Metallome of cerebrovascular endothelial cells infected with *Toxoplasma gondii* using XRF imaging and inductively coupled plasma mass spectrometry

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In this study, we measured the levels of trace elements in human brain microvascular endothelial cells (ECs) infected with *T. gondii*. ECs were infected with tachyzoites of RH strain, and at 6, 24, and 48 hours post infection (hpi), the intracellular concentration of elements was determined using two complementary approaches. A synchrotron radiation-based X-ray fluorescence (SR-XRF) system was used to quantify the concentrations of iron (Fe), zinc (Zn), copper (Cu), and calcium (Ca) in infected and uninfected (control) ECs on the sub-micron spatial resolution. *T. gondii*-hosting ECs contained less Zn levels than uninfected cells. However, levels of Ca, Fe and Cu were not significantly different between infected and control cells. Inductively Coupled Plasma Mass Spectrometry (ICP-MS) analysis revealed infection-specific metallome profiles characterized by significant increase in the levels of Zn, Fe, magnesium (Mg), and cadmium (Cd) compared with control cells at all time points after infection. Intracellular Cu showed significant differences at 6 hr and 48 hr after infection. Zn constituted the largest part (74%) of the total metal composition (metallome) of the parasite. Gene expression analysis showed infection-specific upregulation in the expression of five genes, MT1JP, MT1M, MT1E, MT1F, and MT1X, belonging to the metallothionein gene family. These results point to a possible correlation between *T. gondii* infection and increased expression of MT1 isoforms and altered intracellular levels of Zn, Mg, and Fe. Taken together, a combined SR-XRF and ICP-MS is a promising approach to study the role of elements in mediating host-parasite interaction.

Keywords: *Toxoplasma gondii*, blood-brain barrier, host-pathogen interaction, label-free imaging, X-ray fluorescence spectrometry; ICP-MS

1. Introduction

Toxoplasma gondii is an obligatory intracellular apicomplexan pathogen causing diseases by reiterating their lytic cycle, comprising host cell invasion, parasite replication and parasite egress. The ability this parasite to invade host cells, disseminate through tissues and cause disease depends critically on their ability to locate and engage with the target host tissue, the central nervous system (CNS). The blood-brain barrier (BBB) plays an important role as the biological interface that separates the host neural tissues from circulating blood. Human brain microvascular endothelial cells (ECs) constitute the fundamental component of the BBB, and together with basal lamina, astrocytic foot processes, pericytes, and tight junctions strictly regulate substance entry to neuronal tissue (Abbott 2002; Kniesel and Wolburg 2000; Eyal et al. 2009). Reaching the CNS via overcoming this protective barrier represents a fundamental step in the establishment of brain infection (Elsheikha and Khan, 2010). Metal dyshomeostasis during *Toxoplasma gondii* infection plays a role for metals in mediating parasite-host interaction.

T. gondii invades and survives in host cells by modulating host cell processes and evading innate defenses, but the mechanisms are not fully defined. Iron (Fe), copper (Cu) and zinc (Zn) are essential elements required for a multitude of cellular functions, such as enzymatic reaction, DNA synthesis, metabolic processes, and gene expression (Berg and Shi, 1996). They play key roles in the host response to infection, including the development of immune response and influencing the virulence of the microorganisms (Shankar and Prasad 1998; Weinberg 1999; Schaible and Kaufmann 2004). The intracellular homeostasis of these elements must be tightly regulated, and any alteration in their levels can have adverse impact on the host cell and its ability to respond to microbial infection. *T. gondii* infection has been shown to alter the levels of Zn, Fe, Mg and Cu in the blood of seropositive sheep (Seyerk et al. 2004) and seropositive humans (Al-Khshab and Al-Bakry, 2009), compared to their seronegative counterparts.

Therefore, it is reasonable to hypothesize that host cell elemental content changes in response to *T. gondii* infection especially in tissues with high metabolic demand such as CNS.

In this study, Synchrotron-based X-ray fluorescence microscopy (SR-XRF) was employed, for the first time, to determine changes in the cellular levels of Fe, Cu and Zn, as well as other elements in ECs challenged with *T. gondii*. SR-XRF is a chemical element imaging technique, which can be used to generate x-ray fluorescence 2D elemental maps of biological samples (Yang et al. 2005; Fahrni 2007), with detection sensitivity and spatial resolution well-suited to characterize host-parasite interaction. We compared these results with those obtained from inductively coupled plasma mass spectrometry (ICP-MS) — a current gold standard to determine the average concentration of each element per sample. Our data suggest that *T. gondii* infection of ECs results in a rise of intracellular Zn levels, which may weaken the ability of host cells to limit the parasite proliferation. Thus, limitation of cytoplasmic zinc levels may help to control infection with the intracellular parasite *T. gondii*.

2. Experimental Methods

2.1. Parasite strain

Toxoplasma gondii genotype I (RH strain) tachyzoites were maintained by passage in Madin-Darby Canine Kidney (MDCK) cell cultures grown in complete Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% heat-inactivated featl bovine serum (FBS), 2 mM glutamine, and 1% antibiotic-antimycotic solution at 37°C under a humidified atmosphere of 5% CO_2. The tachyzoites parasite were purified from their feeder MDCK cell cultures by passage through PD-10 desalting columns filled with Sephadex, as described previously (Elsheikha et al., 2006). The purified parasites were centrifuged at 800×*g*, re-suspended in fresh medium, and quantified using a hemocytometer. The final volume of pure tachyzoite suspension was adjusted with fresh RPMI medium.

2.2. Cell culture

Human brain microvascular endothelial cells (ECs) were used at passage 9 and were maintained *in vitro*, as described previously (Elsheikha et al., 2013; 2014). Briefly, ECs were propagated in tissue-culture medium composed of RPMI medium supplemented with 20% (v/v) heat inactivated FBS, 2mM L-glutamine, 1mM sodium pyruvate, 1% MEM non-essential amino acids, 1% MEM vitamins, and 2% penicillin/streptomycin (Invitrogen, GIBCO, UK). Cells were maintained in an incubator in a humidified atmosphere at 37°C and 5% CO_2. Once confluent (~3 days), cells were trypsinized using trypsin-EDTA (Invitrogen, GIBCO, UK). Cells were examined daily under light microscopy and were considered confluent when their expansion had reached a point where cells touched each other on all sides, leaving no intercellular gaps. To exclude whether cell viability has influenced elemental concentration of the ECs or parasite interaction with ECs and any subsequent measurement, the viability of the cells was assessed on a minimum of 100 cells using 0.15% trypan blue exclusion assay prior to use in any experiment.

2.3. Quantification of trace element using ICP-MS

ECs were seeded in T-175 cm^2 tissue culture flasks at 10^{10} cells/flask and grown in RPMI medium, as described above. Once a confluent cell monolayer was formed (~24 h), tachyzoites were added at a multiplicity of infection (MOI) of 2 (i.e., a host:parasite ratio of 1:2). The culture medium was collected 6, 24 and 48 hours post-infection (hpi) from the infected and non-infected (control) cultures and subjected to inductively coupled plasma mass spectrometry (ICP-MS; Varian Ultramass, Melbourne, VIC, Australia) fitted with a direct injection nebulizer

(CETAC, Omaha, NE, USA) using Rh (10 ng/l) as an internal standard in order to quantify the concentrations of the extracellular trace elements. At the same time points after infection (**Supplementary file; Figure S1**), infected and uninfected cells were harvested using a sterile cell scraper, followed by washing three times with deionized water. The cells (infected or uninfected) and purified tachyzoites were centrifuged for 5 min at 800x*g*. The cell or tachyzoite pellets were lyophilized by a Modulyo freeze-dryer (Thermo Savant, USA). These lyophilized pellets were digested for 1 h at ambient temperature using a solution containing 3 ml HNO_3, 3 ml H_2O, and 2 ml H_2O_2. The samples were heated in a microwave for 90 min. The intracellular concentration of the elements was then determined using ICP-MS. The accuracy of the method for element analysis was evaluated using bovine liver standard reference material (SRM) obtained from the National Institute of Standards and Technology (NIST SRM 1577b, USA). A total of 24 elements were measured, including major elements [iron (Fe), manganese (Mn), calcium (Ca), potassium (K), sulfur (S), and barium (Ba)] and trace elements [zinc (Zn), magnesium (Mg), phosphorous (P), boron (B), sodium (Na), vanadium (V), chromium (Cr), cobalt (Co), nickel (Ni), copper (Cu), arsenic (As), selenium (Se), rubidium (Rb), strontium (Sr), molybdenum (Mo), cesium (Cs), cadmium (Cd), and silver (Ag)].

2.4. Cell infection and sample preparation for SR-XRF

Endothelial cells were seeded on quartz slides (UQG Ltd., Cambridge, England) until they formed a monolayer (~ 24 h). *T. gondii* tachyzoites were then added to the cell monolayer at a MOI of 2. Control samples included quartz slides that were also seeded with the same number of cells, but without addition of tachyzoites. At 6, 24 and 48 hpi, three slides from each of the infected and non-infected ECs were washed 3X in sterile phosphate buffered saline (PBS) and fixed in 4% paraformaldehyde for 30 min. The slides were washed again 3X in sterile PBS and left at 4ºC in deionized water (17.8 MΩ-cm) until SR-XRF imaging.

2.5. X-ray fluorescence detection of metals

The synchrotron microprobe of beamline I18 (Mosselmans et al. 2009) at Diamond Light Source, Oxfordshire, UK was utilized to map and quantify the levels of Fe, Cu, Ca, and Zn within the ECs. Control samples were also analyzed to establish a baseline for the levels of these metals. Controls and infected cells with incubation times of 6, 24 and 48 hpi were measured in order to evaluate the metal levels within the host cells at different incubation stages after infection. Elemental maps of the three metals were produced at a resolution of approximately 3 microns covering areas of 400 μm^2. As the levels measured were in the low ppm region, 4 sec/point dwell time was used for improved counting statistics. A thin-film reference material for SR-XRF measurements (AXO Dresden GmbH) was used to evaluate the metal content in terms of ppms. The concentration maps were produced using Pymca (Solé et al. 2007) that allows fitting of the acquired spectra and translating the intensity maps into concentration distributions by taking into account an approximate cellular matrix composition. We assumed 20 micron thickness of the cell monolayer. However, 20 microns cannot replicate the real thickness because the cells have been dried, which would render the levels calculated underestimated. Therefore, the “real ppm” could be much higher than the calculated concentration. From the concentration maps regions of interest (ROI) within cells were identified based on contrast to surrounding using auto-threshold tool in ImageJ. From each ROI an average elemental value was extracted and plotted in the Scatter plots. In an effort to extract the most appropriate information possible, regions that contained single cell monolayers in contrast to overlapping ones were identified and hotspots with excessive elemental counts were excluded from the measurement. However, this approach is prone to pile-up cells and that is why single cell analysis was also performed.

2.6. Elemental analysis at a single cell level

We have developed a data-processing pipeline to quantify the elemental levels in single cells (**Figure 1**). First, we aligned the bright field image to the SR-XRF fluorescent image to ensure that the offset, orientation and resolution of both images were completely matched. From the bright field image, ROI (i.e. individual cells) were selected, and the contour of each cell was manually drawn. These regions were used as masks to crop and extract the information out from the SR-XRF fluorescent images. The individual cells were analyzed separately using connected-component labelling algorithm in MATLAB 2016b (MathWorks, Inc.). Finally, the mean and standard deviation of the element concentrations of individual cells were calculated, and these values were then averaged for at least 20 cells per sample.

2.7. Gene expression analysis

We explored whether *T. gondii* infection-induced metal dyshomeostasis through modulation of metal transporters or metallothionein. EC monolayers were harvested using trypsin and lysed with QIAshredder columns (Qiagen). Total RNA was isolated using an RNeasy Mini Kit (Qiagen), and eluted with nuclease-free water. RNA was stored at −80 °C for 1 week prior to microarray analysis. All subsequent sample handling, labelling and microarray (GeneChip™ Human Gene 2.1 ST, Affymetrix) processing was performed at the Nottingham Arabidopsis Stock Centre (NASC). The RNA concentration and quality was assessed using the Agilent 2100 Bioanalyzer (Agilent Technlogies Inc., Palo Alto, CA) and the RNA 600 Nano Kit (Caliper Life Sciences, Mountain View, CA). Samples with a minimum RNA concentration of 100 ng/µl and RNA Integrity Number (RIN) ≥ 8 were used for gene expression analysis. Single stranded complimentary DNA was prepared from 200 ng of total RNA as per the GeneChip™ WT PLUS Reagent Kit (Applied Biosystems and Affymetrix). Total RNA was first converted to cDNA, followed by *in vivo* transcription to make cRNA. Single stranded cDNA was synthesized, end labeled and hybridized for 16 h at 45°C to GeneChip™ Human Gene 2.1 ST arrays. All steps were performed by a GeneAtlas™ Personal Microarray system (Affymetrix). Gene expression data were analyzed using Partek Genomics Suite 7.0 software (Partek Incorporated). The raw CEL files were normalized using the RMA background correction with quantile normalization, log base 2 transformation and mean probe-set summarization with adjustment for GC content. Differentially expressed genes were considered significant if p-value with FDR was ≤ 0.05 and fold change of >2 or <-2.

2.8. Statistical analysis

The statistical analysis of the SR-XRF data was performed using Microsoft Excel. Statistical significance was evaluated using Student *t*-test (two tailed). A p-value of < 0.05 was considered the cut-off for a significant difference. Differences in the Mass Spectrometry-based trace element concentrations between control and infected cells at different time points were assessed by two-way ANOVA, followed by Tukey's multiple comparison test using Graphpad Prism version 5.0 (Graphpad Software, Inc., San Diego, CA). Because the dataset was not normally distributed, statistical analysis was performed on the transformed data ($\log_{10}$). This ICP-MS dataset was presented as the mean and standard error of the mean (S.E.M) from at least three independent experiments. To classify sample by measurement of one element we applied z-score classifier: mean and standard deviations of infected and uninfected samples were estimated. Under assumption of normality of concentration distribution we found threshold of optimal separation of infected and uninfected groups. This method corresponds to one-dimensional Fisher's linear discriminant. The heatmap of the ICP-MS data was produced to demonstrate the average time courses over the three time points post infection, with the elements ordered according to the cluster analysis. The dendrograms were calculated using Ward's method on

Euclidean distances. The measurements for each element are then considered in turn. For each element, a linear model is fitted with the measurements as the response variable. The covariates are time, treated as a factor with three levels, and the interaction between time and the control/infected condition, giving six parameters. The parameter estimate for the interaction parameters gives an estimate of the difference between the infected and control groups. The standard errors, *t*-values and *p*-values for the estimates were also estimated. After correcting for multiple comparisons, a threshold of $P < 0.00088$ was significant.

3. Results

3.1. Elemental levels in *T. gondii* -infected ECs using ICP-MS

The levels of elements within ECs and in culture medium infected with *T. gondii* compared with uninfected cells were obtained using ICP-MS. Our analysis identified patterns associated with *T. gondii* infection and detected significant differences ($p < 0.001$) between control and infected cultures (**Figure 2**). We also noted that intra and extra cellular measurements often differ by orders of magnitude. The intracellular concentrations of Zn, P, Se, and Rb seemed to increase over time ($p < 0.001$), whereas levels of their counterparts in the extracellular culture medium remained nearly unchanged. In regards to the intracellular levels, Ca and Ag remained flat, whereas the concentrations in the extracellular culture medium decreased over time ($p < 0.001$).

Next, we looked at the differences in individual elements between infected and control cells. The extracellular concentrations of V and Co showed an overlap of intervals of infected and uninfected samples at 6 and 48 hpi and near to overlap at 24 hpi. The intracellular levels of Zn and Mn were significantly different between infected and uninfected cells ($p < 0.001$). Intracellular level of Cu showed significant differences at 6 and 48 hpi ($p < 0.001$). In contrast, the levels of Ca, and the rest of elements were not significantly different between infected and uninfected cells ($p < 0.602$). Accuracies of intracellular z-score classifier showed sensitivity of 90%, 72.4% and 97% at 6, 24 and 48 hpi. Sensitivities of z-score classifier for the intracellular level of Cu were 98.3%, 50% and 99.9% and for the extracellular level were 65.5%, 99.9% and 86.3%, at 6, 24 and 48 hpi, respectively. Also, accuracy of intracellular z-score classifier for the intracellular concentration of Cd was 87.6%, 87.1% and 67.9% at 6, 24 and 48 hpi. The extracellular concentrations of Mo showed strong overlap for 6hr and 48hr and a difference at 24hr.

The volcano plot shows the size of the coefficient (x-axis) *versus* -log (base 10) p-value. For each element and intra/extra-cellular condition we consider the most significant time point (the time point with the smallest *p*-value). The volcano plot (**Figure 3**) shows the effect size, the difference in element levels between control and infected cells, against the corresponding *p*-value for testing significant differences. The horizontal line shows the threshold of significance. We also performed unsupervised hierarchical clustering of the element concentrations. The dendrograms illustrated separation of the samples based on the average concentration of elements in control and infected samples at 6, 24 and 48 hpi. The heatmap provided a graphical display of the temporal changes in the concentration of each element, with one row for the average control (Con) and one row for the average infected (Inf) of each sample. For most elements, the control and the infected cells are paired together (**Figure 4**). Next, the dendrogram (tree) in the heatmap was divided and produced six different groups. The mean (+/- 1 standard deviation) of the individual replicates of the elements that constitute each group were plotted at 6, 24, and 48 hpi. Elements within each of the six cluster groups shared an overall similarity. The difference in the elemental profiles is more easily observed in **Figure 5**.

3.2. Elemental content of purified parasite preparation

The element composition of *T. gondii* tachyzoites was also determined. Zn and Cu were the most abundant elements, representing 74% and 13% of the total parasite elemental content, respectively. Fe, Mn and P constituted 5%, 3% and 2%, respectively, whereas each of Na, S, and Rb accounted for ~1 % (**Figure 6**).

3.3. Trace element levels in *T. gondii*-infected ECs using SR-XFM

The micrometer resolution of synchrotron-based X-ray fluorescence microscopy (SR-XRF) enabled mapping and quantification of the level of elements in ECs infected with *T. gondii*. Maps of Ca and Zn at 6, 24, and 48 hpi of infected and control cells are shown in supplementary files (**Supplementary** files; **Figures S2-3**). Elemental imaging showed that Zn was significantly reduced during infection especially at 24 hpi compared with uninfected cells, whereas Ca was increased at 48 hpi in infected cells (**Figure 7**). Average concentration of the metal content inside single cells was achieved by connected-components labeling algorithm. This approach was sufficiently precise to separate and distinguish metal content inside individual cells in an objective and quantitative manner. The levels of Ca and Zn obtained using this image processing approach showed a similar trend to results based on analysis of hot spots in the cell monolayer (**Figure 8**). Concentration of Cu and other elelemnts concentration was below the detection limit.

3.4. Gene expression microarray data

Affymetrix GeneChip^{TM} Human Gene 2.1 ST microarray analysis of mRNA isolated from ECs, 6, 24, and 48 hpi revealed significant changes in overall gene expression. Principal component analysis (PCA) of gene expression profiles showed distinct transcriptional signatures among the six groups of samples (**Figure 9**). Further analysis showed significant gene expression values (p-value with FDR was ≤ 0.05 and fold change >2 or <-2.) in the metallothioneins (MT) - cysteine-rich, metal-binding proteins. The intensity (LS mean) of the MT1JP, MT1M, MT1E, MT1F, and MT1X genes increased as the time of the infection reached 48 hpi (**Figure 10**). These results were also confirmed by the heatmap and hierarchical clustering dendrogram (**Figure 11**). Differential expression of MT1M and MT1JP genes was detected at 6, 24 and 48 hpi. Interestingly, MT1E, MT1F, MT1X genes showed lower gene expression at 6 hpi after infection compared to MT1M and MT1JP. Gene expression increased gradually at 24 hpi for these three genes. The five genes showed similar gene expression after 48 hpi. These results show that the expression of MT1 isoforms JP, E, F, and X is increased significantly in cells infected with *T. gondii* as compared with uninfected cells.

4. Discussion

The availability and restriction of trace elements are important aspects of pathogen-host interaction (Schaible and Kaufmann, 2004). In the present study, we determined the changes in the concentrations of 23 trace elements in *T. gondii*-infected and uninfected ECs, the main constituent of the BBB, at 6, 24 and 48 hpi using ICP-MS. Significant increases were noted in the levels of Zn, Fe, Mg, and Cd in infected compared with control cells at all time points post infection. The levels of these trace elements were significantly higher at 48 hpi compared with 6 hpi and 24 hpi between infected and control cells. Zn was the most abundant element in the parasite elemental composition. Significant increases were also noted in the levels of P, K, Mn, Cu, Se, Rb, and S at 48 hpi compared with 6 hpi and 24 hpi between infected *versus* control. However, no significant differences in B, Na, Ca, V, Cr, Co, Ni, As, Sr, Mo, Cs, and Ba levels were detected between infected *versus* control or over the course of infection. We also used, for the first time, synchrotron radiation-based X-ray fluorescence (SR-XRF) with micron level spatial resolution to assess the alteration in the levels of Zn, Ca, Fe, and Cu, as well as other

elements in ECs in response to *T. gondii* infection. Elemental mapping analysis showed Zn as the element most likely to be involved in *T. gondii*-EC interaction. We developed an image analysis pipeline to separate individual cells from surrounding cells and to quantify the metal's content inside individual cells using a connected-components labelling algorithm, which assigns a unique label to each separate group of connected pixels. Results obtained by SR-XRF analysis whether based on cell monolayer or single cell levels showed a reduction in the Zn level in infected cells, indicating that single cell analysis is sufficiently sensitive to quantify the intracellular metal concentration. Reduction of Zn in infected cells can be attributed to Zn sequestration by host cells as a host defense strategy against infection with this intracellular parasite.

The difference in the concentration of Zn as revealed by ICP-MS *versus* SR-XRF is probably attributed to the ability of ICP-MS to achieve level of detection many orders of magnitude lower than SR-XRF (McComb et al., 2015). Also, ICP-MS enables absolute quantification of the elements in the entire cell population in the extracted sample. SR-XRF, on the other hand, although enables 2D spatial elemental mapping of the regions of interest, mapped areas might not be representative to the actual content of the metals in the sample due to spatial heterogeneity of the cellular phenotypes that exists even in the same cell population in the same sample. Also, while calculating the elemental concentration we assumed 20 microns thickness of the cell monolayer, which could be render the levels calculated underestimated. Therefore, the "real ppm" could be much higher than the calculated concentration, which would help, qualitatively, to bridge the difference between Zn levels estimated by the two different methods. Microarray analysis of genome-wide gene expression performed on infected and control cells using Affymetrix GeneChip reveled infection-specific upregulation of five genes; MT1JP, MT1M, MT1E, MT1F, and MT1X. These belong to the metallothionein (MT) gene family, encoding small, cysteine-rich, heavy metal-binding proteins, which play an important role in the homeostasis of transition metals (e.g. Fe, Zn and Cu) and detoxification of non-essential trace elements, such as cadmium (Cd) and mercury (Hg) (Klaassen et al., 2009), and cell proliferation (Jin et al., 2004). MTs are also stress response proteins that are induced in response to triggers, such as oxidative stress, infection, inflammation, and heavy metals (Yoshida et al., 2005), to protect against ROS (Ruttkay-Nedecky et al., 2013), via their free radicals scavenging ability (Sato and Bremner, 1993; Andrews, 2000). Because agents that produce reactive oxygen intermediates are known to induce MT, one should anticipate a marked induction of MTs during *T. gondii* infection. Increased expression of these MT genes in infected compared with uninfected cells (<1.5-fold) support the results obtained by ICP-MS, emphasizing the key role of Zn and other transition elements in mediating the host-parasite interaction.

Although the main focus of our work was a search for differential elemental levels in healthy and infected cells, it is also noteworthy that there is a biological link to potential clinical significance of our findings. During *T. gondii* infection, activated immune cells produce reactive oxygen species (ROS) and Th1-derived cytokines, such as Interleukin-2 (IL-2) and interferon-gamma (IFN-γ) in an effort to limit the parasite growth (Elsheikha and Khan, 2010). A possible role for increased level of Zn in infected cells might be related to the anti-inflammatory and antioxidant effects of Zn [Prasad 2000; Prasad et al. 2007; Bao et al., 2010] that can protect host cells from oxidative stress and DNA damage (Oteiza et al., 2000; Ho et al., 2003), known to be associated with *T. gondii* infection (Denkers et al., 2003). Zn can reduce the production of tumor necrosis factor-alpha (TNF-α) and prevent the formation of free radicals as a cofactor of SOD (Prasad et al., 2007). This anti-oxidative stress (OS) effect of Zn has been attributed to down-regulation of gene expression of ROS-producing inflammatory cytokines, such as TNF-α and IL-1β (Prasad et al., 2004). Further, Zn can limit nitric oxide (NO) production in endothelial cells by inhibiting NF-κB-dependent expression of inducible

NO synthase (iNOS) (Cortese-Krott et al., 2014). Zn plays a vital role in maintaining the integrity of vascular endothelial cells, possibly by regulating signaling events to inhibit host cell death (Meerarani et al., 2000). Indeed, increased Zn levels inhibits apoptosis via the modulation of caspase-3 activity (Sunderman, 1995; Beyersmann and Haase 2001). In contrast, reduced Zn level can induce apoptosis via activating caspases-3, -8 and -9, responsible for the proteolysis of several target proteins like poly(ADP-ribose) polymerase or transcription factors and activate apoptosis inducers, such as TNF-alpha (Seve et al., 2002). Zn deficiency can exacerbate the detrimental effects of specific fatty acids, such as linoleic acid, and inflammatory cytokines, such as TNF-α, on vascular endothelial functions. Zinc depletion can also disrupt cell membrane barrier integrity and increases the secretion of IL-8 and neutrophil transmigration (Finamore et al., 2008).

Our results showed elevated levels of Fe in *T. gondii*-infected cells, indicating that Fe is essential for *T. gondii* intracellular growth. Fe is a cofactor of many enzymes involved in diverse cellular processes including respiration and DNA replication (Cassat and Skaar, 2013). Obligatory intracellular parasites, such as *T. gondii* possess mechanisms to obtain Fe from their host to sustain their replication. This parasite hijacks Fe from the host cells by binding to transferrin receptor (TfR) during cell entry. The host expression of TfR was up-regulated in *T. gondii*-infected cells, suggesting that the parasite uses host endocytic pathway and H.O.S.T. structure for Fe acquisition. In the H.O.S.T. structures, host Fe transporters of the Nramp family pumps Fe across the endo-lysosomal membrane where *T. gondii* Fe transporters in the parasitophorous vacuole membrane could transport Fe into the parasitophorous vacuole for uptake by *T. gondii* (Gail et al. 2004). Our data also showed increase in the levels of Mg in *T. gondii*-infected cells compared with uninfected controls. Mg^{2+} and Zn^{2+} ions are required for proper functioning and full activity of *T. gondii* protein farnesyltransferase enzyme, which is responsible for catalysing isoprene lipid modifications (Ibrahim et al., 2001). Mg is also a component of metalloenzymes, which play a role in the normal functioning of the immune system (Tam et al., 2003). Decreased levels of Mg may compromise immune function and increase susceptibility to infection. Rats experimentally infected with *T. gondii*, had significantly reduced levels of Mg in their brain compared to uninfected mice (Geng et al., 2000). Reduced Mg levels were detected in the plasma of patients with chronic toxoplasmosis (Yazar et al., 2003). A marked increase in the levels of Cd was also noted in infected cells compared to controls. Cd can suppress antioxidant enzymes (Bagchi et al., 1996; Chater et al., 2009; Gupta et al., 1991;), leading to increase in the OS and thus increased ability of the parasite to replicate within host cells. These findings show the link between increases in Cd, which led to increases in MT gene expression, and increased levels of intracellular Zn, ultimately leading to maintenance of redox balance and increased resistance to apoptosis, critical processes for to parasite survival. Finally, a significant increase in the levels of P, K, Mn, Se, Rb, and S at 48 hpi compared to 6 hpi and 24 hpi was detected between infected and uninfected cells. These elements mediate numerous cellular activities by acting as a cofactor of many enzymes involved in DNA replication and protein synthesis.

5. Conclusion

Elemental profiling using a combination of non-invasive SR-XRF and highly sensitive ICP-MS, enabled rapid spatial quantification of hot spots of metals in infected cells and quantification of many elements, respectively. Our *in vitro* time course study showed a correlation between *T. gondii* infection and increased expression of MT1 isoforms and increased intracellular levels of Zn, Fe, Mg, Cd, and Co in ECs. Five metallothionein genes; MT1JP, MT1M, MT1E, MT1F, and MT1X, were differentially upregulated in infected cells. These genes should be included in further studies of the effects of *T. gondii* infection on the intracellular dyshomeostasis of transition metals. These findings call for investigation of the

cellular targets affected by metal dyshomeostasis during *T. gondii* infection, in particular, with respect to the role of Zn in modulating OS, DNA damage response/repair pathways and cell proliferation/apoptosis, and regulation of metal transporters. Because maintenance of metal levels is crucial for cellular homeostasis, investigation of the mechanisms that underpin the dysregulation of Zn and other elements in the context of *T. gondii* infection progression may be important in understanding the role of these elements in host defence against *T. gondii*.

Abbreviations

DMSe	Dimethylselenide
GPx	Glutathione peroxidase
GSH	Glutathione
ROS	Reactive oxygen species
XFM X-ray	fluorescence microscopy.

Conflicts of interest

We have no conflicts of interest.

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Figure legends

Fig. 1 Single cell elemental analysis workflow. (a) The bright field image was aligned against (b) the SR-XRF elemental map, and (c) regions of interest (i.e., single cells) in the bright field image were outlined. We then masked the regions of interest on the SR-XRF elemental map (d-f). Individual cells were separated and the average and standard deviation of each element was counted per cell, followed by calculating the average and standard error for each time point (g).

Fig. 2 Plots showing time course dynamic changes in the levels of elements within the cells and in the culture medium in response to infection. Results shown represent the mean value with one standard deviation width interval for each group by taking the arithmetic average across the three replicates available for each element at each time point. X-axis denotes time post infection and Y-axis represents concentrations of the metals. Each panel shows kinetic signature of one of 24 elements detected inside the culture cells or in the supernatant of ECs at 6, 24 and 48 hours post infection (hpi) with *T. gondii* (x-axis). Black lines are controls, red are infected. Solid lines represent the intracellular measurements, dashed lines are the extracellular medium measurements. Notably, the majority of elements did not show a unique trend for both control and infected cells. However, following infection the levels of Zn, P, Se and Rb displayed increasing levels inside the cells, whereas Ca and Ag displayed increasing levels outside the cells ($p = 0.000$).

Fig. 3 Volcano plot showing the differentially abundant elements. Elements are represented as individual circles and plotted along the x-axis by the effect size, the difference between control and infected concentration levels, and on the y-axis by the significance level, $-\log_{10}$ (p value). The horizontal line indicates the significance threshold after correcting for multiple tests, with elements above the line being significant. Only the most significant time point for each element is included.

Fig. 4 A color-scale heatmap and cluster tree representation showing fluctuations in the concentration of elements between infected and uninfected ECs over the course of infection using a colour-scale, with the elements ordered according to the cluster analysis. Average control and infected samples over the course of infection were calculated for each element by taking the mean over control replicates and infected replicates, respectively. Hierarchical cluster analysis combined with a heat map revealed trends across time points after infection (X-axis) and between the measured elements. Each element is represented by two rows, average control (Con) and average infected (Inf), and each time point by a column. The colour scale indicates the concentration value (dark blue indicate higher concentration value, darker red indicates lower elemental concentration values). The dendrograms was calculated using Ward's method on Euclidean distances between the average response vectors.

Fig. 5 Overall trends of the elements in the sub-clusters. The hierarchical clustering in Figure 4 is divided into six groups of elements. The number of elements, including infected (Inf) and control (Con), and intracellular (Intra) and extracellular (Extra), in each cluster is listed in the box next to the corresponding group, with the exception of group 1, which contained the remaining elements. The mean (solid line) is calculated across the individual replicates, which constituted the elements in each group. The kinetic shape templates (mean +/- 1 standard deviation; dashed line) represent the span of all the elemental profiles of similar dynamic patterns in a single cluster. X-axis denotes time post infection.

Fig. 6 Elemental composition of the parasite. Proportions of trace elements in *T. gondii* tachyzoites. Sodium (Na), phosphorous (P), sulfur (S), manganese (Mn), iron (Fe), copper (Cu), zinc (Zn), and rubidium (Rb).

Fig. 7 Scatter plots showing the differences in the levels of Ca and Zn in at different time points after infection, and between infected and control cells. X-axis denotes time post infection and Y-axis represents concentrations of the metals (ppm). Two replicate (indiacted as # 1 and # 2) were for each of infected and uninfected samples and at the three indicated time points.

Fig. 8 The intracellular levels of Ca and Zn as determined by single cell analysis. The level of significant $p < 0.05$ was indicated by (*).

Fig. 9 Principal component analysis (PCA) showing transcriptome differences between expression microarray data of both uninfected and infected ECs. Samples were analyzed at 6 hr, 24 hr and 48 hr after exposure to *T. gondii*. Each sample group is represented by a sphere and is color-coded to indicate the corresponding control/infection category. There was a clear difference in the transcriptome clustering at 48 hr between uninfected and infected samples, suggesting an influence on the transcriptome of the exposure time to *T. gondii*.

Fig. 10 Heatmap and hierarchical clustering dendrogram for five metallothionein genes (MT1X, MT1E, MT1F, MT1M and MT1JP). Results are expressed as fold change of gene expression for infected ECs compared to uninfected ECs at 6 hr, 24 hr and 48 hr after exposure to *T. gondii*. Red color represents upregulation of expression, grey color indicates an unchanged expression level and blue color represents downregulation of expression. The pattern and length of the branches in dendrograms reflect the relatedness of the samples or the genes. Expression of the metallothionein genes is mostly downregulated in all uninfected samples. As the infection starts, the genes become upregulated.

Fig. 11 XY plots showing the intensity of each metallothionein gene (MT1X, MT1E, MT1F, MT1M and MT1JP) across sample groups. Each point (triangle) is the LS mean value of all samples from the same group. The intensity value for the five metallothionein genes increased as the time of the infection progressed compared to controls.

Supplementary Figures

Suppl. Figure 1: Schematic illustration outlining the numbers of groups of samples used to obtain the intracellular elemental levels. Following collection of the culture medium the cultured cells were harvested and extracted in order to quantify the concentrations of the intracellular elements.

Suppl. Figure 2: High-resolution elemental map of Calcium (Ca) in endothelial cells infected with *Toxoplasma gondii* acquired by SXFM at ~3 microns resolution. (A-C) Ca SXRF map (30 μm × 40 μm) at 6 hr, 24 hr, and 48 h post infection compared to the corresponding controls (D-F). The rainbow-colored scale bar reflects the signal intensity measured as micrograms per cm^2 in each pixel, with darker pixels representing areas of low concentration and brighter pixels representing areas of increasing concentration.

Suppl. Figure 3: High-resolution elemental map of Zinc (Zn) in endothelial cells infected with *Toxoplasma gondii* acquired at the SXFM at ~3 microns resolution. (A-C) Zn XRF map (30 μm × 40 μm) at 6 hr, 24 hr, and 48 hr after infection compared to corresponding controls (D-F). The rainbow-colored scale bar reflects the signal intensity measured as micrograms per cm^2 in each pixel, with darker pixels representing areas of low concentration and brighter pixels representing areas of increasing concentration.