Host cell depletion of tryptophan by IFNγ-induced Indoleamine 2,3-dioxygenase 1 (IDO1) inhibits lysosomal replication of Coxiella burnetii

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Abstract

Most intracellular pathogens that reside in a vacuole prevent transit of their compartment to lysosomal organelles. Effector mechanisms induced by the pro-inflammatory cytokine Interferon-gamma (IFNγ) can promote the delivery of pathogen-occupied vacuoles to lysosomes for proteolytic degradation and are therefore important for host defense against intracellular pathogens. The bacterial pathogen Coxiella burnetii is unique in that, transport to the lysosome is essential for replication. The bacterium modulates membrane traffic to create a specialized autophagolysosomal compartment called the Coxiella-containing vacuole (CCV). Importantly, IFNγ signaling inhibits intracellular replication of C. burnetii, raising the question of which IFNγ-activated mechanisms restrict replication of a lysosome-adapted pathogen. To address this question, siRNA was used to silence a panel of IFNγ-induced genes in HeLa cells to identify genes required for restriction of C. burnetii intracellular replication. This screen demonstrated that Indoleamine 2,3-dioxygenase 1 (IDO1) contributes to IFNγ-mediated restriction of C. burnetii. IDO1 is an enzyme that catabolizes cellular tryptophan to kynurenine metabolites thereby reducing tryptophan availability in cells. Cells deficient in IDO1 function were more permissive for C. burnetii replication when treated with IFNγ, and supplementing IFNγ-treated cells with tryptophan enhanced intracellular replication. Additionally, ectopic expression of IDO1 in host cells was sufficient to restrict replication of C. burnetii in the absence of IFNγ signaling. Using differentiated THP1 macrophage-like cells it was determined that IFNγ-activation resulted in IDO1 production, and that supplementation of IFNγ-activated THP1 cells with tryptophan enhanced intracellular replication. Thus, this study identifies IDO1 production as a key cell-autonomous defense mechanism that limits infection by C. burnetii, which suggests that peptides derived from hydrolysis of proteins in the CCV do not provide an adequate supply of tryptophan for bacterial replication.
Author summary

Coxiella burnetii is a mammalian pathogen that can cause a predominantly zoonotic disease called Q-fever. In humans, Q-fever manifests as an acute or chronic illness especially in immunocompromised individuals. C. burnetii is uniquely adapted to live in a lysosome-derived vacuole that degrades proteins and provides nutrients that support intracellular replication. From a cell biological perspective, C. burnetii represents an excellent model to study pathogens that survive in harsh cellular environments. The strategies by which infected cells intrinsically combat C. burnetii are not well-established. In this study, we investigate the underlying mechanism by which IFNγ activates cells and prevents C. burnetii from replicating inside cells. The data presented here demonstrate that IFNγ induces the expression of the enzyme Indoleamine 2,3-dioxygenase 1 (IDO1), which degrades the amino acid tryptophan and restricts the intracellular replication of C. burnetii. The production of IDO1 is sufficient to inhibit replication of C. burnetii, indicating that tryptophan depletion is an effective cell-autonomous defense mechanism against this lysosome-adapted pathogen. In addition, these data imply that the degradative vacuole in which this pathogen resides does not generate a supply of tryptophan sufficient to support intracellular replication.

Introduction

Coxiella burnetii is a gram-negative, obligate intracellular pathogen that causes an infectious disease called Q-fever. Humans are occasionally infected through inhalation of aerosols or through close contact with infected livestock, and the symptoms range from mild flu-like illness to vascular complications and fatal endocarditis (reviewed in [1]). Infection of human cells begins with the phagocytosis of C. burnetii. Phagosomes containing C. burnetii undergo endocytic maturation and fuse with lysosomes, which results in the formation of the Coxilha-containing vacuole (CCV). Acidification of the CCV activates the C. burnetii Type IVB secretion system (T4SS) called Dot/Icm, which promotes the translocation of roughly 100 different bacterial effector proteins into the host cell cytosol [2,3]. Type IV secretion is essential for intracellular replication of C. burnetii and the generation of a spacious CCV that has autophagolysosomal characteristics [3–5]. Individual Type IV effector proteins (T4E) facilitate evasion of innate immune surveillance and acquisition of nutrients and membrane for the CCV (reviewed in [6]). The development of an axenic culture medium and genetic manipulation techniques have made C. burnetii an excellent system to study how pathogens adapt to survive and replicate in a lysosome-derived organelle as well as the cell-autonomous immune strategies in place to control their intracellular replication [7,8].

Adaptive immune responses lead to the production of IFNγ, which is a critical determinant of host protection against C. burnetii in immunocompetent animals [9,10]. IFNγ is a potent pro-inflammatory cytokine secreted by activated lymphocytes during infection. Circulating IFNγ has been reported to be a sensitive and diagnostic biomarker in Q fever patients, which shows that an adaptive cell-mediated immune response has been generated [11,12]. IFNγ receptors, ubiquitously expressed on various cell types, bind to IFNγ and stimulate the Janus kinase- Signal transducer and activator of transcription (JAK-STAT) signaling cascade that activates expression of hundreds of antimicrobial genes that provide cell-autonomous defense against intracellular pathogens. The functions of IFNγ-induced genes include, but are not restricted to, generation of reactive oxygen and nitrogen radicals, antimicrobial peptides, toxic metabolites, activation of immune signaling, immunoproteasome, antigen...
presentation, vesicle traffic, autophagy, immune GTPases, small molecule transporters and production of soluble messengers such as cytokines and chemokines (reviewed in [13]). IFNγ-mediated elimination of intravacuolar pathogens (e.g. Salmonella, Mycobacteria) involves immune GTPase and autophagic-recognition of the pathogen-containing compartment (PCV) and labeling it for lysosomal fusion and degradation [14–16]. In the case of pathogens which rupture their phagosomal vacuole and escape to the cytosol (e.g. Listeria), autophagic response triggers the delivery of the bacteria to the lysosome [17,18]. IFNγ-mediated restriction of C. burnetii replication in professional phagocytic cells has been attributed to phenotypes that include CCV alkalinization, TNF-mediated apoptosis, and generation of reactive nitrogen and oxygen species [19–23]. However, restriction mechanisms against pathogens that have evolved to survive and replicate in hostile lysosomal compartments have not been extensively characterized.

In an effort to identify and characterize specific host proteins that are induced in IFNγ-activated cells and participate in the restriction of C. burnetii intracellular replication, an siRNA screen using a curated set of IFNγ-induced genes was conducted. Data from the screen shows that Indoleamine 2,3-dioxygenase 1 (IDO1) is an IFNγ-induced effector that contributes to the restriction of C. burnetii intracellular replication. IDO1 is an enzyme that catalyzes the conversion of the essential amino acid L-tryptophan to kynurenines, which are then used for the synthesis of the metabolite nicotinamide adenine dinucleotide (NAD+) (reviewed in [24]). Because C. burnetii is a tryptophan auxotroph [25], these data show that one mechanism by which IFNγ restricts the replication of this intracellular pathogen is through IDO1-mediated depletion of an essential nutrient.

Results

IFNγ restricts C. burnetii intracellular replication, CCV size, effector translocation and bacterial infectivity

Macrophages treated with IFNγ will restrict C. burnetii replication by a process that is mediated in part by production of inducible nitric oxide synthase and NADPH oxidase [21–23]. Data demonstrating that macrophages deficient in these enzymes still robustly restrict C. burnetii replication indicates that there must be multiple mechanisms by which mammalian cells restrict intracellular replication of C. burnetii upon stimulation by IFNγ [23]. To identify additional pathways by which IFNγ stimulation restricts intracellular replication of C. burnetii, we examined whether treatment of HeLa 229 cells would restrict intracellular replication of this pathogen. The rationale for using HeLa cells was that these cells have many evolutionarily conserved antimicrobial mechanisms that can restrict the replication of intracellular bacterial pathogens (reviewed in [26]), but HeLa cells may not have as many independent pathways to limit the intracellular replication of C. burnetii upon IFNγ treatment as macrophages given that these are human derived epithelial cells and not professional phagocytes. This would increase the likelihood of identifying host factors important for growth restriction by reducing the possibility of redundancy. In addition, HeLa cells are amenable to genetic manipulation. A layout of the experimental setup is presented in Fig 1A. Luminescence generated by a C. burnetii strain expressing the luxCDABE operon constitutively, served as an indicator of bacterial replication (Fig 1B). HeLa cells were infected with C. burnetii and treated with increasing concentrations of IFNγ at 6h post infection (pi). At concentrations as low as 10 ng/ml, there was a significant decrease in the luminescence values observed as early as d3 pi. Based on these data, d4 pi was used as the standard time-point for subsequent experiments measuring bacterial luminescence (Fig 1B). In agreement with luminescence readouts, C. burnetii genome equivalents (GE) measured by quantitative PCR also showed a significant decline in bacterial
numbers from IFNγ-treated cells (Fig 1C). Of note, GE and luminescence data are graphed on log and linear scales respectively, yet reveal the same trend. Reduced bacterial numbers in IFNγ-treated cells also correlated with smaller CCV sizes on d4 pi (Fig 1D). PFA-fixed cells stained for LAMP1 and C. burnetii were visualized by indirect immunofluorescence microscopy. The CCVs in IFNγ-treated cells were small and the bacteria were packed tightly,
whereas, untreated cells had larger and more spacious CCVs that contained dispersed bacteria (Fig 1D). Quantification showed that the average CCV size in IFNγ-treated cells was reduced significantly compared to that of untreated cells (Fig 1D).

Effector proteins translocated by the Dot/Icm secretion system are required to subvert host vesicle traffic to promote the expansion of the CCV. The observation that the CCVs were smaller in IFNγ-treated cells raised the question of whether IFNγ signaling interferes with the ability of *C. burnetii* to translocate effector proteins. Examination of Dot/Icm-dependent translocation of the effector proteins was measured using the effector Cbu0077 fused to the translocation reporter BlaM encoding a β-lactamase enzyme that will cleave the fluorescent substrate loaded into host cells. These data demonstrated a significant reduction in BlaM-Cbu0077 translocation in IFNγ-treated cells on d2 pi and on d4 pi (Fig 1E). Because unified CCVs were observed in IFNγ-treated cells, which is a phenotype requiring the effector protein Cig2 [27], it is likely that *C. burnetii* are initially capable of translocating early effector proteins in cells that were treated with IFNγ 6h after infection. However, the decrease in translocation of BlaM-Cbu0077 observed at d2 and d4 pi indicates that IFNγ-treatment leads to inhibition of Dot/Icm function at these later times, which is consistent with the decrease observed in bacterial luminescence and replication (Fig 1E).

To determine if *C. burnetii* isolated from IFNγ-treated cells were viable and capable of initiating a secondary infection, a foci-forming unit (FFU) assay was performed. Lysates and supernatants of *C. burnetii*-infected cells that were either untreated or treated with IFNγ were collected on d4 pi. Dilutions of each sample were used to measure the number of bacteria by quantitative PCR (GE) and infect untreated HeLa cells. Infected cells were fixed and stained using an anti-*C. burnetii* antibody on d4 pi and immunofluorescence microscopy was used to determine the FFU value after counting the number of cells containing large CCVs (Fig 1F, left panel). *C. burnetii* collected from untreated cells (primary infection) gave rise to a FFU value that was almost a log higher than that isolated from IFNγ-treated cells infected in parallel. This difference was comparatively larger than that obtained using the GE assay (Fig 1F, right panel). This indicates that IFNγ treatment reduces the viability of *C. burnetii* that were still detectable using the GE assay. Together, these data demonstrate that IFNγ limits the expansion of the CCV, intracellular replication, metabolic activity and viability of *C. burnetii* by inducing cell-autonomous defense mechanisms. Thus, in addition to restricting *C. burnetii* in professional phagocytes [19,20,22,23], these results indicate that IFNγ induces antimicrobial activities that are capable of restricting *C. burnetii* replication in cells that are normally non-phagocytic, which suggests there could be a shared IFNγ-activated antimicrobial activity in these cells.

### Loss of function analysis of IFNγ-induced genes to identify host factors that mediate restriction of *C. burnetii* replication

Depending on the cell type, IFNγ signaling stimulates the expression of hundreds to thousands of genes. Published data sets profiling genes upregulated by IFNγ in HeLa cells and macrophages were used to curate genes that may be involved in restricting *C. burnetii* replication [28–31]. Genes encoding proteins that regulate transport and fusion of membranes were given high priority, as were regulators of nutrient transport and cell metabolism. To identify the specific host factors that mediate restriction, a subset of genes upregulated following IFNγ treatment was silenced individually using siRNA. Cell-surface receptors for the cytokine IFNγ (IFNγR1 and IFNγR2), which signal the kinases of the JAK-STAT signal transduction pathway (JAK1, JAK2) to activate the transcription factor STAT1 were included as positive controls (Fig 2). Because intracellular bacterial luminescence provided a robust and sensitive assay for
C. burnetii replication (Fig 1B), the C. burnetii lux strain was used to monitor the effect of individual gene knockdowns on bacterial replication. Silencing of genes encoding components of the JAK-STAT signal transduction pathway, IFNγR1, IFNγR2, JAK1, JAK2 and STAT1, significantly increased C. burnetii replication in IFNγ-treated cells, which indicated that this screen was sensitive enough to identify potential restriction factor candidates (Fig 2). Among the IFNγ-induced effector genes, silencing of the gene encoding Indoleamine 2,3-dioxygenase 1 (IDO1) resulted in the largest increase in C. burnetii replication in IFNγ-treated cells (Fig 2). These data implicate IDO1 as being a critical effector that mediates restriction of C. burnetii intracellular replication in IFNγ-treated cells.
IDO1 is an IFNγ-induced effector that restricts \textit{C. burnetii} replication

Experiments to validate that IDO1 expression inhibits \textit{C. burnetii} replication were conducted in HeLa cells (Fig 3A). Immunoblot analysis and quantitative RT-PCR demonstrated robust induction of IDO1 in IFNγ-treated cells, and silencing of IDO1 expression by siRNA treatment (Fig 3B). As suggested in the initial screen, IDO1 silencing significantly increased \textit{C. burnetii} luminescence in IFNγ-treated cells (Fig 3C). In STAT1-silenced cells it was found that IFNγ treatment resulted in higher levels of \textit{C. burnetii} luminescence, which indicates that there are additional mechanisms by which IFNγ stimulation of cells restricts intracellular replication of \textit{C. burnetii} in the absence of IDO1 (Fig 3C). IDO1 is a cytosolic enzyme that catabolizes tryptophan, which will reduce cellular levels of this amino acid. This enzyme can be inhibited by 1-Methyl Trp, which is a competitive analogue of tryptophan (Fig 3D). The addition of 1-Methyl Trp enhanced \textit{C. burnetii} luminescence in IFNγ-treated cells to a magnitude similar to that observed when IDO1 was silenced (Fig 3E). Thus, the enzymatic activity of IDO1 is important for restriction of \textit{C. burnetii} in host cells stimulated with IFNγ.

\textbf{Fig 3.} Indoleamine 2,3-dioxygenase 1 (IDO1) enzymatic activity is important for restricting \textit{C. burnetii} replication.

Experimental setup is laid out in A. Induction of IDO1 expression by IFNγ and knockdown efficiency of siRNA were determined by qRT-PCR and western blotting (B). Knocking-down IDO1 by siRNA (C) or blocking the enzymatic activity of IDO1 by 1-Methyl tryptophan (E) allows replication of \textit{C. burnetii lux} in IFNγ-treated cells but does not increase \textit{C. burnetii} luminescence in untreated cells. Mechanism of action of IDO1 is depicted in D.

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IDO1 inhibits *C. burnetii* replication by depleting cellular pools of tryptophan

Because *C. burnetii* is a tryptophan auxotroph it must acquire tryptophan from the host cell [25]. IDO1 expression could inhibit *C. burnetii* replication by depleting tryptophan in the cytosol or by generating kynurenines that have antimicrobial properties. To determine if tryptophan depletion was the primary mechanism by which IDO1 inhibits *C. burnetii* replication, exogenous tryptophan was added to the tissue culture medium to test whether this was sufficient to suppress IDO1-mediated growth restriction. A schematic showing the timing of tryptophan addition and collection of samples is shown in Fig 4A. Tryptophan supplementation increased *C. burnetii* luminescence in IFNγ-treated cells to a magnitude similar to that observed following IDO1 knockdown (Fig 4B). Importantly, tryptophan supplementation did not enhance *C. burnetii* luminescence in IDO1- or STAT1-silenced cells. This indicates that growth restriction mediated by tryptophan depletion is dependent on STAT1 and IDO1. A decrease in *C. burnetii* luminescence was observed in the IFNγ-treated cells 3-days post-infection, consistent with a cessation of luciferase production by intracellular bacteria (Fig 4C). By contrast, *C. burnetii* luminescence values continued to increase over the 5-days of infection when the IFNγ-treated cells were supplemented with tryptophan, which indicates that the intracellular bacteria remained more metabolically active (Fig 4C). Similarly, tryptophan supplementation augmented the function of the type IV secretion system in IFNγ-treated cells as evident from the significant increase in the translocation of the effector protein BlaM-Cbu0077 (Fig 4D). Measurements of CCV area showed that IFNγ treatment resulted in smaller vacuoles containing *C. burnetii*, and this phenotype was suppressed by the addition of tryptophan (Fig 4E). To gain additional insight into the mechanism of IDO1-mediated growth restriction, a GE assay was used to measure *C. burnetii* genome expansion. Similar to the luminescence data, a decrease in *C. burnetii* GE values was observed in the IFNγ-treated cells compared to untreated cells at 4-days post-infection and tryptophan supplementation of cells treated with IFNγ resulted in a significant increase in *C. burnetii* GE values by d7 pi (Fig 4F). Lastly, the FFU assay showed that the addition of tryptophan increased *C. burnetii* viability in the IFNγ-treated cells (Fig 4G). Thus, IDO1 production interferes with *C. burnetii* intracellular replication and survival by depleting free tryptophan in the host cytosol and supplementing the culture medium with excess tryptophan suppresses IDO1-mediated restriction.

**IDO1-mediated tryptophan depletion results in a defect in CCV maintenance**

Transmission electron microscopy (TEM) was used to examine whether IFNγ stimulation had any detectible impact on the morphology of the CCV. Several representative images show that CCVs in untreated cells were spacious, which means the individual *C. burnetii* were dispersed randomly throughout the lumen of the vacuole and were not typically in close contact with each other. Also, numerous vesicles were observed within the lumen of CCVs, which is likely due to robust fusion of autophagosomes with the CCV and formation of internal vesicles by a functional multivesicular body (MVB) pathway (Fig 5). By contrast, CCVs in IFNγ-treated cells were constricted, showed enhanced osmium tetroxide staining, had fewer intraluminal vesicles, and the bacteria inside the vacuole were tightly packed and many showed signs of damage resulting in cellular swelling (Fig 5). Importantly, the membrane of the CCV appeared to be intact and *C. burnetii* were not detected in the cytosol. The CCVs in the IFNγ-treated cells supplemented with tryptophan were similar in appearance to the CCVs in the untreated cells. Thus, tryptophan depletion resulting from IDO1 production interferes with the ability of *C. burnetii* to maintain a spacious CCV that supports intracellular replication.
Fig 4. Tryptophan depletion is the mechanism of IDO1-mediated inhibition of *C. burnetii* replication. Experimental setup is laid out in A. Knocking down *IDO1* by siRNA (B) or supplementing the media with tryptophan (B and C) allows replication of *C. burnetii lus* in IFNγ-treated cells as measured by luminescence. HeLa cells were infected with wt *C. burnetii pBlaM* or pBlaM-77 and left untreated or treated with IFNγ in the presence or absence of additional tryptophan. On d4 pi, cells were loaded with the fluorescent substrate CCF4-AM and translocation of BlaM was assessed by the shift in the fluorescence emission from 535 to 460nm relative to that of uninfected (D). CCV were visualized by indirect immunofluorescence on d4 pi and average CCV sizes were quantified for about 40–50 CCVs per experimental condition (E). Fold increase in GE on d4 and d7 pi, compared to that of d1 pi were measured by qPCR (F). FFU/ml of bacteria derived from HeLa cells infected with *C. burnetii* in the presence or absence of IFNγ and Trp were determined by secondary infection of HeLa cells (G, left panel). As a control, GE was measured after primary infection (G, right panel).
IDO1 expression is sufficient to restrict the intracellular replication of C. burnetii

Although IDO1 was important for restriction of C. burnetii replication in cells stimulated with IFNγ, it was unclear whether IDO1 expression would restrict C. burnetii replication in the absence of other IFNγ-induced genes. To address this question, a HeLa cell line that produces IDO1 under the control of a tetracycline-inducible promoter was created. Immunoblot analysis showed that the IDO1 protein was not produced by unstimulated cells but when cells were stimulated with a tetracycline inducer (doxycycline or anhydrotetracycline) or treated with IFNγ, there was a concentration-dependent increase in IDO1 protein levels (Fig 6A). The IDO1-inducible cell line was infected with C. burnetii lux and IDO1 expression was stimulated by either anhydrotetracycline (referred to as Tet) induction or by stimulation with IFNγ as shown in the schematic (Fig 6B). Anhydrotetracycline induction of IDO1 significantly inhibited C. burnetii replication in the HeLa pTRIPZ-IDO1 cells, but not in the vector control HeLa pTRIPZ-EV cells (Fig 6C, left panel). The addition of tryptophan to the culture medium restored C. burnetii replication in HeLa pTRIPZ-IDO1 cells that had been induced to produce IDO1 (Fig 6C, right panel). The GE assay confirmed data from the luminescence assay and

Fig 5. IDO1-mediated depletion of tryptophan interferes with the size and morphology of the CCV. C. burnetii-infected cells were left untreated or treated with IFNγ in the presence or absence of tryptophan as per the experimental layout in Fig 4A. Representative TEM images from untreated (A–D), IFNγ-treated (E–L) and tryptophan supplemented, IFNγ-treated (M–P) cells on d4 pi are presented here. I, J, K and L are higher magnification images of E, F, G and H respectively. Scale bars are denoted at the bottom right of each image.

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Fig 6. IDO1 expression is sufficient to restrict the intracellular replication of *C. burnetii*. HeLa cells were stably transduced with an empty pTRIPZ lentiviral vector (pTRIPZ-EV) or one where IDO1 was cloned under the control of tetracycline-inducible promoter (pTRIPZ-IDO1). Uninfected pTRIPZ-IDO1 cells were treated with different concentrations of doxycycline (referred to as Dox) or anhydrotetracycline (referred to as Tet) for 3 days or IFNγ for 4 days and lysates tested for IDO1 expression by western blotting (A). Panel B lays out the experimental setup for results shown in C and D. pTRIPZ-EV or pTRIPZ-IDO1 cells were infected with *C. burnetii lux* (C) or wt *C. burnetii* (D) and treated with IFNγ or Tet at indicated time points (B). Luminescence was measured in the absence (left panel) or presence of supplemented tryptophan (right panel) and normalized to that of untreated, pTRIPZ-EV.
showed that induction of IDO1 production in the HeLa pTRIPZ-IDO1 cells was sufficient to restrict *C. burnetii* replication by a mechanism that could be suppressed by the addition of tryptophan to the culture medium (Fig 6D). These data indicate that host cell expression of IDO1 will restrict *C. burnetii* replication in the absence of IFNγ signaling, which validates that depletion of tryptophan in the host cell cytosol is sufficient to disrupt the ability of *C. burnetii* to replicate in the lysosome-derived CCV.

**IDO1 participates in IFNγ-mediated restriction of *C. burnetii* replication in human macrophages**

The THP1 cell line was used to determine whether IDO1-mediated restriction of *C. burnetii* replication is a conserved IFNγ-activated pathway that is operational in human-derived macrophage-like cells. Immunoblot analysis was used to evaluate IDO1 production in undifferentiated THP1 cells and THP1 cells that were differentiated into macrophage-like cells by treatment with PMA. Similar to control HeLa cells, differentiated THP1 cells produced IDO1 protein upon IFNγ stimulation (Fig 7A). Differentiated THP1 cells treated with IFNγ restricted *C. burnetii* replication as determined by measuring bacterial luminescence (Fig 7B). The culture medium was supplemented with increasing amounts of tryptophan to determine if IDO1-mediated tryptophan depletion has a measurable effect on restriction of *C. burnetii* replication in differentiated THP1 cells treated with IFNγ. A dose-dependent increase in *C. burnetii* luminescence was observed upon the addition of tryptophan (Fig 7B). A tryptophan concentration of 0.3125 mM resulted in significantly higher levels of *C. burnetii* luminescence (Fig 7C). Thus, IDO1 is induced and participates in the restriction of *C. burnetii* replication in macrophages.

**Discussion**

Activation of cell-intrinsic defense by the cytokine IFNγ enables mammals to combat a large number of microbial pathogens that are able to survive and replicate in host cells. In this study, *C. burnetii* was used as a model pathogen to advance our understanding of how IFNγ-induced responses enable host cells to defend themselves against pathogens that have evolved the ability to replicate in lysosomes, which are catabolic and hostile organelles for most microbes. The data revealed that IFNγ, even at relatively low doses, stimulated a response that efficiently restricted *C. burnetii* replication in HeLa cells, which indicates that non-phagocytic cells are also equipped with cell-intrinsic antimicrobial mechanisms that can limit replication of this pathogen. The IFNγ-induced enzyme IDO1 was found to be important for restriction of *C. burnetii* replication. IDO1 limits the amount of tryptophan available for *C. burnetii*, which is a tryptophan auxotroph so must acquire this essential nutrient from the host. Data presented here indicate that IDO1-mediated depletion of tryptophan stalls *C. burnetii* infection by inhibiting bacterial metabolism and secretion of effector proteins by the Dot/Icm system. This would explain the impact of IDO1 production on the size, morphology and maintenance of the CCV, which is dependent on Dot/Icm function.

Importantly, IDO1 is an enzyme that remains localized in the host cell cytosol, whereas, *C. burnetii* replicates inside a membrane-bound vacuole. Given that the lysosome-derived CCV is an acidified organelle and retains the ability to hydrolyze proteins, these data suggest that
the pool of tryptophan generated by lysosomal degradation of proteins inside the CCV is not sufficient to maintain the nutritional requirements for *C. burnetii* metabolism. Thus, this organism must have the ability to access metabolites such as tryptophan that are in the cytosol. In addition to tryptophan, *C. burnetii* depends on the host for several other key amino acids, which include arginine, cysteine, histidine, leucine, lysine, phenylalanine, proline, tyrosine, threonine, and valine [25]. An important question for future studies will be to determine which of these essential amino acids can be generated in sufficient quantities by hydrolysis of proteins in the vacuole lumen, and which amino acids must be imported into the vacuole either through the subversion of host transporters or through bacterial transporters that are delivered into the CCV membrane.

Phylogenetic analysis of the *C. burnetii* genome indicates that genome reduction, pseudo-geneization of genes occurred as *Coxiella* evolved and adapted from tick-associated lifestyle to infect mammalian hosts [32–36]. *C. burnetii* tryptophan synthesis genes, in particular, are related to the genes in *Simkania negevensis*, which is in the phylum Chlamydiae and have been suggested to be acquired from Chlamydial ancestors through horizontal transfer [36,37]. A closer analysis of the *C. burnetii trp* genes provides insight into why this pathogen is unable to synthesize tryptophan [32,38]. Multiple frameshift mutations in the *trpDG* genes (CBU1153) renders the biosynthetic pathway incapable of utilizing chorismate as a precursor for tryptophan synthesis [32]. There is also a fusion of the genes encoding phosphoribosyl anthranilate...
apoptotic pathways in infected epithelial cells [53,54], which may be why silencing of the TNF proteins delivered into host cell by the γ-treated cells can restrict the replication of intracellular pathogens. Bacterial effector proteins, superoxide dismutases and peroxiredoxin that neutralize the toxic effect of oxidative radicals [48–52]. Induction of apoptotic cell death pathways is another mechanism by which cells compared to macrophages [47]. In addition, C. burnetii is believed to encode acid phosphatases, superoxide dismutases and peroxiredoxin that neutralize the toxic effect of oxidative radicals [48–52]. Induction of apoptotic cell death pathways is another mechanism by which IFN-γ-treated cells can restrict the replication of intracellular pathogens. Bacterial effector proteins delivered into host cell by the C. burnetii T4SS, however, can block intrinsic and extrinsic apoptotic pathways in infected epithelial cells [53,54], which may be why silencing of the TNF receptor (TNFRSF1A) did not have an effect on C. burnetii growth restriction. Lastly, it is well appreciated that interferon-inducible guanylate binding proteins and the immunity-related GTPase M (GBP1, GBP2, GBP5 and IRGM) target pathogen-containing compartments and cytotoxic bacteria to promote their destruction by cell-autonomous mechanisms (reviewed in [55]). These GTPases were not essential for inhibiting C. burnetii replication. Because C. burnetii-containing phagosomes are transported along the default endocytic pathway to the lysosomes, these GTPases may not recognize the CCV as a modified phagosome that has evaded maturation and should be targeted for lysosomal fusion and degradation. It remains possible that some of these IFN-γ-induced proteins contribute to C. burnetii growth restriction, but because they may have functions that are redundant, there is no enhancement of C. burnetii replication when only one factor is silenced in IFN-γ-treated cells.

Data presented here demonstrate that ectopic production of IDO1 is sufficient to suppress C. burnetii replication in the absence of IFN-γ signaling. IDO1 activity can limit the replication of intracellular pathogens to prevent dissemination, however, is typically not sufficient to kill intracellular microbes and without the participation of other cell autonomous defense pathways, can lead to persistent infection of infected tissues [56,57]. It is likely that other IFN-γ-
dependent mechanisms participate in killing *C. burnetii* once IDO1 renders the bacteria metabolically incapable of interfering with host cell functions. Consistent with IFNγ-activation leading to nutritional depletion, knockdown of SLC11A1, the gene encoding the metal transporter NRAMP1 (Natural resistance-associated macrophage protein 1) that restricts iron and manganese levels in phagosomes, led to a small, but significant increase in *C. burnetii* replication. These data indicate that host modulation of metal concentrations in the CCV could contribute to restriction of intracellular *C. burnetii*. Thus, future studies will be aimed at identifying additional IFNγ-dependent mechanisms that act synergistically to combat intracellular *C. burnetii* replication using cells that are deficient in IDO1.

**Materials and methods**

**Cell lines**

HeLa 229 cells (ATCC) and THP1 (ATCC) were used in experiments. HeLa cells were cultured and maintained in Dulbecco’s Modified Eagle Medium (DMEM, Gibco Cat. 11965–118) supplemented with 10% heat-inactivated fetal bovine serum (FBS). THP1 cells were cultured in Roswell Park Memorial Institute 1640 Medium with ATCC modification (ThermoFisher Scientific, Cat. A1049101) supplemented with 10% heat-inactivated FBS. All cells were maintained at 37˚C with 5% CO₂. During the length of infection experiments, HeLa cells were maintained with 5% FBS whereas THP1 cells were maintained with 10% FBS.

**Coxiella burnetii strains**

Wild-type *Coxiella burnetii* Nine Mile phase II (RSA439) was cultured in ACCM-2 media and used for all experiments [58,59]. A NMII strain that constitutively expresses luminescence was generated and provided by Shawna C. Reed (Table 1). *C. burnetii* strains were grown for 6 days in ACCM-2 at 37˚C, 2.5% O₂ and 5% CO₂ with appropriate antibiotic selection (375μg/ml kanamycin for Kanr strains, 3μg/ml chloramphenicol for Cmr strains) as described [58,59]. Bacterial cultures were centrifuged at 4000 rpm, 4˚C for 15 mins and pellets re-suspended in half the volume with DMEM containing 5% FBS. Bacteria were sonicated for 10’ prior to infection. *C. burnetii* genome equivalents were measured by quantitative PCR as described previously [27].

**Reagents**

Black 96-well plates with clear bottom were purchased from Corning costar (Cat. 3904). Dharmafect (T-2001), 5X siRNA buffer (Cat. B-002000-UB-100) and siGENOME SMARTpool siRNAs as listed in Table 2, were purchased from Dharmacon. Recombinant human IFNγ was obtained from Biolegend (Cat. 570206). L-tryptophan (Trp) and 1-Methyl L-tryptophan (1-Methyl Trp) were obtained from Sigma-Aldrich (Cat. T0254, 447439). Trp was re-

| Table 1. *C. burnetii* strains used in this study. |
|-----------------------------------|-----------------|---------------------------------|-----------------|
| **Strain**                       | **Antibiotic resistance** | **Genotype/Generation**          | **Reference**   |
| Wild-type (wt) Nine Mile phase II (NMII) RSA439 |                      |                                 |                 |
| *C. burnetii lux* (generated and gifted by Shawna C. Reed, Roy Laboratory, Yale University) | Kan’                  | Single copy attTN7::KAN-P311-luxCDABE-TT genomic insertion via plasmid with cbu0311 promoter and a terminator cloned into pMiniTn7T-KAN-luxCDABE (gift from Paul A. Beare, NIAID, NIH, Montana, USA) | [60] |
| *icmL*:Tn C. burnetii             | Kan’                  | Transposon insertion in *icmL*:dotI (cbu1629) | [3] |

https://doi.org/10.1371/journal.ppat.1007955.t001
| Pool Catalog Number | Gene Symbol | Gene Symbol | Gene Symbol | Gene Symbol | Gene Symbol | Gene Symbol | Gene Symbol | Gene Symbol |
|---------------------|-------------|-------------|-------------|-------------|-------------|-------------|-------------|-------------|
| M-011057-00         | IFNGR1      | 3459        | NM_000416   | 4557879     | interferon gamma receptor 1 |
| M-012713-00         | IFNGR2      | 3460        | NM_005534   | 47419933    | interferon gamma receptor 2 |
| M-003146-02         | JAK2        | 3717        | NM_004972   | 13325062    | Janus kinase 2    |
| M-003145-02         | JAK1        | 3716        | NM_002227   | 102469033   | Janus kinase 1    |
| M-011704-01         | IRF1        | 3659        | NM_002198   | 4504720     | interferon regulatory factor 1 |
| M-020858-02         | IRF9        | 10379       | NM_006084   | 82734235    | interferon regulatory factor 9 |
| M-003543-01         | STAT1       | 6772        | NM_007315   | 21536299    | signal transducer and activator of transcription 1 |
| M-003544-02         | STAT3       | 6774        | NM_213662   | 47458819    | signal transducer and activator of transcription 3 |
| M-006423-03         | CEBPB       | 1051        | NM_005194   | 28872795    | CCAAT enhancer binding protein beta |
| M-009240-01         | NOS2        | 4843        | NM_006265   | 24041028    | nitric oxide synthase 2 |
| M-011021-01         | CYBB        | 1536        | NM_003979   | 163854302   | cytochrome b-245 beta chain |
| M-005153-02         | GBP1        | 2633        | NM_002053   | 4503938     | guanylate binding protein 1 |
| M-011867-00         | GBP2        | 2634        | NM_004120   | 38327557    | guanylate binding protein 2 |
| M-028450-01         | IRGM        | 345611      | XM_001127260| 113416797   | immunity related GTPase M |
| M-014116-01         | IFITM3      | 10410       | NM_021034   | 148612841   | interferon induced transmembrane protein 3 |
| M-019496-01         | STX11       | 8676        | NM_003764   | 33667037    | syntaxin 11 |
| M-008317-00         | RAB20       | 55647       | NM_017817   | 8923400     | RAB20, member RAS oncogene family |
| M-028161-01         | RAB43       | 339122      | NM_198490   | 50234888    | RAB43, member RAS oncogene family |
| M-010337-01         | IDO1        | 3620        | NM_002164   | 156071492   | indoleamine 2,3-dioxygenase 1 |
| M-019310-01         | IDO2        | 169355      | NM_194294   | 14859553    | indoleamine 2,3-dioxygenase 2 |
| M-013432-01         | APOL6       | 80830       | NM_030641   | 87162462    | apolipoprotein L6 |
| M-017402-02         | APOL1       | 8542        | NM_003661   | 21735613    | apolipoprotein L1 |
| M-007380-02         | SLC11A1     | 6556        | NM_005078   | 109255240   | solute carrier family 11 member 1 |
| M-009553-00         | RAB36       | 9609        | NM_004914   | 31795534    | RAB36, member RAS oncogene family |
| M-011002-01         | C4A         | 720         | NM_002793   | 67190747    | complement C4A |
| M-005197-00         | TNFRSF1A     | 7132        | NM_001065   | 23312372    | TNF receptor superfamily member 1A |
| M-011511-04         | SOCS1       | 8651        | NM_003745   | 4507232     | suppressor of cytokine signaling 1 |
| M-003502-01         | ICAM1       | 3383        | NM_002001   | 4557877     | intercellular adhesion molecule 1 |
| M-012982-02         | CLIC2       | 1193        | NM_001289   | 66346732    | chloride intracellular channel 2 |
| M-003729-03         | PLSCR1      | 5359        | NM_021105   | 10863876    | phospholipid scramblase 1 |
| M-008322-01         | WARS        | 7453        | NM_004184   | 7710155     | tryptophanyl-tRNA synthetase |
| M-004202-01         | NMI         | 9111        | NM_004688   | 47587813    | N-myc and STAT interactor |
| M-005844-01         | CTSS        | 1520        | NM_004079   | 23110961    | cathepsin S |
| M-007516-01         | SLC2A3      | 6515        | NM_006931   | 5902089     | solute carrier family 2 member 3 |
| M-007402-02         | SLC16A1     | 6566        | NM_003051   | 115583684   | solute carrier family 16 member 1 |
| M-007453-00         | SLC22A2     | 6582        | NM_003058   | 23510441    | solute carrier family 22 member 2 |
| M-007143-01         | DTX3L       | 151636      | NM_138287   | 31377615    | defex E ubiquitin ligase 3L |
| M-018178-00         | GBP5        | 115362      | NM_052942   | 31377630    | guanylate binding protein 5 |
| M-007831-01         | CCL2        | 6347        | NM_002982   | 56191969    | C-C motif chemokine ligand 2 |
| M-006022-01         | PSMB8       | 5696        | NM_148919   | 73747874    | proteasome subunit beta 8 |
| M-011699-01         | IRF8        | 3394        | NM_002163   | 55953136    | interferon regulatory factor 8 |

D-001810-10 ON-TARGETplus Non-targeting Control

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suspended in Tissue Culture grade water and used at 0.3125mM unless otherwise indicated. 1-Methyl Trp was resuspended in 0.1N NaOH and used at 0.2mM. Phenol-red free DMEM and probenecid were obtained from Thermo Fisher Scientific (Cat. 21063029 and P36400). LIVEBLAZer™-FRET B/G loading kit which includes the fluorescent substrate CCF4-AM was purchased from Invitrogen (Cat. K1095).

**siRNA screen and luminescence measurement**

siRNAs were re-suspended in 1X siRNA buffer as 10μM or 2μM stocks and stored at -20°C until use. 48h before infection, HeLa cells (10⁴ cells/well) were reverse-transfected with 25 or 50nM siRNA using Dharmafect (0.2μl/well) in 96-well black, clear bottom plates. As controls, cells were transfected with transfection reagent alone (Mock) or control non-targeting siRNA. HeLa cells were infected with C. burnetii lux at MOI 100 and 6h later, IFNγ was added. Cells were washed and replenished with fresh media without IFNγ on d1 pi and luminescence values measured on specific days post-infection as indicated. Peak C. burnetii luminescence values measured from untreated cells (d4 pi or as indicated) were normalized to 100%. In the experiments performed with Trp and 1-Methyl Trp, C. burnetii-infected cells were supplemented with Trp (0.3125mM) or treated with 1-Methyl Trp (0.2mM) 1h prior to treatment with IFNγ. Trp or 1-Methyl Trp, but not IFNγ, were added back to the media after washing the cells on d1 pi. Bacterial luminescence was measured using TECAN infinite M1000 plate reader on indicated days post-infection.

**Assessing bacterial luminescence in THP1 cells**

THP1 cells were plated at 75000/well in 96-well plates and differentiated with 124ng/ml phorbol 12-myristate 13-acetate (PMA) overnight or left undifferentiated. Cells were infected with MOI 25 and 6h later, treated with IFNγ 100ng/ml in the presence or absence of additional tryptophan (concentrations as indicated). On d1 pi, cells were washed and replenished with IFNγ-free media and supplemented with tryptophan. Bacterial luminescence was measured using TECAN infinite M1000 plate reader on indicated days post-infection.

**BlaM translocation assay**

Effector translocation through the type IV secretion system was assessed by measuring the translocation of β-lactamase (BlaM)-effector fusion protein using a FRET-based assay, as previously described [2]. HeLa cells were plated at 2*10⁴ cells/w in 96w black, clear bottom plates 24h prior to infection. Cells were infected with wt or icmL::Tn C. burnetii expressing BlaM alone or BlaM-77 at MOI 500 as listed in Table 3. Cells were treated with IFNγ 6h later, in the presence or absence of additional tryptophan. On d1 pi, cells were washed and replenished with IFNγ-free media and supplemented with tryptophan where indicated. On d2 or d4 pi, culture medium was replaced with phenol red-free DMEM containing HEPES. Cells were loaded with the fluorescent substrate CCF4/AM using the LIVEBLAZer™-FRET B/G loading kit and probenecid and incubated in dark at RT for 2h. The ratio of signal at 460 and 535nm (blue:green) was measured using the TECAN M1000 plate reader and the response ratio was calculated by normalizing the blue:green ratio of infected cells to that of uninfected control.

**Measurement of bacterial genome equivalents (GE)**

5*10⁴ cells per well were plated in 24-well plates, one day prior to infection. Cells were infected wt C. burnetii NMII at MOI 100. Cells were left untreated or treated with IFNγ in the presence or absence of Trp at 6h pi. Infected cells were washed on d1 pi and replenished with IFNγ-free
fresh media in the presence or absence of additional Trp. Supernatants and cells lysed with distilled water were combined and collected on d1, d4 and d7 pi. Genomic DNA was extracted using Illustra bacterial genomicPrep mini spin kit (Cat no. 28904259, GE) and quantified by qPCR using primers for \( C. burnetii \) dotA gene (Table 4).

### Indirect immunofluorescence

2.5 or 5 \( \times 10^4 \) cells per well were plated in 24-well plates with poly-L-lysine coated coverslips, one day prior to infection. Cells were infected at MOI 100, left untreated or treated with IFN-\( \gamma \) and additional Trp as indicated. On d1 pi, cells were washed and replenished with IFN-\( \gamma \)-free media in the presence or absence of additional Trp. Cells were fixed on d4 pi with 4% paraformaldehyde (PFA) for 20 mins at RT. Coverslips were washed at least 6x times with 1x PBS, permeabilized and blocked with 0.2% saponin, 0.5% BSA and 1% (v/v) heat-inactivated FBS in PBS. Coverslips were stained with primary antibodies- rabbit anti-\( C. burnetii \) [3], mouse anti-LAMP1 (Source: H4A3 Development Studies Hybridoma bank at the University of Iowa) and DAPI (4,6-diamidino-2-phenylindole, Cat no. D9542 from Sigma-Aldrich) at 1:10,000, 1:500 and 1:10,000 dilutions respectively, as previously described [63]. Secondary antibodies goat anti-Rabbit IgG, Alexa Fluor 568 (Life Technologies, A11036) and goat anti-Mouse IgG, Alexa Fluor 488 (Life Technologies, A11029) were used at 1:2000. Stained coverslips were mounted on glass slides using Prolong gold antifade reagent (Life Technologies). Coverslips were

| Plasmid and genotype | Notes | Antibiotic resistance | Reference/ Source |
|----------------------|-------|-----------------------|-------------------|
| pBlaM (pJB-CAT-BlaM) | C. burnetii expression vector for \( \beta \)-lactamase (BlaM) fusion proteins (cbu0069 promoter) | Cm\(^\text{r}\) | [3] |
| pBlaM-77 (pJB-CAT-BlaM:: cbu0077) | C. burnetii expression vector for BlaM-Cbu0077 fusion protein | Cm\(^\text{r}\) | [3] |
| pTRIPZ-EV | Created by annealing the oligos YO-0903 and YO-0904 into the pTRIPZ Inducible Lentiviral shRNA vector (Dharmacon-Horizon Discovery) at Age-I/MluI sites | Amp\(^\text{r}\) | Gift from Dr. Brett Lindenbach, Yale University |
| pTRIPZ-IDOI | Cloned human IDOI gene at EcorI site using ligase-independent cloning | Amp\(^\text{r}\) | This study |
| pVSV-G | Lentiviral envelope plasmid | Amp\(^\text{r}\) | [61] |
| psPAX2 | Lentiviral packaging plasmid | Amp\(^\text{r}\) | [62] |

### Table 3. Primers and oligos used in this study.

| Primer/Oligo | Sequence | Purpose |
|--------------|----------|---------|
| IDOI F | gecctcaaggtttt cacaa | qRT-PCR |
| IDOI R | gacaatatatgcaagaacatgaaaa | qRT-PCR |
| GAPDH F | agcacaactgctgacac | qRT-PCR |
| GAPDH R | gcaactagacacatcgc | qRT-PCR |
| pTRIPZ-IDOI F | acgggacgcaatttaattctgcaaggattcatgcccacacatggcttccc | Cloning IDOI in pTRIPZ vector |
| pTRIPZ-IDOI R | gggcgagcagcacgctgacgaggtgcttctcttttgggttct | Cloning IDOI in pTRIPZ vector |
| dotA F | gccactaagctatcaca | qPCR for \( C. burnetii \) GE |
| dotA R | ccctgccccatattct | qPCR for \( C. burnetii \) GE |
| YO-0903 | cggtgtaaaagggcgccatcatatccgctgtagaatgtagcttcgg | Modifying pTRIPZ |
| YO-0904 | ggcctcaagaagggctgtagaatgtagcttcggcgtactaca | Modifying pTRIPZ |

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visualized with a Photometrics CoolSNAP EZ 20 Mhz digital monochrome camera connected to Nikon Eclipse TE2000-S inverted microscope using Nikon Plan Apo60x objective/ 1.4 numerical aperture. Images were acquired using SlideBook software 6.0 (Intelligent Imaging Innovations), saved as tiff files and resized and labeled using Adobe Illustrator.

FFU assay
To measure the infectivity of C. burnetii in infected cells, Foci Forming Unit (FFU) assay was used. HeLa 229 cells were plated in 6-well plates at 200,000 cells/w one day prior to infection. Cells were infected with wt C. burnetii at MOI 100, left untreated or treated with IFNγ and additional Trp as indicated. On d1 pi, cells were washed and replenished with IFNγ-free media in the presence or absence of additional Trp. Similar to GE assay, supernatants and lysates from infected cells were combined and collected on d4 pi and sonicated for 10'. This culture was serially diluted either in water (to assess primary infection GE by qPCR) or in DMEM containing 5% FBS to infect HeLa cells (plated at 2\(^2\)10\(^4\) cells/well in 96-well plate on the previous day) for a second round of infection. Infected cells in 96-well plates were subsequently washed on d4 pi, fixed with 4% PFA for 20' at RT, permeabilized with 0.5% Triton-X in PBS for 5' and stained with rabbit anti-C. burnetii at 1:10000 in 0.1% Triton-X in PBS. Cells were washed with PBS, stained with DAPI and goat anti-rabbit IgG, Alexa Fluor 488 secondary antibody (Life Technologies, A11034). Foci were visualized by indirect immunofluorescence, manually counted and represented as FFU per ml of the primary infection sample by accounting for the dilution factor.

qRT-PCR
IDO1 expression in HeLa cells was measured by qRT-PCR using primers listed in Table 4. Fold increase in IDO1 expression in IFNγ-treated, Mock or IDO1 siRNA-transfected cells was calculated in comparison to untreated, mock transfected cells by assuming 100% efficiency for primers.

Transmission electron microscopy
HeLa 229 cells were infected with wt C. burnetii at MOI 100 and left untreated or treated with IFNγ in the presence or absence of supplemented Trp at 6h pi. Cells were washed on d1 pi and Trp was supplemented in the appropriate samples. The following method was adapted based on published EM studies with C. burnetii [64] and recommendation from the Yale EM facility. On d4 pi, infected cells in petri dishes were fixed in 2.5% glutaraldehyde in 0.1M sodium cacodylate buffer pH7.4 containing 2% sucrose for 1h, then rinsed in buffer and replaced with 0.1% tannic acid in buffer for another hour. Buffer-rinsed cells were scraped in 1% gelatin and spun down in 2% agar. Chilled blocks were trimmed and post fixed in 1% osmium tetroxide and 1.5% potassium ferrocyanide in buffer for 1 hour. The samples were rinsed in sodium cacodylate and distilled water and en-bloc stained in aqueous 2% uranyl acetate for 1h. This was followed by further rinsing in distilled water and dehydrated through an ethanol series to 100%. The cells were infiltrated with Embed 812 (Electron Microscopy Sciences) resin, placed in silicone molds and baked at 60˚C for 24h. Hardened blocks were sectioned using a Leica UltraCut UC7. 60nm sections were collected on formvar coated nickel grids and stained using 2% uranyl acetate and lead citrate. 60nm Grids were viewed FEI Tencai Biotwin TEM at 80Kv. Images were taken using Morada CCD and iTEM (Olympus) software. Images were resized and scale bars added using Adobe Illustrator.
**Inducible expression of IDO1 in HeLa cells**

IDO1 gene was cloned into an empty lentiviral vector pTRIPZ-EV under the tetracycline-inducible promoter at EcoRI site by ligase independent cloning using the primers listed in Table 4. To derive the lentivirus, HEK293T cells were plated in 10cm dish and transfected with pTRIPZ-EV or pTRIPZ-IDO1 with pVSV-G and psPAX2, as listed in Table 3, using Lipofectamine 2000 (Invitrogen). Lentiviral particles were obtained by collecting the supernatant at 48h and 72h post-transfection, pooled, filtered using 0.45μm low protein binding filter. Lentivirus containing pTRIPZ-EV or pTRIPZ-IDO1 was used to transduce sub-confluent HeLa cells at half the volume of complete media, in the presence of polybrene (8μg/ml). Transduced cells were maintained in culture using puromycin (2.25μg/ml).

**Detection of IDO1 protein by immunoblot analysis**

To test the efficiency of IDO1 knockdown by siRNA, HeLa cells were plated and treated with IDO1 siRNA and IFNγ 10ng/ml as described for the siRNA screen. 24h later, cells were washed and replenished with IFNγ-free media. Cells were collected two days post IFNγ treatment for immunoblotting. To test the expression of IDO1 in pTRIPZ-IDO1 expressing HeLa cells, 10^5 cells/w were plated in 24w plates and treated with increasing concentrations of anhydrotetracycline (referred to as Tet) or Doxycycline for 3 days or IFNγ 10ng/ml for 4 days. Cells treated with IFNγ were washed and replenished with IFNγ-free media 24h post-treatment. To determine if IDO1 is induced in THP1 cells, 4*10^5 cells/w were plated in 12w plates and left untreated or differentiated with PMA overnight. Cells were washed and left untreated or treated with IFNγ 100ng/ml. IFNγ-treated cells were washed and replenished with IFNγ-free media after 24h. Cells were harvested 2 days post IFNγ-treatment. In all cases, cell lysates were prepared using Blue Loading Buffer (Cell signaling, #7722). Anti-IDO1 (BioLegend, W16073A), anti-α-tubulin (Sigma-Aldrich, T9026) prepared at 1:1000 in 5% BSA in 1X TBST and secondary antibodies goat anti-rat or anti-mouse IgG, HRP (Invitrogen, Cat. 62–9520 and 62–6520) were used to detect the proteins by western blotting.

**Data analysis and statistics**

Data presented here are representative of at least 2 experiments. The siRNA screen (Fig 2) represents an average of 3 independent trials. Data were graphed and analyzed using Prism 7 software. Statistical significance was determined by t-test, one-way or two-way ANOVA depending on the number of groups and experimental conditions being compared.

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