Caspase-1/ASC Inflammasome-Mediated Activation of IL-1β–ROS–NF-κB Pathway for Control of *Trypanosoma cruzi* Replication and Survival Is Dispensable in NLRP3<sup>−/−</sup> Macrophages

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Abstract

In this study, we have utilized wild-type (WT), ASC<sup>−/−</sup>, and NLRP3<sup>−/−</sup> macrophages and inhibition approaches to investigate the mechanisms of inflammasome activation and their role in *Trypanosoma cruzi* infection. We also probed human macrophages and analyzed published microarray datasets from human fibroblasts, and endothelial smooth muscle cells for *T. cruzi*-induced changes in the expression genes included in the RT Profiler Human Inflammasome arrays. *T. cruzi* infection elicited a subdued and delayed activation of inflammasome-related gene expression and IL-1β production in mφs in comparison to LPS-treated controls. When WT and ASC<sup>−/−</sup> macrophages were treated with inhibitors of caspase-1, IL-1β, or NADPH oxidase, we found that IL-1β production by caspase-1/ASC inflammasome required reactive oxygen species (ROS) as a secondary signal. Moreover, IL-1β-regulated NF-κB signaling of inflammatory cytokine gene expression and, subsequently, intracellular parasite replication in macrophages. NLRP3<sup>−/−</sup> macrophages, despite an inability to elicit IL-1β activation and inflammatory cytokine gene expression, exhibited a 4-fold decline in intracellular parasites in comparison to that noted in matched WT controls. NLRP3<sup>−/−</sup> macrophages were not refractory to *T. cruzi*, and instead exhibited a very high basal level of ROS (>100-fold higher than WT controls) that was maintained after infection in an IL-1β-independent manner and contributed to efficient parasite killing. We conclude that caspase-1/ASC inflammasomes play a significant role in the activation of IL-1β/ROS and NF-κB signaling of cytokine gene expression for *T. cruzi* control in human and mouse macrophages. However, NLRP3-mediated IL-1β/NFκB activation is dispensable and compensated for by ROS-mediated control of *T. cruzi* replication and survival in macrophages.

Introduction

Chagas disease affects 11–18 million people worldwide [1]. Upon exposure to *Trypanosoma cruzi* (*T. cruzi* or *Tc*), infected individuals exhibit an acute phase of Chagas disease that lasts for a couple of months and is characterized by symptoms such as fever, fatigue, body aches, diarrhea, and vomiting. After the control of parasitemia, a majority of infected patients enter an indeterminate chronic phase that is marked by a lack of clinical symptoms of the acute phase. Ten to thirty years after initial infection, 30–40% of indeterminate phase patients progress to develop chagasic cardiomyopathy [2].

The studies in experimental models have shown that macrophages (mφs), as well as dendritic and natural killer cells, play an important role in control of *T. cruzi* infection [3–5]. The interaction of *T. cruzi* with mφs and other cell types involved in the innate immune response are mediated by pattern recognition receptors (PRRs) such as toll-like receptors (TLRs). Upon recognition of pathogen-associated molecular patterns (PAMPs), TLRs transmit the signal via cytoplasmic domains for the recruitment of cytosolic adaptor molecules, including myeloid differentiation primary-response protein 88 (MyD88), and subsequently induce nuclear factor κB (NFκB) activation, leading to the production of inflammatory cytokines and linking an innate
response to an adaptive immune response (reviewed in [4]). T. cruzi-derived glycosylphosphatidylinositol and mucins have been shown to serve as PAMPs in engaging TLR signaling of a cytokine response. Others have demonstrated that TLR4 and TLR9 are engaged by parasite-derived glycosylphosphatidylinositol phospholipids and DNA, respectively, during the activation of host innate immune response leading to regulation of infection [6,7]. T. cruzi also expresses cruzipain, a kinin-releasing cysteine protease, which induces dendritic cells maturation via activation of bradykinin (BK) B2 receptors [B-R] [8,9].

A newly discovered family of PRRs is named Nucleotide-binding oligomerization domain (NOD) like receptors (NLRs) [10,11]. NLRs have a tripartite domain structure and are characterized by the presence of a central nucleotide-binding oligomerization domain (NOD), also called NACHT domain, present in neuronal apoptosis inhibitor proteins (NAIP) and a C-terminal leucine-rich repeats (LRRs) domain of variable length (20–29 amino acids). The N-terminal effector binding region consists of a protein-to-protein interaction domain, i.e., Pyrin domain (PYD), a caspase recruitment domain (CARD), or baculovirus inhibitor of an apoptosis protein repeat (BIR) domain. Based upon the presence of PYD, CARD and BIR effector domains, NLRs are classified as NLRP, NLRC, and NAIP, respectively [11,12]. Currently known members of the NLR family in humans include seven NLRCs (NLRC1-NLRC5, NLRCX, and CIITA or NLRA), fourteen NLRPs (NLRP1-NLRP14), and seven NAIPs (NAIP1-NAIP7). The multi-meric protein macromolecules formed by NLRs are named inflammasomes. The most studied NLRP1 and NLRP3 inflammasomes recruit ASC (apoptosis-associated, speck-like protein containing a CARD domain) and caspase-1 proteins. The ASC-dependent cleavage and activation of caspase-1 results in the formation of an active complex responsible for converting to active forms of pro-IL-1β (31 kDa to 17 kDa) and pro-IL-18 (24 kDa to 16 kDa) [13] and the activation of the inflammatory cytokine response.

In the context of pathogens invading the heart, it is recognized that besides innate immune cells, both endothelial and vascular smooth muscle cells (VSMCs) can also sense and respond to pathogens (or PAMPs) [14–16]. Cardiomyocytes, the main type of cells in the heart, and heart resident fibroblasts also express TLRs and/or NLRs [17,18]. In this study, we have utilized wild-type (WT), ASC−/− and NLRP3−/− mps and inhibitory approaches to investigate the mechanisms of inflammasome activation and their role in the context of T. cruzi infection. We also probed the RT Profiler PCR Array System to identify the inflammasome-related changes induced by T. cruzi infection of human mps and analyzed the published microarray datasets from T. cruzi-infected fibroblasts, and smooth muscle and endothelial cells for the change in expression of the 94 genes included in the inflammasome arrays. Our data demonstrate that T. cruzi infection, in comparison to treatment with LPS, elicits a subdued activation of inflammatory gene expression and IL-1β production in mps. Yet, caspase-1/ASC inflammasome-dependent activation of the IL-1β – reactive oxygen species (ROS) – NF-κB pathway played an important role in control of T. cruzi replication in mps. Further, NLRP3 controlled the ROS levels in mps, and NLRP3 deficiency resulted in a potent increase in ROS-mediated parasite killing in infected mps. To the best of our knowledge, this is the first study demonstrating a double-edged role of NLRP3 in determining mp activation of ROS and cytokine response, both of which are required for clearance of T. cruzi infection.

**Figure 1.** IL-1β production in macrophages infected by T. cruzi. (A–D) PMA-differentiated THP-1 mps were incubated with T. cruzi trypomastigotes (cell: parasite ratio, 1:3), Tc lysate (10 μg protein/10⁶ cells) or LPS (100 ng/ml) for 3 h (A&C) and 18 h (B&D). In some experiments, ATP was added during last 30 min of incubation (C&D). IL-1β release in supernatants was determined by ELISA. (E–G) IL-1β production in macrophages infected by T. cruzi was measured in the presence or absence of anti-IL-1β antibody for 18 h. (E) SYTO®11 fluorescence as an indicator of parasite uptake (shown by arrows) was determined by using an Olympus BX-15 microscope equipped with a digital camera (magnification 40X). (F) Quantitative PCR analysis of parasite burden in infected mps by using Tc18SrDNA-specific oligonucleotides (normalized to human GAPDH). (G) Addition of anti-IL-1β antibody depletes secreted IL-1β levels in T. cruzi-infected mps. In all figures, data are representative of three independent experiments and presented as mean ± SD. Significance is shown by *normal versus infected and **treated/infected versus infected (*p<0.05, ***p<0.001, and ****p<0.0001).

**Materials and Methods**

**Ethics statement**

All animal experiments were performed according to the National Institutes of Health Guide for Care and Use of Experimental Animals and approved by the UTMB’s Animal Care and Use Committee (protoCol # 08-05-029).

**Mice, parasites, and cells**

C57BL/6 female mice (6–8-weeks old) were purchased from Harlan Labs (Indianapolis, IN). NLRP3−/− and ASC−/− mice...
Table 1. Inflammasome-related differential gene expression in THP-1 macrophages in response to *T. cruzi* infection (± ATP) in comparison to normal controls.

| Gene name     | ddCt log ratio | p value | Gene name     | ddCt log ratio | p value | Gene name     | ddCt log ratio | p value |
|---------------|----------------|---------|---------------|----------------|---------|---------------|----------------|---------|
| CXCL1         | −4.10          | 0.000   | CXCL1         | −4.48          | 0.000   | ACTB         | −2.41          | 0.002   |
| TNF           | −2.86          | 0.000   | TNF           | −3.68          | 0.000   | CXCL2        | −2.21          | 0.001   |
| NFkBIA        | −2.46          | 0.000   | CXCL2         | −5.36          | 0.000   | PTGS2        | −4.73          | 0.020   |
| CXCL2         | −3.14          | 0.000   | NFkBIA        | −3.24          | 0.000   | TNF          | −0.82          | 0.028   |
| CIITA         | 3.07           | 0.001   | TXNIP         | 2.46           | 0.000   | CIITA        | −1.68          | 0.037   |
| Tc vs control at 3h |               |         | Tc + ATP vs control at 3 h |               |         | Tc + ATP vs Tc at 3 h |               |         |
| BF2            | 0.95           | 0.005   | ACTB         | −2.79          | 0.001   | NLRP3        | −0.65          | 0.051   |
| NFkBIA        | −1.03          | 0.007   | RIPK2        | −1.99          | 0.001   |             |               |         |
| TXNIP         | 1.56           | 0.007   | MAPK3        | 1.42           | 0.006   |             |               |         |
| MAPK1         | 0.79           | 0.010   | PSTPIP1      | 1.11           | 0.006   |             |               |         |
| PSTPIP1       | 1.03           | 0.010   | NLRP3        | −0.96          | 0.007   |             |               |         |
| B2M           | 1.44           | 0.023   | BCL2         | 0.86           | 0.009   |             |               |         |
| SUGT1         | 0.86           | 0.026   | SUGT1        | 1.03           | 0.010   |             |               |         |
| TAB2          | 0.66           | 0.032   | PYCARD       | 1.10           | 0.015   |             |               |         |
| CCL5          | −1.12          | 0.042   | IL1B         | −1.75          | 0.018   |             |               |         |
| PEA15         | 0.57           | 0.043   | RPL13A       | 0.78           | 0.025   |             |               |         |
| PYCARD        | 0.86           | 0.049   | IRF1         | 0.78           | 0.034   |             |               |         |
| CCL2          | −2.92          | 0.053   | CCL2         | −2.92          | 0.053   |             |               |         |
| RPL13A        | 0.65           | 0.056   | CCL5         | −1.06          | 0.053   |             |               |         |
| Tc vs control at 18h |         |         | Tc + ATP vs control at 18 h |         |         | Tc + ATP vs Tc at 18 h |         |         |
| CXCL1         | −3.67          | 0.000   | TNF          | −3.97          | 0.000   | NAIP         | −4.79          | 0.001   |
| HSP90B1       | 2.13           | 0.000   | HSP90B1      | 2.41           | 0.000   | MAPK12       | 1.40           | 0.011   |
| CXCL2         | −4.07          | 0.000   | CXCL2        | −4.34          | 0.000   | TIRAP        | 2.57           | 0.076   |
| GAPDH         | 1.61           | 0.000   | PYCARD       | 2.10           | 0.000   |             |               |         |
| MAPK1         | 1.40           | 0.000   | MAPK1        | 1.21           | 0.000   |             |               |         |
| CTSB          | 1.86           | 0.000   | GAPDH        | 1.39           | 0.001   |             |               |         |
| CCL5          | −2.26          | 0.000   | CCL5         | −2.17          | 0.001   |             |               |         |
| NAIP          | 5.05           | 0.001   | TAB2         | 1.20           | 0.001   |             |               |         |
| PYCARD        | 1.64           | 0.001   | CTSB         | 1.56           | 0.001   |             |               |         |
| SUGT1         | 1.21           | 0.003   | MAP3K7       | 1.15           | 0.002   |             |               |         |
| PSTPIP1       | 1.16           | 0.004   | MAPK12       | 1.75           | 0.003   |             |               |         |
| TRAF6         | 1.66           | 0.005   | PSTPIP1      | 1.14           | 0.005   |             |               |         |
| MAPK3         | 1.26           | 0.012   | CHUK         | 1.70           | 0.006   |             |               |         |
| TAB2          | 0.81           | 0.011   | NFkBIA       | −1.26          | 0.008   |             |               |         |
| CFLAR         | −1.77          | 0.016   | B2M          | 1.60           | 0.013   |             |               |         |
| IL1B          | −1.79          | 0.016   | P2RX7        | −2.18          | 0.013   |             |               |         |
| MAP3K7        | 0.86           | 0.015   | CASP4        | 0.79           | 0.018   |             |               |         |
| TNFSF14       | 1.60           | 0.016   | NLRC5        | 3.65           | 0.016   |             |               |         |
| ACTB          | 1.60           | 0.025   | PEA15        | 0.70           | 0.017   |             |               |         |
| CASP4         | 0.70           | 0.032   | TNFSF14      | 1.57           | 0.018   |             |               |         |
| CCL2          | −3.34          | 0.030   | RPL13A       | 0.81           | 0.022   |             |               |         |
| HSP90AA1      | 1.12           | 0.027   | ACTB         | 1.60           | 0.025   |             |               |         |
| IRAK1         | −3.86          | 0.029   | HSP90AA1     | 1.12           | 0.027   |             |               |         |
| P2RX7         | −1.85          | 0.030   | IRF1         | −0.83          | 0.026   |             |               |         |
| RPL13A        | 0.75           | 0.032   | PTGS2        | −4.56          | 0.024   |             |               |         |
Table 1. Cont.

| $Tc$ vs control at 18 h | $Tc$ + ATP vs control at 18 h | $Tc$ + ATP vs $Tc$ at 18 h |
|------------------------|-----------------------------|---------------------------|
| Gene name | ddCt log ratio | p value | Gene name | ddCt log ratio | p value | Gene name | ddCt log ratio | p value |
| PANX1 | 1.00 | 0.034 | TIRAP | 3.29 | 0.028 |
| PEA15 | 0.56 | 0.047 | CARD6 | 2.02 | 0.031 |
| CARD6 | 1.79 | 0.052 | CCL2 | −3.32 | 0.031 |
| IRAK1 | −3.85 | 0.030 |
| PANX1 | 0.98 | 0.035 |
| SUGT1 | 0.81 | 0.035 |
| TRAF6 | 1.12 | 0.040 |
| MAPK3 | 0.91 | 0.055 |

The 96-well RT Profiler Human Inflammasome PCR Arrays (SA Biosciences/Qiagen) were probed in triplicate with cDNA from THP-1 macrophages infected with $T. cruzi$ ($Tc$) for 3 h or 18 h (with or without ATP) as described in Materials and Methods. The Ct values from qPCR data were analyzed by using open source HTqPCR v.1.7 software package (v.2.13). All array data were normalized by Quantile method and filtered to exclude genes that exhibited Ct values $>35$. The relative expression level of each target gene in treated cells was calculated using the formula, fold change $= 2^{-ΔΔCt}$ where $ΔCt$ represents the Ct (sample) - Ct (control). LimmaCt in HT-qPCR package was employed for contrast analysis of all the groups included in experiment and identification of genes that were overall differentially expressed ($p<0.05$).

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Probing the RT Profiler Human Inflammasome PCR Arrays

THP-1 cells, with and without $T. cruzi$ infection and treatments, were harvested with 500 µl Bio-Rad cell lysis/RNA extraction buffer. Total RNA was extracted by using an Aurum RNA isolation kit (Bio-Rad, Hercules, CA) and measured at 260 and 280 nm for determination of purity and concentration. The cDNA probes were generated by reverse transcription of 5 µg total RNA by using the Bio-Rad iScript cDNA synthesis kit.

The 96-well RT Profiler Human Inflammasome PCR Arrays (SA Biosciences/Qiagen, Valencia, CA) containing primer pairs for 84 key genes involved in the function of inflammasomes and NLR signaling were probed with 2 µl of cDNA template in the presence of 6.5 µl of dNTPs, MgCl$_2$, and stabilizers (iQ SYBR Supermix, Bio-Rad), and PCR was carried out on a LightCycler 480 Multiple Plate System. A total of 42 arrays were probed with 14 research samples in triplicate, and datasets were analyzed by Web-based PCR Array Data Analysis software (SA Biosciences) for threshold cycle (Ct) value determination.

The Ct values from qPCR data were analyzed by using the open source HTqPCR v.1.12 software package [19]. Briefly, all array data were imported into HTqPCR, normalized by the quantile method and then filtered to exclude genes that exhibited Ct values $>35$. The relative expression level of each target gene in infected cells was calculated by using the formula, fold change $= 2^{-ΔΔCt}$, where $ΔCt$ represents the Ct ($Tc$-infected or LPS-treated sample) - Ct (control). LimmaCt in the HT-qPCR package was employed for contrast analysis of all groups included in the experiment and for the identification of genes that were overall differentially expressed. LimmaCt utilizes the Linear Model for Microarray data (limma) R package to fit linear models for threshold cycle (Ct) value determination.

In general, primary or cultured macrophages (BM) - derived macrophages from WT, ASC$^{-/-}$ and NLRP3$^{-/-}$ mice (C57BL/6 background) were a gift from Dr V. Dixit (Genentech, San Francisco, CA) and bred at the UTMB animal facility. $T. cruzi$ (SylvioX10/4 strain) trypomastigotes were maintained and propagated by the continuous in vitro passage of parasites in monolayers of C2C12 cells (an immortalized mouse myoblast cell line). $T. cruzi$ isolate and C2C12 cells were purchased from American Type Culture Collection (ATCC, Manassas, VA).

Single-cell suspensions of bone marrow (BM) - derived macrophages from WT, ASC$^{-/-}$ and NLRP3$^{-/-}$ mice (C57BL/6 background) were added to petri dishes ($10^6$ cells/ml) in complete RPMI media containing 20 ng/ml murine macrophage colony stimulating factor (M-CSF, eBioscience, San Diego, CA) and incubated at 37°C in 5% CO$_2$ for 10 days to support differentiation to mφs. The differentiated BM mφs were maintained in the presence of 5 ng/ml M-CSF during experimental use. THP-1 human macrophages were differentiated into mφs by overnight incubation with 50 ng/ml phorbol-12-myristate-13-acetate (PMA), and then rested at 37°C for 24 h in complete RPMI media containing 10% FBS. RAW 266.7 murine mφs were routinely cultured in DMEM with 10% FBS.

In general, primary or cultured mφs ($0.5 - 1 \times 10^6$ cells/ml) were seeded in Nuncl Lab-Tek II chamber slides or 24-well or 6-well plates, infected with $Tc$ trypomastigotes (cell: parasite ratio, 1:3) for 2 h, washed to remove free parasites, and then incubated for 5, 6, 12, and 18 h. In some experiments, 5 mM ATP was added during the last 30 min of incubation. When monitoring the role of inflammasomes or NADPH oxidase (NOX2)-mediated ROS in parasite control, infected mφs were incubated in the presence of 1 µg/ml anti-IL-1β antibody (Santa Cruz, Dallas TX); 20 µM Ac-YYVAD-CHO (caspase-1 inhibitor, Enzo Life Sc., Farmingdale, NY); 100 ng/ml cycloheximide (inhibits protein biosynthesis); 50 µM glibenclamide (inhibits K$^+$ efflux and also inhibits NLRP3 inflammasome activation [Imgenex, San Diego, CA]); 10 µM diphenylene iodonium (DPI); or 30 µM apoCynin (inhibitors of NOX2/ROS); and 1 mM N-acetylcysteine (NAC, ROS scavenger). Macrophages incubated with media alone or LPS (100 ng/ml) were used as controls. Cells and culture supernatants were stored at $-80°C$. 

(C57BL/6 background) were a gift from Dr V. Dixit (Genentech, San Francisco, CA) and bred at the UTMB animal facility. $T. cruzi$ (SylvioX10/4 strain) trypomastigotes were maintained and propagated by the continuous in vitro passage of parasites in monolayers of C2C12 cells (an immortalized mouse myoblast cell line). $T. cruzi$ isolate and C2C12 cells were purchased from American Type Culture Collection (ATCC, Manassas, VA).
statistics which combines all of the t-statistics from all the contrasts to calculate the significance of a gene(s). This F-statistic determines if gene(s) are differentially expressed across any contrast. The p-value is calculated based on the moderated t-statistics and F-statistics followed by an FDR adjustment. LimmaCt with the top table module was used to gain an appreciation of the change in differential expression of a particular gene over time (or treatment), and significance in changes in gene expression was accepted at p<0.05.

In some experiments, quantitative RT-PCR was performed for IL-1β, IL-18, CXCL1, and TNF-α mRNA levels and housekeeping genes (GAPDH and β-actin) with gene-specific primer pairs (Table S1) and iQ SYBR Supermix on a C1000 Touch Thermal Cycler (Bio-Rad). The relative expression level of each target gene was calculated by using the formula, fold change = $2^{\Delta\Delta C_T}$, where $\Delta C_T$ represents the $C_T$ (infected sample) - $C_T$ (control).

**Functional analysis**

Datasets for differential expression of the genes included in RT Profiler Human Inflammasome PCR Arrays in human foreskin fibroblasts (HFF), human microvascular endothelial cells
Table 2. Ingenuity iReport analysis of inflammasome-related datasets in THP-1 macrophages infected by *T. cruzi* for 3 h or 18 h (± ATP).

| Pathways | Gene name | FocuS mols | P value |
|----------|-----------|------------|---------|
| *T. cruzi* infection of THP-1 mφs vs control (3h) | | | |
| **Top biological and molecular functions** | | | |
| 1 | Inflammatory responses/infection | B2M, BCL2, CCL2, CCL5, CXCL1, CXCL2, NFKB1, NFKB1A, PYCARD, RIPK2, TNF, TXNIP | 13 | 5.91E-12–2.49E-04 |
| 1a. Recruitment of neutrophils/phagocytes | CCL2, CCL5, CXCL1, CXCL2, PYCARD, RIPK2, TNF | 7 | 2.77E-10 |
| 1b. Activation of leukocyte/lymphocytes | BCL2, CCL2, CCL5, CXCL1, CXCL2, NFKB1, NFKB1A, PYCARD, TNF | 9 | 1.89E-07 |
| 2 | Cell death of immune cells (decreased) | B2M, BCL2, CCL2, CCL5, CXCL1, CXCL2, MAPK1, NFKB1, NFKB1A, PYCARD, RIPK2, TAB2, TNF, TXNIP | 16 | 4.50E-10 |
| 2a. Macrophage cell death (decreased) | BCL2, CCL2, CCL5, CXCL1, CXCL2, NFKB1, NFKB1A, PYCARD, TNF | 9 | 3.78E-13 |
| **Toxicity analysis** | | | |
| Decreases MPT, mitochondrial swelling | B2M, BCL2, NFKB1, PYCARD, TNF | 5 | 7.27E-08 |
| *T. cruzi* infection of THP-1 mφs vs control (3 h + ATP) | | | |
| **Top biological and molecular functions** | | | |
| 1 | Inflammatory responses/infection | BCL2, CCL2, CCL5, CXCL1, CXCL2, IL1B, IRF1, NFKB1, NFKB1A, NLRP3, PGTSG2, PYCARD, RIPK2, TNF, TXNIP | 16 | 7.54E-14–1.79E-05 |
| 1a. Recruitment/migration of phagocytes/neutrophils | CCL2, CCL5, CXCL1, CXCL2, IL1B, MAPK3, NLRP3, PYCARD, RIPK2, TNF | 9 | 7.69E-09 |
| 1b. Activation of lymphocyte/leukocyte | BCL2, CCL2, CCL5, CXCL1, IL1B, IRF1, NFKB1, PGTSG2, PYCARD, RIPK2, TNF, TXNIP | 12 | 3.44E-12 |
| 2 | Cell death of immune cells/necrosis (decreased) | BCL2, CCL2, CCL5, CXCL1, CXCL2, IL1B, IRF1, MAPK3, NFKB1, NFKB1A, NLRP3, PGTSG2, PYCARD, RIPK2, TNF, TXNIP | 16 | 9.06E-17–1.99E-05 |
| 2a. Cell death of phagocytes, myeloid cells (decreased) | BCL2, CCL2, CCL5, CXCL1, CXCL2, IL1B, IRF1, NFKB1, NFKB1A, PYCARD, TNF | 11 | 9.06E-17 |
| **Toxicity analysis** | | | |
| Decreased MPT, mitochondrial swelling | B2M, BCL2, NFKB1, PYCARD, TNF | 5 | 7.27E-08 |
| *T. cruzi* infection of THP-1 mφs vs control (18 h) | | | |
| **Top biological and molecular functions** | | | |
| 1 | Cell death of immune cells (decreased) | ACTB, CASP4, CCL2, CCL5, CCL5, CHUK, CT5B, CXCL1, CXCL2, CXCL5, GAPDH, HSP90AA1, HSP90B1, IL1B, IRAK1, MAP3K7, MAPK1, MAP3K3, NAPI, P2RX7, PEA15, PYCARD, TAB2, TNF, TNFSF14, TRAF6 | 24 | 1.48E-12–2.60E-04 |
| 1a. Cell death of myeloid/phagocytes | CASP4, CCL2, CCL5, CCL5, CCL5, CXCL1, CXCL1, CXCL2, IL1B, P2RX7, PYCARD, TNF | 10 | 1.72E-12 |
| 2 | Inflammation/infectious disease | CASP4, CCL2, CCL5, CXCL1, CXCL2, HSP90AA1, IL1B, MAPK1, PANX1, P2RX7, PYCARD, TNF, TNFSF14, TRAF6 | 14 | 1.08E-06 |
| 2a. Activation of leukocyte/lymphocytes | CCL2, CCL5, CXCL1, HSP90B1, IL1B, MAP3K7, PYCARD, TNF, TNFSF14 | 9 | 2.95E-07 |
| 2b. Migration of phagocytes/neutrophils | CCL2, CCL5, CT5B, CXCL1, CXCL2, IL1B, P2RX7, TNF | 8 | 2.07E-07 |
| **Toxicity analysis** | | | |
| Gene regulation by PPARz | HSP90AA1, IL1B, MAP3K7, MAPK1, MAP3K3, TNF, TRAF6 | 7 | 4.16E-10 |
| *T. cruzi* infection of THP-1 mφs vs control (18 h + ATP) | | | |
| **Top biological and molecular functions** | | | |
| 1 | Cell death (decreased) | ACTB, B2M, CASP4, CCL2, CCL5, CHUK, CT5B, CXCL1, CXCL2, GAPDH, HSP90AA1, HSP90B1, IL1B, IRAK1, IRF1, MAP3K7, MAPK1, MAPK12, NFKB1A, P2RX7, PEA15, PGTSG2, PYCARD, TAB2, TNF, TNFSF14, TRAF6 | 26 | 6.53E-12–4.09E-04 |
| 1a. Cell death of myeloid/phagocytes | CASP4, CCL2, CCL5, CCL5, CXCL1, CXCL2, IL1B, P2RX7, PYCARD, TNF | 10 | 1.48E-12 |
| 2 | Inflammation/infectious disease | ACTB, B2M, CASP4, CCL2, CCL5, CHUK, CT5B, CXCL1, CXCL2, GAPDH, HSP90AA1, IRAK1, MAP3K7, NFKB1A, P2RX7, PGTSG2, PIRK2, PYCARD, TIRAP, TNF, TNFSF14, TRAF6 | 21 | 3.33E-10–4.43E-04 |
HMVEC), and human vascular smooth muscle cells (HVMSC), infected with T. cruzi for 24 h, were obtained from the HG_U133 plus 2.0 Affymetrix array analysis data posted at Gene Expression Omnibus [20]. The selected differentially expressed gene datasets from Tc-infected HFF, HMVEC, and VSMC cells were submitted to Ingenuity iReport Analysis (Ingenuity Systems, Redwood city, CA). The iReport retrieves a set of biological information such as gene name, sub-cellular location, tissue specificity, function, association with disease, and integrates into networks and signaling pathways with biological meaning and significance. An e-value was calculated by estimating the probability of a random set of genes having a frequency of annotation for that term greater than the frequency obtained in the real set, and a threshold of e-value, $10^{-3}$ was set to retrieve significant molecular functions and biological processes.

Parasite infectivity and replication in mφs

T. cruzi trypomastigotes were labeled with 5 μM SYTO®11 (binds DNA, Molecular Probes-Invitrogen, Eugene, OR) or 5 μM carboxyfluorescein succinimidyl ester (CFSE, binds amines, Invitrogen) for 20 min at 37°C. THP-1- or BM- derived mφs were infected and incubated with labeled T. cruzi trypomastigotes, Table 2. Cont.

| Pathways                                      | Gene name                  | FoCus mols | P value  |
|----------------------------------------------|----------------------------|------------|----------|
| 2a. Activation of leukocytes/lymphocytes     | ↓ B2M, ↑ CCL2, ↑ CCL5, ↑ CXCL1, ↑ CXCL2, ↓ MAP3K7, ↑ TNF | 7          | 9.31E-06 |
| 2b. Migration of phagocytes/neutrophils      | ↑ CCL2, ↑ CCL5, ↓ CHUK, ↓ CTS8, ↑ CXCL1, ↑ TNF, ↓ CXCL2, ↑ NFKB1A, ↑ P2RX7, ↑ PTGS2, ↑ TIRAP | 11         | 2.43E-04 |

Toxicity analysis

| Gene regulation by PPARα                  | ↓ CHUK, ↓ HSP90AA1, ↓ MAP3K7, ↓ MAPK1, ↓ MAP3K7, ↓ NFKB1A, ↑ P2RX52, ↑ TNF, ↑ TRAF6 | 9          | 2.11E-11 |

All differentially expressed proteins identified in THP-1 macrophages infected with T. cruzi for 3 or 18 h (∓ ATP) (listed in Table 1) were uploaded into Ingenuity Pathway Analysis (IPA) to interpret datasets in the context of biological processes and function, and pathway and molecular networks. Presented are the top networks with a p value <0.01 to which maximal number of the differentially expressed proteins identified in chagasic plasma (bolded letters) were associated with. FoCus molecules are the number of differentially expressed plasma proteins associated with an individual network.

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Figure 3. NLRP3/caspase-1 inflammasome is the major source of IL-1β for parasite control in mφs. THP-1 mφs were incubated with T. cruzi in the presence or absence of cycloheximide (CHX), glibenclamide (Glb), Ac-YVAD-CHO, and KCl for 3 h (A) and 18 h (B&C). Macrophages incubated with media alone were used as controls. (A&B) IL-1β release in supernatants was determined by an ELISA. (C) Quantitative PCR analysis of parasite burden in infected macrophages using Tc18SrDNA-specific oligonucleotides.

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Figure 4. Feedback cycle of NOX2/ROS and IL-1β activation in mQs infected by *T. cruzi*. THP-1 mQs were infected with *T. cruzi* as in Fig. 1, and incubated for 3 h (A,C,E) or 18 h (B,D,F,G) in presence of NOX2 inhibitors (diphenylene iodonium (DPI) or apoCynin), ROS scavenger (N-acetylcysteine (NAC)) or IL-1β antibody. (A–D) NOX2 inhibitors decreased ROS and IL-1β levels in infected mQs. Shown are (A&B) DCF fluorescence units and (C&D) IL-1β levels in supernatants determined by an ELISA. (E&F) Treatment with anti-IL-1β antibody decreased the ROS levels in infected mQs. (G) Effect of ROS inhibitors on intracellular parasite burden, as determined by qPCR, in infected mQs. doi:10.1371/journal.pone.0111539.g004

Total DNA from normal and infected cells was isolated by using TRIzol reagent (Life Technologies, Grand Island, NY). Total DNA (100 ng) was used as a template in a quantitative PCR (qPCR) on an iCycler thermal cycler with SYBR Green Supermix (Bio-Rad) and oligonucleotide pairs specific for *Tc*18S ribosomal DNA (Table S1). Data were normalized to murine or human GAPDH, and fold change calculated as $2^{-\Delta\Delta C_{T}}$, where $\Delta C_{T}$ represents the $C_{T}$ (sample) - $C_{T}$ (control).
Activation of mQs by T. cruzi: ROS, nitric oxide (NO) and cytokines levels

mQs were infected with T. cruzi for 1 h, washed to remove free parasites, and then incubated for up to 18 h, as above. Cells were incubated with 5 μM Carboxymethyl-2',7'-dichlorodihydrofluorescein diacetate (CM-H2DCF-DA, from Life Technologies, Ex495/Em527, fluoresces green upon oxidation by intracellular ROS) for 30 min, and the fluorescence was recorded using a SpectraMax M5 microplate reader. In some experiments, cells were incubated for 30 min with 5 μM dihydroethidium (DHE, Ex518nm/Em605nm, fluoresces red upon oxidation and binds DNA). Micrographs of DCF or DHE fluorescence were visualized on an Olympus BX-15 microscope, and images were captured by using a mounted digital camera (magnification 40X).

ROS release in supernatants was determined by an Amplex red assay. Briefly, 50 μL of supernatants from infected mQs were added in triplicate to 96-well, flat-bottomed plates, and mixed with a similar volume of 100 μM 10-acetyl 3,7-dihydroxyphenoxazine (Amplex Red, Life Technologies) and 0.3 U/ml horseradish peroxidase. The H2O2-dependent oxidation of Amplex red to red fluorescent resorufin (Ex510nm/Em590nm) was recorded as above (standard curve: 50 nM - 5 μM sodium nitrite).

The NO level (indicator of iNOS activity) was monitored by the Greiss reagent assay and by using the Nitrate/Nitrite Colorimetric Assay Kit (Cayman, Ann Arbor, MI). Briefly, culture supernatants (50 μl) were reduced with 0.01 unit/100 μl of nitrate reductase, and incubated for 10 min with 100 μl of 1% sulfanilamide prepared in 5% phosphoric acid/0.1% N-(1-naphthyl) ethylenediamine dihydrochloride (1:1, v/v). After incubation for 10 min, formation of diazonium salt was monitored at 545 nm (standard curve: 2-50 μM sodium nitrite).

Culture supernatants from mQs incubated with T. cruzi (± inhibitors) were also utilized for measuring IL-1β and IL-18 release by using OptEIA ELISA kits (eBioscience), according to the manufacturer’s instructions.

Transient transfection and luciferase assay

RAW 264.7 cells were plated in 6-well plates, and, when at 70% confluency, transfected with pGL4.NF-κB-Luc reporter plasmid (3 μg/well, Promega, San Diego, CA) by using JetPEI transfection reagent (Polyplus transfection, New York, NY), according to instructions provided by the manufacturer. The pREP7-Rluc plasmid (500 ng) expressing renilla luciferase was co-transfected into RAW macrophages and used as an internal control reporter. After 30 h of transfection, cells were washed, replenished with complete medium, and infected with T. cruzi (± inflammasome inhibitors) for 18 h (positive control: 10 ng/ml recombinant TNF-α for 6 h). The relative NF-κB transcriptional activity was measured by firefly luciferase activity and normalized to Renilla luciferase activity. The transcriptional activity of NF-κB in normal cells was considered as baseline and valued at 1.

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Figure 5. IL-1β signals NF-κB activation and inflammatory cytokine gene expression in infected mQs. (A) The mRNA levels for IL-1β (panel a), TNF-α (panel b) and CXCL1 (panel c) cytokines were measured in T. cruzi-infected THP-1 mQs at 3h and 18h pi by quantitative RT-PCR. (B) RAW 264.7 macrophages were transiently transfected with pGL4.NF-κB-Luc reporter plasmid and pREP7-Rluc plasmid (transfection efficiency control) as described in Materials and Methods. Transfected cells were infected with T. cruzi and incubated in the presence or absence of anti-IL-1β antibody. MQs incubated with 10 ng/ml recombinant TNF-α for 6 h were used as positive controls. The relative NF-κB transcriptional activity was measured by firefly luciferase activity and normalized to Renilla luciferase activity. The transcriptional activity of NF-κB in normal cells was considered as baseline and valued at 1.

Statistical Analysis

All experiments were conducted at least twice with triplicate observations per sample per experiment. All data were analyzed by using Graph Pad InStat ver.3 software and expressed as mean ± SD. Data were analyzed by the Student’s t test (comparison of 2 groups) and 1-way analysis of variance (ANOVA) with Tukey’s post-hoc test (comparison of multiple groups). Significance is
shown by *p<0.05, **p<0.01, ***p<0.001 (*normal-versus-infected; #infected versus infected/treated).

Results

MØs elicit subdued IL-1β response to T. cruzi infection

THP-1 MØs incubated with T. cruzi trypomastigotes (1:3, cell: parasite ratio) or Tc-lysate exhibited a ~2-fold increase in IL-1β release at 3 h pi that was consistently increased at 6 h and 12 h pi (data not shown) and maximized to a >9.8-fold increase by 18 h pi (Fig.1A&B). Incubation with higher number of parasites (1:4, 1:5 or 1:6, cell: parasite ratio) did not result in a further increase in IL-1β release at 3 h and 18 h pi (data not shown). LPS treatment (100-ng/ml) triggered a substantially higher level of IL-1β release in Tc-infected and LPS-treated cells at 3 h (Fig.1A&C). No significant effect of exogenous ATP on IL-1β release was observed at 18 h post-incubation (Fig.1B&D).

To determine if IL-1β is required for parasite control, MØs were infected with SYTO®11-labeled T. cruzi and incubated in the presence or absence of anti-IL-1β Ab or ROS scavengers (as in Figs.1&4). Shown are IL-1β release measured by an ELISA (A&B), mRNA levels for IL-1β and TNF-α by quantitative RT-PCR (C&D) and Tc18SrDNA signal by qPCR (E&F).

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Figure 6. ASC−/− mØs are compromised in the IL-1β–ROS–NF-kB pathway for control of T. cruzi. Bone-marrow-derived macrophages were isolated from matched WT and ASC−/− mice. Primary mØs were infected with T. cruzi and incubated for 3 h or 18 h in the presence or absence of anti-IL-1β Ab or ROS scavengers (as in Figs.1A&D). Shown are IL-1β release measured by an ELISA (A&B), mRNA levels for IL-1β and TNF-α by quantitative RT-PCR (C&D) and Tc18SrDNA signal by qPCR (E&F).

To determine if IL-1β is required for parasite control, mØs were infected with SYTO®11-labeled T. cruzi and incubated in the presence or absence of anti-IL-1β antibody for 18 h (Fig.1E). The intracellular SYTO®11 fluorescence (indicates parasite presence) was significantly increased in anti-IL-1β antibody - treated THP-1 mØs (Fig.1E,d&f). The qPCR estimation of parasite burden confirmed the microscopic findings and showed a 2-fold increase in Tc18SrDNA signal in infected cells treated with anti-IL-1β antibody (Fig.1F). Antibody efficacy in depleting secreted IL-1β is shown in Fig.1G. Together, these data suggested that a) mØs respond to T. cruzi infection, in comparison to LPS treatment, by a subdued IL-1β release; b) IL-1β release can be enhanced by ATP at an early time-point pi; and c) IL-1β is required for controlling intracellular T. cruzi.

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Inflammasome-related gene expression in m\(\phi\)s infected by \(T.\ cruzi\)

We investigated the mRNA expression levels of various genes involved in the function of inflammasomes and NOD-like receptor (NLR) signaling. THP-1 m\(\phi\)s were incubated with \(T.\ cruzi\) or LPS for 3 h and 18 h (± ATP treatment) and probed for the expression of 95 genes (including house-keeping genes) utilizing the RT\(^2\) Profiler Inflammasome PCR Arrays. The differential mRNA level was captured by qRT-PCR, and HTqPCR software was employed to attain the statistically significant differential expression in \(Tc\)-infected versus control m\(\phi\)s (± ATP) and LPS-treated versus control m\(\phi\)s at 3 h and 18 h (Table 1 & Table S2). Venn diagrams of comparative analysis of gene expression in \(Tc\)-infected and LPS-treated m\(\phi\)s at 3 h and 18 h (± ATP) are shown in Fig.2. Of the 95 genes that were examined, 63 genes exhibited differential expression in one of the studied groups (p<0.05). We noted the differential expression of 18 (8 up-regulated and 10 down-regulated) and 29 (9 up-regulated and 20 down-regulated) genes in \(Tc\)-infected m\(\phi\)s at 3 h and 18 h, respectively, in comparison to that noted in controls (Fig.2A, Table 1). When \(Tc\)-infected m\(\phi\)s were treated with exogenous ATP during the last 30 min of incubation, 22 (15 up-regulated and 7 down-regulated) and 35 (11 up-regulated and 24 down-regulated) of the inflammasome-related genes were differentially expressed at 3 h and 18 h pi, respectively (Fig. 2B & C, Table 1). Only seven and
eight genes were uniquely expressed in \( Tc \)/ATP-treated m\( \text{Q} \)s when compared to \( Tc \)-infected m\( \text{Q} \)s at 3 h and 18 h, respectively. However, other genes, e.g., BCL2, PSTPIP1, and CIITA at 3 h and NAIP1 at 18 h, that were down-regulated by \( T. cruzi \) infection, were up regulated when ATP was provided exogenously (Fig.2B&C, Table 1). In LPS-treated m\( \text{Q} \)s, we noted differential expression of 31 (12 up-regulated and 19 down-regulated) and 27 (11 up-regulated and 16 down-regulated) of the inflammasome-related genes at 3 h and 18 h pi, respectively, and the LPS-induced gene expression profile was not changed by exogenous addition of ATP (Fig.2D&E, Table S2). These data suggest that very few of the inflammasome-related genes are up regulated in response to \( T. cruzi \) infection. Exogenous ATP was effective in enhancing the inflammasome-related gene expression at 3 h, but not at 18 h pi. In comparison, LPS served as a potent activator, as evidenced by a significant up regulation of inflammasome-related gene expression within 3 h post-treatment. These data support the results presented in Fig.1 and suggest that \( T. cruzi \) is a silent invader that elicits low level of inflammatory response in macrophages.

Functional characterization of differentially expressed genes in \( Tc \)-infected cells

The top biological and molecular functional analysis of the differentially expressed gene datasets in \( Tc \)-infected m\( \text{Q} \)s by Ingenuity iReport is presented in Table 2. In infected THP-1 m\( \text{Q} \)s at 3 h pi (\( \pm \) ATP), 13–16 of the differentially expressed genes were indicative of an increase in inflammatory responses and control of macrophages’ cell death (p<0.001, z score: \( -1.195 \) to \( -2.333 \), p<0.001). Exogenous addition of ATP had no significant effect on the top biological functions altered in response to \( T. cruzi \) infection. Toxicity analysis indicated that changes in mitochondrial membrane potential that would likely result in ROS generation would be a key event in inflammatory and cell remodeling/cell death responses (p<0.01). By 18 h pi (\( \pm \) ATP), 14–21 of the differentially expressed genes were involved in inflammation, activation of leukocytes, and/or migration of phagocytes (z score: \( 1.78 \) to \( 2.6 \)) and 24–26 of the differentially expressed genes implicated in cell death were down-regulated (z score: \( -1.23 \) to \( -1.25 \)). The top canonical pathway, toxicity, and upstream regulators analysis suggested the macrophage’s attempt...
to balance inflammation with cellular protection via PPAR-α signaling (p<0.001).

Caspase-1-mediated IL-1β activation is ROS-dependent, and plays a role in control of *T. cruzi* replication in wild-type mφs

To determine if inflammasomes play a role in IL-1β activation in mφs infected by *T. cruzi*, we pre-treated the THP-1 mφs with selective inhibitors of inflammasome activation for 2 h and continued the inhibition pressure during the infection period. The IL-1β release induced at 3 h and 18 h pi was decreased by 100% and 75–95%, respectively, when mφs were treated with cycloheximide (inhibits protein synthesis and thus the inducible arm of the inflammasome pathway), glibenclamide (blocks the caspase-1 and pro-IL-1β maturation), Ac-YVAD-CHO (inhibits caspase-1 activity), or KCl (inhibits K⁺ efflux required for caspase 1 activation) (Fig.3A&B). Importantly, glibenclamide and KCl also blocked the mφs’ ability to control parasite replication, resulting in a 50–70% increase in intracellular *Tc*18SrDNA, as determined by sensitive qPCR (Fig.3C).

Ingenuity iReport analysis of differentially expressed genes in this study (Table 2) and our previously published reports [22] have led us to suggest that infection by *T. cruzi* would elicit ROS by changes in mitoChondrial MPT or NOX2 activation in mφs, and that ROS may serve as a 2nd signal for inflammasome activation [11,23]. We, therefore, determined whether ROS is induced and plays a direct role in IL-1β production in infected mφs. THP-1 mφs exhibited 3.4-fold and 2.7-fold increases in DCF fluorescence (detects intracellular ROS) at 3 h and 18 h pi, respectively (Fig.4A). When NOX2 inhibitors (DPI or apoCynin) were added during incubation with *T. cruzi*, we noted a 21–44% and 68–80% decline in ROS (Fig.4A&B) and 37% and 45% decline in IL-1β release (Fig.4C&D) at 3 h and 18 h pi, respectively. These data suggested that NOX2, at least partially, regulates ROS-dependent IL-1β activation in infected mφs. The observation of a moderate, but a significant (35–45%, p<0.05) decline in DCF fluorescence in infected mφs treated with anti-IL-1β antibody (Fig.4E&F) implied that IL-1β also contributes to activation of ROS production. However, we found no increase in intracellular parasite burden in THP-1 mφs treated with NOX2 inhibitors (Fig.4G). Together, the data presented in Figs.3&4 suggest that a feed-back cycle of caspase-1/IL-1β and ROS activation occurs in response to *T. cruzi* infection in THP-1 mφs and is required for control of intracellular parasite replication. While direct inhibition of IL-1β (Fig.1F) or caspase-1 (Fig.3C) affected the WT mφs ability to control *T. cruzi*, inhibition of ROS-dependent IL-1β was compensated for, likely by activation of other immune defenses capable of controlling the intracellular parasite replication and survival (Fig.4G).

**IL-1β signaling of NFκB in *Tc*-infected mφs**

Because gene expression analysis has identified several of the signaling (e.g. TRAF6, MYD88, NFκB) and cytokine (e.g. CCL2, CG5, CXCL1, TNF) molecules involved in inflammatory responses that were activated in *Tc*-infected mφs (Tables 1 & 2), we determined if IL-1β signals the activation of the nuclear factor kB (NFκB) pathway of cytokine gene expression in mφs. The mRNA levels for IL-1β, TNF-α and CXCL1 in THP-1 mφs were increased by 1.6-fold, 2-fold, and 2.3-fold, respectively, at 3 h pi. At 18 h pi, IL-1β and CXCL1 were increased by 1.6-fold and 5-fold, respectively, while no increase was observed in TNF-α mRNA level (Fig.5A,a–c). Treatment of infected mφs with anti-IL-1β antibody abolished the increase in IL-1β mRNA in infected mφs; possibly indicating that the IL-1β engagement of surface receptors induced the IL-1β mRNA expression. We performed a luciferase reporter assay to verify the role of IL-1β in signaling cytokine gene expression via NFκB in *Tc*-infected mφs. RAW mφs transiently transfected with luciferase reporter plasmid pNFκB-luc with 3×NFκB binding site and pREP7-Rluc (expresses renilla luciferase) were infected with *T. cruzi* for 1 h, and NFκB-dependent luciferase activity (normalized to renilla luciferase) was monitored. We observed a >5-fold and 15-fold increase in luciferase activity, respectively, when mφs were infected with *T. cruzi* or treated with recombinant TNF-α (Fig.5B). Treatment with anti-IL-1β antibody resulted in 70% decline in *Tc*-induced luciferase activity (Fig.5B, p<0.01). Together, the data presented in Fig.5 suggested that IL-1β signals the NFκB activation of cytokine gene expression in mφs infected by *T. cruzi*.

ASC⁻/⁻ mφs were compromised in ROS-dependent IL-1β activation and NFκB-dependent cytokine gene expression, and exhibited pronounced *T. cruzi* replication and survival

To determine if ASC is involved in caspase-1-dependent inflammasome formation and activation of inflammatory proCesses for parasite control, we utilized primary BM-derived mφs from ASC⁻/⁻ mice and matched controls. The ASC⁻/⁻ mφs infected by *T. cruzi* exhibited a significantly compromised IL-1β release (67% and 40% decline at 3 h and 18 h, respectively) when compared to that noted in matched WT controls (Fig.6A&B, p<0.01). Treatment with anti-IL-1β antibody and ROS scavengers (NAC or apoCynin) normalized the IL-1β production in *Tc*-infected ASC⁻/⁻ and WT mφs to control levels. Further, the expression of NFκB-inducible pro-IL-1β and TNF-α mRNAs, that were significantly increased in *Tc*-infected WT mφs, were completely abolished in *Tc*-infected ASC⁻/⁻ mφs (Fig.6C&D). Subsequently, ASC⁻/⁻ mφs exhibited a 3.2-fold and 2.7-fold increase in intracellular parasite burden at 3 h and 18 h pi, respectively, when compared to that noted in matched WT infected mφs (Fig.6E&F). As previously observed in THP-1 mφs (Fig.4G), ASC⁻/⁻ mφs exhibited no further increase in parasite burden upon treatment with ROS scavengers (Fig.6). These data confirmed that the caspase-1/ASC inflammasomes play an important role in parasite control through ROS-dependent IL-1β activation and expression of other inflammatory cytokines in mφs.

**NLRP3⁻/⁻ mφs exhibited increased ROS-dependent control of *T. cruzi***

To specifically determine if NLRP3 inflammasome activation by ASC/caspase-1 is the primary source of protective IL-1β in the context of *Tc* infection, we conducted further studies in primary bone marrow-derived mφs from NLRP3⁻/⁻ mice and matched controls. As expected from studies in ASC⁻/⁻ cells, NLRP3⁻/⁻ mφs lacked the ability to elicit IL-1β activation in response to *T. cruzi* infection (Fig.7A&B, p<0.01). Treatment with anti-IL-1β antibody and ROS scavengers (NAC or apoCynin) normalized the IL-1β production in *Tc*-infected NLRP3⁻/⁻ and WT mφs to control levels (Fig.7B). Further, NFκB-inducible pro-IL-1β, TNF-α and CXCL1 mRNA levels that were increased in *Tc*-infected WT mφs, were almost absent in *Tc*-infected NLRP3⁻/⁻ mφs (Fig.7C&D). However, to our surprise, NLRP3⁻/⁻ mφs exhibited a 12-fold decline in intracellular parasite burden at 18 h, when compared to that noted in matched WT infected mφs (Fig.7E). To determine if NLRP3⁻/⁻ mφs were simply refractory to *T. cruzi*, we incubated the NLRP3⁻/⁻ and matched WT mφs with
CFSE-labeled trypomastigotes for 3 h and analyzed for CFSE fluorescence by flow cytometry. We observed no statistically significant difference in intracellular mean fluorescence intensity (MFI) indicative of the number of parasites/cell [Fig.8A] and the percentage of CFSE+ cells indicative of the number of Tr-infected NLRP3−/− and WT mice [Fig.8B]. Representative micrographs showing efficient uptake of CFSE-labeled parasites in NLRP3−/− and WT primary mice are presented in Fig.8C (panels a&b). These data suggested that NLRP3−/− mice were not refractory to T. cruzi and efficiently phagoCytized parasites in a manner similar to that of the WT mice. Instead, NLRP3−/− mice, in comparison to WT mice, exhibited 5.5-fold higher basal ROS levels (Fig.8D). The ROS production in NLRP3−/− mice was enhanced upon T. cruzi infection and not inhibited by the addition of anti-IL-1β antibody (Fig.8DE). The increase in ROS production in NLRP3−/− mice in response to T. cruzi infection was also evidenced by a significant increase in DHE fluorescence (red, detects intracellular ROS, Fig.8C-e–f). Together, the data presented in Figs.7&8 suggested that NLRP3/ASC/caspase-1 inflammasome mediates IL-1β activation and expression of other inflammatory cytokines in mice infected by T. cruzi; however, NLRP3 deficiency is compensated for by increased ROS levels capable of preventing parasite replication and intracellular survival in NLRP3−/− mice.

**Discussion**

The innate immune response to T. cruzi infection is mediated by diverse PRRs, including receptors of the TLR family [24,25] and ASC-containing inflammasomes (e.g. NOD1) [26], and NLRP3 [27,28]). Genetically modified mice deficient in MYD88 (interacts with TLR-2, -4, -6), TLR-4, NOD1, and ASC exhibited an increased susceptibility to T. cruzi, thus pointing to these PRRs as critical determinants of host resistance to T. cruzi infection [24,26,28,29]. In this manuscript, we have utilized cultured and primary mice and employed inhibitory approaches to investigate the mechanisms of caspase-1/ASC inflammasome activation and their role in the context of T. cruzi infection. We found that mice respond to T. cruzi infection with suboptimal activation of inflammasome-related gene expression and IL-1β production. Functional analysis of the differentially expressed gene datasets in Te-infected mice was indicative of an increase in inflammatory responses and control of macrophages’ cell death. The IL-1β production in Te-infected mice was ROS-dependent and could be enhanced by the exogenous addition of ATP. Studies in WT mice treated with specific inhibitors and ASC−/− mice suggested that caspase-1/ASC inflammasome played a role in activation of the IL-1β–ROS–NF-kB pathway, that when inhibited, resulted in a compromised inflammatory cytokine response and increase in T. cruzi replication and survival in macrophages. However, NLRP3−/− mice, despite an inability to elicit IL-1β activation and inflammatory cytokine gene expression, were capable of parasite control. Thus, our data allow us to conclude that caspase-1/ASC inflammasomes play a significant role in the activation of IL-1β/ROS and NF-kB signaling of cytokine gene expression for T. cruzi control in human and mouse mice. However, NLRP3 balances the mice’s activation of ROS and NFkB/cytokine response, and its deficiency shifted the mice’s responses towards increased ROS-dependent control of T. cruzi. To the best of our knowledge, this is the first study demonstrating a double-edged role of NLRP3 in determining macrophage activation of ROS and cytokine response, both of which are required for clearance of T. cruzi infection.

The NLRs are expressed in most cell types of the immune system, but are also reported to be expressed in other tissues. Based on the expression profile of components of NLRP1, NLRP3, and NLRRC1; blood, placenta and thymus are shown to constitutively express inflammasomes. Other tissues (e.g., heart, vascular tissue, bone marrow) require up regulation of one or two components in order to assemble functional inflammasomes [30]. Our data demonstrated that inflammasome-related gene expression is induced in mps exposed to T. cruzi infection (Table 1) or LPS treatment (Table S2), as well as in non-phagoCytic, human vascular smooth muscle, fibroblast, and microvascular endothelial cells (Table S3). Functional analysis of the gene expression profile indicated that in mps, an inflammatory response to control T. cruzi infection was associated with significant efforts to prevent cell death (Table 2). Infected mps also exhibited suppression of several of the genes involved in PPARγ signaling that induces apoptosis following activation with TNF-α/IFN-γ [31,32]. In non-phagoCytic cells, TLR/MYD88 signaling of NFkB-dependent cytokines’ (IL-6, IFNα/β, IL-12) gene expression and caspase-1/NLRP3-mediated activation of IL-1β gene expression was noted. Canonical analysis, as in mps, also indicated PPARγ regulation of gene expression in HMVEC and HFF cells infected by T. cruzi (Table S4). Our observations allow us to surmise that phagoCytic and non-phagoCytic cells responded to T. cruzi infection by induction of diverse inflammasome-related gene expression. Eventually, all cell types appeared to be overwhelmed by the inflammatory signal and tended to switch to signaling events related to prevention of cell death.

Several studies indicate that ROS are an essential secondary messenger for signaling caspase-1/ASC inflammasome activation [11,34]. The use of ROS scavengers controlled IL-1β activation by virtually all agonists of the NLRP3 inflammasome [33,34]. Besides ROS, extracellular ATP, through activation of the P2X7 (puriogenic ionotrophic ATP-gated cation channel), triggers K+ efflux, which, in turn, triggers pore formation by pannexin, thereby allowing the delivery of pathogen products into the cytosol, resulting in caspase-1/ASC inflammasome activation [35]. In agreement with the literature, we also observed ROS-dependent IL-1β activation mediated by caspase-1/ASC inflammasome in mps exposed to T. cruzi (Figs.1–4). The exogenous addition of ATP resulted in a higher level of IL-1β activation in infected mps at 3 h pi, as also noted by others [28]. The lack of an effect of exogenous ATP on the extent of IL-1β activation in infected mps at 18 h pi may mean that other signals were generated. We speculate that either T. cruzi kDNA released by dying T. cruzi within mps or mtDNA released due to bystander damage in infected mps served as a secondary signal in activating caspase-1/ASC inflammasomes at 18 h pi. This notion is supported by the observation of similar levels of IL-1β activation in mps incubated with live as well as dead T. cruzi and requires further investigation.

Importantly, we made a novel observation and noted a feed-back cycle of IL-1β signaling of ROS activation in infected mps (Figs.3&4). The molecular mechanism for the ROS production by IL-1β remains to be elucidated. In previous studies, IL-1β was found to stimulate phospholipase A2, promoting release of arachidonic acid. Since arachidonic acid can activate NADPH oxidase to produce superoxide, it is possible that this fatty acid may serve as an intermediate in the IL-1β-induced activation of enzymes, leading to the production of ROS [36,37]. However, several non-NADPH oxidase-dependent sources, including mito-Chondrial electron transport and arachidonate metabolism, may also be involved in the cytokine-induced ROS generation, to be investigated in future studies.

It is intriguing that both ASC−/− and NLRP3−/− mice were equally invaded by T. cruzi, lacked IL-1β production, and elicited ineffective, NF-kB-mediated cytokine gene expression (Figs.6–8);
yet only ASC−/− mps were restrictive in their capacity to control T. cruzi infection [Fig. 6]. ASC−/− and caspase-1−/− mice have been documented to exhibit a higher incidence of mortality, cardiac parasitism and heart inflammation, meaning that ASC/caspase-1 inflammasomes are critical determinants of host resistance to infection with T. cruzi [29]. Like ASC−/− mice and in vitro cultured ASC−/− mps, NOD1−/− mice and BM-derived derived mps from NOD1−/− mice also showed an impaired induction of NF-κB-dependent products and failed to restrict T. cruzi infection [26]. Our finding that a deficiency of NLRP3 did not affect mps ability to control parasites allows us to surmise that the caspase-1/ASC requirement for effective control of T. cruzi is delivered via formation and activation of inflammasomes with other NLRs, e.g., NLRP1, AIM2 or NLRC4. The ability of NLRP3−/− mps to efficiently manage parasite killing via enhanced ROS release (Figs. 7 & 8) suggests that NLRP3 suppresses NOX2-dependent ROS efficiently in infected BM-derived monoCyttes from NLRP3−/− mice that were differentiated to mps [30]. 

Our finding that a deficiency of NLRP3 did not affect mps' ability to kill T. cruzi. Caspase-1, upon activation by NLRP3/ASC, locoCilizes to phagosomes and disturbs NOX2 control of pH, thereby triggering acidification and microbicidal activity of phagosomes in mps infected by StaphyloCoCcus aureus [39]. We surmise that caspase-1 lcoCilization to phagosome and suppression of NOX2/ROS enhances the bactericidal activity of mps. However, NLRP3 interaction with NOX2 resulting in low level of ROS production likely maintains the alkalization of the phagosomal lumen that prevents the mps' ability to directly kill T. cruzi, but plays a critical role in allowing mps to function as specialized phagoCytes adapted to proCess antigens for cross presentation and elicitation of adaptive immunity. 

Other investigators have shown a severe defect in nitric oxide (NO) production and impairment in mps-mediated T. cruzi killing in NLRP3−/− mice and isolated mps [27]. In this study, we utilized the BM-derived monoCyttes from NLRP3−/− mice that were differentiated to mps by M-CSF treatment, and infected with T. cruzi (SylvioX10) for 3 h and 18 h. Others utilized peritoneal mps obtained 4 days after intra-peritoneal injection of 1% starch solution in NLRP3−/− mice and infected these mps with the Y strain of T. cruzi for 48 h for all the studies [27]. We propose that NLRP3 deficiency is compensated for by over activation of NOX2/ROS that effectively controlled the early invasion and replication of T. cruzi in mps, as observed in this study. However, a lack of NF-κB-mediated cytokine response (Fig. 7) and iNOS/NO activation [27] prevented the NLRP3−/− mps from sustained, long-term control of the parasites, resulting in increased susceptibility. It is also plausible that the differences in the source of mps, parasite isolates, and the time course of infection may explain the observed differences in the ability of NLRP3−/− mps to control T. cruzi infection in our and published studies. 

In summary, we have demonstrated that T. cruzi interfered with the potent activation of caspase-1/ASC inflammasome-related gene expression and cytokine response in mps to ensure its survival. We found that caspase-1/ASC inflammasomes played a role in the activation of the IL-1β-ROS-NF-κB pathway, that when inhibited, resulted in an increase in T. cruzi replication and survival in mps. However, NLRP3−/− mps were compensated for by increased NOX2/ROS activation capable of parasite killing. Our data suggest that the NLRP3/caspase/ASC inflammasome balances the mps' activation of ROS and NFκB/cytokine response and provide the first evidence for NLRP3 regulation of NOX2 function as an effector mechanism contributing to parasite persistence.

Supporting Information

Table S1 Oligonucleotides used in this study.

(DOCX)

Table S2 Inflammasome-related differential gene expression in THP-1 macrophages in response to LPS treatment (± ATP) in comparison to normal controls.

(DOC)

Table S3 Inflammasome-related differential gene expression in non-phagoCytes at 24 h infection by Trypanosoma cruzi.

(DOCX)

Table S4 Ingenuity iReport analysis of inflammasome-related datasets in non-phagoCytes infected by T. cruzi.

(DOCX)

Author Contributions

Conceived and designed the experiments: NJG, JE ND. Performed the experiments: ND MS SG BAL. Analyzed the data: NJG MS SG MNG. Contributed reagents/materials/analysis tools: NJG ND BAL RF. Wrote the paper: NJG MS BAL ND.

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