The vacuolar iron transporter mediates iron detoxification in *Toxoplasma gondii*

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Abstract

Iron is essential to living cells, acting as a cofactor in a number of important enzymes in metabolism; however in the absence of correct storage iron forms dangerous oxygen radicals. In both yeast and plants, iron is stored in a membrane-bound vacuole through the action of a vacuolar iron transporter (VIT). This transporter is conserved in the apicomplexan family of obligate intracellular parasites, including in *Toxoplasma gondii*, a pathogen of medical and veterinary importance. Here, we assess the role of VIT, and iron storage, in *T. gondii*. We show that iron is restricted to a compartment in the parasite that does not overlap with zinc. By deleting VIT we find a slight growth defect *in vitro*, however the absence of VIT leads to hypersensitivity to iron, confirming its essential role in iron detoxification in the parasite. This hypersensitivity can be rescued by scavenging of oxygen radicals. In the absence of VIT, parasites store less iron and are at a growth disadvantage when moving into an iron-depleted environment. We show parasite VIT expression is regulated by iron levels at both the transcript and protein level, and by altering the distribution of VIT within the cell. In the absence of VIT, we find that *T. gondii* responds by altering expression of genes with a role in iron metabolism and by increasing the activity of the antioxidant protein catalase. We also show that iron detoxification has an important role both in parasite survival within macrophages and in virulence in a mouse model. Together, by demonstrating a critical role for VIT during iron detoxification in *T. gondii*, we reveal the importance of iron storage in the parasite and provide the first insight into the machinery involved.

Introduction

The obligate intracellular parasite *Toxoplasma gondii* is an unusually promiscuous pathogen, able to infect most warm-blooded species, almost all nucleated cells and virulent in multiple tissue types. This lifestyle makes it an important pathogen of humans and livestock where it is a major cause of miscarriage and economic loss (Stelzer et al., 2019). However, the indiscriminate lifestyle of *T. gondii* also suggests that the parasite is able to adapt to different environments to ensure a sufficient supply of key nutrients. One key element required by the parasite is iron. Iron plays a central role in proteins essential for respiration in almost all living cells. In common with most eukaryotes, elemental analysis found that around 5% of the atoms of *T. gondii* atoms are iron (Al-sandaqchi et al., 2018). This iron is required for a number of essential cellular processes, including heme biosynthesis (Bergmann et al., 2020; Kloehn et al., 2021), iron sulphur (Fe-S) cluster biogenesis (Aw et al., 2021), and as a cofactor in several important proteins such as catalase (Kwok et al., 2004; Odberg-Ferragut et al., 2000), prolyl hydroxylases (Florimond et al., 2019) and the deoxyribonucleotide synthesis enzyme ribonucleotide reductase.

However, the ease with which iron gains and loses electrons makes it potentially dangerous in the cellular environment (Galaris et al., 2019; Imlay et al., 1988). Free iron reacts with H_2O_2 , produced by aerobic respiration, via the Fenton reaction to form highly reactive hydroxyl and hydroperoxyl radicals ($HO\cdot$ and $HOO\cdot$, collectively reactive oxygen species (ROS)) which can cause extensive damage to the cell. To avoid this fate, mammalian cells safely store the majority of iron in the cytosol within protein cages of ferritin (Arosio et al., 2015). Ferritin has been highly conserved through evolution, with homologs found in plants and bacteria (Arosio et al., 2017). However, outside of the Metazoa, organisms including plants, yeast and protists, instead transport iron into membrane-bound vacuoles through the action of an H^+ -dependent antiporter, named CCC1 in yeast or the vacuolar iron transporter (VIT) in plants (Li et al., 2001; Sorribes-Dauden et al., 2020). The apicomplexan family, including *T. gondii* and other key human and veterinary parasites such as *Plasmodium* sp. (cause of malaria), *Cryptosporidium* sp. (cause of diarrheal disease) and *Eimeria* (important chicken pathogen), lack ferritins entirely and so presumably rely on storage of iron within a membrane bound compartment.

In support of this hypothesis, a homologue of the plant iron transporter *vacuolar iron transporter 1* (VIT1) was described in *Plasmodium* and shown to be a functional iron transporter that complemented a $\Delta ccc1$ yeast strain (Labarbuta et al., 2017; Sharma et al., 2021; Slavic et al., 2016). Deletion of VIT in *Plasmodium* demonstrated that the gene was essential for replication in both blood and liver stage parasites. Further, absence of VIT rendered *Plasmodium* more susceptible to iron overload and more resistant to iron removal (Slavic et al., 2016).

However, *Plasmodium* sp. undergo their asexual lifecycles in the unusual environment of the red blood cell, with very high levels of iron in the form of haemoglobin. In contrast, the ubiquitous pathogen *T. gondii* is a generalist and so faces different iron stresses through its lifecycle. We were interested in how *T. gondii* stores iron, and the effects disrupting iron storage has on the parasite. Based on homology to the *Plasmodium* and plant vacuolar iron transporters, we identified VIT in *T. gondii* and investigated the role of VIT and iron storage in the parasite. We show that iron is localised within a compartment of the parasite, separate from zinc, supporting the hypothesis that iron is stored in a vacuole. By knocking out VIT, we find that it is not essential for replication, however expression of VIT confers a survival advantage during *in vitro* culture. VIT has a highly dynamic localisation throughout the cell cycle. We see transient

colocalisation with a marker of the vacuolar associated compartment (VAC), suggesting this as the location of stored iron. We show that deletion of VIT makes the parasites significantly more sensitive to exogenous iron, and that this effect is mediated through an increase in reactive oxygen species (ROS) upon iron treatment. Further, VIT expression is regulated changes in exogenous iron in *T. gondii*. We also find that parasites lacking VIT are more susceptible to killing by activated macrophages, and this is likely a key factor in their inability to cause pathogenesis in mice.

Results

Deletion of VIT leads to a moderate growth defect

The presence of a VIT homolog in *T. gondii* suggests that iron is stored in a membrane-bound compartment. To examine this, we looked at iron localization by X-ray fluorescence microscopy (XFM). We found that iron in extracellular and recently invaded parasites localised to a distinct region of the cell, which did not significantly overlap with either zinc or calcium (Fig. 1a). Instead, zinc, calcium and phosphorus appeared to closely overlap, supporting previous work proposing that zinc is stored in compartments with similarities to acidocalcisomes (Chasen et al., 2019; Luo et al., 2001). This also complements previous work in *Plasmodium* showing that zinc and iron appear to be stored in the same compartment (Marvin et al., 2012). VIT from *T. gondii* was previously identified through homology to plant and *Plasmodium* transporters (Slavic et al., 2016). To confirm the conservation of key residues, we aligned the protein sequence of *T. gondii* VIT (TGGT1_266800) with sequences from *Plasmodium*, yeast (CCC1) and plants (*Arabidopsis thaliana* and *Eucalyptus grandis*, as the crystal structure of EgVIT was recently published (Kato et al., 2019)) (Fig. 1b). The *T. gondii* VIT had 33% identity with AtVIT1, including conservation of key residues D43 and M80, recently shown to be essential for iron binding (Kato et al., 2019). To investigate the function of VIT in *T. gondii*, the coding region was deleted using two small guide RNAs targeting the 5' and 3' regions of the open reading frame (see Table 1) and replaced with a cassette encoding mNeonGreen under the control of the SAG1 promotor (Fig. 1c) in a Δ Ku80 parental cell line. Integration of the cassette and loss of the native ORF was confirmed by PCR (Fig. 1d). Δ VIT parasites were viable in culture and able to form cleared areas on a monolayer of host cells at 6 days post infection (Fig. 1e). A genome-wide essentiality screen predicted that loss of VIT had a mild effect on parasite growth (phenotype score -1.22) (Sidik et al., 2016). To examine the growth of the parasites in more detail, we quantified the number of parasites within a parasitophorous vacuole at 24 h post infection (Fig. 1f). The Δ VIT line had fewer parasites per vacuole (significant at the 2 cell, $p = 0.0005$ and the 8 cell stage, $p = 0.0037$, multiple t tests corrected by Holm-Sidak), demonstrating that VIT has a role in parasite replication under normal growth conditions. We also examined the parasite's ability to survive extracellularly. Mechanically released parasites were incubated extracellularly at 37 °C for the indicated time before allowing invasion of fresh cells and quantifying the number of plaques formed after 6 days. We found no difference in extracellular survival between the parental and Δ VIT parasites ($p > 0.05$, multiple t tests corrected by Holm-Sidak) (Fig. 1g), demonstrating that VIT does not have a role in parasite survival outside of the host cell. As an iron transporter, it could be expected that deletion of VIT would lead to a change in the distribution of iron within the cell. Using XFM we examined iron distribution in Δ VIT parasites shortly after invasion. Although the signal was heterogeneous, we generally saw less iron within cells compared to the parental line. Some cells appeared to have no or very little iron while in other cells we saw a more fragmented signal which did not overlap with the other elements detected (Fig. 1h). Overall, we determined that VIT has a small role in parasite replication and that it may have a role in iron distribution within the cells.

Δ VIT parasites are hypersensitive to excess iron and have lower cellular iron levels

In yeast and *Plasmodium*, deletion of CCC1 or PbVIT leads to hypersensitivity to excess iron (Li et al., 2001; Slavic et al., 2016). To examine this in *T. gondii*, we performed a plaque assay under increasing concentrations of ferric ammonium chloride (FAC). We found that Δ VIT parasites were noticeably more sensitive to excess FAC, forming fewer and much smaller plaques than the parental line (Fig. 2a). To confirm this result, we performed a competition assay under standard conditions and with excess FAC. To serve as controls, two new lines were constructed in Rh Δ HX, where the coding region of *ku80* was replaced with a cassette expressing mNeonGreen (Δ Ku80::mNeon, called mNeon) or tdTomato (Δ Ku80::tdTomato) (Fig. S1a), resulting in two lines which expressed fluorescent proteins in place of the endogenous *ku80* gene. We confirmed that these lines were fluorescent using flow cytometry and the loss of the *ku80* gene by PCR (Fig. S1b). To assess the fitness defect of the Δ VIT strain, Δ VIT or mNeon parasites were mixed with tdTomato in an equal ratio and untreated or treated with 200 μ M FAC. The proportion of green parasites (either mNeon or Δ VIT) in the population was assessed at each passage by flow cytometry. The proportion of mNeon parasites did not change over the experiment, however, the Δ VIT line was significantly ($p = 0.002$, t test, Holm-Sidak corrected) outcompeted by three days post infection (Fig. 2b). Addition of FAC exacerbated this phenotype, Δ VIT parasites were significantly ($p = 0.001$, t test, Holm-Sidak corrected) outcompeted by two days post infection in the presence of excess iron and were almost undetectable by four days post infection. This shows that VIT is

required for growth under normal conditions, and lack of VIT sensitises parasites to exogenous iron. To confirm this, we constructed a second Δ VIT strain by replacing the *vit* gene with a DHFR cassette (**Fig. S2a**), named Δ VIT::DHFR_{TS}. We confirmed integration by PCR (**Fig. S2b**) and confirmed these parasites were also hypersensitive to excess iron by plaque assay (**Fig. S2c and d**).

To quantify iron hypersensitivity, we infected host cells in 96-well plates with mNeon or Δ VIT parasites and treated with increasing concentrations of FAC for four days before quantifying parasite fluorescence, normalized to untreated parasites (Fig. 2c). The Δ VIT parasites were significantly ($p < 0.0001$, extra sum of squares F-test) more sensitive to FAC treatment, with an EC₅₀ of 0.004 μ M (95% C.I. 0.001 to 0.018 μ M), compared to the mNeon EC₅₀ of 102 μ M (95% C.I. 20 to 490 μ M), an increase in sensitivity of around 25,000-fold.

VIT homologs from various species have been shown to transport other metals, including manganese (Li et al., 2001) and zinc (Zhang et al., 2012). To test if deletion of VIT altered the sensitivity of *T. gondii* to metals other than iron, we treated parasites as above with increasing concentrations of zinc, copper, cadmium, and manganese and determined the EC₅₀. We saw that the absence of VIT did not affect the response to treatment with excess zinc, copper, and manganese (t test, p values > 0.05) (Fig. 2d and S3a-d). While this is not direct evidence that *T. gondii* VIT is unable to transport these substrates, it does show that VIT is not required for the detoxification of the selected metals. However, we did see a significant shift in the sensitivity to the heavy metal cadmium (EC₅₀ of parental line 3.41 μ M (95% C.I. 1.63 to 6.34 μ M), EC₅₀ of Δ VIT 0.04 μ M (95% C.I. 0.008 to 0.149 μ M), $p = 0.0009$, extra sum of squares F-test) (Fig. 2d and S3c). Cadmium has complex effects on mammalian cells and tissues (Templeton and Liu, 2010) and there is evidence that cadmium can lead to increased ROS production (Shaikh et al., 1999). To determine if the Δ VIT parasites were more susceptible to general increases in oxidative stress, we treated the parasites with sodium arsenite which induces oxidative stress through a number of mechanisms (Hu et al., 2020) and has been successfully used in *T. gondii* to induce oxidative stress (Augusto et al., 2021). The concentrations of Ars used did not affect host cell viability as measured by Alamar blue assay (**Fig. S3e**). We saw no significant change in the sensitivity between parental line and the Δ VIT parasites to Ars treatment (Fig. 2e), demonstrating that the effect of VIT on cadmium toxicity is somewhat specific, and that Δ VIT parasites do not have a general sensitivity to oxidative stress. VIT from the distantly related diatom *Phaeodactylum tricornutum* is transcriptionally regulated by cadmium (Brembu et al., 2011) where the authors suggest that Cd²⁺ may also be stored in a vacuole. Responses to cadmium have not previously been examined in the Apicomplexa, however our results here may suggest some link between iron and cadmium detoxification in *T. gondii*.

In *Plasmodium*, deletion of VIT also led to a change in the sensitivity to the iron chelator deferoxamine (DFO) (Slavic et al., 2016). However, in *T. gondii* we saw no change in the ability of the parasites to replicate in the presence of increasing concentrations of iron chelator (Fig. 2E) (EC₅₀ of mNeon 160 μ M (95% C.I. 65.9 to 362.2 μ M) and Δ VIT 101.3 μ M (95% C.I. 44.9 to 233.5 μ M), $p > 0.05$, extra sum of squares F-test) over the four days of the experiment.

If VIT is responsible for storing iron, it is possible that in its absence, parasites would be disadvantaged in replication when moving to an iron-poor environment. To simulate this, we pretreated host cells with DFO for 24 h to deplete iron, before adding mNeon or Δ VIT parasites and fixed after only 14 h post invasion. We then quantified the average number of parasites/vacuole. We found that pretreatment with DFO significantly reduced the average parasites/vacuole in both the mNeon and the Δ VIT parasite lines (Fig. 2f), demonstrating the importance of iron to parasite replication. Although we do see a replication defect in the Δ VIT strain (Fig. 1f), at this early point in infection it was not significant. However, in an iron-poor environment there was a significant decrease in the average parasite/vacuole in the Δ VIT strain compared to the mNeon strain, suggesting that the potential disruption of iron storage inhibits replication in an iron-depleted environment.

The above results suggested a loss of stored iron. To assess if iron levels changed in the Δ VIT::DHFR_{TS} parasites, we quantified total parasite-associated iron using inductively coupled plasma-mass spectrometry (ICP-MS). Using this method, we found a mean of 2.6×10^{-5} ppb of Fe/parasite in the parental parasite line (Fig. 2g). In the absence of VIT, we saw a significant ($p = 0.035$, t test) reduction in iron to approximately 1.5×10^{-5} ppb/parasite, approximately 60% of the parental level. As a control, we saw no change in the levels of zinc between the parasite lines (**Fig. S2e**). A reduction in total iron has been observed before upon disruption of vacuolar iron transporters in yeast and plants (Kim et al., 2006; Li et al., 2001), and confirms the role of VIT in *T. gondii* iron storage.

VIT has a dynamic localisation throughout the lytic cycle

In *Plasmodium*, VIT colocalized with markers of the endoplasmic reticulum (ER) throughout the parasite's lifecycle (Slavic et al., 2016). To determine the localisation of VIT in *T. gondii*, we first overexpressed VIT with a C-terminal Ty tag from the TUB promotor. Transient overexpression of VIT-Ty resulted in localisation of several foci within the cell with some expression at the parasite periphery (Fig. 3a).

The central foci did not colocalise with the apicoplast marker HSP60 but did show a degree of overlap with the marker of the vacuolar associated compartment (VAC), cathepsin protease L (CPL). However, overexpression of VIT may be toxic as we did not see any parasites past the two-to-four cell stage. To determine the native localisation, we endogenously tagged VIT using 3xHA tags at the C-terminus (Fig. 3b). We saw that endogenously tagged VIT-HA colocalized with the overexpressed VIT-Ty (Fig. S4a) although we saw less expression at the periphery of the parasite. We also confirmed the expression of a single band of VIT-HA at the expected size (35 kDa) by western blot (Fig. 3c). Upon IFA, we observed VIT-HA expression as a single point in extracellular parasites. This localisation was maintained at 1 h post infection, but between 1 and 6 h post infection this point appeared to fragment and by 24 h post infection VIT-HA signal was seen in multiple small structures throughout the cytoplasm of the parasite (Fig. 3d). We quantified this fragmentation using an automated ImageJ macro and saw a significant increase ($p < 0.0001$, one way ANOVA with Tukey correction) in the number of VIT-HA foci per parasite between 1 and 6 h post infection (Fig. 3e). We also assessed the amount of VIT-HA by western blot and found that the change in localisation was not associated with a significant change in protein level (Fig. 3f and g). Dynamic localisation through the lytic cycle has previously been observed in proteins localizing to the vacuolar associated compartment (VAC) (Thornton et al., 2019; Warring et al., 2014) and as we saw some overlap with CPL with the overexpressed VIT-Ty, we tested if VIT-HA colocalised with CPL. However, we did not see colocalisation of VIT-HA and CPL at 1 h post infection (Fig. 3h). In case the epitope tag was affecting the colocalisation, we endogenously tagged VIT with a Myc epitope and saw the same dynamic localisation (Fig. S4b). Interestingly, we observed frequent colocalization between VIT-Myc and CPL (Fig. 3h). To investigate this more fully, we imaged extracellular parasites using immunoelectron microscopy (Fig. 3i). We found VIT-HA signal frequently at the vacuolar associated compartment (Fig. 3i i and ii), although occasionally it was also seen on electron-dense vesicular structures (Fig. 3i iii) or, more surprisingly, the rhoptries (Fig. 3i iv). If VIT localises, even transiently, to the VAC it is possible that the VAC is the site of iron storage in the cell. To determine how the VAC responded to excess iron, we treated parasites with excess iron (2.5 mM FAC) to be sure of overwhelming any host cell buffering effects and determined the area of the VAC in an unbiased manner using an ImageJ macro and the luminal VAC marker CPL. We found that the total area of CPL signal significantly increased (Fig. 3j) when parental parasites were treated with high exogenous iron ($p = 0.0008$, one way ANOVA with Dunnett's correction). We saw no change in VAC area between the parental and Δ VIT parasites under normal conditions, but interestingly the area of the VAC did not change when we treated Δ VIT parasites with excess iron (Fig. 3j). These results suggest that VAC morphology is altered by excess iron through the actions of VIT, providing supporting evidence that VIT is localized to this compartment in *T. gondii*.

VIT expression is regulated by iron availability

The expression, localisation and activity of transporters are frequently regulated by the availability of the substrate. This has been well established in model organisms (Conte and Walker, 2011; Gao and Dubos, 2021; Lee et al., 2020; Sorribes-Dauden et al., 2020) and was recently reported for the arginine transporter ApiAT1 in *T. gondii* (Rajendran et al., 2021). To determine if iron affects expression of VIT, we performed qRT-PCR on *vit*. Unexpectedly, we found that transcript levels were significantly decreased ($p = 0.007$, one sample *t* test) (Fig. 4a) at 24 h upon treatment with excess iron. This is the opposite to the situation seen in yeast, where excess iron upregulates CCC1 (Li et al., 2008). In contrast, removal of iron by DFO treatment did not lead to a change in VIT RNA. As transcript and protein levels do not always correlate, we examined changes at the protein level by western blotting using VIT-HA. We found that treatment with FAC also led to a variable but significant ($p = 0.043$, one sample *t* test) decrease in VIT protein levels (Fig. 4b and c), however interestingly, removal of iron by DFO also led to a decrease ($p = 0.032$, one sample *t* test) in VIT-HA levels despite no change in RNA levels. As well as regulation at the mRNA and protein level, the function of transporters can also be regulated by changes in localisation (Lee et al., 2020). We assessed the localisation of VIT-HA upon changes in iron levels. As described above, we quantified the number of VIT-foci after treatment with FAC or DFO at 6 and 24 h post infection. At 6 h post infection we saw a significant ($p = 0.0004$, one way ANOVA with Tukey) decrease in the number of foci/parasite upon treatment with DFO but not FAC (Fig. 4d). By 24 h post infection we saw a significant decrease in foci number under both conditions ($p < 0.0001$, one way ANOVA with Tukey) (Fig. 4e). This may show a different response to high or low iron, or may reflect the speed at which the treatments take effect within the cells. In either case, changes in iron levels appear to alter the transcription, protein level and localisation of VIT within the parasite.

Transcriptional response to the absence of VIT

The finding that VIT appears regulated by iron led us to examine how the transcriptome changes in the absence of VIT and correct iron storage. RNAseq was performed on the parental and Δ VIT strains in triplicate. Under these conditions, 70 genes were downregulated upon deletion of VIT ($\log_2$ fold change (LFC) < -1 , adjusted p value (Padj) < 0.05) and 62 were upregulated ($\log_2$ fold change > 1 , adjusted p value < 0.05) under standard growth conditions (Fig. 4f, Table S1). We also saw significant upregulation of the potential plasma membrane ABC-type transporter (TGME49_239020). This gene had strong structural homology ($E = 7.1 \times 10^{-135}$, HHPRED (Söding

et al., 2005)) to multidrug efflux transporters and is named multidrug resistance protein 1 (MDR1) in *Plasmodium* (Koenderink et al., 2010). Interestingly, work in mammalian cancer cells has shown that removal of iron led to a decrease in MDR1 levels (Fang et al., 2010) and disrupting iron storage through increased expression of ferritin led to an increase in MDR expression (Epsztejn et al., 1999). Upregulation of this transporter may thus be an adaptation by the cell to attempt to remove excess iron from the parasite, in the absence of correct storage.

There are two well studied pathways involving iron in *T. gondii*, the heme biosynthesis pathway and iron-sulfur cluster biogenesis. The contribution of VIT-based iron storage to these pathways is currently unknown. We examined the transcriptional changes in the heme biosynthesis pathways (**Fig. S5a**) and found there was a general upregulation of several components of the pathway upon VIT deletion, most significantly PBGD. The only gene in this pathway which directly interacts with iron – ferrochelatase – was not altered, however there was a significant upregulation in a cytochrome c heme lyase, an important destination for heme in the parasite. *Toxoplasma* encodes three iron-sulfur cluster biogenesis pathways, localised to the cytosol, mitochondrion and apicoplast. Interestingly, we saw opposing changes in the mitochondrial ISC pathway, with downregulation of the scaffold IscU and upregulation of the acceptor IscA (**Fig. S5b**). In both mammalian and yeast cells, IscU expression is iron dependent, suggesting that the altered iron storage in the Δ VIT parasites is altering iron-dependent processes within the cell (Garland et al., 1999; Tong and Rouault, 2006). Overall, we saw significant changes in transcription upon deletion of VIT, with changes in transcription of genes and pathways known to require iron.

Iron overload leads to ROS accumulation in the absence of VIT

Ferrous iron can lead to the production of reactive oxygen species (ROS) through the Fenton reaction (Imlay et al., 1988) and loss of CCC1 in yeast is associated with increased oxidative stress (de Llanos et al., 2016). To determine if absence of VIT affected ROS accumulation in *T. gondii*, we stained parasites with CellROX Deep Red, which fluoresces in the presence of ROS, and quantified fluorescence by flow cytometry. In the parental line, treatment with high concentrations of FAC (2 mM) did not significantly increase the ROS levels, suggesting the presence of effective mechanisms for controlling iron toxicity in these cells. Although there was no increase in ROS levels under normal conditions in the Δ VIT strain, upon FAC treatment ROS levels were significantly ($p = 0.004$, one way ANOVA with Sidak's correction) higher than in the parental line (**Fig. 5a**).

To determine if the increase in ROS induced by FAC in the Δ VIT line is responsible for the increased sensitivity, we treated the parasites with the ROS scavenger N-acetyl-cysteine (NAC) (Charvat and Arrizabalaga, 2016). Upon 5 mM NAC treatment, the FAC EC₅₀ for the parental line increased slightly (from 88.28 μ M, 95% C.I. 24.4 to 307 μ M to 109.7 μ M, 95% C.I. 15 to 136 μ M), but not significantly (**Fig. 5b and c**). However, scavenging of ROS by NAC led to a significant ($p < 0.0001$, extra sum of square F test) protective effect (EC₅₀ 3.8 μ M, 95% C.I. 0.08 to 5.5 μ M, compared to 0.018 μ M, 95% C. I. 0.0021 to 0.1 μ M) to excess iron in the Δ VIT parasites (**Fig. 5b and c**). This demonstrates that a significant driver of the increased sensitivity of the Δ VIT parasites to FAC is the increased production of ROS.

Although the Δ VIT parasites have a small growth defect *in vitro*, we were intrigued to see that there was no increase in ROS under normal conditions. The parasite has various lines of defence against ROS, including thioredoxins, two SODs expressed in tachyzoites and a cytosolic catalase (Alves et al., 2021; Kwok et al., 2004; Xue et al., 2017). Although the RNAseq did not show any changes in SOD, SOD2 (**Fig. S6a**) or catalase transcription (**Fig. S6b**), we investigated the activity of these enzymes. SOD activity was visualised using a native page in gel activity assay, however saw no obvious difference between the parental and Δ VIT parasites (**Fig. S6c**). *T. gondii* encodes three SOD enzymes, two of which are expressed in tachyzoites and localised to the cytosol and mitochondria (Kwok et al., 2004; Sibley et al., 1986) and we were only able to distinguish one from the host cell SOD enzymes. Given this, we cannot exclude the possibility that SOD activity overall did change, but we were unable to visualise this. We also assessed the enzyme activity of catalase using a colorimetric assay (Hadwan, 2018; Portes et al., 2015). We found that deletion of VIT led to a significantly ($p = 0.0009$, Welch's one-way ANOVA with Dunnett's correction) increased catalase activity compared to the parental line under normal growth conditions (**Fig. 5d**). This suggests that to maintain growth, Δ VIT parasites require higher catalase activity. This is despite the lower amounts of parasite-associated iron, potentially due to the mislocalisation of iron in these parasites.

In *T. gondii*, catalase is cytosolic (Kwok et al., 2004) and would not protect the parasite's mitochondrion from ROS. To examine if excess iron changes in mitochondrial ROS (mROS) accumulation, we stained parasites with the mitochondrial-specific ROS probe MitoSOX (**Fig. 5e and f**). We found that excess iron led to an increase in mROS in the parental line, however this was significantly exacerbated in Δ VIT parasites. Interestingly we also saw a significantly higher percentage of MitoSOX-high cells in the Δ VIT under normal growth conditions, in contrast to the cytosolic ROS results (**Fig. 5a**). Despite the higher levels of mROS, we did not see a significant change in mitochondrial membrane potential as measured by MitoTracker (**Fig. S6d**). Upon examination of the literature, we found that

overexpression of a mitochondrial iron transporter can compensate somewhat for the lack of CCC1 in yeast (Li et al., 2010). Through reciprocal blast searches, we found the parasite homolog of the mitochondrial iron transporter, TGME49_277090 (**Fig. S7a**). Based on homology and in common with mammals, we called this putative transporter mitoferrin (mtf) and endogenously tagged this gene in the parental and Δ VIT parasite lines (**Fig. S7b**). Mtf-HA localised to the mitochondrion, as demonstrated by co-localisation with the mitochondrial marker TOM40 (Fig. 5g) (van Dooren et al., 2016). Interestingly, we saw an apparent accumulation of Mtf-HA in the Δ VIT line. We quantified this from immunofluorescence images and saw an increase in Mtf-HA staining in the Δ VIT line compared to TOM40 (as an internal control) (Fig. 5h). These results suggest that in the Δ VIT line, parasites upregulate Mtf at the protein level, perhaps in an attempt to remove iron from the cytosol.

VIT is required for virulence *in vivo*

The increased activity of catalase and the reduced growth in cells suggested that VIT has an important role in survival of *T. gondii* *in vitro*. To determine if VIT had a role in causing disease, we infected five Swiss Webster mice peritoneally with either 20 or 100 tachyzoites of the parental or Δ VIT::DHFR_{TS} parasites and assessed mouse survival over time (**Fig. 6a**). Deletion of VIT led to significantly increased survival after infection with both 20 ($p < 0.0001$, log-rank test) and 100 parasites ($p = 0.0027$, log-rank test) demonstrating that VIT has an important role in virulence *in vivo*.

Given the relatively small growth defect seen under normal growth conditions, we were surprised by the magnitude of the effect *in vivo*. In order to cause disease, *T. gondii* must evade the host immune system and colonise various tissues. One important bottleneck is the ability of the parasite to survive in activated phagocytic cells (Lykens et al., 2010; Wang et al., 2020). To determine if loss of VIT affected the parasite's ability to survive, we prestimulated the murine monocytic cell line RAW 264.3 with LPS and interferon- γ (IFN γ) before infecting them at an MOI of 5 for 48 h. We quantified fluorescence and normalized levels in stimulated macrophages to unstimulated cells. Δ VIT parasites demonstrated less fluorescence even in unstimulated macrophages (**Fig. 6b**), possibly related to the reduced ability of these parasites to replicate (e.g. Fig. 1f). However, after normalisation we saw that Δ VIT parasites had a significantly ($p = 0.01$, t test) reduced ability to survive in activated macrophages, with a survival of around 12 % compared to 21 % survival in the mNeon line (**Fig. 6c**). Although virulence is a complex process, it is possible that the reduced survival of the Δ VIT parasites in macrophages contributed to their inability to cause severe disease in mice.

Discussion

Iron plays a central role in metabolism; however its potential toxicity means that transport and storage are tightly regulated. Cells lacking the ability to correctly detoxify and store iron typically develop iron hypersensitivity, excess ROS and are frequently unable to survive other stresses (Li et al., 2001; Mesquita et al., 2020; Ravet et al., 2009; Ruangkiattikul et al., 2012; Zhang et al., 2012). Here, we have investigated the role of VIT in iron storage and detoxification in *T. gondii*. Deletion of VIT leads to a decrease in stored iron within the cells and iron hypersensitivity. We find that VIT localises to a dynamic compartment within the cell, which changes in distribution throughout the lytic cycle. VIT is regulated at the transcript and protein level by iron availability and the absence of VIT-mediated iron detoxification leads to transcriptional changes in the cell, including in pathways and genes known to rely on iron. We show that in the absence of VIT, parasite hypersensitivity to excess iron can be reversed by scavenging of excess ROS, suggesting that iron-generated ROS is the major cause of parasite death and that parasites lacking VIT can compensate in multiple ways, by raising antioxidant defences and possibly moving iron into alternative organelles. Finally, we show that VIT is required for survival in activated macrophages, and in pathogenesis *in vivo*, confirming the importance of iron detoxification to the parasite.

Lack of VIT causes hypersensitivity (over 20,000 fold) to excess iron in *T. gondii*. This dramatic phenotype is mostly specific to iron, as no change in the sensitivity to other metals was seen (with the exception of cadmium). This supports previous data from *Plasmodium* showing that apicomplexan VIT is a selective iron transporter (Slavic et al., 2016), unlike the homologues from rice or yeast which additionally transport zinc and manganese (Li et al., 2001; Zhang et al., 2012). Further supporting VIT specificity, we saw no changes in parasite-associated zinc upon VIT deletion and using XFM we saw that zinc and iron appeared to localise in separate compartments within the cells. In *T. gondii*, lack of VIT was also associated with decreased parasite-associated iron and a decreased ability to replicate in acute iron deprivation. These results are congruent with studies on VIT transporters of yeast and plants which also showed that the absence of CCC1/VIT was associated with reduced cellular iron (Gollhofer et al., 2014; Li et al., 2001). However, in *Plasmodium*, deletion of VIT was associated with an increase in chelatable, or labile, iron in the cells (Slavic et al., 2016). We were not able to assess the labile iron in our cells using a similar method. However, our results are likely consistent with data from *Plasmodium*, as although the total

parasite-associated iron is reduced, we believe that the labile iron in the cytoplasm increases in the absence of VIT. Supporting this, we also see increased cytosolic ROS upon iron treatment compared to the parental line.

VIT had a highly dynamic localisation within the cell, existing as a single point in extracellular parasites and shortly after invasion before fragmenting as parasite replication was initiated. We saw limited co-localisation between VIT and cathepsin protease L (CPL), a marker of the vacuolar-associated compartment (VAC) (Miranda et al., 2010). The VAC is a dynamic, poorly-defined vesicular compartment which appears to act as a site for ingested protein digestion (Dou et al., 2014; Liu et al., 2014). Localisation of VIT to the VAC fits well with previous observations that VIT is an iron/H⁺ antiporter (Kato et al., 2019; Labarbuta et al., 2017) as the VAC is acidified (Liu et al., 2014; Miranda et al., 2010). Further, using X-ray fluorescence microscopy (XFM) to assess subcellular elemental distribution, we saw iron localising to what appears to be a single structure, which did not overlap with zinc or calcium. This supports previous reports that zinc is stored within acidocalcisomes within the cell (Chasen et al., 2019) and suggests that iron is stored within a separate compartment.

In contrast to our results, in *P. berghei*, VIT-GFP was found to co-localise with markers for the ER throughout the parasite's lifecycle (Slavic et al., 2016). It is possible that this differential localisation was influenced by the tags used (e.g. the presence of GFP can alter the localisation of proteins (Weill et al., 2018)). However, the differences in the localisation observed between *T. gondii* and *P. berghei* may also reflect fundamental differences in the biology of these parasites. *Plasmodium* parasites replicate asexually within red blood cells which contain large amounts of iron-bound hemoglobin. For this reason, in *Plasmodium* the vast majority of parasite-associated iron is sequestered as hemozoin crystals within the digestive vacuole (Kapishnikov et al., 2017; Marvin et al., 2012). In contrast, *T. gondii* lives in the relatively iron-poor environment of nucleated cells. This difference in environmental conditions may be driving the differences in localisation observed. However, the phenotype of VIT in both species suggests a surprising overlap in mechanisms of iron detoxification. Further studies, examining the localisation of VIT in greater detail in *T. gondii* and *Plasmodium* may be helpful to understand the location and dynamics of iron storage in both species.

Our data reveal that VIT levels drop transcriptionally upon iron excess, and at the protein level both upon iron depletion and excess. Transcriptional and post-transcriptional control of iron uptake and storage have been examined in detail in bacteria, yeast, plants and mammalian cells (Andrews et al., 2003; Gao and Dubos, 2021; Li et al., 2008; Philpott et al., 2012; Wang and Pantopoulos, 2011), and was recently also demonstrated in the kinetoplastid parasite *Trypanosoma brucei* (Carbajo et al., 2021). However, iron-mediated regulation has not previously been observed in the Apicomplexa. Interestingly, upon excess iron we saw a drop in transcript and protein levels of VIT, the opposite response to the predicted upregulation as seen in yeast (Li et al., 2008). This may suggest that *T. gondii* VIT has other functions within the cell, however this will be investigated in future work. Recently, expression of an amino acid transporter, ApiAT1, has been shown to be regulated by substrate availability via an upstream small ORF in *T. gondii* (Rajendran et al., 2021). VIT now joins ApiAT1 as the only substrate-regulated transporters identified in the Apicomplexa, although our results suggest that VIT may be regulated at multiple levels, including by modulating localisation. Understanding when and how each method of regulation is used by the parasite, and the pathways controlling this, remains to be unpicked.

As we were able to make a clean knockout of VIT, we were able to assess how the parasites compensated for the lack of iron storage. It has been shown that in bacteria, yeast, and some plants under stressful conditions, cells will induce iron sparing programs, reducing the production of some iron-containing proteins while prioritising others, often orchestrated at the transcriptional level (Blaby-Haas and Merchant, 2013; Lin et al., 2011; Philpott et al., 2012; Sanvisens et al., 2011). Our findings provide the first evidence of such programs in *T. gondii*. In the absence of VIT, *T. gondii* represses IscU while upregulating IscA. Transcriptional control of IscU by iron levels has been observed in a number of organisms (Garland et al., 1999; Tong and Rouault, 2006) and regulation of an IscA homolog by iron has been observed in bacteria (Andrews et al., 2003). Given the recent interest in iron sulfur biogenesis in *T. gondii* as a target for drug development (Aw et al., 2021; Pamukcu et al., 2021), these findings suggest a currently unknown mechanism regulating this essential metabolic pathway. Beyond transcription, we found that under normal conditions, Δ VIT parasites attempted to compensate by increasing the activity (although not expression) of catalase and possibly other antioxidants, however this increase was not sufficient to prevent ROS accumulation upon further stresses. The importance of iron storage and detoxification across the tree of life (Arosio et al., 2015; de Llanos et al., 2016; Li et al., 2010; Ravet et al., 2009) highlights the dangers of iron to almost all cells. We also saw an increased expression of a putative mitochondrial iron transporter, Mtf, in the absence of VIT. We suggest that in the absence of correct iron storage, *T. gondii* upregulates this transporter, increasing the iron (and ROS) levels in the mitochondrion, to help remove iron from the cytosol. A similar pathway has been proposed in yeast (Li et al., 2010) and suggests a level of metabolic flexibility in *T. gondii*. The response of the parasites to oxidative stress and its role in the lytic life cycle has of been of interest recently (Alves et al., 2021; Augusto et al., 2021) and how iron uptake and storage are linked with these pathways will be an interesting future area of research.

VIT also has an important role in survival in activated macrophages. Activation of macrophages by IFN γ leads to not only induction of pathogen killing programs, but also nutrient restriction, including reduced levels of iron (Abreu et al., 2020; Adams et al., 1990; Appelberg, 2006). Further, a previous study on IFN γ -activated enterocytes saw that *T. gondii* growth restriction was reversible by addition of excess iron (Dimier and Bout, 1998). Given that the Δ VIT parasites are more susceptible to acute iron loss, this may be enough to restrict parasite replication, although we cannot rule out that the parasites are more susceptible to the stressful conditions of the phagosome.

In conclusion, we provide the first study of an iron transporter in *T. gondii*. We show that it has a role, although is not essential, for growth under normal conditions, but is vital in detoxification of excess iron. This work provides a basis for the future study of iron within *T. gondii* where many questions remain, including how iron enters the parasite, how it moves between the organelles and the mechanisms of iron-mediated regulation. The conservation of VIT between the Apicomplexa demonstrates that, despite the very different environments that the parasites make home, they utilise common mechanisms to limit environmental toxicity.

Materials And Methods

T. gondii and host cell maintenance

Toxoplasma gondii tachyzoites were maintained at 37°C with 5% CO $_2$ and were grown in human foreskin fibroblasts (HFFs) cultured in Dulbecco's modified Eagle's medium (DMEM) that was supplemented with either 3% (D3) or 10% heat-inactivated foetal bovine serum (D10) (FBS), 2mM L-glutamine and 10 μ g ml $^{-1}$ gentamicin.

T. gondii strain construction

The Δ Ku80::mNeon and Δ Ku80::tdTomato strains were generated by transfection of 40 μ g of plasmids containing Cas9 with the required sgRNA (sgRNA sequences are listed in Table 1) along with 30 μ g of purified PCR product (mNeon green expressing cassette) - amplified using primers p1 and p2 (see Table 2 for primer sequences) into the RH Δ HX line for the Δ Ku80::mNeon line and with 30 μ g of purified PCR product (tdTomato red expressing cassette) - amplified using primers p3 and p4 for the Δ Ku80::tdTomato line. Parasites were transfected into the parental RH Δ HX line using a Gene Pulse BioRad electroporator as previously described (Herm-Götz et al., 2007). Fluorescent parasites were isolated by FACS using an Aria III (BD Biosciences), and sorted directly into a 96 well plate pre-seeded with HFFs and incubated at 37°C with 5% CO $_2$ for 5-7 days. Positive fluorescent plaques were then identified using microscopy. Absence of *ku80* was confirmed by PCR using primers p7 and p8

The Δ VIT strain was constructed by co-transfection of two Cas9 guides targeted to the 5' and 3' ends of the genomic sequence with a repair template consisting of an mNeonGreen cassette (including a strong SAG1 promotor) in the opposite orientation from *vit*. Parasites were sorted and cloned out as above and integration of the repair cassette confirmed by PCR using primers 21 and 22 for the 3' junction, primers 23 and 24 for the 5' junction and 24 and 25 to amplify the endogenous gene. The Δ VIT::DHFR $_{TS}$ (Reynolds et al., 2001) was constructed by co-transfection of two Cas9 guides targeted to the 5' and 3' ends of the genomic sequence (Table 1) with a repair template consisting of the coding region of the pyrimethamine resistance cassette using primers p13 and p14. Transgenic parasites were selected with pyrimethamine, cloned by limiting dilution, and confirmed by Sanger sequencing.

The VIT-HA strain was constructed by transfection of a Cas9 guide targeting the 3' end of the genomic sequence (Table 1) and repaired with an annealed synthetic oligonucleotide (IDT) encoding 40 base pairs of homology and inserting three copies of the HA epitope sequence prior to the stop codon. Fifty million tachyzoites were transfected by electroporation in a 4 mm gap cuvette using a Bio-Rad Gene Pulser II with an exponential decay program set to 1500 V, 25 μ F capacitance and no resistance and immediately added to a confluent HFF monolayer in a T25 flask. Parasites were selected with 50 μ g/mL phleomycin at 24 h post transfection for 6 h and subsequently added to a confluent HFF monolayer in T25 flask. Parasites were cloned by serial dilution and insertion of the HA tag was confirmed by IFA and western blot.

The VIT-Myc strain was constructed by transfection of a Cas9 guide targeting the 3' end of the genomic sequence. The 50 bp upstream and downstream homologous regions around the stop codon of the VIT gene were encoded in the forward and reverse primers to generate the repair template by PCR (using primers p15 and p16, Table 2), which flanks the homologous regions at both ends of the 3xMyc epitope tag and chloramphenicol resistance cassette. The plasmid encoding the *TgVIT*-targeting guide RNA and Cas9-GFP and the repair template were co-transfected into RH Δ *ku80* parasites. The stop codon of *vit* was replaced by the 3xMyc epitope tag and

chloramphenicol resistance cassette. The transfectants were selected through multiple rounds of chloramphenicol to generate a stable population. The VIT-Myc fusion protein was confirmed by immunoblotting analysis.

The VIT-Ty overexpression plasmid was constructed from the MyoAtil-GFP overexpression construct (Harding et al., 2016). The MyoAtil-GFP was removed by digestion with EcoRI and PacI and *vit* amplified from cDNA using primers 19 and 20 (Table 2), which contained a Ty epitope tag. The insert was cloned into the vector using Gibson assembly (NEB) and the correct sequence confirmed by sequencing.

The Mtf-HA strain was constructed by transfection of a Cas9 guide targeting the 3' end of the genomic sequence (Table 1). The 50 bp upstream and downstream homologous regions around the stop codon of the *mtf* gene were encoded in the forward and reverse primers to generate the repair template by PCR (using premier p26 and p27, Table 2), which flanks the homologous regions at both ends of the 3xHA epitope tag and chloramphenicol resistance cassette. Parasites were transfected as above and selected using chloramphenicol prior to cloning and the insertion of the HA tag verified by PCR and immunofluorescence.

Immunofluorescence Assays

Indirect immunofluorescence assays (IFA) were performed on both infected HFF monolayers or naturally egressed extracellular tachyzoites. Intracellular *T. gondii* parasites that were grown on HFFs on coverslips were fixed with 4% paraformaldehyde (PFA) for 20 minutes at room temperature and then washed with 1X phosphate buffered saline (PBS). Extracellular parasites for immunofluorescence assays were spun onto poly-L-lysine (Advanced BioMatrix) coated coverslips and fixed with 4% PFA for 20 minutes at room temperature and washed once in PBS. Cells were permeabilized and blocked in PBS/0.2% Triton X-100/2% Bovine Serum Albumin (BSA) (PBS/Triton/BSA), for 30 minutes at room temperature or 4°C overnight. The slides were then incubated in a wet chamber with primary antibodies (rat anti-HA, Merk (11867423001) 1:1000 and guinea pig anti-CDPK, a kind gift of Dr Sebastian Lourido (Whitehead Institute), 1:5000, rabbit anti-CPL, a kind gift from Dr. Vern Carruthers (University of Michigan), 1:500, mouse anti-MYC (LifeTechnologies) 1:400) in the PBS/Triton/BSA mixture for 1 hour at room temperature. After washing, slides were incubated in a wet chamber with the secondary antibodies (Alexa Fluor Goat anti-Rat 594, Invitrogen, 1:1000 and Alexa Fluor Goat anti-Guinea Pig, Invitrogen, 1:1000) in PBS/Triton/BSA for 1 h at room temperature in the dark. After further washes, cells were then mounted onto slides with Fluoromount with DAPI (Southern Biotech). Micrograph images were obtained using a DeltaVision microscope (Applied Precision) and processed and deconvolved using SoftWoRx and FIJI software.

X-ray Fluorescence Microscopy (XFM)

Samples for X-ray fluorescence microscopy were prepared following the protocol outlined by Finney and Jin (Finney and Jin, 2015). Briefly SiN windows (Norcada, Edmonton, Alberta, Canada) were attached to the bottom of a 35mm tissue culture dish and HFFs were seeded overnight and infected with RHΔ*Ku80*Δ*HXG* (parental) parasites or ΔVIT::DHFR_{TS} parasites at an MOI of 3. Parasites were allowed to invade for 1 hour at 37°C with 5% CO₂, fixed with 4% PFA for 20 minutes at RT, and washed with TRIS-Glucose buffer (260 mM glucose, 9 mM acetic acid and 10 mM Tris buffer, pH 7.4). Elemental mapping was performed at the Advanced Photon Source Synchrotron X-ray microprobe at Sector 2, beamline station 2-ID-E with a beam focused to 0.3 micron using Fresnel Zone Plates using hard X-rays of 10.5 keV, provided by Kohzu double-crystal monochromator. Fluorescence signal from the raster-scanned samples was collected with a 4-element Vortex-ME4 Silicon Drift Detector (Hitachi High-Tech Science America, Chatsworth, California, US) positioned at 90° to the incident beam. Per pixel elemental fitting and quantification was obtained using the thin-film reference sample AXO 1X (AXO DRESDEN, GmbH, Germany). Analysis was performed using MAPS software (Vogt, 2003).

Plaque assays

Plaque assays were performed as previously described (Sidik et al., 2016). Briefly, 500 parental or ΔVIT parasites were applied onto confluent monolayers of HFF cells. Parasites were allowed to replicate for 7 days undisturbed before being washed with PBS, fixed with ice cold 70% ethanol and stained using crystal violet stain (12.5 g crystal violet in 125 ml ethanol, diluted in 500 ml 1% ammonium oxalate) for 10 mins at room temperature before washing with distilled H₂O and imaging.

Extracellular survival

To assess extracellular survival, parasites were mechanically released from host cells, counted, and diluted to 1 parasite/μl in D3 and incubated at 37 °C for the indicated time. 100 μl of parasites were then placed onto two wells of a 12 well plate of confluent HFF cells

and allowed to form plaques (as above) for 5 - 7 days. Plaques were counted and normalised to parasites not incubated extracellularly.

Competition assay

Δ Ku80::mNeon, Δ Ku80::tdTomato and Δ VIT extracellular parasites were counted and normalized to 2×10^7 cells/ml in HBSS + 1% FBS. Equal numbers of Δ Ku80::mNeon and Δ Ku80::tdTomato parasites, and Δ Ku80::tdTomato and Δ VIT parasites were co-inoculated in a 1:1 ratio into host cells with either DMEM alone or DMEM supplemented with 200 μ M FAC (2 technical replicates per condition). The mixed populations were subsequently passaged every 2 or 3 days for about 10 days. At every passage, parasites were filtered and collected for analysis using a BD Celesta, data acquired using FACSDiva software (BD Biosciences). Data were processed using FlowJo v10 (BD Biosciences).

Fluorescent growth assay

Growth assays of fluorescent cells were performed using mNeon-expressing parasites in black, clear bottomed 96 well plates, preseeded with HFFs. Host cells were infected with 1,000 either Δ Ku80::mNeon or Δ VIT tachyzoites per well and were allowed to invade for 2 hours at 37°C with 5% CO₂. After 2 hours, the media was supplemented with either FAC, DFO, Ars, ZnSO₄, CuSO₄, CdCl₂ at appropriate concentrations and incubated at 37°C with 5% CO₂ for 4 days. 5 mM N-acetyl-cysteine (NAC, Sigma A7250) was added to all wells where appropriate. Fluorescence (at 594 nm emission) for each well was recorded at 4 days using a PHERAstar FS microplate reader (BMG LabTech). All experiments performed in triplicate wells, at least three independent biological replicates. Uninfected wells served as blanks and fluorescence values were normalised to infected, untreated wells. Dose-response curves and EC₅₀s were calculated using GraphPad Prism 9 software.

ICP-MS

Total parasite-associated iron was quantified using ICP-MS. Confluent HFF monolayers in D150 culture flasks were infected with either mock, parental, or Δ VIT::DHFR_{TS} parasites and cultured for 30h. Parasites were harvested by mechanical lysis and filtration through 0.3 μ m filters and quantified on a hemocytometer. Parasites were washed with chelexed PBS and digested in 30% Nitric acid at 85°C for 3h. Digested samples were diluted in 2% Nitric acid with 0.025% Triton X-100. A four-point calibration curve was generated (5, 10, 25, and 50 ppb Fe) and ⁵⁶Fe was quantified using Scandium (⁴⁵Sc) as an internal standard.

Alamar blue assay

HFF cells were grown in black opaque 96-well plates until confluent and were treated with indicated concentrations of sodium arsenite (Ars). This was then followed by an Alamar Blue assay with 0.5 mM resazurin at 37°C with 5% CO₂ for 4 hours. PHERAstar FS microplate reader (BMG LabTech) was used to determine fluorescence at 530/25 excitation and 590/25 emission. Host cell viability was determined by normalising values to untreated HFFs. The experiment was performed in triplicate for three biological replicates.

RT-qPCR

RT-qPCR was used to assay relative *vit* expression. Human foreskin fibroblasts were incubated in either D3 or D3 supplemented with ferric ammonium citrate (5 mM) for 6 hours prior to infection with *T. gondii* (RH Δ Ku80). Parasites were cultured for 24 hours prior to collection by scraping the HFF monolayer, passing the cell suspension through a 26G needle six times and filtration to remove host cell debris. Parasites were then pelleted by centrifugation at 1500 x g for 10 min, the media was then removed and cell pellets frozen and stored at -80°C. Total RNA was extracted from parasite pellets using the RNAeasy Mini kit (Qiagen) and DNase I (Invitrogen) treated for 15 mins at room temperature followed by DNase denaturation at 65°C for 20 mins. cDNA synthesis was performed with the High Capacity cDNA RT kit (Invitrogen) according to manufacturer's instructions.

RT-qPCR was carried out on the Applied Biosystems 7500 Real Time PCR system using Power SYBRgreen PCR master mix (Invitrogen), 2 ng of cDNA per reaction and the following cycling conditions: 95°C for 10 minutes, 40 cycles of 95°C for 15 seconds, 60°C for 1 minute. Primer sequences can be found in Table 2. Reactions were run in triplicate from three independent biological experiments. Relative fold changes for treated vs. untreated cells was calculated using the 2^{- $\Delta\Delta$ Ct} method (Livak and Schmittgen, 2001) using actin as a housekeeping gene control. Graphpad Prism 9 was used to perform statistical analysis. Specifically, *t* tests testing the null hypothesis - that for each treatment $\text{ddCt}_{\text{average}} = 0$, were used to examine the statistical significance of the observed fold changes.

Immunoelectron Microscopy

For immunolabeling, the samples were fixed in phosphate buffer, pH 7.2, containing 4% freshly prepared formaldehyde. After several washes in the same buffer, they were dehydrated in ascending ethanol series and embedded in LR White resin (Agar Scientific). Ultrathin sections (70 nm thick) were obtained using an ultramicrotome (Leica Microsystems). The sections were collected on formvar-coated nickel grids and then blocked in PBS containing 3% bovine serum albumin for 1 hour. After this time, they were incubated in the presence of anti-HA (Cell Signalling (C29F4)). Then they were washed several times in blocking buffer, and incubated with 15 nm gold-conjugated Protein A (Aurion). The grids were washed several times in the blocking buffer, dried and contrasted with 4% uranyl acetate, and observed using a JEOL 1200 EX transmission electron microscope operating at 80kV.

RNAseq

T75 flasks of human foreskin fibroblasts were incubated in either standard D3, D3 supplemented with 5 mM FAC or 100 μ M DFO for 24 h prior to infection. Each T75 was then infected with $6-7 \times 10^6$ cells of wild type (RH Δ Ku80) or Δ VIT. Infected monolayers were cultured at 37°C, 5% CO₂ for 24 hours prior to parasite collection. Parasites were pelleted by centrifugation at 1500 x g for 10 min and stored at -80°C as dry pellets until required. RNA was extracted from the pellets using the RNeasy kit (Qiagen) according to the manufacturer's instructions. RNA libraries were then prepared using Illumina Stranded mRNA library preparation method and sequenced at 2 x 75 bp to an average of more than 5 million reads per sample. Raw sequencing data (FASTQ format) was processed using the Galaxy public server hosted by EuPathDB (<https://veupathdb.globusgenomics.org/>). FastQC and Trimmomatic were used for quality control and to remove low quality reads (where Q < 20 across 4bp sliding windows) and adapter sequences (Bolger et al., 2014). The filtered reads were aligned to the *T. gondii* ME49 genome using HISAT2 (Kim et al., 2015). These sequence alignments were used to identify reads uniquely mapped to annotated genes using HTseq-count. Differential expression analysis was performed in R using DESeq2 (Love et al., 2014). Additional packages EnhancedVolcano (Blighe K et al., 2021), pheatmap and ggplot2 were used to generate plots. All processed data is in Table S2 and raw FASTA files are available at <http://www.ncbi.nlm.nih.gov/bioproject/>, bioproject ID: PRJNA754376.

Western blotting

Parasites were filtered and collected by centrifugation at 1500xg for 10 minutes. Samples were lysed with RIPA lysis buffer (150 mM sodium chloride, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS) and 50 mM Tris, pH 8.0) for 15 minutes on ice. Samples were then resuspended with 4X SDS loading dye with 5% w/v beta-mercaptoethanol, boiled at 95°C for 5 minutes and separated on a 10% SDS-PAGE gel for 1.2 h at 160 v. EZ-Run Prestained protein (Fisher) ladder was used as a molecular weight marker. Proteins were then semi-dry transferred to nitrocellulose membrane in Towbin buffer (0.025 M Tris, 0.192 M Glycine, 10% methanol) for 30 minutes at 190 mA and blocked at room temperature in 5% milk in 0.1% Tween/PBS. Blots were then stained with antibodies for 1 h at room temperature: primary rat anti-HA (Merk) at 1:500 and rabbit anti-Tom40 (van Dooren et al., 2016) at 1:2,000 followed by secondary fluorescent antibodies: goat anti-rat coupled to IRDye 800 (1:10,000, LI-COR) and goat anti-rabbit coupled to IRDye 680CW (1:10,000, LI-COR) and visualized using the Odyssey LCX.

Flow cytometry

CellROX staining was used to quantify ROS as previously described (Matta et al., 2018). Briefly, Δ Ku80::mNeonGreen or Δ VIT parasites were untreated or treated with 2 mM FAC overnight, collected and washed once with PBS. Cells were stained with 10 μ M CellROX Deep red (Thermo Fisher, C10422), 5 μ M MitoSOX (Thermo Fisher, M36008) or 50 nM MitoTracker RedCMXRos (Thermo Fisher, M7512) in PBS and incubated at 37 °C in the dark for 1 h (CellROX) or 10 mins (MitoSOX and MitoTracker). Parasites, including unstrained controls, were then analysed on a BD Celesta analyser and data acquired using FACSDiva software (BD Biosciences). Parasites were gated on forward and side scatter and on green fluorescence. All data was analysed using FlowJo v10 (BD Biosciences). For CellROX, the geometric mean of CellROX fluorescence from at least 5000 parasites in the Cy5 channel was determined.

Catalase activity assay

Parental and Δ VIT parasites were untreated or treated overnight with 2 mM FAC and the activity of catalase assessed as described previously (Hadwan, 2018; Portes et al., 2015). Briefly, parasites were collected, filtered, pelleted by centrifugation, and resuspended in 80 μ l of molecular grade H₂O with 20 μ l of PBS. Parasites were lysed by freeze-thawing three times on dry ice before 50 μ l of lysate was mixed with 100 μ l of 10 mM H₂O₂ and incubated at 37 °C for 2 mins. The lysate was then mixed with 600 μ l of cobalt buffer (3.5 mM Co(NO₃)₂·6H₂O, 3.2 mM sodium hexametaphosphate, (NaPO₃)₆ and 850 mM sodium bicarbonate) and incubated in the dark at room

temperature for 10 mins. Blank samples (only cobalt buffer) and standard (no lysate) controls were included. Absorbance was read at 440 nm using a PHERAstar plate reader and activity was calculated using the following formula:

$$\text{Catalase Activity of test kU} = 2.303(\text{time}) \times \log \frac{\text{standard (absorbance)}}{\text{sample (absorbance)}}$$

In gel SOD activity assay

Parasite SOD activity was assessed as previously described with some modifications (Sibley et al., 1986). Parental and Δ VIT parasites were scraped, syringed, filtered, and pelleted. Uninfected host cells were also scraped and syringed but the filtering step was omitted. Cell pellets were resuspended in 100 mM KPO₄ buffer supplemented with cOmplete, EDTA-free protease inhibitors (Sigma) and incubated on ice for 5 mins. 10 μ l was then reserved for western blotting while the remaining was mixed with native loading buffer (2 M sucrose, 0.5% bromophenol blue) and loaded onto a polyacrylamide gel in the absence of SDS. The gel was ran at 150 v for 2-3 h at 4 °C before being washed gently in water and incubated in development solution (50 ml of 100 mM KPO₄, 50 mg Nitro Blue Tetrazolium, 7.5 mg riboflavin, 162 μ l TEMED) for 45 mins in the dark. The gel was then exposed to light and washed in distilled water 2 - 3 times before imaging.

Phylogenetic analysis of Mtf

Mitoferrin homologs were identified by reciprocal BLAST search against representative apicomplexan and model eukaryotic genomes. TGME49_235650 was used as an outgroup. Protein sequences were extracted, aligned using ClustalW and the phylogenetic tree generated by neighbour-joining and visualized using SeaView (v5.0.4) (Gouy et al., 2010).

Macrophage survival assay

2.5 x 10⁶/ml RAW 264.7 macrophages (ATCC TIB-71) were seeded onto a black, clear bottomed 96 well plate and allowed to adhere for 2 - 4 h. Macrophages were then activated by the addition of 0.2 μ g/ml LPS and 0.1 ng/ml murine recombinant interferon- γ (IFN γ) (both ThermoFisher Scientific) for 24 h. Macrophages were infected with Δ Ku80::mNeon or Δ VIT parasites at an MOI of 5 and fluorescence quantified at 48 h post infection using a PHERAstar plate reader. Survival was normalised to the fluorescence of naive macrophages. The experiment was performed in technical triplicate at least three times.

In vivo infection

Groups of five female Swiss Webster (Jackson) female mice, aged 6 weeks, were infected with either 20 or 100 tachyzoites intraperitoneally in 150 μ l of PBS. The number of injected parasites was confirmed by plaque assay. Surviving mice were tested for seropositivity by ELISA. Animal studies described here adhere to a protocol approved by the Committee on the Use and Care of Animals of the University of Michigan. Results were plotted in Graphpad Prism 9 and differences in survival assessed by the Log-rank (Mantel-Cox) test.

Declarations

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Tables

gRNA sequences

Table 1	
Name	Sequence (5'-3')
<i>ku80</i> 5'	cacttgggacggcgttgaat
<i>ku80</i> 3'	cgcggccgtcatcgagttt
<i>vit</i> 5'	gtccctttttccacgaatt
<i>vit</i> 3'	AACTTGACTGCCGCCTAGAG
<i>mtf</i> 3'	gtgaacgctctcccgatttg

Table 1. A list of the gRNA sequences used in this publication

Primer sequences

Table 2		
Name	Sequence (5'-3')	Notes
P1	aggatgcccaccccgctgccaacccgagcttgcttgcAAGCTTTTACATCCGTTGCC	<i>mNeon for vit KO FW</i>
P2	ccgaaagcttgaccgtgtgtgtcgacagacgtcaacaagaCCCTcgggggggcaagaatt	<i>mNeon for vit KO RV</i>
P3	CGCGTCTTCTGGAGGTGCCTCTTCGCCGTCTCCTCTTTCTCGAG GAAGCTTTTACATCC	<i>mNeon for ku80 KO FW</i>
P4	GATCGAGTAAGTCATCAACGTCATGCGGATCTAAGGGTCTCCCT CGGGGGGGCAAGAATT	<i>mNeon for ku80 KO RV</i>
P5	CGCGTCTTCTGGAGGTGCCTCTTCGCCGTCTCCTCTTTCCATG CATGTCCCGCTTCGT	<i>tdTomato for ku80 KO FW</i>
P6	GATCGAGTAAGTCATCAACGTCATGCGGATCTAAGGGTCTGTGT CATGTAGCCTGCCAGA	<i>tdTomato for ku80 KO RV</i>
P7	GGCGCTTCCTGGGCAAAGCTTCAAG	<i>ku80 FW</i>
P8	CCGTCAATGGCGTCACTCCGATTCCG	<i>ku80 RV</i>
P9	CGCCCAGTCCATGGTTGATA	<i>VIT RTqPCR FW</i>
P10	TGAACATGACCAAGCCTCTCT	<i>VIT RTqPCR RV</i>
P11	GGGACGACATGGAGAAAATC	<i>Actin RTqPCR FW</i>
P12	AGAAAGAACGGCCTGGATAG	<i>Actin RTqPCR RV</i>
P13	CAGACGTCAACAAGATCCAAATTCGTGGAAAAAAGGG ACAATGCAGAAACCGGTGTGTCT	<i>DHFR_{TS} for vit ko FW</i>
P14	GCCACCCCGCCTGCCAACCCGAGCTTGGCTTGAGCC ACTCTAGACAGCCATCTCCATCT	<i>DHFR_{TS} for vit ko FW</i>
P15	ATGCCATGGTGTGCTCGCAGCTCGTCGTCGGTGCAAA CTTGACTGCCGCCATTAAATTTGGAAGTGGAGGACGG	<i>forward primer for the epitope-tagging of VIT with 3xmyc epitope at its C-terminus</i>
P16	CCCAGGATGCGCCACCCCGCCTGCCAACCCGAGCTTG GCTTGACAGCCACTGCTCTAGAACTAGTGATC	<i>reverse primer for the epitope-tagging of VIT with 3xmyc epitope at its C-terminus</i>
P17	GTGCTCGCAGCTCGTCGTCGGTGCAAACCTTACTGCCG CCTACCCATACGATGTTCCAGATTACGCTGGCTATCCCT ATGACGTCCCGGACTATGCAGGATCCTATCCATATGACG TTCCAGATTACGCTTAGAGTGGCTGCAAGCCAAGCTCGG GTTGGCAGGCGGGGTGG	<i>Repair template for generation of VIT-HA FW</i>
P18	CCACCCCGCCTGCCAACCCGAGCTTGGCTTGAGCCA CTCTAAGCGTAATCTGGAACGTCATATGGATAGGATCCT GCATAGTCCGGGACGTCATAGGGATAGCCAGCGTAATCT GGAACATCGTATGGGTAGGCGGCAGTCAAGTTTGACCG ACGACGAGCTGCGAGCAC	<i>Repair template for generation of VIT-HA RV</i>
P19	CGTTGGCATTTTTCTTGGACAAAATGCAGATGCCAGCTAGCGGAGCAGG	<i>Amplification of vit from cDNA FW</i>
P20	AGCACAACGGTGATTAATTAATCGAGCGGGTCTGGTTC	<i>Amplification of vit from cDNA RV</i>

	GTGTGGACCTCCAAaaAGGCGGCAGTCAAGTTTG	
P21	gggtgtctttcgtaccccgttgc	<i>Amplification of the 3' vit junction FW</i>
P22	ctttataatggggcacatgc	<i>Amplification of the 3' vit junction RV</i>
P23	ggacttcagggttaactcctcataac	<i>Amplification of the 5' vit junction FW</i>
P24	gccgacctccatgcattttgatatc	<i>Amplification of the 5' vit junction RV</i>
P25	GTTCTTAGAAGGTCTCTATGCCATGG	<i>Confirmation of presence of vit gene FW</i>
P26	gttcctctggaacggttcaacgttcagatgggaatatgTACCCGTACGACGTCCCGGA	<i>Repair template for generation of mtf-HA FW</i>
P27	tcgccacactgtctacagagagctgactagaaagttacacCTGATAGCTGTCTTGTGTAT	<i>Repair template for generation of mtf-HA RV</i>
P28	ggtctcgctacctgcgtttctctgcg	<i>Amplification of the 3' mtf junction RV</i>
P29	GGATCCGAGCACGCGCAGTAAAG	<i>Amplification of the 5' mtf junction RV</i>

Table 2. A list of the primer sequences used in this publication

Figures

Figure 1

Iron localization and construction and analysis of a Δ VIT parasite line. **a.** X-ray fluorescence microscopy of extracellular and intracellular *T. gondii*. No overlap between Fe and Zn was observed, however overlap between Zn, Ca and P was observed in both intracellular and extracellular parasites. Line indicates the outline of the parasites. **b.** Alignment of VIT1 using Clustal Omega from *T. gondii* (TGGT1_266800), *P. falciparum* (PF3D7_1223700), *P. berghei* (PBANKA_1438600), *S. cerevisiae* (CCC1), *A. thaliana* (AtVIT1) and *E. grandis* (EgVIT1). Identity (*) and similarity (., :) indicated. Key residues for iron binding highlighted in orange. **c.** Schematic indicating the method used to replace the endogenous *vit* gene with the mNeonGreen cassette. **d.** PCR reactions confirming the replacement of the endogenous gene, expected sizes indicated on schematic. * represents secondary bands, possibly unspecific. **e.** Plaque assay demonstrating that the Δ VIT line is viable. Representative of two independent experiments. **f.** Quantification of number of parasites/vacuole at 24 h post infection. Results are the mean of $n = 3$, $\pm$ SD. p value from multiple t tests, corrected by Holm-Sidak. **g.** Quantification of extracellular survival. There was no change in the percentage of plaques formed after incubation at various times extracellularly (normalised to 0 h) between the parental and the Δ VIT parasite line. Bars are the mean of $n = 3$, $\pm$ SD. **h.** X-ray fluorescence microscopy examining elemental composition of intracellular Δ VIT parasites. No change was seen in Zn, Ca, P or S however Fe appeared either depleted and more fragmented. Line indicates the outline of the parasites.

Figure 2

ΔVIT parasites are more susceptible to iron overload. **a.** Plaque assay performed under indicated concentrations of iron (ferric ammonium chloride, FAC). ΔVIT parasites showed increased susceptibility to exogenous iron and made smaller plaques. **b.** Red tdTomato parasites were mixed with mNeon and ΔVIT parasites in a 1:1 ratio and untreated or treated with 200 μM FAC. ΔVIT parasites were significantly outcompeted by three days post infection, or two days in the presence of excess iron. Points are the mean of $n = 4$, $\pm$ SD. * $p < 0.01$, ** $p < 0.005$, t test between mNeon and ΔVIT, ++ $p < 0.005$, +++ $p < 0.0001$, t test between mNeon + 200 μM FAC and ΔVIT + 200 μM FAC, corrected with Holm-Sidak. **c.** Dose response curve showing ΔVIT parasites are significantly more sensitive to excess FAC than the mNeon line. Points are the mean of $n = 3 \pm$ SEM. **d.** Graph showing mean EC_{50} for iron, zinc, copper, cadmium, and manganese for mNeon and ΔVIT parasites, each point represents an individual experiment performed in triplicate. Bars at mean, $\pm$ SD. p values from t test. **e.** Treatment of mNeon and ΔVIT parasites with sodium arsenite, a ROS generator, showed no difference in sensitivity between the two lines. Points are the mean of $n = 3$, $\pm$ SEM. **f.** ΔVIT parasites did not show any significant change in the EC_{50} upon treatment with the iron chelator DFO. Results are $n = 3$, $\pm$ SEM. **g.** mNeon and ΔVIT parasites were allowed to invade HFF cells untreated, or pretreated with DFO for 24 h. At 14 h post invasion, average parasite/vacuole were quantified. Results mean of $n = 3 \pm$ SD, at least 100 vacuoles counted/experiment. **h.** ICP-MS quantification of parental and ΔVIT::DHFR_{TS} parasites. Bars are at the mean of $n = 3 \pm$ SD, p value from t test.

Figure 3

VIT has a dynamic localisation which alters through the lytic cycle **a.** Transient overexpression of VIT-Ty in the parental parasite line. VIT-Ty (magenta) showed colocalisation with the periphery of the parasite (IMC1) and the VAC marker CPL, but not the apicoplast marker HSP60 (all green). Scale bar 5 μ m. **b.** VIT was endogenously tagged with 3xHA tags at the C-terminus. **c.** Western blot against HA epitope demonstrated a single band at around 35 kDa. **d.** IFA of VIT-HA showing a dynamic localisation through the lytic cycle, VIT-HA exists at a single point in extracellular and 1 h post invasion parasites before fragmenting at 6 - 24 h post invasion to a number of small foci throughout the parasite. Scale bar 5 μ m. **e.** Violin plot of number of foci/parasite from $n = 3$, 100 parasites quantified/experiment. Bars at mean and quartiles. p values from one way ANOVA with Holm-Sidak correction. **f.** Western blot showing VIT-HA at indicated time points, TOM40 used as a loading control. **g.** Quantification of VIT-HA levels from three independent experiments, ns - no significant change. **h.** VIT-HA does not co-localise with the VAC marker CPL at 1 h post invasion, however overlap was seen between endogenously tagged VIT-myc and CPL. Scale bar 5 μ m. **i.** Extracellular VIT-HA parasites imaged by immunoelectron microscopy. VIT-HA signal was frequently seen at the vacuole (VAC) (**i** and **ii**) with occasional staining at structures resembling the acidocalcisomes (**iii**) and the rhoptries (R) (**iv**). Scale bar 500 nm. **j.** Violin plot of parental and Δ VIT parasites, treated for 1 h with excess FAC and area of the VAC (as assessed by CPL staining) was quantified. Results of $n = 2$, bar at mean $\pm$ quartiles.

Figure 4

VIT expression is regulated by the changes in iron levels a. qRT PCR on *vit* transcripts after treatment with FAC or DFO, normalised to actin. Points represent $n = 4$, bars at mean $\pm$ SD. p values from one sample t -test. **b.** Western blot showing levels of VIT-HA after 24 h treatment with 100 μ M DFO or 5 mM FAC. TOM40 used as a loading control. **c.** Quantification of VIT-HA levels from three independent experiments, p values from one sample t test. **d.** VIT-HA foci at 6 h post invasion after treatment with FAC or DFO. There was no significant change upon FAC treatment, however there was a significant decrease in the number of foci upon DFO treatment. Results from $n = 3$, at least 100 parasites/experiment. p values from one way ANOVA with Tukey correction. **e.** As above, but quantified at 24 h post invasion. There was a significant decrease in the number of foci after treatment with both FAC and DFO, p values from one way ANOVA with Tukey correction. . Results from $n = 3$, at least 100 parasites/experiment. p values from one way ANOVA with Tukey correction. **f.** Volcano plot from RNAseq data comparing the parental line to Δ VIT. See text for more details.

Figure 5

Iron overload in absence of VIT leads to ROS accumulation. a. mNeon and Δ VIT parasites were treated with FAC overnight, and CellROX Deep Red fluorescence quantified using flow cytometry. FAC treated parental cells had no significant effect on CellROX fluorescence, however in Δ VIT parasites, CellROX was significantly ($p = 0.004$, one way ANOVA with Sidak correction). Each point represents geometric mean fluorescence of over 10,000 cells from $n = 4$, ns - not significant. Bars are at mean, $\pm$ SD. **b.** Treatment with NAC (5 mM) rescued Δ VIT parasite hypersensitivity to FAC. Points are the mean of $n = 3$, $\pm$ SEM. **c.** Graph showing the mean EC_{50} for FAC of mNeon and Δ VIT parasites, with or without NAC treatment. Each point represents an individual experiment, bars at the mean of $n = 3$, $\pm$ SD. p values from extra sum of square F test. **d.** Δ VIT parasites had significantly higher catalase activity compared to the parental line. Each

point represents an independent experiment, $n = 3$, bar at mean $\pm$ SD. p value from one way ANOVA with Sidak correction. **e.** mNeon and Δ VIT parasites were treated as in (**a**), and MitoSOX fluorescence quantified. Overlapping histogram from a single experiment showing change in fluorescence distribution. **f.** % of cells MitoSOX positive from $n = 3$, bars at the mean $\pm$ SD. p value from one way ANOVA with Tukey correction. **g.** Immunofluorescence showing localisation and expression of parental and Δ VIT parasites endogenously expressing Mtf-HA. Scale bar 5 μ m. **h.** Quantification of Mtf-HA signal as a ratio of TOM40. Points represent individual vacuoles from $n = 3$, bar at mean $\pm$ SD. p value from one way ANOVA with Tukey correction.

Figure 6

VIT is required for parasite survival in macrophages and for pathogenesis *in vivo* **a.** 10 mice were infected with 20 or 100 tachyzoites of the indicated strain and survival monitored over the course of the experiment. p values from Log-rank (Mantel-Cox) test. **b.** mNeon and Δ VIT parasites were used to infect macrophages at an MOI of 5 and fluorescence (arbitrary units) monitored after 72 h. Results are the mean of $n = 4$, performed in triplicate, $\pm$ SD. p values from t test. **c.** After normalisation of the fluorescence in the unstimulated macrophages (from **b**) there was a significant decrease in survival of the Δ VIT parasites in stimulated (IFN γ /LPS) macrophages. Results are the mean of $n = 4$, performed in triplicate, $\pm$ SD. p values from t test.

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