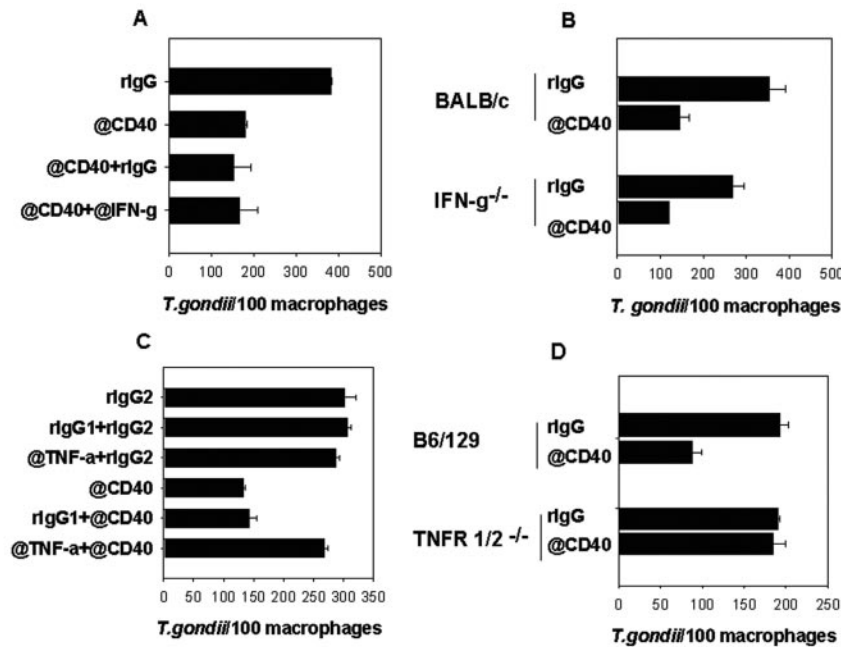


FIG. 3. CD40 induces anti-*T. gondii* activity in mouse macrophages independently of IFN- $\gamma$  but requires TNF- $\alpha$  signaling. (A) Peritoneal macrophages from BALB/c mice were cultured with either isotype control or stimulatory anti-CD40 MAb in the presence of a neutralizing anti-IFN- $\gamma$  or control MABs. Monolayers were examined by light microscopy 18 h after addition of *T. gondii*. (B) Peritoneal macrophages from BALB/c and IFN- $\gamma$ <sup>-/-</sup> mice were cultured with either isotype control or stimulatory anti-CD40 MABs. (C) Peritoneal macrophages from BALB/c mice were cultured with either isotype control or stimulatory anti-CD40 MAB in the presence of a neutralizing anti-TNF- $\alpha$  or control MABs. (D) Peritoneal macrophages from B6/129 and TNFR1/2<sup>-/-</sup> mice were cultured with either isotype control or stimulatory anti-CD40 MAB. Results of one representative experiment out of three are shown.

crease in the number of tachyzoites per 100 macrophages, parasite load remained unaffected in TNFR1/2<sup>-/-</sup> macrophages incubated with anti-CD40 MAB ( $P = 0.34$ ,  $n = 3$ ). Thus, CD40 induces the anti-*T. gondii* activity of mouse macrophages through TNF- $\alpha$  signaling.

**Mouse macrophages are heterogeneous in regard to CD40 expression and acquisition of anti-*T. gondii* activity.** The number of tachyzoites per infected macrophage remained unchanged in CD40-stimulated monolayers (Fig. 1), suggesting that not all macrophages acquire anti-*T. gondii* activity in response to CD40 stimulation. Indeed, flow cytometric analysis revealed that CD40 is expressed by only  $72.7\% \pm 5.6\%$  of resident peritoneal macrophages from BALB/c mice ( $n = 4$ ). To study the induction of antimicrobial activity by subpopulations of mouse macrophages, resident peritoneal macrophages were incubated with transgenic *T. gondii* cells expressing YFP, followed by staining for membrane CD40 by immunofluorescence. At 1 h postinfection, the percentages of infected cells were similar in CD40<sup>+</sup> and CD40<sup>-</sup> macrophages in both control and CD40-stimulated monolayers (Fig. 4A) ( $P > 0.2$ ,  $n = 3$ ). The numbers of tachyzoites per 100 macrophages were also comparable among all groups ( $P > 0.2$ ). After 18 h, the infection rates and parasite loads remained similar in CD40<sup>+</sup> and CD40<sup>-</sup> macrophages in control monolayers and in CD40<sup>-</sup> macrophages in CD40-stimulated monolayers ( $P > 0.7$ ). In contrast, CD40<sup>+</sup> macrophages from the CD40-stimulated groups exhibited a  $61.8\% \pm 4.2\%$  decrease in infection rate compared to that at the 1-h time point and a  $64.0\% \pm 4.2\%$  decrease in parasite load compared to that for the rest of the

experimental groups ( $P < 0.003$ ). While  $52.45\% \pm 2.95\%$  of infected cells at 18 h postchallenge were CD40<sup>+</sup> in control monolayers, this percentage decreased to  $18.57\% \pm 2.6\%$  after CD40 stimulation (Fig. 4B).

To confirm that only CD40<sup>+</sup> macrophages acquire antimicrobial activity, bone marrow-derived macrophages ( $29.9\% \pm 5.2\%$  CD40<sup>+</sup> prior to sorting) were sorted into CD40<sup>+</sup> and CD40<sup>-</sup> cells, followed by challenge with *T. gondii*. Addition of anti-CD40 MAB did not affect parasite load in CD40<sup>-</sup> macrophages (Fig. 5). In contrast, CD40 stimulation of sorted CD40<sup>+</sup> macrophages resulted in a marked decrease in the number of tachyzoites per 100 macrophages ( $64.2\% \pm 5\%$  inhibition;  $n = 3$ ,  $P = 0.001$ ). Similar results were obtained when macrophages were stimulated with recombinant mouse CD154 ( $69.5\% \pm 3.3\%$  inhibition in parasite load;  $P = 0.001$ ) (data not shown). Thus, mouse macrophages are heterogeneous for CD40 expression and only CD40<sup>+</sup> macrophages acquire anti-*T. gondii* activity in response to CD40 stimulation.

**CD40 signaling induces macrophage anti-*T. gondii* activity independently of RNI.** Antimicrobial activity mediated by NOS2 activation and production of RNI is a hallmark of IFN- $\gamma$ -activated mouse macrophages. To further address the distinctiveness of CD40-activated macrophages compared to that of IFN- $\gamma$ -activated macrophages, we determined if CD40 signaling induces antimicrobial activity through the production of RNI. Supernatants were collected 24 h after macrophages were incubated in CM with or without control or anti-CD40 MABs or IFN- $\gamma$  plus LPS. Nitrite could not be detected in supernatants obtained from macrophages incubated in CM

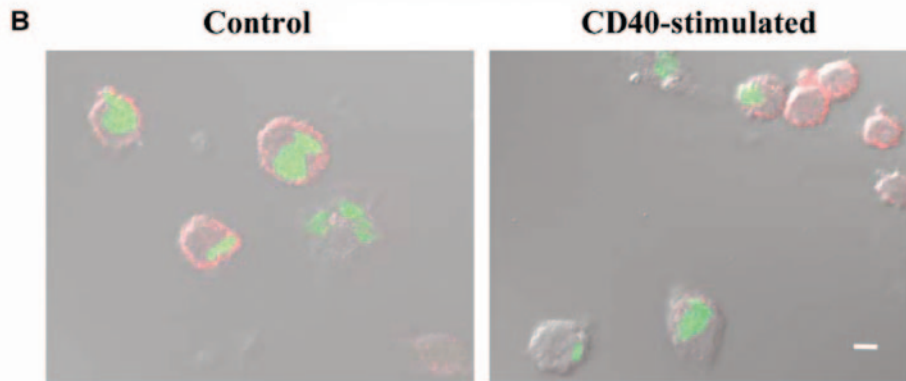
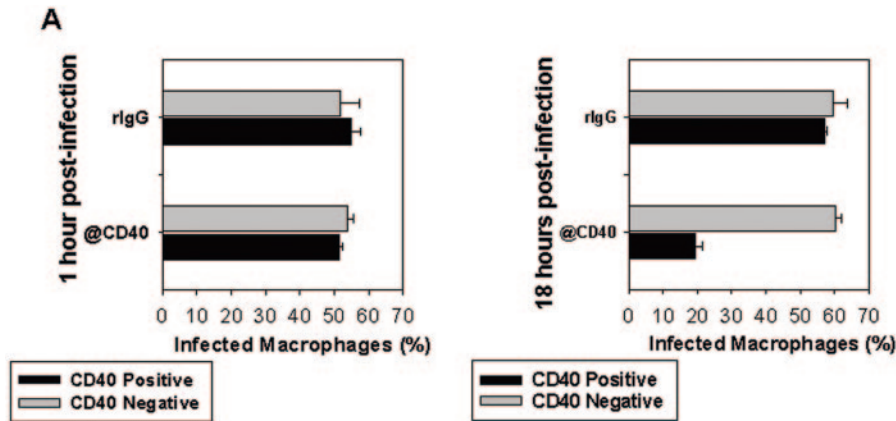


FIG. 4. Only the CD40<sup>+</sup> subpopulation of macrophages acquires anti-*T. gondii* activity in response to anti-CD40 MAb. Peritoneal macrophages cultured with anti-CD40 or control MABs were infected with *T. gondii* expressing YFP. At 1 and 18 h postinfection, expression of membrane CD40 was analyzed using a biotinylated anti-CD40 MAB and Alexa 568-conjugated streptavidin. Cells were examined by confocal immunofluorescence microscopy. (A) Percentages of infected cells in CD40<sup>+</sup> and CD40<sup>-</sup> macrophages at 1 and 18 h postinfection are shown. (B) Representative fluorescence and differential interference contrast microphotograph taken 18 h postinfection reveals that CD40 stimulation causes a decrease in the percentage of infected cells among CD40<sup>+</sup> macrophages. Bar, 5  $\mu$ m. Results from one representative experiment out of three are shown.

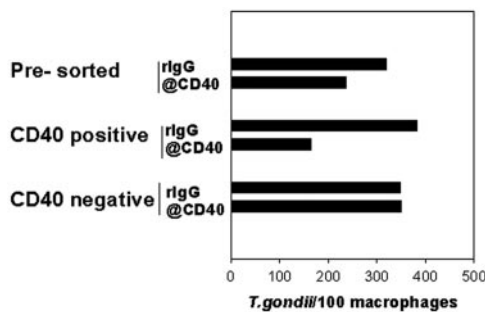


FIG. 5. Only purified CD40<sup>+</sup> macrophages acquire anti-*T. gondii* activity in response to anti-CD40 MAB. Unsorted and sorted CD40<sup>+</sup> and CD40<sup>-</sup> bone marrow-derived macrophages were cultured with anti-CD40 or control MABs. Monolayers were examined by light microscopy 18 h after *T. gondii* challenge. Results of one representative experiment out of three are shown.

alone or in the presence of anti-CD40 or control MABs (Fig. 6A). In parallel experiments, high concentrations of nitrite were detected in supernatants collected from macrophages stimulated with IFN- $\gamma$ -LPS ( $32.8 \pm 3.9 \mu$ M;  $n = 3$ ). Lack of nitrite production was also noted when supernatants from CD40-stimulated macrophages were collected 18 h after challenge with *T. gondii* (data not shown).

Next, we determined if blockade of RNI secretion affects the induction of anti-*T. gondii* activity in CD40-activated macrophages. Addition of the NOS2 inhibitor NMA caused a  $92.6\% \pm 7.4\%$  inhibition ( $P = 0.001$ ,  $n = 3$ ) in the production of nitrite by macrophages stimulated with IFN- $\gamma$ -LPS (Fig. 6A). In parallel experiments, NMA significantly inhibited ( $91.9\% \pm 4.9\%$  inhibition;  $P = 0.001$ ,  $n = 3$ ) the anti-*T. gondii* activity of IFN- $\gamma$ -LPS-treated macrophages (Fig. 6B). This confirms the previous demonstration that IFN- $\gamma$  induces anti-*T. gondii* activity in mouse macrophages through RNI production (1). In contrast, NMA had no effect on the reduction of parasite load in CD40-stimulated macrophages (Fig. 6B) ( $P = 0.6$ ,  $n = 4$ ). The NADPH inhibitor DPI blocks the production not only of

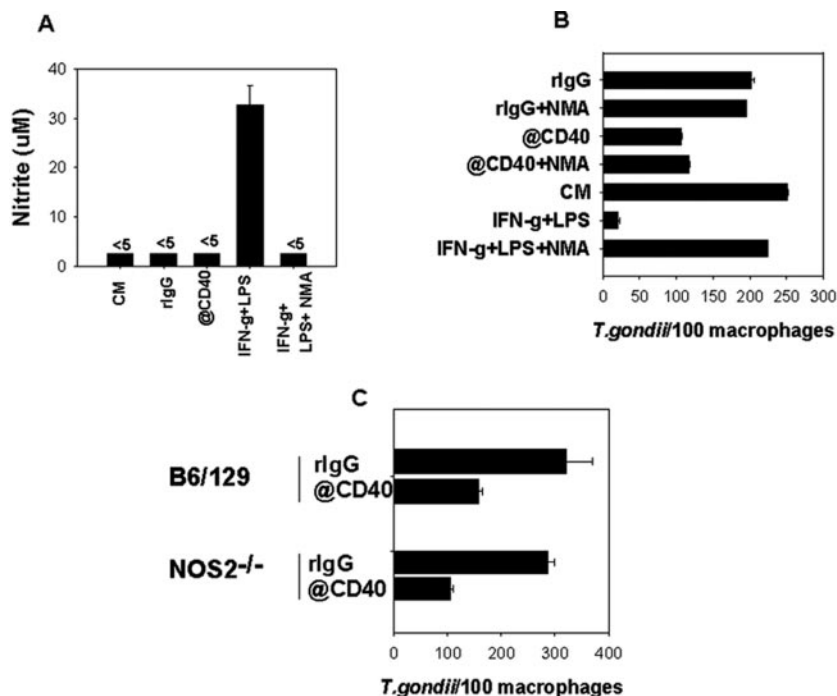


FIG. 6. CD40 induces macrophage anti-*T. gondii* activity independently of RNI. Panels A and B show that sorted CD40<sup>+</sup> bone marrow-derived macrophages from BALB/c mice were incubated with either isotype control or stimulatory anti-CD40 MAb or with IFN- $\gamma$  plus LPS with or without NMA as described in Materials and Methods. (A) Cell-free supernatants were collected 24 h poststimulation with MABs or IFN- $\gamma$  plus LPS and were used to measure nitrate concentrations. (B) Monolayers were examined microscopically at 18 h postchallenge. (C) Peritoneal macrophages from B6/129 or NOS2<sup>-/-</sup> mice were incubated with either control or stimulatory anti-CD40 MAB. Monolayers were examined microscopically at 18 h postchallenge with *T. gondii*. Results of one representative experiment out of three are shown.

reactive oxygen intermediates (ROI) but also of RNI (30). Addition of DPI for the first 1 h of stimulation with IFN- $\gamma$ -LPS caused a 92.7%  $\pm$  7.3% inhibition of anti-*T. gondii* activity of IFN- $\gamma$ -LPS-treated macrophages ( $P = 0.01$ ). DPI had no effect on the anti-*T. gondii* activity of macrophages stimulated with anti-CD40 MAB ( $P = 0.9$ ,  $n = 3$ ) (data not shown).

To further determine if CD40 signaling induces macrophage antimicrobial activity independently of RNI production, we ascertained if macrophages from NOS2<sup>-/-</sup> mice acquire anti-*T. gondii* activity in response to CD40 stimulation. Peritoneal macrophages from NOS2<sup>-/-</sup> mice activated with anti-CD40 MAB remarkably reduced the number of tachyzoites/100 macrophages compared to macrophages incubated with isotype control MAB (Fig. 6C) (47.2%  $\pm$  5.0% inhibition;  $P < 0.00$ ,  $n = 3$ ). Parasite load decreased to similar extents in both NOS2<sup>-/-</sup> and wild-type control macrophages incubated with stimulatory anti-CD40 MAB ( $P = 0.7$ ,  $n = 3$ ). All things considered, CD40 signaling does not trigger RNI secretion by macrophages and these metabolites do not mediate the anti-*T. gondii* activity of macrophages stimulated through CD40.

**CD40 signaling and expression of NOS2 in mouse macrophages.** NOS2 can be regulated at transcriptional and post-transcriptional levels (41, 43). In addition, production of RNI can be influenced by the availability of substrates and cofactors and the presence of endogenous NOS2 inhibitors (21). Thus, we determined if CD40 signaling induces NOS2 expression despite a lack of production of RNI. NOS2 protein was readily detected in cell lysates obtained from bone marrow macro-

phages stimulated with IFN- $\gamma$ -LPS for 24 h. In parallel experiments, macrophages incubated with anti-CD40 MAB failed to express NOS2 (Fig. 7A). Similar results were obtained when lysates were collected 18 h postinfection with *T. gondii* (data not shown). Increased levels of NOS2 transcripts were restricted to IFN- $\gamma$ -LPS-activated macrophages (Fig. 7B). Considering all things together, CD40 signaling triggers macrophage anti-*T. gondii* activity independently of NOS2 activation and RNI production.

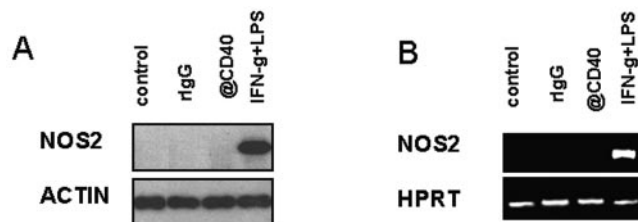


FIG. 7. CD40 does not induce NOS2 expression in mouse macrophages. (A) Bone marrow-derived macrophages were cultured in CM alone or in the presence of isotype control or stimulatory anti-CD40 MAB or IFN- $\gamma$  plus LPS. Total cell lysates were obtained after 24 h and were used to determine levels of NOS2 and actin by immunoblot staining. (B) RNA was obtained from bone marrow-derived macrophages after 6 h and used to generate cDNA. Levels of NOS2 and HPRT mRNA were determined by reverse transcription-PCR. Results of one representative experiment out of three are shown.

## DISCUSSION

There is significant interest in identifying the gamut of mechanisms that lead to macrophage activation and induction of antimicrobial activity. Here, we report that macrophages with the effector phenotype of antimicrobial activity against an intracellular pathogen can be generated by CD40 signaling in the absence of priming with IFN- $\gamma$  and RNI production, two central features of classically activated macrophages (9, 19). Thus, these data indicate that there is heterogeneity in the biology of macrophages that exhibit antimicrobial activity against intracellular pathogens.

Studies in IFN- $\gamma$ <sup>-/-</sup> and IFN- $\gamma$ R<sup>-/-</sup> mice and in humans with congenital defects in IFN- $\gamma$  signaling indicate that TNF- $\alpha$  mediates IFN- $\gamma$ -independent host protection against *L. monocytogenes*, *L. major*, *H. capsulatum*, and *T. gondii* (13, 36, 38, 45). We report that CD40 signaling triggers TNF- $\alpha$ -dependent antimicrobial activity in mouse macrophages in the absence of IFN- $\gamma$ . In contrast to recombinant mouse CD154 or stimulatory anti-CD40 MAb, CD154<sup>+</sup> T cells induced anti-*T. gondii* activity in macrophages previously infected with the parasite. These findings are likely explained by the superior capacity of membrane CD154 to trigger CD40 signaling (8). In addition, suboptimal engagement of CD40 as a result of a short (4-h) incubation with stimulatory anti-CD40 MAb likely explains the lack of induction of anti-*T. gondii* activity in macrophages reported in another study (25). Given that macrophages are central to the control of intracellular pathogens, our results suggest that CD40 is an important activator of TNF- $\alpha$  signaling that mediates the control of intracellular pathogens when there is an IFN- $\gamma$  deficiency. Indeed, CD40 signaling enhances the in vivo control of *T. gondii* in IFN- $\gamma$ <sup>-/-</sup> mice independently of T cells (C. S. Subauste and M. Wessendarp, manuscript in preparation).

CD40-CD154 interaction is crucial for resistance against a wide variety of intracellular pathogens. This signaling pathway mediates host protection, at least in part, through the stimulation of IL-12-IFN- $\gamma$  production (7, 14, 27, 34). CD40 has also been reported to stimulate the antimicrobial activity of IFN- $\gamma$ -primed mouse macrophages against *Leishmania* and *T. gondii* (14, 25, 27). In the case of *Leishmania amazoniensis*, the effect of CD40 signaling plus IFN- $\gamma$  is mediated by RNI production (27). We demonstrate that CD40 signaling alone induces antimicrobial activity independently of RNI. One study reported that CD40 signaling induces the NOS2-dependent killing of *L. major* amastigotes in mouse macrophages (3). The discrepancy with our results can be explained by the fact that these studies were conducted with preactivated (thioglycolate-induced) peritoneal macrophages rather than resting macrophages and/or the use of a different pathogen. Studies using a model of allogeneic stimulation support our contention that CD40 signaling alone does not activate RNI production. These studies revealed that IFN- $\gamma$  is necessary for T-cell-dependent RNI production by macrophages and that CD40 stimulation alone or in the presence of TNF- $\alpha$  is unable to induce this response (5).

RNI is pivotal for mediating the effects of IFN- $\gamma$  on the antimicrobial activity of mouse macrophages. Production of RNI is required for in vitro macrophage killing and in vivo resistance against pathogens such as *Leishmania*, *L. monocy-*

*togenes*, and *Trypanosoma cruzi* (1, 4, 6, 11, 16, 17, 28, 40, 42). CD40 signaling enhances the production of RNI by IFN- $\gamma$ -activated macrophages. Indeed, it has been proposed that CD40 controls *L. amazoniensis*, *Candida albicans*, and *Cryptococcus neoformans* through the stimulation of RNI production (22, 27, 39). While RNI are important mediators of host protection, there is evidence of the existence of RNI-independent mechanisms of resistance against intracellular pathogens (12, 26). In this regard, whereas IFN- $\gamma$ <sup>-/-</sup> mice are susceptible to acute infection with *T. gondii*, NOS2<sup>-/-</sup> mice survive the acute phase of infection with this pathogen (26). On the contrary, NOS2 is required for control of the chronic phase of *T. gondii* infection since NOS2<sup>-/-</sup> mice develop toxoplasmic encephalitis. Interestingly, IFN- $\gamma$  and NOS2 are essential for control of *T. gondii* in the brain and yet neither factor is sufficient for host protection since TNFR1/2<sup>-/-</sup> and CD154<sup>-/-</sup> mice develop toxoplasmic encephalitis despite the upregulation of IFN- $\gamma$  and NOS2 in the brain (25, 44). Taken together, these data strongly suggest that there are IFN- $\gamma$ - and RNI-independent mechanisms that lead to control of *T. gondii*. Indeed, studies in interferon regulatory factor 1-deficient mice revealed that the administration of exogenous IL-12 enhances protection against *T. gondii* through a mechanism that appears independent of IFN- $\gamma$  (15). The importance of macrophages as effectors of host protection, together with the central role of the CD40-CD154 pathway as a major mediator of this activation, raises the possibility that this pathway may control IFN- $\gamma$ - and RNI-independent host protection.

One of the interesting aspects of the biology of CD40 is the fact that this receptor is expressed not only on antigen-presenting cells in peripheral tissues but also on cells of the central nervous system: microglia, neurons, and possibly astrocytes (23, 37). Thus, defective induction of antimicrobial activity may be one of the mechanisms that explain the development of toxoplasmic encephalitis in CD154<sup>-/-</sup> mice. The fact that NOS2<sup>-/-</sup> mice are susceptible to toxoplasmic encephalitis suggests that either both RNI-dependent and -independent host protections need to be operative in the brain to control the parasite or that CD40<sup>+</sup> cells in the central nervous system do not possess RNI-independent mechanisms of pathogen control after CD40 stimulation. Future studies should address these possibilities.

Production of ROI has been linked to the anti-*T. gondii* activity of macrophages (20), although the in vivo relevance of this response is uncertain. Our studies with DPI, an NADPH inhibitor, indicate that CD40 mediates anti-*T. gondii* activity through a mechanism independent of ROI production. This is supported by studies that revealed that scavengers of O<sub>2</sub><sup>-</sup> and H<sub>2</sub>O<sub>2</sub> do not affect the induction of anti-*T. gondii* activity in CD40-activated human and mouse macrophages (R. M. Andrade, M. Wessendarp, and C. S. Subauste, unpublished observations). In addition, studies using human and mouse macrophages showed that supplementation of tryptophan is also without effect on CD40-stimulated macrophages (Andrade, Wessendarp, and Subauste, unpublished). Studies are under way to identify the effector mechanism by which CD40 signaling induces anti-*T. gondii* activity in macrophages.

Defective induction of CD40-dependent macrophage antimicrobial activity may play a role in pathogen immune evasion and in the susceptibility to opportunistic infections in certain

immunodeficiencies. *L. major* has been reported to impair CD40 signaling through p38 mitogen-activated protein kinase in macrophages, and this mechanism has been proposed to contribute to susceptibility in mice (3). It is likely that the susceptibility of patients with X-linked Hyper IgM syndrome to opportunistic infection is in part explained by defective macrophage activation (2). In the case of human immunodeficiency virus type 1 (HIV-1) infection, CD4<sup>+</sup> T cells have defective induction of CD154 in response to opportunistic pathogens (33). In addition, HIV-1-infected patients have defects in CD40 signaling in B cells (18) and dendritic cells infected with HIV-1 do not mature in response to CD40 stimulation (10). It remains to be determined whether CD40 signaling is impaired in macrophages from HIV-1-infected patients and whether such a defect contributes to immunodeficiency and susceptibility to opportunistic infections.

In summary, our studies indicate that the heterogeneity of macrophage biology also extends to macrophages that exhibit antimicrobial activity against an intracellular pathogen. We show that macrophages with this function can be induced by CD40 in the absence of IFN- $\gamma$  and that they control an intracellular pathogen independently of RNI. A better understanding of the biology of macrophage activation may lead to new approaches to enhance host resistance against intracellular pathogens.

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#### REFERENCES

- Adams, L. B., J. B. Hibbs, Jr., R. R. Taintor, and J. L. Krahenbuhl. 1990. Microbiostatic effect of murine-activated macrophages for *Toxoplasma gondii*. Role for synthesis of inorganic nitrogen oxides from L-arginine. *J. Immunol.* **144**:2725–2729.
- Andrade, R. M., M. Wessendarp, and C. S. Subauste. 2003. CD154 activates macrophage antimicrobial activity in the absence of IFN- $\gamma$  through a TNF- $\alpha$ -dependent mechanism. *J. Immunol.* **171**:6750–6756.
- Awasthi, A., R. Mathur, A. Khan, B. N. Joshi, N. Jain, S. Sawant, R. Boppana, D. Mitra, and B. Saha. 2003. CD40 signaling is impaired in *L. major*-infected macrophages and is rescued by a p38MAPK activator establishing a host-protective memory T cell response. *J. Exp. Med.* **197**:1037–1043.
- Beckerman, K. P., H. W. Rogers, J. A. Corbett, R. D. Schreiber, M. L. McDaniel, and E. R. Unanue. 1993. Release of nitric oxide during the T cell-independent pathway of macrophage activation. Its role in resistance to *Listeria monocytogenes*. *J. Immunol.* **150**:888–895.
- Bingaman, A. W., T. C. Pearson, and C. P. Larsen. 2000. Role of CD40L in T cell-dependent nitric oxide production by murine macrophages. *Transplant Immunol.* **8**:195–202.
- Boockvar, K. S., D. L. Granger, R. M. Poston, M. Maybodi, M. K. Washington, J. B. Hibbs, Jr., and R. L. Kurlander. 1994. Nitric oxide produced during murine listeriosis is protective. *Infect. Immun.* **62**:1089–1100.
- Campbell, K. A., P. J. Owendale, M. K. Kennedy, W. C. Fanslow, S. G. Reed, and C. R. Maliszewski. 1996. CD40 ligand is required for protective cell-mediated immunity to *Leishmania major*. *Immunity* **4**:283–289.
- Fanslow, W. C., S. Srinivasan, R. Paxton, M. G. Gibson, M. K. Spriggs, and R. J. Armitage. 1994. Structural characteristics of CD40 ligand that determine biological function. *Semin. Immunol.* **6**:267–278.
- Gordon, S. 2003. Alternative activation of macrophages. *Nat. Rev. Immunol.* **3**:23–35.
- Granelli-Piperno, A., A. Golebiowska, C. Trumpfheller, F. P. Siegal, and R. M. Steinman. 2004. HIV-1-infected monocyte-derived dendritic cells do not undergo maturation but can elicit IL-10 production and T cell regulation. *Proc. Natl. Acad. Sci. USA* **101**:7669–7674.
- Green, S. J., M. Meltzer, J. B. Hibbs, Jr., and C. A. Nacy. 1990. Activated macrophages destroy intracellular *Leishmania major* amastigotes by an L-arginine-dependent killing mechanism. *J. Immunol.* **144**:278–283.
- Igietseme, J. U., L. L. Perry, G. A. Ananaba, I. M. Uriri, O. O. Ojior, S. N. Kumar, and H. D. Caldwell. 1998. Chlamydial infection in inducible nitric oxide synthase knockout mice. *Infect. Immun.* **66**:1282–1286.
- Janssen, R., A. Van Wengen, E. Verhard, T. De Boer, T. Zomerdijk, T. H. M. Ottenhoff, and J. T. van Dissel. 2002. Divergent role for TNF- $\alpha$  in IFN- $\gamma$ -induced killing of *Toxoplasma gondii* and *Salmonella typhimurium* contributes to selective susceptibility of patients with partial IFN- $\gamma$  receptor 1 deficiency. *J. Immunol.* **169**:3900–3907.
- Kamanaka, M., P. Yu, T. Yasui, K. Yoshida, T. Kawabe, T. Horii, T. Kishimoto, and H. Kikutani. 1996. Protective role of CD40 in *Leishmania major* infection at two distinct phases of cell-mediated immunity. *Immunity* **4**:275–281.
- Khan, I. A., T. Matsuura, S. Fonseka, and L. H. Kasper. 1996. Production of nitric oxide (NO) is not essential for protection against *Toxoplasma gondii* infection in IRF-1<sup>-/-</sup> mice. *J. Immunol.* **156**:636–643.
- Langermans, J. A. M., M. E. B. Van der Hulst, P. H. Nibbering, P. S. Hiemstra, L. Franssen, and R. Van Furth. 1992. IFN- $\gamma$ -induced L-arginine-dependent toxoplasmastatic activity in murine peritoneal macrophages is mediated through endogenous tumor necrosis factor- $\alpha$ . *J. Immunol.* **148**:568–574.
- MacMicking, J. D., C. Nathan, G. Hom, N. Chartrain, D. S. Fletcher, M. Trumbauer, K. Stevens, Q. Xie, K. Sokol, N. Hutchinson, et al. 1995. Altered responses to bacterial infection and endotoxic shock in mice lacking inducible nitric oxide synthase. *Cell* **81**:641–650.
- Malaspina, A., S. Moir, S. Kottilli, C. W. Hallahan, L. Ehler, S. Liu, M. Planta, T.-W. Chun, and A. S. Fauci. 2003. Deleterious effect of HIV-1 plasma viremia on B cells costimulatory function. *J. Immunol.* **170**:5965–5972.
- Mosser, D. M. 2003. The many faces of macrophage activation. *J. Leukoc. Biol.* **73**:209–212.
- Murray, H. W., C. W. Juangbhanich, C. F. Nathan, and Z. A. Cohn. 1979. Macrophage oxygen-dependent antimicrobial activity. II. The role of oxygen intermediates. *J. Exp. Med.* **150**:950–964.
- Nathan, C., and Q. W. Xie. 1994. Regulation of biosynthesis of nitric oxide. *J. Biol. Chem.* **269**:13725–13728.
- Netea, M. G., J. W. M. van der Meer, I. Verschueren, and B. J. Kullberg. 2002. CD40/CD40 ligand interactions in the host defense against disseminated *Candida albicans* infection: the role of macrophage-derived nitric oxide. *Eur. J. Immunol.* **32**:1455–1463.
- Nguyen, V. T., and E. N. Benveniste. 2000. IL-4-activated STAT-6 inhibits IFN- $\gamma$ -induced CD40 gene expression in macrophages/microglia. *J. Immunol.* **165**:6235–6243.
- Ottenhoff, T. H. M., D. Kumararatne, and J. L. Casanova. 1998. Novel human immunodeficiencies reveal the essential role of type-1 cytokines in immunity to intracellular bacteria. *Immunol. Today* **19**:491–494.
- Reichmann, G., W. Walker, E. N. Villegas, L. Craig, G. Cai, J. Alexander, and C. A. Hunter. 2000. The CD40/CD40 ligand interaction is required for resistance to toxoplasmic encephalitis. *Infect. Immun.* **68**:1312–1318.
- Scharton-Kersten, T., G. Yap, J. Magram, and A. Sher. 1997. Inducible nitric oxide is essential for host control of persistent but not acute infection with the intracellular pathogen *Toxoplasma gondii*. *J. Exp. Med.* **185**:1261–1273.
- Soong, L., J. C. Xu, I. S. Grewal, P. Kima, J. Sun, B. J. Longley, Jr., N. H. Ruddle, D. McMahon-Pratt, and R. A. Flavell. 1996. Disruption of CD40-CD40 ligand interactions results in an enhanced susceptibility to *Leishmania amazonensis* infection. *Immunity* **4**:263–273.
- Stenger, S., N. Donhauser, H. Thuring, M. Rollinghoff, and C. Bogdan. 1996. Reactivation of latent leishmaniasis by inhibition of inducible nitric oxide synthase. *J. Exp. Med.* **183**:1501–1514.
- Stenger, S., H. Thuring, M. Rollinghoff, and C. Bogdan. 1994. Tissue expression of inducible nitric oxide synthase is closely associated with resistance to *Leishmania major*. *J. Exp. Med.* **180**:783–793.
- Stuehr, D. J., O. A. Fasehun, N. S. Kwon, S. S. Gross, J. A. Gonzalez, R. Levi, and C. Nathan. 1991. Inhibition of macrophage and endothelial cell nitric oxide synthase by diphenyleioidonium and its analogs. *FASEB J.* **5**:98–103.
- Subauste, C. S., J. Y. Chung, D. Do, A. H. Koniaris, C. A. Hunter, J. G. Montoya, S. Porcelli, and J. S. Remington. 1995. Preferential activation and expansion of human peripheral blood  $\gamma\delta$  T cells in response to *Toxoplasma gondii* in vitro and their cytokine production and cytotoxic activity against *T. gondii*-infected cells. *J. Clin. Investig.* **96**:610–619.
- Subauste, C. S., A. H. Koniaris, and J. S. Remington. 1991. Murine CD8<sup>+</sup> cytotoxic T lymphocytes lyse *Toxoplasma gondii*-infected cells. *J. Immunol.* **147**:3955–3959.
- Subauste, C. S., M. Wessendarp, J.-A. C. Portillo, R. M. Andrade, L. M. Hinds, F. J. Gomez, A. G. Smulian, P. A. Grubbs, and L. A. Haglund. 2004. Pathogen-specific induction of CD154 is impaired in CD4<sup>+</sup> T cells from human immunodeficiency virus-infected individuals. *J. Infect. Dis.* **189**:61–70.
- Subauste, C. S., M. Wessendarp, R. U. Sorensen, and L. Leiva. 1999. CD40-CD40 ligand interaction is central to cell-mediated immunity against *Toxoplasma gondii*: patients with hyper IgM syndrome have a defective type 1



- immune response that can be restored by soluble CD40 ligand trimer. *J. Immunol.* **162**:6690–6700.
35. **Svetic, A., F. D. Finkelman, Y. C. Jian, C. W. Dieffenbach, D. E. Scott, K. F. McCarthy, A. D. Steinberg, and W. C. Gause.** 1991. Cytokine gene expression after in vivo primary immunization with goat antibody to mouse IgD antibody. *J. Immunol.* **147**:2391–2397.
36. **Szalay, G., C. H. Ladel, C. Blum, and S. H. E. Kaufmann.** 1996. IL-4 neutralization or TNF- $\alpha$  treatment ameliorate disease by an intracellular pathogen in IFN- $\gamma$  receptor-deficient mice. *J. Immunol.* **157**:4746–4750.
37. **Tan, J., T. Town, T. Mori, D. Obregon, Y. Wu, A. DelleDonne, A. Rojiani, F. Crawford, R. A. Flavell, and M. Mullan.** 2002. CD40 is expressed and functional on neuronal cells. *EMBO J.* **21**:643–652.
38. **Taylor, A. P., and H. W. Murray.** 1997. Intracellular antimicrobial activity in the absence of interferon- $\gamma$ : effect of interleukin-12 in experimental visceral leishmaniasis in interferon- $\gamma$  gene-disrupted mice. *J. Exp. Med.* **185**:1231–1239.
39. **Vecchiarelli, A., C. Retini, D. Pietrella, C. Monari, and T. R. Kozel.** 2000. T lymphocyte and monocyte interaction by CD40/CD40 ligand facilitates a lymphoproliferative response and killing of *Cryptococcus neoformans* in vitro. *Eur. J. Immunol.* **30**:1385–1393.
40. **Vespa, G. N., F. Q. Cunha, and J. S. Silva.** 1994. Nitric oxide is involved in control of *Trypanosoma cruzi*-induced parasitemia and directly kills the parasite in vitro. *Infect. Immun.* **62**:5177–5182.
41. **Vodovotz, Y., C. Bogdan, J. Paik, Q. W. Xie, and C. Nathan.** 1993. Mechanisms of suppression of macrophage nitric oxide release by transforming growth factor beta. *J. Exp. Med.* **178**:605–613.
42. **Wei, X., I. G. Charles, A. Smith, J. Ure, G. Feng, F. Huang, D. Xu, W. Muller, S. Moncada, and F. Y. Liew.** 1995. Altered immune response in mice lacking inducible nitric oxide synthase. *Nature* **375**:408–411.
43. **Xie, Q. W., R. Whisnant, and C. Nathan.** 1993. Promoter of the mouse gene encoding calcium-independent nitric oxide synthase confers inducibility by interferon  $\gamma$  and bacterial lipopolysaccharide. *J. Exp. Med.* **177**:1779–1784.
44. **Yap, G. S., T. Scharton-Kersten, H. Charest, and A. Sher.** 1998. Decreased resistance of TNF receptor p55- and p75-deficient mice to chronic toxoplasmosis despite normal activation of inducible nitric oxide synthase in vivo. *J. Immunol.* **160**:1340–1345.
45. **Zhou, P., G. Miller, and R. A. Seder.** 1998. Factors involved in regulating primary and secondary immunity to infection with *Histoplasma capsulatum*: TNF- $\alpha$  plays a critical role in maintaining secondary immunity in the absence of IFN- $\gamma$ . *J. Immunol.* **160**:1359–1368.

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