NFATc1 Mediates Toll-Like Receptor-Independent Innate Immune Responses during Trypanosoma cruzi Infection

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Abstract

Host defense against the intracellular protozoan parasite Trypanosoma cruzi depends on Toll-like receptor (TLR)-dependent innate immune responses. Recent studies also suggest the presence of TLR-independent responses to several microorganisms, such as viruses, bacteria, and fungi. However, the TLR-independent responses to protozoa remain unclear. Here, we demonstrate a novel TLR-independent innate response pathway to T. cruzi. Myd88−/− Trif−/− mice lacking TLR signaling showed normal T. cruzi-induced Th1 responses and maturation of dendritic cells (DCs), despite high sensitivity to the infection. IFN-γ was normally induced in T. cruzi-infected Myd88−/− Trif−/− innate immune cells, and further was responsible for the TLR-independent Th1 responses and DC maturation after T. cruzi infection. T. cruzi infection induced elevation of the intracellular Ca2+ level. Furthermore, T. cruzi-induced IFN-γ expression was blocked by inhibition of Ca2+ signaling. NFATc1, which plays a pivotal role in Ca2+ signaling in lymphocytes, was activated in T. cruzi-infected Myd88−/− Trif−/− innate immune cells. T. cruzi-infected Nfatc1−/− fetal liver DCs were impaired in IFN-γ production and DC maturation. These results demonstrate that NFATc1 mediates TLR-independent innate immune responses in T. cruzi infection.


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Introduction

The host defense against invasion of intracellular pathogens relies on Th1 cell-derived IFN-γ that activates macrophages to kill the engulfed pathogens [1]. Toll-like receptor (TLR)-mediated recognition of pathogens has been established to induce activation of innate immune cells such as dendritic cells (DCs) and subsequent development of Th1 cells [2,3]. However, recent evidence also indicates the presence of TLR-independent mechanisms for the recognition of microorganisms such as bacteria, viruses, and fungi [4,5]. Accordingly, TLR-independent mechanisms for Th1 development have been demonstrated in several infectious models such as fungal and bacterial infections [6,7]. However, TLR-independent recognition of protozoa remains unknown.

Trypanosoma cruzi is an intracellular protozoan parasite that causes Chagas’ disease, a chronic disorder characterized by cardiomyopathy and malformation of the intestine [8]. Several components of T. cruzi have been shown to induce TLR-dependent activation of innate immunity and subsequent development of Th1 cells [9–14]. The absence of TLR-dependent activation of innate immunity results in high susceptibility to T. cruzi infection [15,16] due to defective type I interferon (IFN)–mediated induction of the GTPase IFN-inducible p47 (IRG47) [17]. Invasion of infective metacyclic trypomastigotes of T. cruzi into host cells induces a close interaction between the parasites and the host, because T. cruzi utilize several host-derived factors in order to establish the infection. These include activation of Ca2+ signaling pathways and phosphatidylinositol-3-kinases [18–20]. However, it remains unclear how T. cruzi-mediated activation of host cytoplasmic signaling pathways is regulated and whether it is TLR-dependent or -independent.

In T cells, the nuclear factor of activated T cells (NFAT) family of transcription factors has been shown to mediate production of cytokines including IFN-γ [21,22]. The NFAT family of proteins comprises four closely related members (NFATc1, NFATc2, NFATc3, and NFATc4) that are activated by Ca2+ signaling, and NFAT5 that is regulated by osmotic stress. The role of NFAT proteins in T cells has been well characterized [21,22]. However,
**Author Summary**

*Trypanosoma cruzi* is an intracellular protozoan parasite that causes Chagas diseases in humans. Invasion of *T. cruzi* into the host is sensed by Toll-like receptors (TLRs), which recognize microbial components that are present in microbes but not in the host. TLRs are essential for the initiation of immune responses against pathogens. Recent evidence indicates the presence of TLR-independent mechanisms for the recognition of microbes, such as bacteria, viruses, and fungi. However, TLR-independent recognition of protozoa remains unknown. We found that immune responses against *T. cruzi* were induced even in the absence of TLR signaling. The TLR-independent responses were found to be mediated by IFN-γ production in innate immune cells. Furthermore, the TLR-independent IFN-γ production was revealed to be mediated by Ca²⁺-dependent activation of NFAT1c, which has been shown to play a pivotal role in cytokine production in T lymphocytes. Therefore, we screened genes that were normally induced in *T. cruzi*-infected DCs (about 4000 genes) were MyD88 dependent, as the TLR-independent IFN-γ induction in wild-type DCs was reduced in *Trif*-deficient DCs. Some of the genes that were normally induced in *Myd88*-/− DCs, but not in *Myd88*-/−/Trif−/− DCs (MyD88/Trif-dependent genes; 14% of genes that were induced in wild-type DCs) are known to be induced by type I IFNs. In addition, a majority of the small number of genes that were induced even in *Myd88*-/−/Trif−/− DCs (6%) were IFN-γ-inducible genes (Figure S3). In order to corroborate that IFN-γ-inducible genes are normally induced in *T. cruzi*-infected *Myd88*-/−/Trif−/− DCs, we analyzed mRNA expression of *Ifng* and IFN-γ-inducible genes, including *Stat1* and *Irgm*, by real-time RT-PCR (Figure 2A). *T. cruzi* infection resulted in robust induction of *Ifng*, *Stat1*, and *Irgm* in wild-type, *Myd88*-/−, and *Myd88*-/−/Trif−/− mice infected with *T. cruzi* trypomastigotes for 6 h, then were washed, cultured for 48 h, and analyzed for expression of MHC class II, CD40, and CD86 by flow cytometry (Figure 1D). *T. cruzi* infection resulted in enhanced expression of these molecules in wild-type BMDCs. Expression was also increased in BMDCs derived from *Myd88*-/−/Trif−/− mice after *T. cruzi* infection, indicating normal maturation of *T. cruzi*-infected DCs of *Myd88*-/−/Trif−/− mice. Thus, Th1 cell development and DC maturation were induced during *T. cruzi* infection even in the absence of TLR-dependent activation of innate immunity.

**Results**

Normal Th1 response in *T. cruzi*-infected *Myd88*-/−/Trif−/− mice

Previously, we demonstrated that mice lacking both MyD88 and TRIF, in which TLR-dependent activation was abolished, are highly sensitive to infection with *T. cruzi* [17]. Because TLRs have been shown to control development of Th1 cells, we analyzed Th1 responses in *T. cruzi*-infected mice. Mice were intraperitoneally (i.p.) infected with *T. cruzi* trypomastigotes, and at 6 days of infection CD4⁺ T cells were isolated from the spleen and stimulated with anti-CD3 antibody (Ab) (Figure 1A). In *T. cruzi*-infected wild-type mice, there was considerable production of IFN-γ compared with that in non-infected control mice, indicating induction of potent Th1 responses. In *Myd88*-/− and *Myd88*-/−/Trif−/− mice, IFN-γ production was similar to that in wild-type mice following *T. cruzi* infection. Next, we analyzed the antigen-specific Th1 response at 0, 4, 6, and 10 days after *T. cruzi* infection by stimulating CD4⁺ T cells with freeze-thawed *T. cruzi* in the presence of antigen presenting cells (APC) (Figure 1B). This stimulation induced marked production of IFN-γ at 6 and 10 days of the infection in wild-type mice. Even in CD4⁺ T cells derived from *T. cruzi*-infected *Myd88*-/− and *Myd88*-/−/Trif−/− mice, antigen-specific production of IFN-γ was induced to levels similar to that of wild-type mice. Thus, the antigen-specific Th1 response was not impaired in *Myd88*-/− and *Myd88*-/−/Trif−/− mice. We also analyzed IFN-γ production from CD4⁺ T cells by intracellular staining (Figure 1C, Figure S1A). The number of IFN-γ-producing CD4⁺ T cells was almost equally elevated in wild-type, *Myd88*-/− and *Myd88*-/−/Trif−/− mice at 6 days (Figure 1C) as well as at 10 days (Figure S1A) after infection. Consistent with previous studies [25,26], the number of IFN-γ producing CD8⁺ T cells and NK1.1⁺ natural killer cells was not increased at 10 days after *T. cruzi* infection (Figure S1B).

Development of Th1 cells is critically controlled by DCs [27,28]. In addition, stimulation of TLRs induces maturation of DCs [3]. Therefore, we analyzed expression of MHC class II and co-stimulatory molecules on *T. cruzi*-infected DCs. Bone marrow-derived DCs (BMDCs) were infected with *T. cruzi* trypomastigotes for 6 h, then were washed, cultured for 48 h, and analyzed for expression of MHC class II, CD40, and CD86 by flow cytometry (Figure 1D). *T. cruzi* infection resulted in enhanced expression of these molecules in wild-type BMDCs. Expression was also increased in BMDCs derived from *Myd88*-/−/Trif−/− mice after *T. cruzi* infection, indicating normal maturation of *T. cruzi*-infected DCs of *Myd88*-/−/Trif−/− mice. Thus, Th1 cell development and DC maturation were induced during *T. cruzi* infection even in the absence of TLR-dependent activation of innate immunity.

IFN-γ induction in *T. cruzi*-infected *Myd88*-/−/Trif−/− DCs and macrophages

*T. cruzi* infection induced maturation of DCs in the absence of TLR signaling. Therefore, we screened genes that were normally induced in *T. cruzi*-infected DCs of *Myd88*-/−/Trif−/− mice. BMDCs from wild-type, *Myd88*-/−, and *Myd88*-/−/Trif−/− mice were infected with *T. cruzi* trypomastigotes for 6 h, then mRNA was extracted and used for DNA microarray analysis. Approximately 80% of genes that were induced in *T. cruzi*-infected wild-type DCs (about 4000 genes) were MyD88-dependent, as the *T. cruzi*-mediated induction was reduced in *Myd88*-/− DCs (Figure S2). Some of the genes that were normally induced in *Myd88*-/− DCs, but not induced in *Myd88*-/−/Trif−/− DCs (MyD88/Trif-dependent genes; 14% of genes that were induced in wild-type DCs) are known to be induced by type I IFNs. In addition, a majority of the small number of genes that were induced even in *Myd88*-/−/Trif−/− DCs (6%) were IFN-γ-inducible genes (Figure S3). In order to corroborate that IFN-γ-inducible genes are normally induced in *T. cruzi*-infected *Myd88*-/−/Trif−/− DCs, we analyzed mRNA expression of *Ifng* and IFN-γ-inducible genes, including *Stat1* and *Irgm*, by real-time RT-PCR (Figure 2A). *T. cruzi* infection resulted in robust induction of *Ifng*, *Stat1*, and *Irgm* in wild-type, *Myd88*-/−, *Trif*−/−, and *Myd88*-/−/Trif−/− DCs. *T. cruzi*-induced expression of *Stat1* and *Irgm* in wild-type DCs was inhibited by addition of a de novo protein synthesis inhibitor, cycloheximide (CHX) (Figure 2B). In contrast, CHX did not inhibit *T. cruzi*-induced *Ifng* expression (Figure 2C). Next, in order to analyze whether the expression of the IFN-γ-inducible genes was secondary to induction of *Ifng*, we used BMDCs derived from *Ifng*−/− mice in which the IFN-γ-mediated response was abolished (Figure 2D, E). In *Ifng*−/− BMDCs, *T. cruzi*-mediated induction of *Stat1* and *Irgm* was reduced, whereas induction of *Ifng* was unimpaired. These data indicate that *Ifng* was induced primarily in response to *T. cruzi* infection, and *Stat1* and *Irgm* were induced secondary to *Ifng* induction. In peritoneal Mφ, similar patterns of *T. cruzi*-mediated gene expression were observed (Figure S4). Recently, the CD11c⁺ B220⁺ NK1.1⁺ subset of cells was identified as a natural killer (NK) cell subset with a high capacity for IFN-γ production in response to IL-12 or a TLR9 ligand [29–31]. To exclude the possibility of contamination of these cells in preparation of BMDCs or Mφ, we purified CD11c⁺ B220⁺ NK1.1⁺ population from the spleen, and analyzed for IFN-γ expression (Figure S5A). CD11c⁺ B220⁺ NK1.1⁺ cells
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A

\[
\begin{array}{c}
\text{IFN-\(\gamma\) (ng/ml)} \\
\text{wild-type} \quad \text{Myd88}^{+/-} \quad \text{Myd88}^{-/-} \quad \text{Trif}^{-/-} \\
\end{array}
\]

\(\alpha\)-CD3

B

\[
\text{IFN-\(\gamma\) (ng/ml)} \\
0 \quad 4 \quad 6 \quad 10 \text{ (days)} \\
\text{wild-type (PBS)} \quad \text{Myd88}^{+/-} \text{(PBS)} \quad \text{Myd88}^{-/-} \text{Trif}^{-/-} \text{(PBS)} \\
\text{wild-type (T. cruzi)} \quad \text{Myd88}^{+/-} \text{(T. cruzi)} \quad \text{Myd88}^{-/-} \text{Trif}^{-/-} \text{(T. cruzi)}
\]

C

T. cruzi infected (day 6)

<table>
<thead>
<tr>
<th></th>
<th>uninfected</th>
<th>wild-type</th>
<th>Myd88(^{+/-})</th>
<th>Myd88(^{-/-})Trif(^{-/-})</th>
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</thead>
<tbody>
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<td>3.07</td>
<td>9.14</td>
<td>13</td>
<td>12.4</td>
</tr>
<tr>
<td>IFN-(\gamma)</td>
<td></td>
<td></td>
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</tbody>
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D

\[
\text{wild-type} \quad \text{Myd88}^{+/-} \text{Trif}^{-/-} \\
\text{CD40} \quad \text{CD86} \quad \text{I-A}^b
\]

\(\square\) T. cruzi
showed very low levels of IFN-γ expression in response to IL-12/IL-18 stimulation compared with the NK cell subset (Figure S5B). However, these cells from wild-type and Myd88−/−/Trif−/− mice expressed IFN-γ in response to T. cruzi infection (Figure 2F). Flow cytometric analysis further demonstrated that T. cruzi-infected CD11chigh splenic DCs expressed IFN-γ protein (Figure 2G). These findings indicate that T. cruzi infection induces IFN-γ production in DCs.

IFN-γ-mediated DC maturation and Th1 responses in T. cruzi-infected Myd88−/−/Trif−/− mice

Next, we analyzed whether IFN-γ was involved in TLR-independent DC maturation and Th1 responses during T. cruzi infection. In BMDCs derived from Ifng−/− mice, T. cruzi-induced enhancement of CD40, CD86, and MHC class II was partially reduced (Figure 3A). Furthermore, expression of these molecules was completely abolished in T. cruzi-infected DCs of Myd88−/−/Trif−/−/Ifng−/− mice. Enhanced expression of these molecules in response to exogenous IFN-γ was not observed in Ifng−/− BMDCs (Figure S6A). These findings indicate that IFN-γ produced from T. cruzi-infected DCs mediated DC maturation. We also analyzed IFN-γ production from splenic CD4+ T cells of T. cruzi-infected mice (Figure 3B). In both Ifng−/− and Myd88−/−/Trif−/−/Ifng−/− mice, T. cruzi antigen-dependent production of IFN-γ was severely reduced. Importance of IFN-γ production was further underscored by the finding that Ifng−/− mice were more sensitive to T. cruzi infection than Myd88−/−/Trif−/− mice (Figure S6B). These results demonstrate that IFN-γ mediates TLR-independent DC maturation and Th1 development during T. cruzi infection.

Importance of IL-12 in Th1 cell development has been established [32]. Indeed, IL-12p40-deficient mice were highly susceptible to T. cruzi infection with severely reduced Th1 responses [33,34] and Figure S7A). In addition, IL-12p40 concentration in the serum was decreased in T. cruzi-infected Ifng−/− mice (Figure S7B). In T. cruzi-infected Myd88−/−/Trif−/− mice, IL-12p40 production was severely reduced, but still induced [17], suggesting that IL-12 is produced via TLR-dependent and -independent pathways. Thus, IFN-γ, which is produced via the TLR-independent pathways, might induce IL-12p40 to activate T cells to fully differentiate into Th1 cells.

Involvement of Ca2+ signaling in IFN-γ induction in T. cruzi-infected Myd88−/−/Trif−/− cells

Next, we analyzed the molecular mechanisms for TLR-independent induction of IFN-γ after T. cruzi infection. In Myd88−/−/Trif−/− DCs, T. cruzi-induced phosphorylation of MAP kinases such as ERK, p38, and JNK, as well as degradation of IκBα was not observed at all (Figure S8A). In addition, T. cruzi infection did not induce DNA binding activity of NF-κB in Myd88−/−/Trif−/− DCs (Figure S8B). Thus, T. cruzi-mediated activation of NF-κB and MAP kinases was not induced in the absence of TLR signaling. Next, we stimulated DCs with T. cruzi trypomastigotes killed by repeated freeze-thaw steps. Live T. cruzi, but not killed parasites, induced Ifng expression (Figure 4A). Because many studies have demonstrated that T. cruzi utilize the host Ca2+ signaling to establish the infection [35], we assessed the intracellular Ca2+ concentration in T. cruzi-infected BMφ using a fluorescent Ca2+ indicator Fluo-4 AM (Figure 4B, Figure S9A, B). T. cruzi infection led to rapid increase in intracellular Ca2+ level in both wild-type and Myd88−/−/Trif−/− BMφ, which returned to the basal level after 18 min of the infection. Epimastigotes, which are not able to invade the host cells, did not induce the elevation of Ca2+ concentration in BMφ (Figure S9C). These results prompted us to examine whether Ca2+ mobilization induced by intracellular invasion of T. cruzi contributed to the TLR-independent Ifng induction. Accordingly, we treated wild-type and Myd88−/−/Trif−/− BMDCs with an intracellular Ca2+ chelator, bis-(o-aminophenoxy)-ethane-N,N,N’,N’-tetraacetic acid tetra (acetoxymethyl) ester (BAPTA-AM), and infected with T. cruzi. In BAPTA-AM pre-treated DCs, T. cruzi-induced Ifng expression was severely reduced, although lipopolysaccharide (LPS)-induced response was not impaired (Figure 4C). In this condition, T. cruzi-induced elevation of intracellular Ca2+ concentration was severely reduced (Figure S10). In addition, stimulation with both phorbol myristate acetate (PMA)/Ca2+ ionophore or Ca2+ ionophore alone, which mimics Ca2+ signaling, induced expression of Ifng in both wild-type and Myd88−/−/Trif−/− BMφ (Figure 4D). Taken together, these findings indicate that T. cruzi-dependent intracellular Ca2+ mobilization mediates TLR-independent Ifng induction.

NFATc1 activation in T. cruzi-infected Myd88−/−/Trif−/− cells

In the host cells, especially in T lymphocytes, Ca2+ mobilization induces activation of cytokine genes via calcmodulin/calcineurin-dependent activation of the transcription factor NFAT. Therefore, we treated Myd88−/−/Trif−/−/Mφ with FK506 to block calcineurin activation, and infected with T. cruzi. Treatment of FK506 resulted in a marked decrease in T. cruzi-induced expression of Ifng, despite normal LPS-induced response (Figure 5A). Among NFAT members, Nfatc1, Nfatc3, and Nfat5 mRNA were abundantly expressed in BMDCs (Figure S11). A previous study has shown that NFATc1 increased anti-CD3/anti-CD28-induced IFN-γ promoter activity in T cells [36]. Furthermore, it has been demonstrated that IFN-γ production was normal in NFATc3-deficient splenocytes [37]. In addition, NFAT5 has been shown to be activated by osmotic stress, but not by Ca2+ signaling [38]. Thus, we focused on NFATc1. In wild-type and Myd88−/−/Trif−/− BMφ, T. cruzi trypomastigotes infection induced nuclear translocation of NFATc1 (Figure 5B). T. cruzi-infected nuclear translocation of NFATc1 was blocked by the pre-treatment with BAPTA-AM in wild-type and Myd88−/−/Trif−/− BMφ (Figure 5A). These results indicate that NFATc1 is activated in response to T. cruzi infection in a TLR-independent manner. Next, we analyzed whether NFATc1 was involved in the T. cruzi-induced IFN-γ production. We obtained RAW264.7 macrophage clones expressing different levels of NFATc1.
**Discussion**

In the present study, we analyzed TLR-independent innate immune responses against the intracellular protozoan parasite *T. cruzi*. *T. cruzi*-infected Myd88<sup>−/−</sup> Trif<sup>−/−</sup> mice displayed normal Th1 responses and normal DC maturation. A comprehensive analysis of gene expression profiles of *T. cruzi*-infected DCs identified IFN-γ as a TLR-independent gene which mediated DC maturation and Th1 responses even in the absence of TLR signaling. *T. cruzi* infection induced an increase in intracellular Ca<sup>2+</sup> level in DCs and macrophages, which led to NFATc1 activation and IFN-γ induction in a TLR-independent manner. In *Nfatc1<sup>−/−</sup>* DCs, *T. cruzi*-induced IFN-γ production and DC maturation was impaired. These findings demonstrate that NFATc1 is responsible for TLR-independent innate immune responses during *T. cruzi* infection.

The family of TLRs has been established to be critical for the innate recognition of *T. cruzi* [14]. TLR signaling pathways consist of two major components mediated by MyD88 and TRIF [33,34]. Myd88<sup>−/−</sup> mice show a high susceptibility to *T. cruzi* infection [15,16], while mice deficient in both MyD88 and TRIF are even more susceptible to *T. cruzi* infection [17]. These findings indicate that TLR-dependent recognition of *T. cruzi* is crucial to the host defense against the parasite. In this regard, TLR-dependent induction of IFN-β might be responsible for higher susceptibility to *T. cruzi* infection in Myd88<sup>−/−</sup> Trif<sup>−/−</sup> mice in spite of the normal Th1 responses [17].

A previous study showed the MyD88-dependent IFN-γ production in *T. cruzi*-infected mice [16]. However, surprisingly, we found that Myd88<sup>−/−</sup> Trif<sup>−/−</sup> mice exhibited normal Th1-dependent IFN-γ production. Discrepancy between both studies might be due to distinct experimental protocols. IFN-γ is known to facilitate IL-12 production. Indeed, IL-12p40-deficient mice were highly susceptible to *T. cruzi* infection with severely reduced Th1 responses [33,34]. In *T. cruzi*-infected Myd88<sup>−/−</sup> Trif<sup>−/−</sup> mice, IL-12p40 production was severely reduced, but still induced [17], suggesting that IL-12 is produced via TLR-dependent and -independent pathways. Considering that *T. cruzi*-infected *Ifng<sup>−/−</sup>* mice showed decreased level of serum IL-12p40 and that *T. cruzi*-infected *Nfatc1<sup>−/−</sup>* FLDCs exhibited reduced expression of IL-12p40, NFATc1-dependent IFN-γ production may facilitate IL-12p40 production. Alternatively, the direct involvement of NFATc1 in activation of IL-12p40 gene has been also shown [23]. Collectively, *T. cruzi* infection might cause not only the TLR-dependent IL-12p40 production, but also the NFATc1-mediated (TLR-independent) production of IFN-γ and IL-12p40, coordinating the host Th1 response.

IFN-γ was identified as a gene induced in *T. cruzi*-infected Myd88<sup>−/−</sup> Trif<sup>−/−</sup> DCs. IFN-γ production by DCs was first demonstrated in IL-12-stimulated CD8<sup>+</sup> lymphoid DCs [44]. Subsequently, CD11c<sup>lo</sup>B220<sup>−</sup>NK1.1<sup>−</sup> cells were shown to produce high amounts of IFN-γ in response to IL-12 or a TLR9 agonist.
Figure 3. IFN-γ dependent DC maturation and Th1 response in *T. cruzi* infection. (A) Bone marrow DCs of wild-type, *Ifngr1*<sup>−/−</sup>, and *Myd88*<sup>−/−</sup> *Trif*<sup>−/−</sup> *Ifngr1*<sup>−/−</sup> mice were infected with *T. cruzi* for 6 h, then washed and cultured for 48 h. The cells were analyzed for expression of CD40, CD86, and I-A<sup>b</sup> using flow cytometry. Representative results are shown from three independent experiments. (B) Wild-type, *Ifngr1*<sup>−/−</sup>, and *Myd88*<sup>−/−</sup> *Trif*<sup>−/−</sup> *Ifngr1*<sup>−/−</sup> mice were infected with *T. cruzi*. At six days after infection, CD4<sup>+</sup> T cells were isolated from the spleen, and then stimulated with freeze-thawed *T. cruzi* in the presence of antigen presenting cells. After 24 h, supernatants were collected and assayed for IFN-γ production by ELISA. The values are the means±s.d. of four independent experiments each carried out in triplicate. #<em>P</em> < 0.05.

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Figure 4. Ca\(^{2+}\) signaling-dependent IFN-\(\gamma\) production in \(T. cruzi\)-infected DCs. (A) Bone marrow DCs were stimulated with live \(T. cruzi\) or \(T. cruzi\) killed by repeated freeze-thaw steps for the indicated periods. Next, total RNA was isolated and analyzed for \(Ifng\) expression by real-time RT-PCR. The fold differences of each sample relative to EF1\(\alpha\) are shown. *: not detected. (B) Bone marrow M\(\phi\) from wild-type and \(Myd88^{-/-}\) \(Trif^{-/-}\) mice were treated with Fluo-4AM for 30 min, and washed. Then, cells which were infected or non-infected with \(T. cruzi\) were analyzed by fluorescence microscopy at the indicated periods. Representative of three independent experiments. (C) Bone marrow DCs from wild-type and \(Myd88^{-/-}\) \(Trif^{-/-}\) mice were pre-treated with BAPTA-AM (100 \(\mu\)M) for 30 min, and washed. Then, cells were infected with \(T. cruzi\) for 3 h. Expression of \(Ifng\) was analyzed by real-time RT PCR. Bone marrow DCs from wild-type mice were stimulated with LPS (100 ng/ml) for 3 h, and analyzed for expression of \(Il6\) and \(Tnf\). (D) Bone marrow M\(\phi\) from wild-type and \(Myd88^{-/-}\) \(Trif^{-/-}\) mice were stimulated with 5 \(\mu\)M Ca\(^{2+}\) ionophore plus 50 ng/ml PMA or 5 \(\mu\)M Ca\(^{2+}\) ionophore for the indicated periods, and analyzed for expression of \(Ifng\). Data are mean \(\pm\) s.d., and representative one of three independent experiments. *: not detected.

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Figure 5. T. cruzi-induced activation of NFATc1 in Myd88<sup>−/−</sup> Trif<sup>−/−</sup> DCs and Mφ. (A) Wild-type and Myd88<sup>−/−</sup> Trif<sup>−/−</sup> bone marrow Mφ were infected with T. cruzi or stimulated with LPS for 6 h in the presence or absence of FK506 (0.5 µM or 2.5 µM). Next, total RNA was isolated and analyzed for Ifng, Tnf or Il6 expression by real-time RT-PCR. The fold differences of each sample relative to EF1<sub>a</sub> are shown. *: not detected. (B) Bone marrow Mφ from wild-type and Myd88<sup>−/−</sup> Trif<sup>−/−</sup> mice were transfected with the NFATc1 expression plasmid. Cells were then infected with T. cruzi for 30 min., and stained with anti-NFATc1 antibody (red) and DAPI (blue). (C) RAW 264.7 cell clones (designated #3, #7, and #9) transfected with the NFATc1 expression plasmid were analyzed for expression of NFATc1 by immunoblot with antibodies specific for NFATc1 and β-actin. (D) RAW 264.7 cells expressing NFATc1 were infected with T. cruzi for 3 h. Next, total RNA was extracted and used for real-time RT-PCR analysis using primers specific for Ifng, Stat1, and Irgm. (E) RAW 264.7 cells expressing NFATc1 (clone #9) were infected with T. cruzi for 3 h in the presence or absence of FK506, and total RNA was extracted. Real-time RT-PCR analysis was performed using primers specific for Ifng. *: not detected. Data are mean±s.d., and a representative result of at least three independent experiments.

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NFATC1 induced expression in these cells (our unpublished data). In addition, T. cruzi survival within the host cells [19]. In addition, the elevation of intracellular Ca\(^{2+}\) concentration in the host cells to promote gene expression [51]. The role of NFAT family of transcription factors is involved in these processes.

Protozoan parasites including T. cruzi require Ca\(^{2+}\) for their survival within the host cells [19]. In addition, T. cruzi evokes elevation of intracellular Ca\(^{2+}\) concentration in the host cells to establish the invasion [49,50]. Our findings indicate that T. cruzi-induced activation of host Ca\(^{2+}\) signaling mediates IFN-\(\gamma\) production. In the host cells, the family of NFAT transcription factors, which is activated by calmodulin/calciuemin, is known to bridge Ca\(^{2+}\) to promote gene expression [51]. The role of NFAT proteins has been well characterized in T lymphocytes, and can induce activation of the Ifng gene [22,52]. However, the role of NFAT proteins in innate immune cells remains unclear. Several reports indicate that NFAT proteins are activated in macrophages [24,33]. In addition, cyclosporin A, which blocks calcineurin-independent NFAT activation, has been shown to inhibit DC functions [54,55]. In accordance with these reports, in the present study NFATC1 was activated in Mydd88\(^{-/-}\), Trif\(^{-/-}\), and Mydd88\(^{-/-}\) Trif\(^{-/-}\) mice were infected with T. cruzi for 3 h, and analyzed for Tbx21 expression by real-time RT-PCR. Data are mean+s.d., and a representative result of at least three independent experiments. (B) RAW 264.7 cells were transiently transfected with either T-bet or NFATc1 expression vector, or both expression vectors together with the IFN-\(\gamma\) reporter plasmid. After 18 h of transfection, the cells were infected with T. cruzi for 18 h and activity of the reporter analyzed by luciferase assay. Data indicate mean+s.d., and a representative result of at least three independent experiments.

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Figure 6. NFATC1 and T-bet had a synergistic effect on activation of the Ifng promoter. (A) Peritoneal M\(\phi\) from wild-type, Mydd88\(^{-/-}\), Trif\(^{-/-}\), and Mydd88\(^{-/-}\) Trif\(^{-/-}\) mice were infected with T. cruzi for 3 h, and analyzed for Tbx21 expression by real-time RT-PCR. Data are mean+s.d., and a representative result of at least three independent experiments. (B) RAW 264.7 cells were transiently transfected with either T-bet or NFATc1 expression vector, or both expression vectors together with the IFN-\(\gamma\) reporter plasmid. After 18 h of transfection, the cells were infected with T. cruzi for 18 h and activity of the reporter analyzed by luciferase assay. Data indicate mean+s.d., and a representative result of at least three independent experiments.

In summary, in the present study we revealed a new TLR-independent mechanism for the interaction between protozoan parasites and host innate immunity. Ca\(^{2+}\) is critical for both living organisms, and therefore the parasite utilizes host Ca\(^{2+}\) for its benefit. On the host side, Ca\(^{2+}\) signaling leads to activation of NFATc1 to eliminate the parasite. It would be interesting in the future to analyze the precise role of NFATc1 in protozoan parasite infection using innate immune cell-specific NFATc1-deficient mice.

Materials and Methods

Mice

All animal experiments were conducted in accordance with guidelines of the Animal Care and Use Committee of Osaka University and Kyushu University. Mydd88\(^{-/-}\), Trif\(^{-/-}\), Ifng\(^{f/f}\), and Nfatc1\(^{f/f}\) mice were generated as previously described [17,42]. Each mouse strain was backcrossed to C57BL/6 for at least five generations, and then used to generate double or triple-mutant mice.
**Reagents**

PE-conjugated anti-CD11c, PE-conjugated anti IFN-γ, APC-conjugated anti-CD11c, APC-conjugated anti-CD40, FITC-conjugated anti-NK1.1, FITC-conjugated anti-CD40, FITC-conjugated anti-CD86, FITC-conjugated anti-I-Ab and Pacific Blue-conjugated anti-B220 antibodies were purchased from BD Pharmingen. Anti-NFATc1 and anti-β actin antibodies were purchased from Santa Cruz. Ca²⁺ ionophore A23187 (C7522), PMA (P1585), and FK506 (F4679) were purchased from Sigma. Fluo-4 AM was purchased from Invitrogen. BAPTA-AM was from Calbiochem. Cycloheximide was from Nacalai tesque.

**Preparation of macrophages (Mϕ), dendritic cells (DCs), and antigen presenting cells (APC)**

To isolate peritoneal Mϕ, mice were i.p. injected with 2 ml of 4% thioglycolate medium (Sigma), and peritoneal exudate cells were isolated from the peritoneal cavity at three days post injection. The cells were incubated for 24 h in the presence or absence of regeneration IFN-γ. Surface expression of CD40, CD86, and I-A^b^ was analyzed by flow cytometry. Data are shown mean±s.d. of triplicate samples and a representative of four independent experiments.

**Figure 7. Defective T. cruzi-induced IFN-γ response in Nfatc1^−/−^ DCs.**

(A, B) Wild-type and Nfatc1^−/−^ FLDCs were infected with T. cruzi for the indicated periods. Next, total RNA was extracted, and analyzed for expression of *Ifng*, *Tnf* and *Il6*. *: not detected. (C) Wild-type and Nfatc1^−/−^ FLDCs were infected with T. cruzi for 6 h, then washed and cultured for 24 h in the presence or absence of recombinant IFN-γ. Surface expression of CD40, CD86, and I-A^b^ was analyzed by flow cytometry. Data are shown mean±s.d. of triplicate samples and a representative of four independent experiments.

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and 10 ng/ml GM-CSF (Pepro Tech) or 30% L cell culture supernatant, respectively. After six days, the cells were used as bone marrow DCs or bone marrow Mφ in experiments. To prepare fetal liver-derived DCs (FLDCs), fetal liver (FL) were obtained from 12.5 days post-coitum murine embryos, and FL cells were dissociated by pipetting, passed through a nylon mesh, and then cultured in RPMI 1640 medium supplemented with 10% FBS, 100 μM 2ME, 20 ng/ml GM-CSF, 10 ng/ml Flt3 ligand (Pepro Tech), and 10 ng/ml SCF (Pepro Tech) as described [57]. After eight days, the floating cells were used as FLDCs in experiments. RAW 264.7 cells were transfected with the NFATc1 (pcDNA3) expression plasmid. The cells expressing NFATc1 were selected in the presence of 0.4 mg/ml G418 and cloned. Splenocytes from wild-type mice were irradiated (30 Gy) and used as APC.

Parasite and experimental infection

The trypomastigote stage of T. cruzi Tulahuen strain was maintained in vivo in Ifng−/− mice by passages every other week or in vitro in LLC-MK2 cells by passages every four days. For in vivo experiments, 5×10^5 Mφ or DCs were infected with 1.5×10^6 trypomastigotes. For in vivo experiments, mice were i.p. injected with 6×10^6 trypomastigotes or PBS. Epimastigotes of Tulahuen strain were grown at 26°C in liver infusion tryptose liquid medium, supplemented with 2.5% hemoglobin and 10% fetal calf serum.

Real-time RT-PCR

Total RNA was isolated with TRIzol reagent (Invitrogen), and 1–2 μg of RNA was reverse transcribed using M-MLV reverse transcriptase (Promega) and random primers (Toyobo) after treatment with RQ1 DNase I (Promega). Real-time RT-PCR was performed on an ABI 7300 (Applied Biosystems) using the TaqMan Universal PCR Master Mix (Applied Biosystems). All data were normalized to the corresponding gene expression, and the fold difference relative to the EF-1α was calculated. Amplification conditions were: 50°C (2 min), 95°C (10 min), 40 cycles of 95°C (15 s), and 60°C (60 s). Primers of Tbx21, Stat1, Il12b, and Tnf were purchased from Assay on Demand (Applied Biosystems). Sequences for EF-1α forward primer 5′-aagtagggaaggccgtggtt-3′ and reverse primer 5′-actggaagccacaaccagtg-3′, reverse primer 5′-ggcttcgctggccccagataaa-3′, B6 probe 5′-cttcttgggactgatgctggtgaca-3′, forward primer 5′-gcaaaagaatgaccccttccttta-3′, reverse primer 5′-agatggaggaggtggtctttg-3′, and Ifng probe 5′-gctaacccttcgctggcttcttt-3′, forward primer 5′-tcaagtggcatagatgtggaagaa-3′, and reverse primer 5′-tcaagtggcatagatgtggaagaa-3′.

Intracellular cytokine staining

Splenic cells were isolated from T. cruzi-infected mice at the indicated time point and stimulated with PMA and ionomycin for 4 h in the presence of 10 μg/ml brefeldin A. In experiments to detect IFN-γ production from CD11c+ cells, splenic cells were infected with T. cruzi (1:1) for 12 h, and further cultured for 6 h in the presence of 10 μg/ml brefeldin A. After staining of surface CD11c, CD4, CD8 or NK1.1, the cells were fixed with CytopermCytofix (BD Biosciences) for 20 min and incubated with PE-conjugated anti-IFN-γ Ab. Flow cytometric analysis was performed on FACS Canto II (BD Biosciences).

Measurement of cytokine production

For in vivo experiments, mice were i.p. injected with T. cruzi, and CD4^+ T cells were isolated from the spleen at the indicated days after the infection. 2.5×10^6 CD4^+ T cells were stimulated with anti-CD3 Ab or freeze-thawed T. cruzi in the presence of 2.5×10^5 APC for 24 h. The culture supernatants were collected and diluted at 1:5, ELISA was performed with anti-mouse IFN-γ Ab, avidin-HRP, and TMB solution purchased from eBioscience. Optical densities were determined at 450 nm wavelengths with reference at 570 nm. Levels of IFN-γ were calculated from the standard curve by using purified mouse IFN-γ purchased from eBioscience.

Flow cytometry

Bone marrow DCs or FLDCs were infected with T. cruzi for 6 h, washed and then cultured for 24 or 48 h. The T. cruzi-infected cells were stained with the combination of PE-conjugated anti-CD11c and the indicated antibodies at 4°C for 20 min, and washed. Flow cytometric analysis was performed on FACSCalibur or FACScant II flow cytometer (BD Biosciences) and using FlowJo software (Tree Star). CD11c^high^ cells and NK cells were sorted using FACS Aria (BD Biosciences). The instrumental compensation was set in each experiment using single color, 2-color or 4-color stained samples.

Intracellular calcium measurements

This assay was performed as described [58]. In brief, bone marrow Mφ were plated on glass-bottom dishes were incubated in serum-free RPMI 1640 supplemented with 2 μM Fluo-4 AM, the increase in fluorescent intensity of which indicates increased Ca^{2+} level, at 37°C for 30 min. The cells were then washed to remove the free extracellular dye, and were maintained in culture medium during the whole experiment. The analysis of changes of basal intracellular calcium concentrations in response to T. cruzi infection was performed using an IX71 fluorescence microscope (Olympus).

Immunofluorescence microscope

Bone marrow Mφ were transfected with pcDNA3-NFATc1 by nucleofection (mouse macrophage nucleofector kit; Amaxa). After 24 h, the cells were infected with T. cruzi for 30 min, washed with Tris-buffered saline (TBS), and then fixed with 3.7% formaldehyde in TBS for 15 min at room temperature. After permeabilization with 0.2% Triton X-100, cells were washed with TBS, incubated with anti-NFATc1 antibody in TBS containing 1% bovine serum albumin, then incubated with Alexa Fluor 594-conjugated goat anti-mouse immunoglobulin G (Molecular Probes). To stain the nucleus, cells were cultured with 0.5 mg/ml 4, 6-diamidino-2-phenylindole (DAPI; Wako). Stained cells were analyzed using an LSM510 confocal microscope (CarlZeiss).

Luciferase assay

RAW 264.7 cells were transfected with the indicated expression plasmids together with the reporter plasmid IFN-γ-Luc and the internal control plasmid pHRG-TK by Nucleofection (Nucleofector Kit V; Amaxa). After 18 h, the cells were infected with T. cruzi for 18 h, and the luciferase activities of whole cell lysates were measured using the Dual-luciferase reporter assay system (Promega) and Lumat LD 9507 (Berthold).

Statistical analysis

Differences between control and experimental groups were evaluated by the Student’s t-test.

Supporting Information

Figure S1 IFN-γ production from lymphocytes after T. cruzi infection. (A) Splenocytes were isolated from wild-type, Myd88^−/− and Myd88^−/−/Trif^−/− mice at 10 days after T. cruzi infection, and
stimulated with 1 μg/ml ionomycin plus 50 ng/ml PMA. After surface staining with APC-conjugated anti-CD4 Ab, the cells were permeabilized and then stained with PE-conjugated anti-IFN-γ Ab, and analyzed by flow cytometry. Representative results are shown from four independent experiments. The percentages of IFN-γ-producing CD4+ cells of individual mice are shown. (B) Splenocytes were isolated from wild-type and Myd88−/−Trif−/− mice at 10 days after T. cruzi infection, and stimulated with 1 μg/ml ionomycin plus 50 ng/ml PMA. After surface staining with FITC-conjugated anti-CD11c and the indicated antibodies at 4°C, the cells were permeabilized and then stained with PE-conjugated anti-IFN-γ Ab, and analyzed by flow cytometry. Representative results are shown from two independent experiments. The percentages of IFN-γ-producing NK1.1+ or CD69+ cells of individual mice are shown.

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**Figure S2** Microarray analysis of T. cruzi-infected DCs. Bone marrow DCs from wild-type, Myd88−/− and Myd88−/−Trif−/− mice were infected with T. cruzi for 6 h. Then, microarray analysis was performed using 5 μg of total RNA. Data are shown in fold-increase of T. cruzi-infected cells compared with non-infected cells. Genes shown by yellow colored boxes have been reported as IFN-γ inducible genes.

Found at: doi:10.1371/journal.ppat.1000514.s002 (0.04 MB PDF)

**Figure S3** TLR-independent expression of IFN-γ-inducible genes in T. cruzi-infected DCs. Bone marrow DCs from wild-type, Myd88−/− and Myd88−/−Trif−/− mice were infected with T. cruzi for 6 h. Then, microarray analysis was performed using 5 μg of total RNA. Data are shown in fold-increase of T. cruzi-infected cells compared with non-infected cells. Genes shown by yellow colored boxes have been reported as IFN-γ inducible genes.

Found at: doi:10.1371/journal.ppat.1000514.s003 (0.04 MB PDF)

**Figure S4** Expression of IFN-γ-inducible genes in T. cruzi-infected peritoneal Mφ. (A, B, C) Peritoneal Mφ from wild-type, Myd88−/−, Trif−/−, Myd88−/−Trif−/− and Ifngr1−/− mice were infected with T. cruzi for the indicated periods. Total RNA was extracted, and used for real-time RT-PCR analysis using primers specific for Ifng, Stat1 and Irgm. All data were normalized to the corresponding gene Eif1al encoding elongation factor-1α (EF1α) expression, and the fold difference relative to the E1Fα was shown. Data are a representative of three independent experiments. *; not detected.

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**Figure S5** Low level of IFN-γ expression in IL-12/IL-18 stimulated CD11c+ cells. (A) Spleenic CD11c+ cells (CD11c+ B220− NK1.1−) and NK cells (CD11c− B220− NK1.1+) were sorted by FACS (BD Bioscience). Numbers indicate percentages of CD11c+ NK1.1− and CD11c− B220− cells. (B) These cells were stimulated with 10 ng/ml IL-12 plus 10 ng/ml IL-18 for 6 h. Total RNA was isolated, and then Ifng mRNA expression was quantified by real-time RT-PCR and normalized to the level of EF1α. Data indicate mean±s.d. and a representative result of two independent experiments. *; not detected.

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**Figure S6** IFN-γ-dependent DC maturation and host defense in T. cruzi infection. (A) Bone marrow DCs from wild-type and Ifngr1−/− mice were stimulated with 10 ng/ml murine IFN-γ for 48 h. IFNγ-stimulated DCs were stained with the combination of PE-conjugated anti-CD11c and the indicated antibodies at 4°C for 20 min, and washed. Flow cytometry analysis was performed on FACS Canto II (BD Bioscience). (B) Wild-type (n = 9), Myd88−/−Trif−/− (n = 5) and Ifngr1−/− (n = 11) mice were intraperitoneally infected with 1×10⁴ T. cruzi. Serum numbers of trypanostigotes were monitored at the indicated times after infection. Note that many of Ifngr1−/− mice died before 15 days of the infection.

Found at: doi:10.1371/journal.ppat.1000514.s006 (0.03 MB PDF)

**Figure S7** IL-12 dependent Th1 response in T. cruzi infection. (A) Wild-type (n = 4) and Ifngr1−/− (n = 4) mice were intraperitoneally infected with 60 T. cruzi. At 6 days after infection, CD4+ T cells were isolated from the spleen, and then stimulated with freeze-thawed T. cruzi in the presence of antigen presenting cells. After 24 h, supernatants were collected and assayed for IFN-γ production by ELISA. #:P<0.00066. *; not detected. (B) Wild-type (n = 2) and Ifngr1−/− (n = 2) mice were intraperitoneally infected with 60 T. cruzi. At 3 days postinfection with T. cruzi, concentrations of IL-12p40 in the sera from infected mice were quantified by ELISA. *; not detected.

Found at: doi:10.1371/journal.ppat.1000514.s007 (0.02 MB PDF)

**Figure S8** Impaired activation of MAP kinases and NF-xB in T. cruzi-infected Mφ. (A) Bone marrow DCs were infected with T. cruzi for the indicated periods. Cell lysates were analyzed by Western blot analysis using antibodies specifically recognizing the indicated proteins. (B) Bone marrow DCs were infected with T. cruzi for the indicated periods. Nuclear extracts were subjected to EMSA using a radiolabeled oligonucleotide containing the murine xB site of the TNF promoter.

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**Figure S9** T. cruzi-dependent increase in Ca²⁺ concentration in Mφ. (A) Cells showing bright fluorescence at 15 min after T. cruzi infection were counted. Average of numbers of cells with bright fluorescence (% in total cells counted) in twelve fields from three independent experiments (4 fields in each experiment) in ×400 magnification is shown. (B, C) Bone marrow Mφ from wild-type and Myd88−/−Trif−/− mice were incubated with Fluo-4AM for 30 min, then washed and infected with trypomastigotes or epimastigotes for the indicated periods. The cells were analyzed by IX71 fluorescence microscope (Olympus). Epimastigotes of Tulahuen strain were grown at 26°C in liver infusion tryptose liquid medium, supplemented with 2.5% hemoglobin and 10% fetal calf serum.

Found at: doi:10.1371/journal.ppat.1000514.s009 (0.23 MB PDF)

**Figure S10** Effect of Ca²⁺ chelator on T. cruzi-induced response in Mφ. (A) Peritoneal Mφ from wild-type mice were pre-incubated with 100 μM BAPTA-AM for 30 min in the presence of Fluo-4AM, then washed and infected or none-infected with T. cruzi for the indicated periods. Then, cells were analyzed by fluorescence microscopy. Representative of five independent experiments. (B) Cells showing bright fluorescence were counted at 10 min after T. cruzi infection, and average of total fifteen fields (from five independent experiments) is shown.

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**Figure S11** Expression of NFAT family member in bone marrow DCs. Total RNA was isolated from bone marrow DCs of wild-type and Myd88−/−Trif−/−. Microarray analysis was performed from 5 μg of total RNA. Levels of mRNA expression of NFAT family members are shown as average difference.

Found at: doi:10.1371/journal.ppat.1000514.s011 (0.01 MB PDF)

**Figure S12** Ca²⁺-dependent nuclear translocation of NFATc1 in T. cruzi-infected Mφ. Bone marrow-derived macrophages from wild-type mice (A) and Myd88−/−Trif−/− mice (B) were transfected with the NFATc1 expression plasmid. Cells were treated with BAPTA-AM (100 μM) for 30 min and washed, and...
then infected with *T. cruzi* for 30 min. *T. cruzi*-infected cells were stained with anti-NFATc1 antibody (red) and DAPI (blue). The right panels show the percentage of nuclear translocated NFATc1 in total cells (mean ± SEM) in each experiment. (A) Fetal liver DCs from 12.5 d.p.c. wild-type and NFATc1/−/− embryos were cultured with 20 ng/ml GM-CSF, 10 ng/ml Flt3 ligand, and 10 ng/ml SCF for 8 days. Expression of CD11c was analyzed and shown to be comparable between both genotypes. CD11c+ cells were enriched by MACS (Miltenyi Biotec) and used for experiments as FLDCs.

**Figure S13** Generation of CD11c+ cells from NFATc1/−/− fetal liver cells. Fetal liver cells from 12.5 d.p.c. wild-type and *T. cruzi*-infected cells were cultured with 20 ng/ml GM-CSF, 10 ng/ml Flt3 ligand, and 10 ng/ml SCF for 8 days. Expression of CD11c was analyzed and shown to be comparable between both genotypes. CD11c+ cells were enriched by MACS (Miltenyi Biotec) and used for experiments as FLDCs.

**Figure S14** Response of NFATc1/−/− FLDCs to LPS and *T. cruzi*. (A) Fetal liver DCs from 12.5 d.p.c. wild-type and NFATc1/−/− embryos were stimulated with 100 ng/ml LPS for 24 h. LPS-stimulated FLDCs were stained with the combination of PE-conjugated anti-CD11c and the indicated antibodies at 4°C for 30 min. (B) Fetal liver DCs from 12.5 d.p.c. wild-type and NFATc1/−/− embryos were infected with *T. cruzi* for the indicated periods. Total RNA was extracted, and used for real-time RT-PCR analysis using primers specific for *Il12b*. All data were normalized to the corresponding gene *Esf1α* encoding elongation factor-1α (*Eef1a1*) expression, and the fold difference relative to the *Esf1α* is shown.

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**Author Contributions**

Conceived and designed the experiments: HK RK KT. Performed the experiments: HK RK KA TK. Analyzed the data: HK RK KH MY. Contributed reagents/materials/analysis tools: HK RK MO TWM SU SA HT. Wrote the paper: HK KT.

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