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Assays to Evaluate *Toxoplasma*–Macrophage Interactions

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Abstract

The obligate intracellular protozoan parasite *Toxoplasma gondii* can infect any nucleated cell from a warm-blooded host. However, its interaction with host macrophages plays a critical role in shaping the immune response during infection. Therefore, assessing *Toxoplasma*–macrophage interactions at a cellular level is important. In this chapter, we describe assays that can be used to characterize *Toxoplasma*–macrophage interactions. These assays can also be used to evaluate other host–pathogen interactions. We describe multiplex approaches for measuring arginase activity, indoleamine 2,3 dioxygenase activity, cell death, and parasite growth during *Toxoplasma*–macrophage interactions. These assays can be used to compare how different *Toxoplasma* strains differ in their interaction with macrophages, and we describe how to properly assess *Toxoplasma* strain differences in *Toxoplasma*–macrophage interactions.

Keywords

Arginase; GRA15; IDO; Inflammasome; LDH; Macrophage; Macrophage polarization; MTS; ROP16; *Toxoplasma*

1 Introduction

Cells of the macrophage–monocyte lineage are extremely plastic in nature and can be polarized into different subtypes by different exogenous stimuli [1]. The plasticity of macrophages plays a pivotal role in many diseases, including infectious, allergic, and autoimmune diseases [1]. When macrophages are stimulated by interferon gamma (IFNγ) and/or lipopolysaccharide (LPS) the NF-κB and the signal transducer and activator of transcription 1 (STAT1) signaling pathways are activated leading macrophages to become polarized toward classically activated or M1 macrophages. To get the full spectrum of macrophage microbicidal activity, it is important to stimulate macrophages with both IFNγ and LPS or IFNγ and tumor necrosis factor alpha (TNFα) [2, 3]. M1 macrophages are characterized by secretion of proinflammatory cytokines and enhanced pathogen killing ability [4]. On the contrary, macrophages that are stimulated with interleukin (IL)-4 or IL-13, which activate STAT6, become polarized toward alternatively activated or M2

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macrophages. M2 macrophages have more capacity for tissue repair and are less effective in killing intracellular pathogens [4]. Phenotypically, M1 macrophages are characterized by generation of high levels of nitric oxide (NO), reactive oxygen species (ROS), high activity of indoleamine 2,3 dioxygenase (IDO), and secretion of IL-12, which enhances type I T helper cell (Th1) responses. M2 macrophages are characterized by possessing high arginase activity, expression of mannose receptor, and secretion of cytokines such as IL-10, which enhances Th2 responses [4]. Thus, many intracellular pathogens suppress M1 macrophage polarization and activate M2 macrophages. *Toxoplasma* is an intracellular protozoan parasite that is estimated to infect ~30% of humans worldwide [5]. Many *Toxoplasma* strains exist, but in North America and Europe the majority of *Toxoplasma* strains belong to the clonal type I, II, III, and XII lineage [6]. These clonal lineages differ in many phenotypes including virulence in mice, in vitro growth rate, migration, modulation of host signaling pathways, and susceptibility to host immune responses [7]. *Toxoplasma* type II strains induce M1 macrophages in vitro with enhanced IL-12 production, whereas type I and III strains activate M2 macrophages characterized by high arginase activity, expression of the mannose receptor, and low IL-12 production [8]. This strain-specific induction of macrophage polarization is largely dependent on *Toxoplasma* proteins that are secreted into the host cell called ROPs and GRAs, which are secreted from *Toxoplasma* secretory organelles called rhoptries and dense granules. We have shown that two *Toxoplasma* proteins play a major role in the modulation of macrophage function and thereby determine the level of immune-induced inflammation. These proteins are ROP16 (a secreted kinase) and GRA15 (a secreted protein with no characterized motifs). ROP16 activates the STAT6 transcription factor resulting in M2 macrophage activation and the repression of inflammation. GRA15 activates the NF-kB transcription factor resulting in M1 macrophage activation and the activation of an inflammatory response [8]. Type I and III strain parasites have a more active version of ROP16 compared to type II strain parasites, while type II parasites have a more active GRA15 or higher expression of GRA15 compared to type I and type III strain parasites. These strain differences in ROP16 and GRA15 determine *Toxoplasma* strain differences in macrophage polarization [8–10]. It was recently shown that GRA24 also contributes to M1 macrophage polarization by activating the p38 mitogen-activated protein kinase (MAPK) pathway. Unlike the strain-specific activities of GRA15 and ROP16, GRA24 from all strains tested so far can activate p38 MAPK [11]. Thus, strain differences in GRA15 and ROP16 plays a pivotal role in determining macrophage polarization upon *Toxoplasma* infection. For many *Toxoplasma* strains the mechanism by which they modulate macrophage activation remains unknown. Furthermore, it is likely that other parasite gene products can modulate macrophage function and polarization. Thus, assessing macrophage polarization upon infection with different *Toxoplasma* strains with an easy assay is important. For this, the combined assessment of IL-12, NO, or IDO as markers for M1 activation and arginase activity as a marker for M2 activation from a single experiment can provide direct evidence for macrophage polarization by *Toxoplasma.* We will describe assays that can determine if macrophages infected with different *Toxoplasma* strains differ in their polarization profile. IL-12 can be measured from the supernatant using any commercially available ELISA kit. The arginase and IDO assay that will be described in the following section is easy to set up and perform (~2–3 h) in a 96-well plate format. Because the method described here determines the arginase activity from the cell lysate, the assay can be combined with other

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assays that use cell culture supernatant (e.g., assays to measure NO production or quantitate cytokines present in the supernatant).

2 Measurement of Arginase Activity from Macrophage Lysates Using a Microplate-Based Method

2.1 Materials

Prepare all solutions using deionized water and analytical or molecular biology grade reagents. Prepare and store all reagents at room temperature, unless otherwise mentioned.

2.2 Cell Culture Reagents

Macrophages are extremely sensitive to even very low levels of lipopolysaccharide (LPS) (endotoxin) from gram-negative bacteria. All solutions, buffers and media should be made with sterile, tissue culture grade, endotoxin-tested water. To avoid LPS contamination use disposable sterile plastics rather than laboratory glassware for all steps in this protocol and for storing solutions. LPS contamination can be determined with the Limulus Amebocyte Lysate assay [12]. Additionally, all the cells should be devoid of mycoplasma contamination as it can affect cell physiology, and often leads to erroneous results. Therefore, all the cells used in the study should be checked for mycoplasma contamination every 3–4 weeks either by PCR (most sensitive) or by indirect immunofluorescence assays looking for DNA-positive staining outside the host cell nucleus. For the PCR detection the following primers can be used: Fw: 5′ GGGAGCAAACAGGATTAGATACCCT 3′; Rv: 5′ TGCACCATCTGTCACTCTGTTAACCTC 3′. The following program should be used with the primers mentioned above-initial denaturation (1 min at 98 °C), denaturation (30 s at 98 °C), annealing (30 s at 55 °C), polymerization (2 min 30 s at 72 °C), repeat the cycle for 32 times and finish with a final extension (7 min at 72 °C). All mycoplasma-positive reagents should be discarded and autoclaved.

1. DMEM (Dulbecco’s Modified Eagle Medium) with high glucose supplemented with 10% heat-inactivated fetal bovine serum (HI-FBS), L-Glutamine (2 mM), 100 U/mL Penicillin, 100 μg/mL Streptomycin, 20 μg/mL Gentamycin, and HEPES (1 M) (complete DMEM) is used to culture the Raw264.7 macrophage cell line. For bone marrow derived macrophages (BMDM) it is recommended to make a small batch of media containing complete DMEM complemented with 20% L929 mouse fibroblast cell culture supernatant (*see* Note 1), 1× nonessential amino acids (NEAA), and 1 M sodium pyruvate. To make 100 mL of medium, 20 mL of L929 medium should be added to 80 mL of complete DMEM that was supplemented with NEAA and sodium pyruvate (1 M final concentration). It is not recommended to store the BMDM medium for more than 5 days because the cytokines present in the L929 culture supernatant (*see* Note 1) can degrade at 4 °

[1]. Differentiation of bone marrow-derived macrophages (BMDM) from bone marrow in vitro requires macrophage colony-stimulating factor (M-CSF). L929 is a murine fibroblast cell line that produces and secretes M-CSF and the filtered supernatant is described as L929 conditioned media, which needs to be added for the differentiation of BMDMs. Because the amount of M-CSF in L929 conditioned media can differ from batch to batch, it is recommended that the M-CSF is made in a large batch and its concentration is measured by ELISA. This batch should be frozen as 50 mL aliquots at −20 °C.

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C. For human peripheral blood mononuclear cells (PBMCs) and monocytes, RPMI1640 media supplemented with 10% HI-FBS, L-Glutamine (2 mM), 100 U/mL Penicillin, 100 μg/mL Streptomycin, and 20 μg/mL Gentamycin should be used. *Toxoplasma* parasites are usually maintained (*see* Note 2) in human foreskin fibroblasts (HFFs) in complete DMEM medium. All cells (and *Toxoplasma*) are cultured at 37 °C with 5% CO_2.

2. Sterile phosphate buffer saline (PBS).

3. Recombinant IL-4 of mouse or human origin, final concentration to be added to cells is 50 μg/mL.

4. Sterile endotoxin-free centrifuge tubes ranging from 0.5 to 50 mL volume.

5. Flat bottom 96- and 24-well plates.

6. Sterile serological pipettes 5–50 mL.

7. Cell culture incubator maintained at 37 °C with 5% CO_2.

8. Centrifuge at 4 °C with adjustable acceleration and deceleration.

2.3 Assay Reagents

1. 0.1% v/v Triton X-100 solution (this will work as a cell lysis agent; dilution should be made in deionized water). For one 96-well plate, 2.5 mL of 0.1% Triton X-100 solution is sufficient.

2. Halt protease inhibitor cocktail without EDTA (Thermo Scientific #1861284), 100× (final concentration will be 1× and dilution should be made with 0.1% Triton X-100). To make 2.5 mL of lysis buffer for one 96-well plate, 25 μL of Halt protease inhibitor cocktail and 2.5 μL of 100% Triton X-100 should be added to 472.5 μL of deionized water.

3. 10 mM Manganese chloride ($MnCl_2$) dissolved in 50 mM Tris–HCl, pH 7.5 (Can be used until the solution turns black, usually 1 week). To make 30 mL of $MnCl_2$, dissolve 0.059 g of $MnCl_2$ in 50 mM Tris–HCl, pH 7.5.

4. 1 M L-arginine, pH 9.7 (should be made fresh). To make a 3 mL solution of 1 M L-arginine, add 0.5226 g L-arginine to 3 mL of deionized water and vortex the solution to dissolve the L-arginine. Keeping the solution at 37 °C for 5–10 min will help with dissolving the L-arginine.

5. A solution of an acid mixture containing sulfuric acid/phosphoric acid/water ($H_2SO_4/H_3PO_4/H_2O$, v/v/v) in a ratio of 1:3:7 (can be stored at room temperature for at least 1 month). To make 110 mL acid mixture, add 10 mL of sulfuric acid and 30 mL phosphoric acid to 70 mL of deionized water.

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2. *Toxoplasma gondii* is an obligate intracellular parasite; therefore, it is usually cultured in a host cell for instance in HFFs. To harvest a good number of viable parasites from the host cells, it is important to harvest parasites in large vacuole from intact HFFs (especially when working with non-RH parasites that are less viable outside a host cell). These infected cells can be lysed while passing the cells through a 27 and 30 G needle consecutively after scraping the cells from a T25 flask.

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6. 9% 2-isonitrosopropiophenone (ISPF) diluted in 100% ethanol (should be made fresh). For 1.5 mL solution add 0.135 g of 2-isonitrosopropiophenone to 1.5 mL of 100% ethanol and vortex the mixture to dissolve it. The solution has a semicrystalline appearance.

7. 1 M urea solution in deionized water (can be stored at 4 °C for at least 1 month). To make a 50 mL 1 M urea solution, dissolve 3 g of urea into 50 mL of deionized water and keep it at 4 °C.

2.4 Method

1. Seed 1×10^5 macrophages [13] in each well of a 96-well plate in 100 μL medium specific for the macrophage type (mentioned in cell culture reagents point 1) for at least 3 h to ensure cell adherence. For Raw264.7 macrophages, if cells are seeded the day before, seed at a density of 5×10^4 cells/well. For each condition, seed the cells in triplicate. All the outer wells should contain sterile PBS to prevent evaporation of the medium from the wells (Fig. 1a).

2. Once the parasites have grown sufficiently to create many large vacuoles that are about to lyse out of the cell, harvest parasites from infected HFFs by syringe lysis (27 and 30 G needle) should be used for maximum lysis) method (*see* Note 2). Remove medium from the parasite pellet after centrifugation ($570 \times g$ for 7 min) and resuspend the pellet in 1 mL of medium (same as used for culturing of macrophages). Count the parasites using a hemocytometer.

3. Infect the macrophages with *Toxoplasma* (*see* Note 3) using a multiplicity of infection (MOI) of 0.5–10 depending on the strain type (*see* Subheading 6) for 20–24 h. Make sure to keep the total volume per well constant by using macrophage media to adjust the total volume (Fig. 1a).

4. Keep three wells for uninfected cells and three wells for cells stimulated with 50 μg/mL recombinant IL-4 (positive control) (*see* Note 4, Fig. 1a).

5. It is good practice to set up a plaque assay to determine the viability of the parasites, especially when comparing different parasite strains. The arginase activity and IL-12 production of macrophages are dependent on the infection level of the macrophages. To be able to interpret results obtained from different parasite strains or performed at different days it is therefore important to know the infectivity of your parasites. To do so, plaque assays in 24-well plates seeded with HFFs in triplicate can be used to determine the viability of the parasites (*see* Note 5). Without plaque assay results, potential differences between different

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[3]. *Toxoplasma* type I (RH), type II (ME49), and type III strains (CEP) expressing GFP and luciferase can be used for the assay [15]. RH always grows faster than the other two types, so to synchronize the growth of parasite strains, always pass more parasites from previous flasks of parasites for type II and III compared to the RH strain. Also, during the infection type I RH parasites have a higher infectivity than type II and III; therefore, the MOIs used for RH should be lower than for type II and III. For instance, an MOI 3/5/7 for type II and III whereas for type I RH an MOI of 1/2/3 is used. A better choice for a type I strain might be the GT1 strain, as it is more similar to the type II and III strains in terms of infectivity.

[4]. IL-4 is a cytokine that when bound to its prototype receptor IL4R alpha (IL4Rα) triggers the phosphorylation and activation of STAT6. STAT6 activation induces macrophages to convert to M2 macrophages. STAT6 induces the expression of arginase in macrophages and dendritic cells [1].

parasites (e.g., different strains or knockout *vs.* wild type) on arginase activity or IL-12 production of macrophages cannot be distinguished from potential influences of differences in viability.

6. After 20–24 h of infection, take the plate from the CO_2 incubator and look under the microscope to determine if there is any cell lysis caused by parasite growth. If a high degree of cell lysis occurs in some wells or with certain MOIs, it has to be noted, as this may influence the results. Following that, spin down the plates at 440 × *g* for 5 min.

7. Transfer the supernatant to another 96-well low protein binding plate (normal 96-well tissue culture plates are OK) using a multichannel pipette and use for cytokine ELISA, lactate dehydrogenase (LDH) release assay, or NO assay.

8. Once the medium is removed, add 25 μL Triton X-100 with 1× protease inhibitor cocktail to each well of the plate with the macrophages. Place the plate at −80 °C and proceed the next day with 2–3 freeze–thaw cycles, or freeze-thaw 2–3 times to ensure complete lysis ofthe cells and proceed same day. During freeze thaw, put two heating blocks at 60 °C.

9. Check under microscope that cells are lysed. They will appear as ghost transparent bodies.

10. Following cell lysis, add 25 μL 50 mM Tris–HCl containing 10 mM $MnCl_2$.

11. Incubate the plate containing cell and parasite lysate for 10 min at 60 °C (by sandwiching the plate between two heat blocks preheated to 60 °C).

12. After 10 min, add 25 μL 1 M L-arginine to the wells containing the 50 μL lysate.

13. After that, incubate the plate for 60 min in a 37 °C incubator (*see* Note 6).

14. During this 60 min incubation time, dilute 1 M urea to the following concentrations 1500–750-375–187.5–93.8–46.9–23.4–0 μM each for triplicate wells as standards (50 μL per well). Also turn the temperature of the heating blocks up to 95 °C.

15. After 60 min, remove the plate and add 175 μL H_2SO_4–H_3PO_4–H_2O (1:3:7) to all wells. Add 200 μL to the urea dilution series in the same plate (Fig. 1a).

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5. Because *Toxoplasma* rapidly dies extracellularly (especially non-RH strains) the intended MOI determined by counting parasites with a hemocytometer often does not represent the number of parasites that are capable of infecting a cell. When comparing strains, it is therefore important to set up a plaque assay to get an indication of the infectivity of the parasites because many phenotypes are correlated with the infection load. For the plaque assay, usually 100 parasites for type I strain (RH), and 200–300 parasites for non-RH strains are added to 24-well plates with monolayers of HFFs and incubated for 4/5 days (RH) or 6/7 days (non-RH strains) after which the number of plaques is counted. Here it is important to note that if all the wells of the 24-well plates need to be used it is better to keep the plate in a sterile box with wet tissue papers because the medium from boundary wells can evaporate due to prolonged incubation time (4–6 days) of the assay. The infectivity of the parasites for each strain is determined from this plaque number (keeping in mind that each plaque is derived from a single parasite). Because macrophages were infected with different MOIs the “real MOI” for each strain can be matched and used to compare the arginase activity (Fig. 1c, d). For example, in the given example (Table 1), from the plaque assay if 100 parasites of the RH strain generate 70 plaques it means that the viability is 70%, that is, intended MOI of 1 in reality is 0.7. On the other hand, if 250 type II parasites form 40 plaques, it means that the parasite viability is 0.16%, that is, intended MOI of 5 means real MOI of 0.8, the MOI 1 of RH strain should be compared with MOI 5 of type II strain (Table 1).

6. It is important to keep the incubation time fixed because the enzyme activity in international unit (IU) is calculated using the number of min used for incubation.

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16. Add 12.5 μL 9% ISPF to all wells (precipitation will occur).

17. Place a high temperature resistant microplate sealing film (usually PCR plate cover) on the 96-well plate and put the 96-well plate cover on top of this.

18. Incubate for 30–60 min at 95 ° C on top of a heating block with another heating block inverted on it (to prevent evaporation) and incubate till precipitation is gone and development of pink color can be seen. The high temperature of the plates will cause damage to the plates, so keep another microplate ready (does not need to be tissue culture grade) to transfer the liquid from the heated plate to that plate.

19. After removing the plate from heating block, incubate for 10 min at room temperature in the dark. If samples are "cloudy" read later (keep plate in the dark).

20. Read the absorbance at OD at 540 and 650 nm and export the data to .txt file (*see* Note 7).

21. Open the txt file with excel and calculate the concentration of urea in μM from the standard curve (Fig. 1b). Subtract absorbance at OD 650 from OD 540. Determine the sample values from the urea standard curve (Fig. 1c).

3 Measurement of Host Cell Viability and *Toxoplasma* Growth in a Multiplex Assay

3.1 Introduction

Host cell death is one of several immune mechanisms for inhibiting *Toxoplasma*'s growth by destroying its replicative niche. It has been observed in human primary fibroblasts and Lewis rat macrophages [14]. IFNγ plays a pivotal role in the immune response against *Toxoplasma* in both mice and humans. Host cell death is often accompanied with restriction of parasite growth and can differ depending on the infecting *Toxoplasma* strain type [14]. Herein, we will describe a method for measuring host cell death and parasite growth from a single experiment and from the same wells using a 96-well plate assay. This assay will measure cell death by quantifying the release of the host cytoplasmic enzyme lactate dehydrogenase (LDH) into the cell culture supernatant upon cell death and measure parasite growth by measuring luciferase activity from luciferase expressing parasites. However, it should be noted that using luciferase measurements for total parasite growth has limitations as luciferase is a very stable protein and therefore signal from dead parasites could contribute to the signal. Therefore, parasite/vacuole counting should also be considered (*see* Note 8 and Ref 14). Our example will be for HFFs, but the same assay can be performed on macrophages.

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7. A monochromator-based plate reader can be used where all the wavelengths including 540 and 650 nm can be set. Otherwise a filter-based plate reader could be used, where a filter of 550 and 670 nm (for the background absorbance) is present.

8. If nonluciferase strains are used for infection, then to measure parasite growth in parallel, parasite/vacuole counting can be performed as a representative assay for parasite replication. This assay is done in a 24-well plate using coverslips as described by Iaconetti et al. [20].

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3.2 Materials

Prepare all solutions using deionized water and analytical or molecular biology grade reagents. Prepare and store all reagents at room temperature, unless otherwise mentioned.

3.3 Cell Culture Reagents

1. For culturing macrophages all the reagents and equipment must be free of endotoxins as stated in the arginase assay protocol. The macrophage culture medium is the same as mentioned in the arginase assay protocol. For culturing HFFs complete DMEM should be used.
2. Any parasite strain (e.g., type I/II/III) or any transgenic strain that will be used in this assay should be genetically engineered to express luciferase [15] (*see* Note 3).
3. Sterile phosphate buffer saline (PBS).
4. Recombinant IFNγ of either human or mouse origin. The IFNγ should be dissolved in sterile complete DMEM media as a 100× concentrated stock solution and kept at −80 °C for storage in small aliquots to avoid repeated freeze–thaw cycles. To activate the full toxoplasmacidal activity of macrophages either LPS (10 ng/mL) or recombinant TNFα (20–100 U/mL) should be added together with IFNγ (*see* Note 9).
5. Sterile endotoxin-free centrifuge tubes ranging from 0.5 to 50 mL.
6. Flat bottom 96- and 24-well plates.
7. Sterile serological pipettes 5–50 mL.
8. Cell culture incubator maintained at 37 °C and with 5% CO_2.
9. Centrifuge at 4 °C with adjustable acceleration and deceleration.

3.4 Assay Reagents

1. LDH cytotoxicity detection kit (Roche, Sigma). This system is a microplate-based method where LDH released from the dying cells can be measured from 50 to 100 μL cell culture supernatant (*see* Note 10).

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[9] The activity of IFNγ and TNFα usually varies between manufacturers. Thus, it is recommended that for the first time, a broad range of IFNγ and TNFα concentrations be tested to find the effective dose (EC_{50}, concentration at which parasite growth is reduced to 50%) for the experiment. Usually the working concentration of IFNγ and TNFα ranges between 10 and 100 U/mL. Commonly, recombinant IFNγ and TNFα comes at 100 μg to 1 mg per vial. To achieve the concentration in "Units/mL" the following formula can be used:

$$\text{Concentration in Unit/mL} = \text{specific activity in Units/mg} \times \text{Concentration in mg/mL}$$

The IFNγ and TNFα stock concentration should be made 10^4 or 10^5 U/mL. It is recommended that each time an aliquot is taken out from −80 °C, the IFNγ and TNFα concentration that gives the same or similar result as in the first experiment be determined. This is important as the activity of IFNγ and TNFα can deteriorate over time. LPS should be dissolved in endotoxin-free sterile water at a concentration of 1–10 mg/mL and should be aliquoted and stored in −20 °C for 6 months. Avoid repeated usage of IFNγ, TNFα, and LPS from same aliquot.

[10] The usual recommendation for measurement of LDH using the kit from Roche, Sigma is to use 100 μL culture supernatant with 100 μL of LDH reagent. The important feature here is to keep the culture supernatant and LDH reagent ratio at 1:1.

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2. Complete DMEM medium plus 2% v/v Triton X-100. This solution is used to lyse all the cells, which will be the control for maximal LDH release. To make 50 mL of this medium, 1 mL of 100% Triton X-100 should be added to 49 mL of complete DMEM medium.

3. Cell lysis buffer for the luciferase assay. To make the buffer, 1× PBS containing 2 mM Dithiothreitol (DTT) (*see* Note 11), 10% v/v glycerol, and 1% v/v Triton X-100 should be used. To make 50 mL of lysis buffer, 5 mL of 100% glycerol stock solution, 0.5 mL of 100% Triton X-100, and 0.1 mL of 1 M DTT solution (final concentration will be 2 mM) should mixed with 1× PBS to make the volume 50 mL. Alternatively, a 5× Luciferase Cell Culture Lysis Reagent can be purchased from Promega (Cat. Number E1500), which should be stored at −20 °C. Each time, the desired volume can be made by diluting the 5× reagent in deionized water (*see* Note 12).

4. Luciferase assay buffer: To make a 2× luciferase assay buffer the following reagents are required:

   a. 500 mM (100×) magnesium chloride ($MgCl_2$) solution. To make 10 mL solution, weigh 476.05 mg of $MgCl_2$ and dissolve in deionized water. Solution can be stored at room temperature for at least 1 month.

   b. 15 mM (100×) ATP solution. To prepare 10 mL solution, weigh 82.67 mg of ATP and dissolve in deionized water. Make aliquots and store at −20 °C.

   c. 400 mM (4×) Tris–HCl, pH 7.8 buffer. Weigh 4.85 g of Tris base and dissolve in 85 mL of deionized water. Adjust pH with 1 M HCl to pH 7.8 and make the volume up to 100 mL.

   d. To make 1 mL of fresh 2× assay buffer, add the following in order: 0.5 mL Tris–HCl (4×) solution, 0.02 mL of $MgCl_2$ (100×), 0.02 mL of ATP (100×) and adjust the volume to 1 mL with deionized water. The 2× assay buffer can also be made in a large quantity and stored in small aliquots at −20 °C. The day of measuring luminescence, aliquots can be thawed and diluted to 1× with deionized water.

5. D-Luciferin solution: To make a 100× solution (15 mg/mL) of K-Luciferin, (alternatively, Na-luciferin can also be used) dissolve 90 mg of K-luciferin in deionized water and store as small aliquots at −80 °C for up to 6 months. Aliquots can also be stored be stored at −20 °C for 2–3 weeks. Add 2 μL of D-luciferin substrate (100×) to 0.198 mL of 1× luciferase assay buffer on the day of measurement.

11. A stock solution of 1 M DTT should be made by dissolving 1.54 g of DTT to 8 mL of deionized water and then make up to 10 mL. Following that, small aliquots of 0.1 mL could be made in small centrifuge tubes, wrapped in aluminum foil, and kept at −20 °C to avoid repeated freeze–thaw cycles.

12. If the luciferase assay will be performed later, it is recommended to add protease inhibitor cocktail 1× (Halt protease inhibitor cocktail from Thermo Fisher, without EDTA) to the lysis buffer. Add 25 μL of this lysis buffer to each well of the 96-well plates.

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3.5 Method

1. Seed the host cells in 96-well tissue culture treated plates using 2×10^4 HFFs or 1×10^5 macrophages/well (volume = 100 μL/well) as shown in Fig. 2a. All the boundary wells should contain sterile PBS to keep the plate in moist condition for good health of the host cells and less chance of evaporation of the medium from the wells.

2. Following that, add the stimuli of interest and incubate the cells for another 16–24 h at 37 °C. IFNγ should be used at 10–100 U/mL but the activity can vary from batch to batch (*see* Note 13). In addition, LPS could also be added at a concentration of 10 ng/mL (the stock concentration usually is 1–10 mg/mL) or alternatively TNFα could also be added at a concentration of 10–100 U/mL (stock concentration at 10,000 U/mL).

3. After incubation with the stimuli, add luciferase expressing parasites at three different MOIs, that is, for type I (RH) strain use MOIs of 1/2/3 and for non-RH strains use MOIs of 3/5/7 (*see* Fig. 2a). Set up a plaque assay in parallel to get the real MOI (*see* Note 13). Let the infection proceed for 20–24 h in a 37 °C tissue culture incubator. Make sure to keep the total volume per well constant.

4. After 20–24 h of infection, remove the medium from the wells that will serve as the control for maximum cell lysis (Fig. 2a) and replace it with complete DMEM with 2% Triton X-100 media (check under the microscope that the cells have all lysed: host cell nuclei will appear very distinct with a clear background).

5. Before taking out the medium from each well, plates should be centrifuged at $440 \times g$ for 5 min at room temperature to make sure the medium is free of parasites or cells.

6. Transfer the culture supernatant to a different 96-well plate using a multichannel pipette. To three wells add cell culture medium as a control as FBS present in medium can contain different levels of LDH (*see* Note 14). This plate will be used to measure cell death by measuring the amount of LDH present in the culture supernatant.

7. For the LDH assay from the culture supernatant, add the freshly prepared LDH reagent in a ratio of 1:1 to the 100 μL culture supernatant according to the manufacturer's instructions (Roche, Sigma) and incubate for 25–30 min in the dark, at room temperature.

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[13]. For all the assays, comparison between strain types or between wild-type vs. knockout strains should be interpreted with MOI matching by plaque assay as stated in arginase assay protocol. For the example given in Fig. 2b, c, it can be clearly seen that at different MOI the values are different. It is therefore recommended to keep a near constant MOI for repeated experiments (Fig. 2b, c). Therefore, MOI matching should be done using the plaque assay results as shown in Table 1.

[14]. The culture supernatant can be stored at 4 °C for 1–2 weeks for LDH measurement. However, it is better to measure it the day of collection. The plates should never be stored in −20 °C as this can decrease the LDH enzyme activity. Another important consideration is the use of media with FBS. FBS contains LDH, so using 10% FBS containing media can lead to high background compared to 1% FBS containing media. However, 10% FBS containing media is better for host cell health and metabolism. Thus, keeping media only wells at the end of incubation is important as a control.

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8. The absorbance should be measured at 490 nm using a microplate reader having a filter of 490 nm or a monochromator-based plate reader. The working formula for measuring LDH release is as follows:

$$\%\text{LDH release} = \frac{[(\text{sample O.D} - \text{blank O.D}) - (\text{control O.D} - \text{blank O.D})]}{[(\text{lysis control O.D} - \text{blank O.D}) - (\text{control O.D} - \text{blank O.D})]} \times 100$$

Blank O.D.= O.D. of medium only with LDH reagent.

Control O.D. = O.D of medium from untreated and unstimulated host cells, this is supposed to have minimum absorbance if the cells are healthy.

Lysis control O.D. = Host cells treated with 2% Triton X-100 containing media which ruptures the cell membrane and releases maximum LDH. These wells will have maximum absorbance.

9. To measure the parasite growth by luciferase, add 25 μL lysis buffer mentioned in assay reagents point 3 to the cells from which the medium was removed. Seal the plate with a plate sealer and keep it at −20 °C for 1 week or at −80 °C for at least 1 month. Alternatively, freeze-thaw for 2–3 times and process the same day.

10. To proceed the same day for luciferase assay, after freeze thawing the lysate plate, plates should be kept at 37 °C for 30 min before reading. Two approaches can be taken for reading luminescence, either by using a luciferase plate reader or using a single channel luminometer. For plate reader-based measurement, transfer all the lysate to a white-walled plate for luminescence (e.g., Corning Costar plate, Cat. No. #3912) using a multichannel pipette followed by addition of 50 μL of luciferase assay buffer with D-Luciferin (made fresh) as described in points 4 and 5 in assay reagents. The program for measuring luminescence should be used with 10s delay at room temperature. If a manual single channel luminometer will be used, then 50 μL of freshly made luciferase assay buffer with D-Luciferin should be first added to 1.5 mL clear centrifuge tubes (the number of tubes will be dependent on the number of wells in the plate, one tube/well). Colored centrifuge tubes should not be used. Following this, 25 μL of the lysate from one well should be added to one tube and luminescence should be recorded using 10 s delay program. This should be done sequentially for all tubes one at a time. For analysis of the result, luciferase read of non-IFNγ treated wells will be considered as 100% and relative growth inhibition of IFNγ-treated wells will be calculated accordingly. To graphically represent, either % relative growth or % relative growth inhibition (% relative growth in non-IFNγ wells – % relative growth in IFNα-treated wells) can be plotted as shown in Fig. 2d.

4 MTS-PMS–Based Assay for Host Cell Viability: An Alternative Approach

4.1 Introduction

In addition to measuring host cell death with the LDH release assay, one can also measure the number of viable host cells. A method that is commonly used to do this is known as the MTS/PMS, [(3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-

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sulfophenyl)-2H-tetrazolium inner salt)/phenazine methosulfate) assay [16]. This assay measures the reduction of the MTS tetrazolium compound by viable cells to generate a colored soluble formazan product. This conversion is carried out by mitochondrial dehydrogenase enzymes of metabolically active cells. The soluble formazan dye produced by viable cells can be quantified by measuring the absorbance at 490 nm [16]. This assay is rapid and can be efficiently used for measuring macrophage viability upon infection with *Toxoplasma gondii* [17].

4.2 Materials

Prepare all solutions using deionized water and analytical or molecular biology grade reagents. Prepare and store all reagents at room temperature, unless otherwise mentioned.

4.3 Cell Culture Reagents

1. For culturing macrophages all the reagents and equipment must be free of endotoxins as stated in the arginase assay protocol. The macrophage culture medium is the same as mentioned in the arginase assay protocol. For culturing HFFs complete DMEM should be used.
2. Any parasite strain (e.g., type I/II/III) or any transgenic strain that will be used in this assay should be genetically engineered to express luciferase [15].
3. Sterile phosphate buffer saline (PBS).
4. Sterile endotoxin-free centrifuge tubes ranging from 0.5 to 50 mL.
5. Recombinant IFNγ of either human or mouse origin. The IFNγ should be dissolved in sterile complete DMEM media as a 100× concentrated stock solution and kept at −80 °C for storage in small aliquots to avoid repeated freeze–thaw cycles. (*For IFNγ concentrations and stability please see* Note 1 *of LDH assay method*).
6. Flat bottom 96- and 24-well plates.
7. Sterile serological pipettes 5–50 mL.
8. Cell culture incubator maintained at 37 °C and with 5% CO_2.
9. Centrifuge at 4 °C with adjustable acceleration and deceleration.

4.4 Assay Reagents

1. MTS stock reagent preparation: the final MTS stock concentration is 2 mg/mL to be made in sterile 1× PBS. To make 20 mL of the reagent, weigh 40 mg of the MTS powder and mix with 20 mL of sterile 1× PBS. To dissolve, vortex the solution for till the MTS is completely dissolved. When the MTS is completely dissolved, the solution will appear yellowish. Wrap the tube with aluminum foil as the solution is light sensitive. For storage, make small aliquots wrap them with aluminum foil and keep them in a cardboard box at −20 °C.
2. Preparation of PMS solution: a stock solution of PMS can be prepared in 1× sterile PBS. The concentration of the PMS stock solution is 9.2 mg/mL. To make

10 mL of solution, weigh 92 mg of PMS and mix with 10 mL of 1× sterile PBS. Small aliquots can be made, wrapped with aluminum foil, and kept in a cardboard box at −20 °C. This concentration of PMS is a 100× solution.

3. MTS-PMS reagent preparation: on the day of the experiment, take one aliquot each of MTS and PMS. First, make a 10× dilution of the PMS stock solution to get a 10× concentration (0.92 mg/mL). Mix MTS and PMS at a ratio of 10:1 in which the final concentration of PMS will be 0.092 mg/mL (1×). For instance, to make 1650 μL of final solution, mix 1500 μL of MTS with 150 μL of 0.92 mg/mL of PMS solution (10×). From this final mixture of MTS-PMS, 20 μL should be added to 100 μL of cell suspension.

4. LPS (*see* Note 15): Dissolve the LPS in endotoxin-free water to make a stock concentration of 1 mg/mL. Make small aliquots and keep them at −20 °C for 6 months with full functional activity. Avoid repeated freeze thawing.

5. Nigericin: Dissolve nigericin in 100% ethanol. The stock concentration of Nigericin is 1 mg/mL. Make small aliquots and keep them at −20 °C for 1 year with full functional activity. Avoid repeated freeze thawing.

4.5 Method

1. Seed the host cells in 96-well tissue culture treated plates using 1×10^5 macrophages/well (volume = 100 μL/well) as shown in Fig. 2e. All the boundary wells should contain sterile PBS to keep the plate in moist condition for good health of the host cells and less chance of evaporation of the medium from the wells.

2. Following cell seeding for 2–4 h, add the stimuli of interest and incubate the cells for another 16–24 h at 37 °C. IFNγ should be used at 10–100 U/mL but the activity can vary from batch to batch (*For IFNγ concentrations and stability please see* Note 1 *of LDH assay method*).

3. After incubation with the stimuli, add parasites at three different MOIs, that is, for type I (RH) strain use MOIs of 1/2/3 and for non-RH strains use MOIs of 3/5/7 (Fig. 1a). Set up a plaque assay in parallel to get the real MOI. Let the infection proceed for 20–24 h in a 37 °C tissue culture incubator. Make sure to keep the total volume per well constant.

4. After 20 h of infection, add LPS (final concentration will be 10 ng/mL) to the designated well (Fig. 2e) for 4 h and then add nigericin (5 μM final concentration) for the final 1 h. before addition of MTS-PMS reagent (*see* Note 16). These wells will serve as a positive control for cell death. Add freshly

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15. It is important to use ultrapure LPS (ligand for Toll like receptor-4/TLR-4) as standard LPS can contain lipoproteins, which can also activate TLR-2 along with TLR-4 and thus can influence the result of the experiment.

16. LPS is an agonist of TLR-4 and activates the nuclear factor κB (NF-κB) transcription factors, leading to upregulation of the expression of the nucleotide-binding oligomerization domain, leucine-rich repeat, and pyrin domain containing protein (*NLRP)* 3 inflammasome gene (signal 1). Nigericin, a potassium ionophore, acts as a second signal for assembly of the NLRP3 inflammasome complex and activation and cleavage of caspases 1 and 4 followed by cleavage and activation of gasdermin D, which makes pores in the cell membrane. This causes the cells to die in a process known as pyroptosis [21]. Thus, wells treated with LPS and nigericin together will act as a positive control for cell death.

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prepared 20 μL of MTS-PMS solution to each well and wrap the plate with aluminum foil, and put it back in the 37 °C tissue culture incubator. Incubate for 1–2 h. Here it is important to check the color formation every 45 min to 1 h. If the red-purple formazan color is formed, measure absorbance at 490 nm. Alternatively, plate can be incubated longer for more color development. Background absorbance, which comes from spontaneous reduction of MTS-PMS by dissolved oxygen in the media, should not be more than 0.2.

5. To calculate the percentage viability of host cells the following formula can be used:

$$\%\text{viabiliy} = \frac{[\text{sample O.D} - \text{blank O.D}]}{[\text{control O.D} - \text{blank O.D}]} \times 100$$

   a. *Blank O.D.* = O.D of medium only with MTS-PMS reagent.

   b. *Control O.D.* = O.D of medium from untreated and unstimulated host cells, this is supposed to have maximum absorbance if the cells are healthy.

5 Measurement of Indoleamine 2,3 Dioxygenase (IDO) Activity from IFNγ-Stimulated *Toxoplasma*-Infected Host Cells

5.1 Introduction

IFNγ is the major mediator of the immune response against *Toxoplasma* irrespective of host organism and cell type [18]. In humans, the mechanism of IFNγ-mediated *Toxoplasma* growth inhibition varies between cell types [14]. Among the various effector mechanisms induced by IFNγ, activation of L-tryptophan catabolism by induction of indoleamine 2,3 dioxygenase (IDO) plays an important role because *Toxoplasma* is an auxotroph for L-tryptophan [19]. Therefore, degradation of L-tryptophan is an effective mechanism for limiting parasite growth inside host cells. To assay L-tryptophan catabolism, additional L-tryptophan needs to be added to the culture media, but it should be noted that this might make it more difficult to measure parasite growth restriction due to L-tryptophan degradation. Therefore, if parasite growth restriction also needs to be assessed, culture media without additional L-tryptophan should be used in parallel. IDO is the first and rate-limiting enzyme of tryptophan catabolism through the kynurenine pathway and considered as an immunoregulatory enzyme of M1 macrophages [4]. Herein, we will describe a 96-well plate–based method for determination of IDO activity that can be combined with other assays such as cytokine measurements, cytotoxicity assays, and also parasite growth measurements. This assay will determine the L-tryptophan catabolic product Kynurenine from the culture supernatant, which correlates directly with IDO activity of the cells. Although our example is for HFFs, the same assay can be performed on macrophages.

5.2 Materials

Prepare all solutions using deionized water and analytical or molecular biology grade reagents. Prepare and store all reagents at room temperature, unless otherwise mentioned.

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5.3 Cell Culture Reagents

For culturing host cells (macrophages and HFFs) all the reagents and equipment must be free of endotoxins as stated in the arginase and LDH assay protocol. The medium should contain 0.6 mM L-tryptophan. Usually DMEM contains 0.08 mM L-tryptophan; therefore, 0.52 mM L-tryptophan should be added to the medium to enhance the signal of the assay. To make 100 mL of medium, add 10.62 mg of L-tryptophan and mix and dissolve the L-Tryptophan by warming the medium in a 37 °C water bath for 10 min and then mixing again with a 25 mL serological pipette. This step must be repeated 2–3× times to completely dissolve the tryptophan in the medium. Finally, filter-sterilize the medium through a 0.22 μm filter. This medium is stable for to 2–3 weeks at 4 °C (*see* Note 17).

1. If parasite growth is measured by the luciferase assay, any parasite strain (e.g., type I/II/III) or any transgenic strain that will be used in this assay should be genetically engineered to express luciferase.
2. Sterile phosphate buffer saline (PBS).
3. Recombinant IFNγ of either human or mouse origin. The IFNγ should be dissolved as stated in the LDH assay protocol.
4. Sterile endotoxin-free centrifuge tubes ranging from 0.5 to 50 mL.
5. Flat bottom and V bottom 96-well plates and 24-well plates.
6. Sterile serological pipettes 5–50 mL.
7. Cell culture incubator maintained at 37 °C and with 5% CO_2.
8. Centrifuge at 4 °C with adjustable acceleration and deceleration.

5.4 Assay Reagents

1. 30% w/v trichloroacetic acid (TCA). To make 100 mL of solution of TCA add 30 mL of 6.1 N TCA (Sigma-Aldrich, cat. number T0699) to 70 mL of deionized water. This solution is stable 4 °C for at least 1 month.
2. 5 mM L-Kynurenine solution. This solution will be diluted to prepare the standard curve for the assay. To prepare a 5 mL solution of 5 mM L-Kynurenine solution, weigh 5.2 mg and dissolve it in deionized water. Make aliquots and store them at −20 °C. Solution is stable for 1 year at −20 °C.
3. Ehrlich's reagent: Ehrlich's reagent is 1.2% p-dimethylaminobenzaldehyde in glacial acetic acid solution. This reagent should be made fresh on the day of the assay. To make 25 mL of Ehrlich's reagent, weigh 300 mg of p-dimethylaminobenzaldehyde and dissolve it in 25 mL of glacial acetic acid solution. This solution is light sensitive and has a light green color. So, after preparation, wrap the tube with aluminum foil.

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[17]. The stock solution of L-tryptophan is dissolved in 0.1 N NaOH. This stock solution can subsequently be added directly into the media. In this case, control wells containing the appropriate amount of NaOH should also be added to the experimental setup.

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5.5 Method

1. Seed the host cells in 96-well tissue culture treated plates using 2×10^4 HFFs or 1×10^5 macrophages/well (volume = 150 μL/well) as shown in Fig. 3a (*see* Note 18). All the boundary wells should contain sterile PBS to keep the plate in moist condition for good health of the host cells and less chance of evaporation of the medium from the wells.

2. Following this, add the stimuli of interest and incubate the cells for a further 16–24 h at 37 °C. IFNγ should be used at 10–100 U/mL (*see* Note 9).

3. After incubation with the stimuli, add luciferase expressing parasites at three different MOIs, that is, for type I (RH) strain use MOIs of 1/2/3 and for non-RH strains use MOIs of 3/5/7. Set up a plaque assay in parallel to get the real MOI (*see* Note 5). Infection should proceed for 18–24 h at 37 °C in a tissue culture incubator. Make sure, to keep the total volume per well constant. The total volume should be 200 μL per well (Fig. 3a).

4. After 20–24 h of incubation, centrifuge the plate at 500 × *g* for 5 min at room temperature to make sure the medium is free of parasites and cells.

5. Transfer the 200 μL culture supernatant to a different 96-well plate using a multichannel pipette. In the original plate, where the cells and parasites have been incubated, add 25 μL lysis buffer mentioned in assay reagents point 3 (LDH assay protocol) to the wells to measure the parasite growth by luciferase. Seal the plate with a plate sealer and keep it in −20 °C for 1 week or at −80 °C for at least 1 month. Alternatively, freeze-thaw for 2–3 times and process the same day.

6. Now, transfer 150 μL of the culture supernatant to a 96-well V. bottom plate and add 20 μL of TCA to all the wells of the V bottom plate. After sealing the plate with a plate sealer, incubate the plate at 50 °C for 30 min using a heating block. This incubation is to hydrolyze the N-formyl kynurenine produced from the catabolism of L-tryptophan to L-kynurenine. During this time, to the remaining 50 μL of the culture supernatant, add 50 μL of freshly prepared LDH reagent for measuring cell death or that plate could be stored at 4 °C for 1–2 weeks to measure the LDH activity later.

7. After 30 min of incubation, take out the plate and centrifuge the plate for 10 min at 600 × *g*. During this time dilute 5 M L-kynurenine to the following concentrations: 1500–750–375–187.5–93.8–46.9–23.4–11.72–5.86–0 μM each for triplicate wells as standards (100 μL per well). A typical standard curve of L-kynurenine is shown in Fig. 3b where the concentration of L-kynurenine is indicated on the *X*-axis and the net OD is indicated on the *Y*-axis.

8. Following centrifugation, transfer 100 μL of supernatant, taking care to not touch the bottom of the plate, to a new 96-well flat bottom plate. This is important because at the bottom of the wells, TCA precipitated proteins will be present.

[18] To determine the specificity of IFNγ-mediated induction of IDO activity, an inhibitor of IDO, 1-methyl-L-tryptophan (1-MT), can also be used at a concentration of 1 or 2 mM [22].

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Therefore, to avoid protein contamination, it is recommended not to touch the bottom of the wells.

9. Add 100 μL of Ehrlich's reagent (1:1 ratio with the supernatant) to the wells and incubate for 10 min.

10. Measure the absorbance at 490 nm using a plate reader. The concentration of L-Kynurenine can be determined using the standard curve of L-Kynurenine (Fig. 3c, d).

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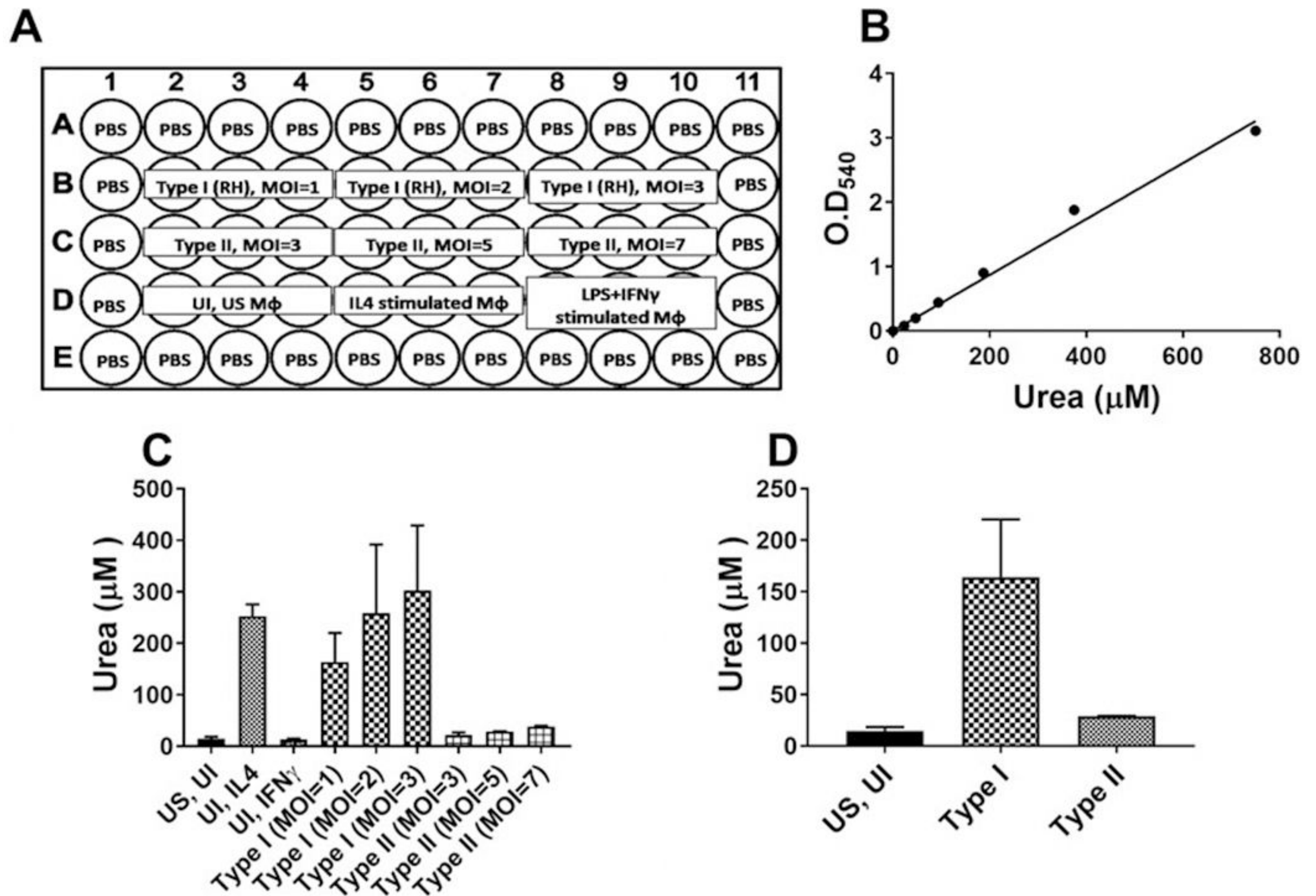

Fig. 1.

Arginase assay. (**a**) A 96-well format for measuring arginase activity from macrophages. All the conditions should be performed in triplicate wells as depicted. *Mφ* macrophages, *LPS* lipopolysaccharide, *UI* uninfected, *US* unstimulated. (**b**) A standard curve of urea from which the amount of urea produced by *Toxoplasma*-infected macrophages or cytokine-treated macrophages can be derived. (**c**) Amount of urea produced from macrophages is shown where IL-4-treated cells served as positive control and IFNγ-treated cells as negative control. Different strains of *Toxoplasma* at different MOIs induced different amounts of urea. (**d**) Urea production by macrophages infected with different *Toxoplasma* strains at a similar MOI matched by plaque assay as shown in Table 1

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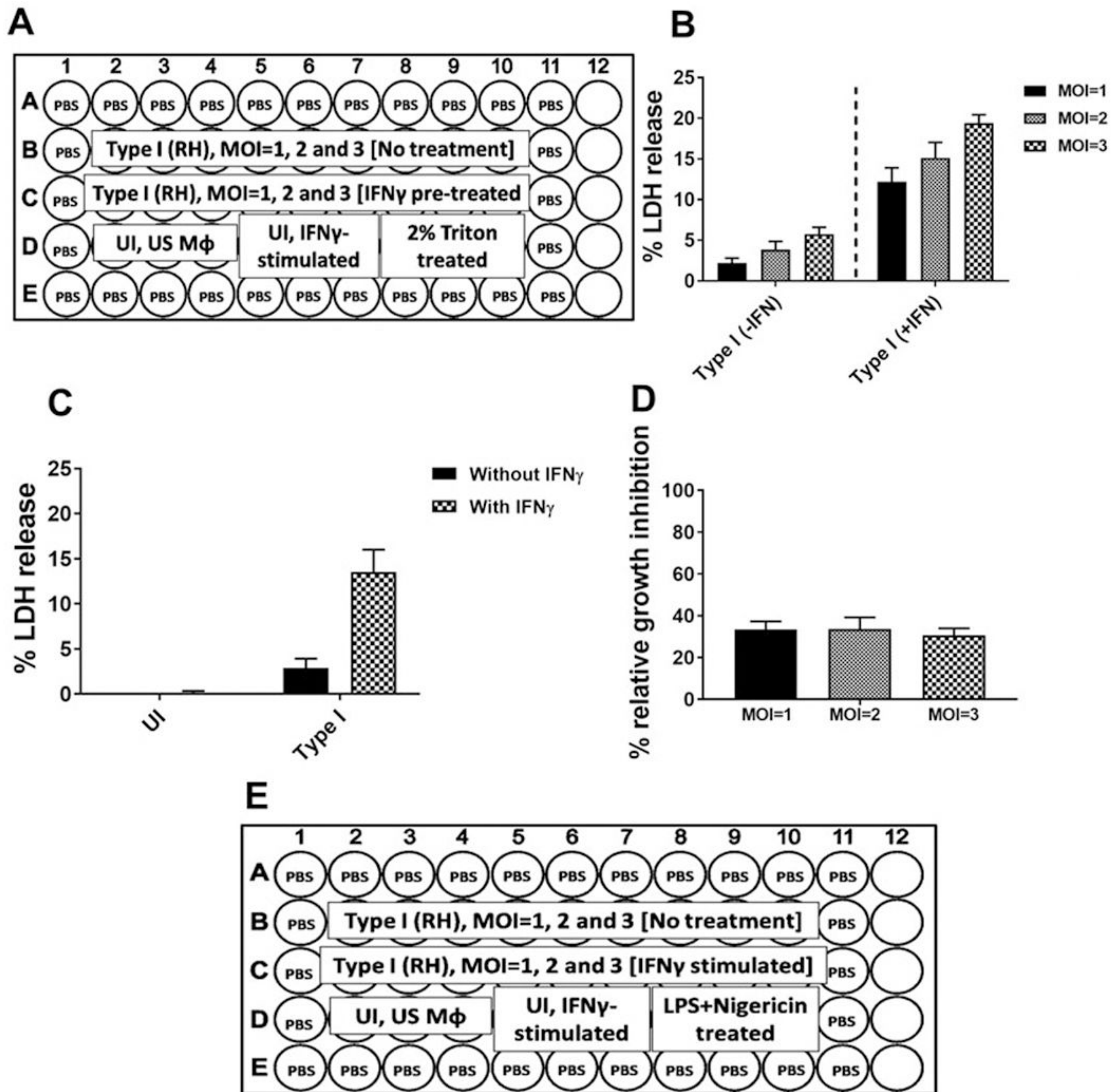

Fig. 2.

Cell death assay determined by LDH release, cell viability determined by MTS-PMS assay, and parasite growth assay determined by luciferase activity measurement. (**a**) A format for 96-well plate–based multiplex assay for cell death and parasite growth measurement. *UI* uninfected, *US* unstimulated. (**b**) Bar diagram showing the normalized values of LDH release (normalized to untreated and uninfected HFFs) from the *Toxoplasma*-infected HFFs with and without IFNγ treatment (10 U/mL). (**c**) Amount of LDH release by *Toxoplasma*-infected (at a matched MOI of 1 from 3 different experiments, as determined by plaque

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assay) IFNγ-stimulated HFFs. (**d**) Measurement of % relative growth inhibition at MOI 1–3 of *Toxoplasma* in IFNγ-stimulated and unstimulated (US) HFFs. Here the mean luciferase reading of the triplicate wells from unstimulated cells are considered as 100% and the relative % of growth in IFNγ-stimulated cells was calculated from this value. (**e**) A format for 96-well plate–based MTS-PMS–based assay for measuring cell viability *UI* uninfected, *US* unstimulated

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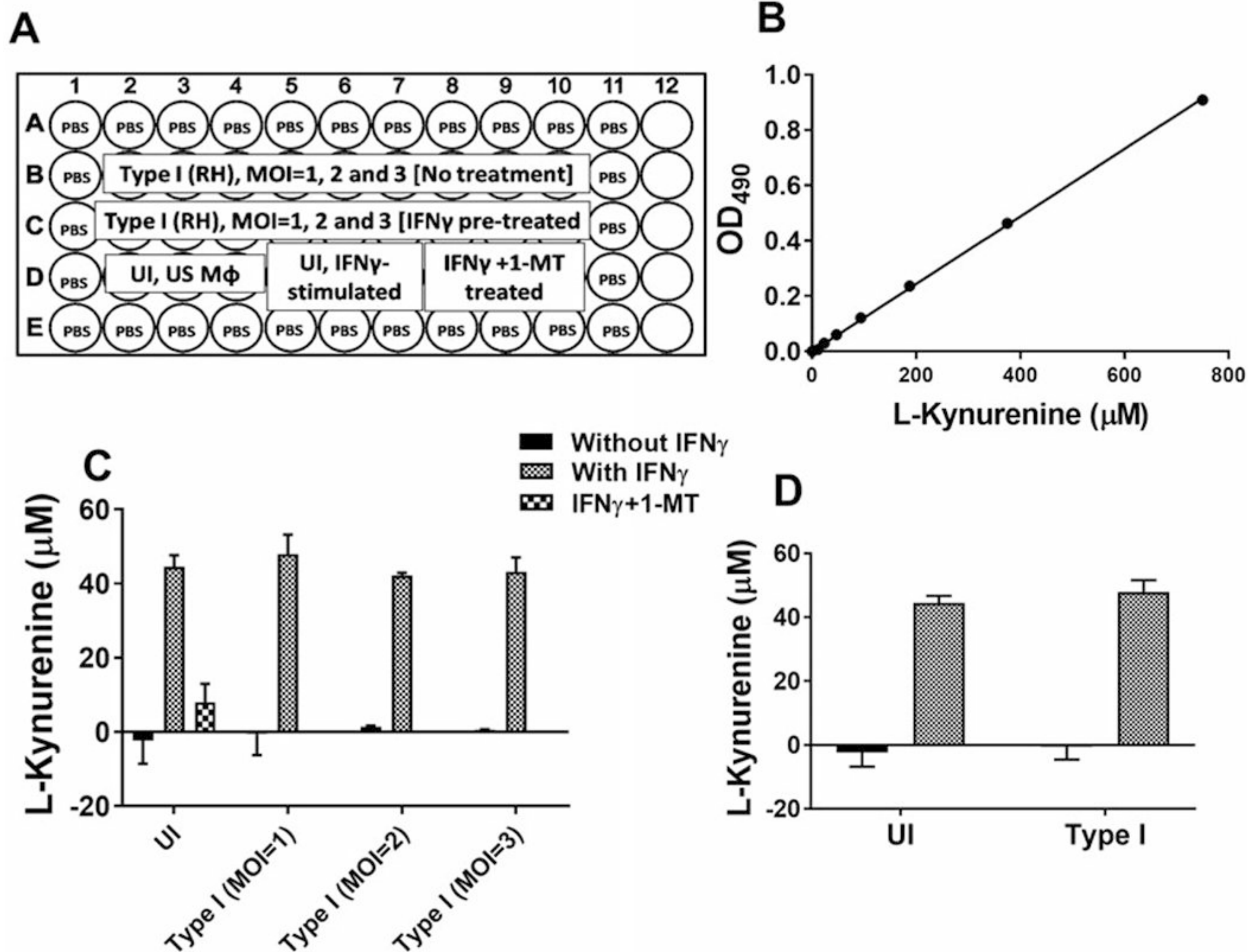

Fig. 3.

Measurement of indoleamine 2,3 dioxygenase assay from the culture supernatant of IFNγ-stimulated cells. (**a**) A 96-well plate format for designing the IDO measurement experiment in IFNγ-stimulated *vs.* unstimulated *Toxoplasma*-infected cells. (**b**) Standard curve of L-kynurenine for calculation of L-kynurenine values from the supernatants collected from IFNγ-treated and untreated *Toxoplasma*-infected cells. (**c**) Bar diagram showing the amount of L-kynurenine produced from IFNγ-treated and untreated Toxoplasma-infected cells. (**d**) Comparison of L-kynurenine production between IFNγ-treated uninfected HFFs with IFNγ-treated and *Toxoplasma*-infected (at MOI of 1) HFFs

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Table 1

Plaque assay result and viability matching

						Intended MOIs				
	Plaque number					**1**	**2**	**3**	**5**	**7**
Strain type	**Well A**	**Well B**	**Well C**	**Average**	**% viability**	**Real MOIs**				
Type I (100 parasites)	62	71	77	70	70	**0.7**	1.4	2.1		
Type II (250 parasites)	46	40	34	40	16			0.5	**0.8**	1.1

Values marked in bold are viability-matched MOIs