Interferon-γ Restricts Toxoplasma gondii Development in Murine Skeletal Muscle Cells via Nitric Oxide Production and Immunity-Related GTPases

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Abstract

The apicomplexan parasite Toxoplasma gondii is regularly transmitted to humans via the ingestion of contaminated meat products from chronically infected livestock. This route of transmission requires intracellular development and long-term survival of the parasite within muscle tissue. In this study, we determined the cell-autonomous immunity of mature primary embryonic or C2C12 skeletal muscle cells (SkMCs) to infection with T. gondii. Non-activated SkMCs and control fibroblasts sustained parasite replication; however, interferon (IFN)-γ significantly inhibited parasite growth in SkMCs but not in fibroblasts. Intracellular parasite replication was diminished by IFN-γ whereas host cell invasion was not affected. Tumor necrosis factor (TNF) did not further increase the IFN-γ-triggered host defense of SkMCs against Toxoplasma. Remarkably, IFN-γ alone or in combination with TNF decreased the high level of T. gondii bradyzoite formation being observed in non-activated SkMCs. Stimulation of SkMCs with IFN-γ strongly triggered expression of inducible nitric oxide synthase (iNOS) transcripts, and induced significantly higher levels of nitric oxide (NO) in SkMCs than in fibroblasts. Consequently, pharmacological inhibition of iNOS partially abrogated the IFN-γ-induced toxoplasmal activity of SkMCs. In addition, SkMCs strongly up-regulated immunity-regulated GTPases (IRGs) following stimulation with IFN-γ. IRGs accumulated on Toxoplasma-containing vacuoles in SkMCs in a parasite strain-dependent manner. Subsequent vacuole disruption and signs of degenerating parasites were regularly recognized in IFN-γ-treated SkMCs infected with type II parasites. Together, murine SkMCs exert potent toxoplasmal activity after stimulation with IFN-γ and have to be considered active participants in the local immune response against Toxoplasma in skeletal muscle.

Introduction

Skeletal muscle plays a critical role in the transmission of the zoonotic parasite Toxoplasma gondii to humans. Between 30% and 63% of human infections have been related to the consumption of undercooked or cured meat products as revealed by a multi-centre study involving acutely infected pregnant women and non-infected controls [1]. Although infection is mostly asymptomatic or benign, the parasite is a significant threat for individuals with a premature immune system and can lead to severe and life-threatening toxoplasmosis. Transmission of T. gondii to humans via the ingestion of contaminated meat products may depend on the ingestion of chronically infected livestock and poultry. We have shown previously that these cells, after differentiation in vitro to mature myotubes, indeed provide a niche which sustains intracellular development and differentiation to the bradyzoite stage of the parasite [2].

During embryogenesis or following muscle injury, SkMCs transform from proliferating and fusogenic stem cells, i.e. myoblasts to multinucleated myotubes which further differentiate to large syncytial muscle fibers [3]. Mature SkMCs provide a unique immunological environment for the development of pathogens, with no detectable expression of major histocompatibility complex (MHC) class I and class II expression under physiological conditions [4]. Furthermore, expression of HLA-G or the B7 homologue B7-H1 (PD-L1) by human myoblasts fulfills tolerizing or even suppressive functions within muscle tissue [5,6]. Limited immune reactions in skeletal muscle may thus facilitate long-term survival of Toxoplasma and make this organ to one of the preferred body sites where tissue cysts persist until orally ingested by a new host [7]. Under certain conditions, i.e. after activation by proinflammatory cytokines in vitro or during inflammatory myopathies in vivo, however, muscle cells can express MHC class I and II antigens and present antigens to T cells [8,9]. In addition, they express a variety of cell surface receptors and soluble immune mediators and thus fulfill requirements for immunocompetence. During reactivation of chronic toxoplasmosis or during acute disease following recent infection, the occurrence of polymyositis has been well established in both humans and animals [10,11,12,13,14]. Although immune cells infiltrating the infected muscle tissue certainly mediate much of the local inflammatory response, this supports the view that SkMCs contribute to the immune response to T. gondii within muscle tissue and may be...
pivotal during toxoplasmic myopathies. However, the impact of SkMCs in the local host response to Toxoplasma and host factors or molecular mechanisms which might limit parasite development in SkMCs have not yet been determined.

Resistance to infection with obligate intracellular Toxoplasma parasites largely depends on Th1-type cell-mediated immune responses. Interferon (IFN)-γ released from CD4+ and CD8+ T lymphocytes is the most critical mediator of immunity against T. gondii [15,16,17,18]. Early during infection, natural killer (NK) cells are the main producers of IFN-γ and are important mediators of innate immunity [19]. IFN-γ activates effector cells of both hematopoietic and non-hematopoietic origin to exert anti-Toxoplasma activity [20]. Tumor necrosis factor (TNF), interleukin (IL)-1 and IL-6 synergize with IFN-γ to strengthen the anti-parasitic response [21,22]. They exert anti-parasitic activity by up-regulating the expression of effector molecules in various cell types. Depending on the host species, control of intracellular T. gondii is mediated by production of nitric oxide (NO) by the inducible NO synthase (iNOS) [23,24], disruption of the parasitophorous vacuole by immunity-related GTPases (IRGps; formerly called p47 GTPases) and p65 guanylate-binding proteins (GBPps; also called p65 GTPases) [25,26,27], tryptophan starvation via up-regulation of the indoleamine 2,3-dioxygenase (IDO) [28], production of oxygen radicals [29], and activity of P2X7 receptors [30].

In this study, we determined the impact of IFN-γ and TNF on the development of T. gondii in mouse SkMCs that have been differentiated in vitro to mature myotubes. The results show that IFN-γ readily activates muscle cells to restrict parasite replication but does not trigger differentiation from the rapidly replicating tachyzoite to the slowly replicating bradyzoite stage. NO production mediated by iNOS and disruption of the Toxoplasma PV by IRG activity may be instrumental in restricting parasite propagation in SkMCs. These results establish SkMCs as immunocompetent effector cells in the response to Toxoplasma within skeletal muscle.

Results

In vitro differentiation of SkMCs

Differentiation of primary embryonic skeletal muscle cells after in vitro cultivation for 6 days has been shown previously by the presence of multinucleated myotubes and the up-regulation of muscle-specific transcription factors MyoD and Myf5 [2]. Here, we also determined the differentiation of C2C12 mouse myoblasts to mature myotubes. The results show that transfer of C2C12 myoblasts into differentiation medium induced significant levels of myogenin mRNA 72 hours after seeding which further increased during the following 6 days (Fig. 1A). Up-regulation of mRNA of the basic helix-loop-helix transcription factor MyoD was slightly delayed as compared to myogenin mRNA but also continuously increased starting from 120 hours post seeding until the end of the observation (Fig. 1A). Immunoblotting confirmed the expression of muscle-specific transcription factors myogenin and MyoD during cultivation of C2C12 cells in differentiation medium, with the highest levels being observed between 72 and 168 hours after seeding the cells (Fig. 1B). In addition, myosin heavy chain (MyHC) which is indicative for terminal differentiation of SkMCs was expressed from 72 hours after seeding onwards with highest levels being observed from 120 hours onwards (Fig. 1B). Immunofluorescence microscopy confirmed expression of MyHC in an increasing number of cells (Fig. 1C). Most of these cells were highly elongated and contained multiple nuclei indicating formation of mature myotubes. NIH/3T3 control fibroblasts did not express any of the muscle-specific proteins MyoD, Myf5 or MyHC as expected (Fig. 1B).

IFN-γ restricts T. gondii replication in SkMCs

In order to unravel the impact of IFN-γ and TNF on Toxoplasma development within muscular tissue, we determined parasite propagation in in vitro differentiated SkMCs and control
fibroblasts. Since mouse-virulent type I *T. gondii* strains are able to resist distinct defense mechanisms [31,32], we first used *lacZ*-transgenic parasites on the type I RH background. This enabled us to determine whether SKMCs are able to even restrict replication of relatively resistant parasite strains. Following treatment of primary mouse-derived myotubes with 100 U/ml IFN-γ, β-galactosidase reporter activity at 2 and 4 days post infection was significantly reduced as compared to untreated controls (*p*<0.05; Student's *t*-test; Fig. 2A). IFN-γ-mediated inhibition of parasite propagation was even more pronounced in differentiated C2C12 cells leading to β-galactosidase activity of only ~52% (*p*<0.05) and ~22% (*p*<0.01) of untreated controls at 2 or 4 days p.i., respectively (Fig. 2B). Although IFN-γ also restricted parasite growth in control fibroblasts, this reduction was statistically not significant (Fig. 2C). Dose-response analyses of the effect of IFN-γ at 2 and 4 days of treatment indicated an increasing anti-parasitic activity of the cytokine from as little as 0.1 U/ml to 100 U/ml, with the highest concentration largely abrogating parasite propagation (Fig. 2D). TNF enhanced IFN-γ activity only to a minor extent in C2C12 SKMCs and NIH/3T3 fibroblasts, but had no synergistic effect in primary SKMCs (Fig. 2A–C) suggesting that TNF is not of major relevance for limiting parasite propagation in SKMCs. Further experiments using wild type NTE parasites confirmed the ability of IFN-γ-activated SKMCs to restrict *Toxoplasma* replication and extended the above findings to mouse-avirulent type II strains. Since these parasites have a longer duplication time as compared to type I parasites, their use also enabled us to extend the observation periods without extensive host cell lysis. Immunoblot analyses confirmed a strong anti-*Toxoplasma* activity of IFN-γ in SKMCs. The abundance of soluble *Toxoplasma* proteins including the predominant surface antigen SAG1 indeed strongly increased in non-activated C2C12 myotubes, but not in those concomitantly activated with IFN-γ (Fig. 3A). In contrast, the levels of parasite proteins in infected NIH/3T3 similarly increased in IFN-γ-treated NIH/3T3 as in untreated controls. We also recognized that following treatment of differentiated SKMCs with IFN-γ, expression of myosin HC decreased considerably. Together, the data established that IFN-γ fulfills anti-parasitic activity against *T. gondii* in SKMCs and also influences the expression of muscle-specific marker proteins.

Propagation of *T. gondii* in IFN-γ-stimulated SKMCs can be restricted by decreased invasion or decreased intracellular replication. In order to distinguish between these possibilities, we fluorescently labeled *T. gondii* (strain NTE) following infection of SKMCs or fibroblasts for different periods of time and analyzed them microscopically. The results show significantly lower average numbers of parasites per parasitophorous vacuole in IFN-γ-treated primary SKMCs or C2C12 cells as compared to the untreated controls (*p*<0.05, Student's *t*-test; Fig. 3B). In contrast, the number of parasites per vacuole was only slightly reduced by IFN-γ in NIH/3T3 cells at 2 days post infection (*p*>0.05). TNF did not further reduce IFN-γ-regulated *T. gondii* replication in SKMCs or fibroblasts (Fig. 3B).

As reported previously, *T. gondii* efficiently invaded non-activated SKMCs [2], and this was similarly observed at 4 hours post infection in IFN-γ-activated SKMCs (Fig. 3C–D). From 48 hours onwards, the percentages of infected primary SKMCs and C2C12 cells strongly increased in the absence of IFN-γ, which reflected considerable parasite replication (see above), subsequent host cell lysis and infection of new host cells (Fig. 3C–D). In sharp contrast, following activation with IFN-γ alone or in combination with TNF, the percentages of infected cells slightly decreased.

Furthermore, the percentages of infected cells increased in both non-activated and activated fibroblasts although the increase was clearly lower after activation with IFN-γ or IFN-γ plus TNF as compared to non-activated controls (Fig. 3E). These results collectively established a major impact of IFN-γ but not TNF on the parasite replication in differentiated SKMCs.

**Bradyzoite formation is not accelerated in activated SKMCs**

In distinct cell types, proinflammatory cytokines including IFN-γ and TNF are appropriate triggers to induce stage differentiation from fast-replicating tachyzoites to dormant bradyzoites. We therefore wondered whether reduced replication of *T. gondii* in IFN-γ-treated SKMCs is accompanied by conversion towards the bradyzoite stage. For these experiments we used the NTE strain of *T. gondii*, since type II strains convert more readily to bradyzoites than type I strains. Double immunofluorescence microscopy using stage-specific antibodies showed that differentiated C2C12 SKMCs cultivated in the absence of any exogenous stimulus triggered high levels of bradyzoite formation already at 2 days after infection (Fig. 4A), thus confirming previous findings [2]. Differentiation towards the bradyzoite stage was most often observed in vacuoles which harbored 2 to 8 parasites after two days of infection, whereas larger vacuoles and single parasites mostly did not react with the C22 antibody (Fig. 4B, upper panel). Remarkably, the percentage of bradyzoite-containing vacuoles was considerably lower when SKMCs had been previously activated with IFN-γ or IFN-γ along with TNF (Fig. 4A). This was likely due to an increased appearance of vacuoles containing only one parasite (Fig. 4B) the latter being unable to convert to the bradyzoite stage [33,34]. Lower levels of bradyzoite-containing vacuoles after IFN-γ or IFN-γ/TNF activation of SKMCs as compared to non-activated SKMCs persisted until 144 hours after infection. We also recognized a general reduction in the number of bradyzoite-containing vacuoles during extended infection times and this was partially related to parasite-mediated host cell lysis and infection of new SKMCs. In conclusion, IFN-γ alone or combined with TNF appears inappropriate to enhance *T. gondii* bradyzoite formation in differentiated SKMCs.

**IFN-γ-triggered iNOS activity restricts *T. gondii* development in SKMCs**

Depending on the host species and the cell type, several effector mechanisms including the production of reactive nitrogen intermediates contribute to the anti-parasitic activity of IFN-γ in *Toxoplasma*-infected cells [24,33]. We therefore examined whether expression and activity of iNOS, the IFN-γ-regulated isoform of nitric oxide synthases might also contribute restricting *T. gondii* propagation in differentiated murine SKMCs. Stimulation of primary embryonic SKMCs or C2C12 SKMCs with IFN-γ led to much higher levels of iNOS mRNA at 24, 48 and 96 hours as compared to fibroblasts (Fig. 5A). Consequently, nitric oxide (NO) was produced by IFN-γ-activated C2C12 SKMCs at significantly higher concentrations than by IFN-γ-treated fibroblasts as revealed by measuring nitrite, a stable end product of NO (Fig. 5B; *p*<0.01). Additional stimulation with TNF increased IFN-γ-triggered NO production in both SKMCs and fibroblasts slightly, and NO levels were again higher in SKMCs than in fibroblasts (*p*<0.01). In contrast, only low levels of NO were produced by non-activated SKMCs and fibroblasts, and these did not differ significantly between both cell types.

In order to unravel the functional importance of IFN-γ-triggered NO production in restricting *Toxoplasma* development in SKMCs, iNOS activity was inhibited using L-NIL. Control experiments indicated that 100 μM L-NIL indeed completely
Toxoplasma is a parasite that can infect a variety of cell types, including muscle cells and fibroblasts. Research has shown that cell type-specific expression of IRG proteins plays a crucial role in restricting parasite development. In specifically differentiated muscle cells (SkMCs), IFN-γ significantly upregulates Irga6, a protein that localizes to parasite-containing vacuoles.

**Figure 2.** IFN-γ restricts *T. gondii* development in differentiated SkMCs more vigorously than in fibroblasts. *In vitro* differentiated primary embryonic SkMCs (A) or C2C12 SkMCs (B, D) and control NIH/3T3 fibroblasts (C) were infected or not with transgenic RH parasites expressing β-galactosidase (RH βGal; parasite-host cell ratio 2:1). (A–C) At the time of infection, cells were stimulated with 100 U/ml IFN-γ alone (cross-hatched bars) or along with 100 pg/ml TNF (black bars), or were left non-stimulated (open bars). At 2 or 4 days post infection (dpi), growth of β-galactosidase-expressing *T. gondii* was quantitated by a colorimetric CPRG assay. Bars represent means ± S.E.M. from at least three independent experiments. Significant differences between non-stimulated and stimulated cells are indicated (*p<0.05; **p<0.01). (D) Infected C2C12 SkMCs (open symbols) or non-infected controls (closed symbols) were stimulated with 0–100 U/ml IFN-γ for 2 or 4 days. Parasites were quantitated as described above. Results are means ± S.E.M. from 5 independent experiments.

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The expression of Irga6 in SkMCs, as compared to fibroblasts, was found to be significantly higher, indicating a more effective response to IFN-γ. This suggests a role for iNOS activity in restricting parasite development in these muscle cells. Moreover, the percentage of Irga6-positive vacuoles increased with time, correlating with the restriction of parasite replication. These findings provide insights into the mechanisms of immune response against *Toxoplasma* in muscle cells, highlighting the importance of cell type-specific expression of IRG proteins in the innate immune response.
completely surround the parasite as described previously in IFN-γ-treated astrocytes [25]. Furthermore, punctate structures that clearly reacted with anti-Toxoplasma serum became evident suggesting disintegration of the parasite (Fig. 6D, small arrowheads).

Despite the loading of distinct IRGs on PVs harboring type I, i.e. virulent T. gondii, they nevertheless avoid vacuole disruption [37,39], at least in part by phosphorylating IRGs through the parasite kinase ROP18 [41,42]. We therefore next compared loading of Irgb6 on vacuoles containing NTE and RHβGal T. gondii, since its presence at PVMs correlates with subsequent PV disruption and parasite death [37,39,40]. The results show that Irgb6 was efficiently deposited on vacuoles containing NTE but not RHβGal parasites (Fig. 7A). Loading of PVs with Irgb6 was already detected at 2 hours after infection/activation with IFN-γ and the percentage of Irgb6-positive PVs rose thereafter in NTE-infected SKMCs to approximately 45% (Fig. 7B). In contrast, in RHβGal-infected SKMCs, the percentage of Irgb6-positive vacuoles did not exceed 5%. We nevertheless observed a small proportion of SKMCs with RHβGal-containing PVs that also accumulated Irgb6 and that showed signs of PV disruption, e.g. rough or even disrupted PVM labeling (Fig. 7C) [25]. Together, the data strongly suggested that IRG-mediated disruption of parasite-containing vacuoles may contribute to the IFN-γ-mediated defense of SKMCs primarily but not exclusively against avirulent T. gondii.

Lysis of Toxoplasma vacuoles by IRG activity and parasite death can subsequently lead to necrotic death of the host cell [37]. Therefore, we next examined whether IFN-γ alone or in combination with TNF induces cell death in SKMCs or fibroblasts. IFN-γ or IFN-γ plus TNF indeed increased LDH activity in the supernatants of non-infected SKMCs until 4 days of stimulation as compared to untreated controls thus indicating a loss of cell viability (Fig. 8A). More importantly, however, IFN-γ did not specifically decrease the cell viability in Toxoplasma-infected SKMCs, as it would have been expected if IRG-mediated disruption of parasite PVs had led to host cell death (Fig. 8B). Instead, infection of C2C12 cells with T. gondii led to an increase of LDH activity particularly at later time points of infection, and this occurred in both cells that had been activated with IFN-γ or not. LDH activity also increased time-dependently in control fibroblasts irrespective of whether cells had been infected or not with T. gondii and had been treated or not with IFN-γ or IFN-γ plus TNF (Fig. 8C,D). Thus, host cell viability was not compromised as a result of IRG activity and disruption of parasite-containing PVs in IFN-γ-activated SKMCs.

Discussion

Skeletal muscle is one of the preferred tissues that sustains the development and long-term survival of Toxoplasma tissue cysts [7]. Here, we have explored the cell-autonomous immune responses of

Figure 3. Impact of IFN-γ and TNF on Toxoplasma development in SKMCs. Primary embryonic SkMCs or C2C12 SkMCs were differentiated to mature myotubes. They were, together with control NIH/3T3 fibroblasts, infected with T. gondii NTE tachyzoites [parasite-host cell ratio 2:1]. At the time of infection, cells were stimulated or not with 100 U/ml IFN-γ alone or combined with 100 pg/ml TNF. (A) At different time points post infection (p.i.), C2C12 and NIH/3T3 cells were lysed, and soluble proteins were separated by SDS-PAGE and transferred to nitrocellulose membranes. Membranes were probed with anti-T. gondii SAG1, anti-mouse myosin HC, or anti-mouse actin and appropriate HRP-conjugated secondary antibodies. Immune complexes were visualized by enhanced chemiluminescence. (B) Forty-eight hours after infection, cells were fixed and permeabilized, and T. gondii was visualized by immunofluorescence staining. The total cell population was stained with propidium iodide. The number of parasites was counted in at least 100 parasitophorous vacuoles (PV) per sample. Data represents the mean number of parasites per PV ± S.E.M. from at least 3 independent experiments; significant differences between non-stimulated and stimulated cells are indicated (*p<0.05), (C–E) At the time of infection, primary SkMCs (C), C2C12 (D) or NIH/3T3 (E) cells were stimulated with IFN-γ alone (open squares), IFN-γ combined with TNF (open triangles), or were left untreated (closed symbols). Cells were fixed at different time points after infection, and T. gondii and total cells were visualized by immunolabelling and propidium iodide staining, respectively. After inspection of at least 500 cells per sample, the percentages of infected cells were calculated. Results are means ± S.E.M. from at least 3 independent experiments.

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primary and permanent mouse-derived SkMCs after infection with *T. gondii*. The results for the first time establish the immunocompetence of mature muscle cells during infections with both avirulent and virulent *T. gondii* strains. IFN-γ, but not TNF is pivotal in activating innate defense mechanisms in SkMCs and in restricting parasite propagation. We provide evidence that NO production by iNOS and loading and subsequent disruption of *Toxoplasma* containing PVs by IRGs (i.e. p47 GTPases) fulfill anti-parasitic activities in SkMCs after stimulation with IFN-γ. Remarkably, inhibition of parasite replication in SkMCs after stimulation with IFN-γ does not accelerate bradyzoite formation. These results strongly suggest that during toxoplasmosis, SkMCs are active players rather than passive targets of the local immune response towards *Toxoplasma* thereby determining the host-parasite interaction in muscle tissue.

IFN-γ-activated, mononuclear phagocytes are the major mediators of resistance to *T. gondii* [43]. However, IFN-γ-responsiveness of non-hematopoietic cells is also required for mice to survive a *Toxoplasma* infection [29]. In this study, we prove that SkMCs are capable to considerably inhibit propagation of *T. gondii* after stimulation with IFN-γ. They may thus actively contribute to the local host defense in muscle tissue. While this is rather unlikely to be critical for host survival, immune responses to *T. gondii* in skeletal muscle may however significantly impact the ability of the parasite to persist within this tissue. Although species-specific differences in effector molecules of the host response clearly exist (see below), we nevertheless assume that our findings in general also apply for the immune responses in SkMCs from animals used for human consumption such as pigs, sheep, goats, poultry and others. Anti-parasitic cell-autonomous immunity in SkMCs from livestock may thus have major implications for transmission of *T. gondii* to humans via the ingestion of undercooked or cured meat products. In addition, it also establishes SkMCs as active participants during inflammatory myopathies, e.g. polymyositis or dermatomyositis, during toxoplasmosis as described in humans and animals [10, 11, 12, 13, 14].

The capability of SkMCs to restrict parasite propagation after activation with IFN-γ is in sharp contrast to neurons which is another preferred cell type for *Toxoplasma* long-term persistence [44, 45], and which failed to inhibit the parasites’ replication after activation with IFN-γ and/or TNF [46]. This indicates that responsiveness of the infected host cell to IFN-γ and/or TNF appears not to be of major relevance for the preferential formation of tissue cysts and long-term persistence of *T. gondii* within brain and muscle tissues. This view is further supported by the finding that treatment of SkMCs with IFN-γ alone or combined with TNF even diminished the conversion from tachyzoites to potentially persisting bradyzoites. The latter contrasts to the triggering of bradyzoite formation in mouse macrophages after stimulation with IFN-γ [33]. In distinct cell types including SkMCs and neurons, however, cell intrinsic factors may instead favor stage differentiation and persistence of *Toxoplasma* tissue cysts as also suggested earlier [2, 47]. Recently, it has been indeed shown that upregulation of the cell division autoantigen-1 following treatment of human fibroblasts with a substituted pyrrole, triggers stage differentiation and *Toxoplasma* cyst formation [48]. It should be mentioned that from the present study we cannot completely rule out the possibility that long-term stabilization of tissue cysts within SkMCs is achieved by activation with IFN-γ.

Our data shows that IFN-γ exerts its effect primarily on the parasite replication in SkMCs but not the parasite invasion into SkMCs since initial infection rates were similar irrespective of IFN-γ stimulation. Only at later time points of infection, IFN-γ-dependent differences in the proportion of infected SkMCs...
became apparent which were most likely due to the reduction in parasite replication after IFN-γ activation, thereby avoiding host cell lysis and subsequent invasion of new host cells. *Toxoplasma* gains access into its host cell by an active parasite-driven process and we confirm that this remains unaffected by stimulation of the host cell with IFN-γ or not. Instead, IFN-γ-induced iNOS expression and activity contributed to the toxoplasmacidal effect in SkMCs as revealed by pharmacological inhibition. Reactive nitrogen intermediates including NO exert important toxoplasmacidal activities against both type I (mouse-virulent) and type II (mouse-avirulent) parasites in mice in vitro and in vivo [23,24,35,36,49], and our results suggest that this holds also true for the local immune response in muscle tissue. Stimulation of SkMCs with IFN-γ led to much higher levels of iNOS transcripts and NO as compared to fibroblasts. Consequently, inhibition of iNOS partially abrogated the effect of IFN-γ on parasite replication in SkMCs but not in fibroblasts. Inflammatory cytokines including IFN-γ also triggered iNOS activity in cardiomyocytes from mice and rats and exerted microbicidal activity against a flagellate parasite, *Trypanosoma cruzi* [50,51]. Thus, iNOS and NO appear to represent a general defense mechanism of muscle cells against intracellular pathogens at least in mice. Since NO production also modulates the expression of chemokines in muscle cells [52] it will be interesting to see whether iNOS activity may also be involved in the pathogenesis of inflammatory myopathies during toxoplasmosis.

Another mechanism that we describe exerting anti-*Toxoplasma* activity in SkMCs is the loading of parasitophorous vacuoles with IFN-γ-dependent IRG proteins and subsequent disruption of vacuoles. This protein family plays a pivotal role in the immune defense against *Toxoplasma* and other intracellular pathogens. Importantly, several reports indicate that the IRG protein family only confers resistance against mouse-avirulent *T. gondii* strains whereas virulent *T. gondii* evade IRG-mediated destruction [37,38,39,40], at least partially via phosphorylation by the rhoptry kinase ROP18 [41,42]. Hence, mice deficient in individual members of the IRGs rapidly succumb during acute infection with an avirulent *T. gondii* strain [53,54,55]. IRGs can be induced in a variety of tissues [53] and expression in both hematopoietic and non-hematopoietic cells is required for resistance against *Toxoplasma* [56]. Here, we show for the first time that Irga6 and Irgm3 were strongly induced in SkMCs following treatment with IFN-γ. More importantly, both Irga6 and Irgb6 rapidly accumulated on the membrane of vacuoles containing avirulent NTE parasites and together with other IRGs may subsequently mediate disruption of the parasitophorous vacuole as also suggested herein.

Figure 5. IFN-γ-triggered expression and activity of iNOS partially restricts *Toxoplasma* propagation in SkMCs. Primary embryonic SkMCs (pSMC) or C2C12 SkMCs were differentiated to mature myotubes. Differentiated SkMCs and control NIH/3T3 fibroblasts were infected at a parasite-host cell ratio of 2:1 with transgenic *T. gondii* RH tachyzoites expressing β-galactosidase as a reporter (C–E), or were left non-infected (A,B). Cells were stimulated or not with 100 U/ml IFN-γ alone or combined with 100 pg/ml TNF. (A) Total RNA was isolated at the indicated time points, mRNA was reverse transcribed, and iNOS or β-actin cDNA was amplified by quantitative real-time PCR. Data represents the mean induction of iNOS mRNA by IFN-γ normalized to β-actin; data is from a representative experiment out of two. (B) Nitrite was measured by the Griess reaction in culture supernatants obtained from non-infected C2C12 (black bars) and non-infected NIH/3T3 cells (cross-hatched bars) at the indicated time points. Data represents means ± S.E.M. from at least 5 independent experiments; significant differences between C2C12 and NIH/3T3 cells are indicated (**p<0.01). (C–E) Primary SkMCs (C), C2C12 (D) and NIH/3T3 cells (E) were treated with L-NIL (open bars), or were left untreated (black bars) at 1 hour prior to infection and cytokine stimulation. At different time points after infection/cytokine stimulation, growth of β-galactosidase-expressing *T. gondii* was quantitated by a colorimetric CPRG assay. The inhibition of parasite growth triggered by IFN-γ alone or in combination with TNF was calculated by subtracting OD570 values of cytokine-stimulated cultures from that of non-stimulated control cultures; bars represent means ± S.E.M. from at least two independent experiments.

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Vacuoles containing virulent RHβGal, on the other hand, efficiently accumulated only Irga6 but not Irgb6, and signs of vacuole disruption were also rarely observed. Coating of PVs with IRGs in a consistent hierarchy is necessary and supposedly also sufficient [38] to mediate vacuole disruption and parasite death. Loading of PVs with Irgb6 is an early marker of subsequent vacuole disruption [37,38,39], largely discriminates between vacuoles containing virulent type I or avirulent type II and III T. gondii [37,38,39], and is necessary for efficient defense of primed macrophages against Toxoplasma [42]. Our data is thus consistent with the view that the IRG system is a critical strain-dependent defense mechanism of mice against T. gondii. In our study, we extend these previous findings by demonstrating that it also operates in IFN-γ-activated SkMCs infected with type II and presumably also type III parasites. We indeed provide circumstantial evidence for disrupted Toxoplasma vacuoles in IFN-γ-activated SkMCs infected with NTE parasites and we propose that this contributes to the host defense against T. gondii in muscle tissue. It should be mentioned that in activated SkMCs, we occasionally also observed Irgb6-positive vacuoles harboring virulent RHβGal parasites and that we also recognized signs of disrupting RHβGal PVs. Thus, evasion of IRG-mediated destruction is not an absolute trait of virulent T. gondii parasites as also reported by others [37,38]. Although present throughout the vertebrates, the IRG protein family has been lost or largely reduced in birds and humans, respectively [55]. It remains to be determined whether in these species the loss of a functional IRG system is compensated by another family of GTPases, i.e. the p65 guanylate-binding proteins (GBPs) [57]. In addition, a possible contribution of GBPs in the local immune response to Toxoplasma in SkMCs from IRG-proficient animals, e.g. mice [27], deserves closer attention.

The parasite and host cell fate after IRG-mediated disruption of the PV is a matter of debate and far from having been solved [55]. In IFN-γ-primed mouse astrocytes, parasites released from disrupted PVs directly degenerate in the host cell cytosol [25,58] or – within a certain time frame post infection – can egress from the host cell [58]. After IRG-mediated PV disruption in activated fibroblasts, degenerating parasites in the host cell cytosol have been shown to induce a necrotic death of the host cell [37]. In
Figure 7. Differential targeting of GTPase Irgb6 to parasitophorous vacuoles of type I and type II *Toxoplasma* strains in SkMCs. Differentiated C2C12 SkMCs were infected with *T. gondii* RHβGal or NTE parasites and were activated with 100 U/ml IFN-γ at the time of infection. After fixation at different time points p.i., Irgb6 (green fluorescence) and *T. gondii* (red fluorescence) were immunolabeled and analysed by confocal laser scanning microscopy. (A) Representative images of PVs of NTE and RHβGal parasites from one out of two independent experiments are depicted. (B) Percentages of Irgb6-positive parasitophorous vacuolar membranes (PVM) were determined microscopically by examining at least 100 vacuoles per sample; data represents means ± S.E.M. (n = 2). (C) Irgb6-positive RHβGal vacuoles could be rarely observed; a RHβGal vacuole showing early signs of disruption is indicated. doi:10.1371/journal.pone.0045440.g007

Figure 8. IFN-γ does not accelerate necrotic cell death in *Toxoplasma*-infected SkMCs. Differentiated C2C12 SkMCs (A,B) and NIH/3T3 fibroblasts (C,D) were either infected with *T. gondii* (NTE strain, parasite-host cell ratio 2:1; B,D), or were mock-infected (A,C). At the time of infection, cells were stimulated with IFN-γ alone (open squares) or combined with TNF (open triangles), or were left non-stimulated (closed circles). Lactate dehydrogenase (LDH) activity was monitored in culture supernatants at different time points of infection. Maximal LDH activity of each sample was determined in parallel by complete cell lysis. LDH activities in the supernatants of experimental samples are expressed as percentages of the maximal activity; data represents means ± S.E.M. from three independent experiments. doi:10.1371/journal.pone.0045440.g008
IFN-γ-activated SkMCs, we did not recognize any signs of parasite egress or a decrease of host cell viability that could be attributed to IRG activity. This can be due to the fact that we activated the SkMCs at the time point of infection instead of using IFN-γ-primed cells for infection as in the studies described above [37,50]. In addition, there is clear evidence for cell type-specific differences in the mechanisms of IRG-mediated resistance [55]. Thus, the exact modes of IRG-mediated host resistance in SkMCs remains to be determined.

In conclusion, SkMCs have to be considered potent effector cells of the local innate immune response to *T. gondii*. After stimulation with IFN-γ, they considerably restrict parasite replication by inducing iNOS- and IRG-mediated activities without accelerating bradyzoite formation. Thus, IFN-γ-regulated cell-autonomous immunity of SkMCs rather limits parasite abundance than facilitating tissue cyst formation during *Toxoplasma* colonization of muscle tissue. Differences in the SkMCs immune response to the parasite may also determine the significance of different host species for transmission of *Toxoplasma* to humans via undercooked or cured meat products.

### Materials and Methods

**Ethics statement**

All animal work has been conducted according to relevant national and international guidelines and was approved by the Niedersächsisches Landesamt für Verbraucherschutz und Lebensmittelsicherheit [Project 509.42502/01-T-23.05].

**Isolation of primary SkMC and differentiation of C2C12 cells**

Primary SkMC were isolated from NMRI mouse embryos 18–19 days after conception as described previously [2]. Briefly, thigh muscles were minced in Ringer solution and digested with 0.05% trypsin, 0.01% EDTA in PBS, pH 7.2. After addition of DMEM, 10% heat-inactivated fetal calf serum (FCS) and centrifugation, cells were incubated for 45 min in DMEM supplemented with 9% horse serum, 5% FCS, 2.5 mM CaCl₂, 100 U/ml penicillin and 100 μg/ml streptomycin in tissue culture flasks. Non-adherent cells enriched for myoblasts were then seeded at 1.5 × 10⁴ per well in 96-well plate containing 13-mm glass cover slips or at 1.5 × 10⁵ per well in 6-well plates. In order to facilitate adherence of SkMC, glass cover slips were pre-coated using 0.5 mg/ml poly-L-ornithine hydrobromide in 150 mM boric acid/NaOH, pH 8.3 and 15 μg/ml mouse laminin. Unless stated otherwise, cells were cultured for 6 days in order to enable the formation of myotubes.

C2C12 myoblasts (European Collection of Animal Cell Cultures (ECACC), Salisbury, UK) were propagated in DMEM supplemented with 10% FCS, 100 U/ml penicillin and 100 μg/ml streptomycin. Cells were seeded at 1 × 10⁶ per well in 96-well tissue culture plates, at 2.5 × 10⁴ per well in 24-well plates containing 13-mm glass cover slips or at 1.5 × 10⁵ per well in 6-well plates. In order to facilitate adherence of SkMC, glass cover slips were pre-coated using 0.5 mg/ml poly-L-ornithine hydrobromide in 150 mM boric acid/NaOH, pH 8.3 and 15 μg/ml mouse laminin. Unless stated otherwise, cells were cultured for 6 days in order to enable the formation of myotubes.

**SDS-PAGE and immunoblotting**

In order to analyze the differentiation of SkMCs and the intracellular development of *T. gondii*, parasite-infected or non-infected SkMCs and fibroblasts were isolated by EDTA/trypsin treatment and equal amounts of cells were lysed for 1 hour in 1% Triton X-100, 0.15 M NaCl, 50 mM Tris/HCl, pH 8.0, 50 mM NaF, 5 mM sodium pyrophosphate, 1 mM PMSF, 1 mM EDTA, 1 mM sodium orthovanadate, and 5 μg/ml each of leupeptin, aprotinin and pepstatin. After centrifugation, soluble proteins were separated by standard SDS-PAGE and transferred to nitrocellulose by semidyblotting. Non-specific binding sites were blocked for 2 hours with 5% dry skimmed milk, 0.2% Tween-20, 0.02% NaN₃ in PBS, pH 7.4. Membranes were incubated overnight at 4°C with 1.5 μg/ml mouse monoclonal anti-MycD, 1.5 μg/ml mouse monoclonal anti-myogenin (both from BD Biosciences, Heidelberg, Germany), rabbit polyclonal anti-myosin heavy chain (H-300, 1:1,000; Santa Cruz Biotechnology, Heidelberg, Germany), mouse monoclonal anti-actin (clone C4; kindly provided by J. Lessard, Cincinnatti, OH), rabbit polyclonal anti-β-tubulin [57,58], mouse monoclonal anti-actin (clone C4; kindly provided by J. Lessard, Cincinnatti, OH), rabbit polyclonal anti-β-tubulin.
(1:10,000) or rabbit polyclonal anti-SAG1 (1:1,000) diluted in 5% dry skimmed milk, 0.05% Tween-20 in PBS, pH 7.4. After washing, primary antibodies were detected with horseradish peroxidase-conjugated anti-mouse or anti-rabbit IgG (Dianova, Hamburg, Germany). Thereafter, enhanced chemiluminescence reagent (GE Healthcare, Freiburg, Germany) was added and digital images obtained using a LAS-4000 luminescent image analyzer (Fujifilm, Dusseldorf, Germany).

Immunofluorescence staining and confocal microscopy
The intracellular development of *T. gondii* as well as the expression of host cell proteins was determined by immunofluorescence microscopy. Infected SkMCs and fibroblasts or non-infected controls were fixed with 4% paraformaldehyde in 0.1 M sodium cacodylate, pH 7.4 for 1 hour and quenched for 10 minutes in 50 mM NH₄Cl in PBS, pH 7.4. Thereafter, cells were permeabilized and unspecific binding sites blocked for 1 hour using 0.1 mg/ml saponin, 1% BSA in PBS. Cells were then incubated for 1 hour with 5 µg/ml anti-MyoD1, 5 µg/ml anti-myogenin or 5 µg/ml anti-desmin mouse monoclonal antibodies (all from BD Biosciences), rabbit polyclonal anti-myosin heavy chain (1:100; Santa Cruz Biotechnology), or with rabbit polyclonal anti-*T. gondii* (1:1,000) alone or combined with rat monoclonal CC2 recognizing bradyzoite-containing but not tachyzoite-containing PVs [62], or with mouse polyclonal anti-*T. gondii* (1:100) combined with rabbit polyclonal anti-Irga6 (1:3,000) or anti-Irgb6 (1:4,000; anti-IRGs kindly provided by J. Howard, Cologne, Germany) diluted in 1% BSA, 0.1 mg/ml saponin in PBS. The cells were washed and were then incubated for 1 hour with Cy2-conjugated or Cy3-conjugated donkey F(ab')₂ fragment anti-rabbit IgG or anti-mouse IgG (Dianova, Hamburg, Germany). In some experiments, the total cell population was labeled with 5 µg/ml propidium iodide in PBS. The cells were mounted with Mowio4-88 (Calbiochem-Novabiochem, Bad Soden, Germany) and were then examined by confocal laser scanning microscopy using Leica TCS SP2.

Griess reaction
Nitrite as a reliable marker for NO production was measured by the Griess assay as described previously [24]. Briefly, 100 µl of cell culture supernatants from *T. gondii*-infected or non-infected SkMCs or NaNO₂ standards (1–128 µM) were incubated in duplicate with an equal volume of 0.5% sulfanilamide, 0.05% naphthylamine dihydrochloride in 5% H₃PO₄. The absorbance was read at 560 nm after 5 minutes in a microplate reader.

Cytotoxicity assay
The integrity of *T. gondii*-infected or non-infected SkMCs and control fibroblasts after stimulation with IFN-γ was determined using a colorimetric Cytotoxicity Detection KitPLUS as recommended by the manufacturer (Roche Diagnostics, Mannheim, Germany). Briefly, the activity of lactate dehydrogenase (LDH) in cell culture supernatants was measured in triplicate by co-incubation for 30 min with an equal volume of reaction mixture, consisting of a diaphorase/NAD⁺ mixture in the assay dye solution. Optical density was determined at 490 nm. The LDH activity in supernatants from experimental samples was expressed as the percentage of the maximal activity which was determined by disrupting the cells in lysis solution (Roche) prior to measuring LDH activity.

Statistical analysis
Results are expressed as means ± S.E.M. of at least three independent experiments unless otherwise indicated. Significant differences between mean values were identified by the Student’s t-test. P values of less than 0.05 were considered significant.

Supporting Information

Figure S1 Immunity-related GTPase Irga6 target parasitophorous vacuoles containing transgenic RHβGal parasites in IFN-γ-activated SkMCs. Differentiated C2C12 SkMCs were infected with *T. gondii* RHβGal or NTE parasites at a parasite-host cell ratio of 2:1 as indicated and were concomitantly activated with 100 µM IFN-γ or were left non-activated. C2C12 cells were fixed at different time points after infection, and Irga6 (green fluorescence) and *T. gondii* (red fluorescence) were immunolabeled. (A) Representative images of RHβGal-infected SkMCs were recorded by confocal laser scanning microscopy. (B) Percentages of Irga6-positive parasitophorous vacuolar membranes (PVM) were determined microscopically by examining at least 100 vacuoles per sample in C2C12 SkMCs infected with either NTE or RHβGal parasites. Data represents means ± S.E.M. from two independent experiments. (TIF)

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Author Contributions
Conceived and designed the experiments: ACT CGKL. Performed the experiments: ACT IJS. Analyzed the data: ACT IJS CGKL. Wrote the paper: ACT IJS CGKL.
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