Transcriptomic analysis reveals *Toxoplasma gondii* strain-specific differences in host cell response to dense granule protein GRA15

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Abstract Growth and replication of the protozoan parasite *Toxoplasma gondii* within host cell entail the production of several effector proteins, which the parasite exploits for counteracting the host's immune response. Despite considerable research to define the host signaling pathways manipulated by *T. gondii* and their effectors, there has been limited progress into understanding how individual members of the dense granule proteins (GRAs) modulate gene expression within host cells. The aim of this study was to evaluate whether *T. gondii* GRA15 protein plays any role in regulating host gene expression. Baby Hamster Kidney cells (BHK-21) were transfected with plasmids encoding GRA15 genes of either type I GT1 strain ($GRA15_{I}$) or type II PRU strain ($GRA15_{II}$). Gene expression patterns of transfected and nontransfected BHK-21 cells were investigated using RNA-sequencing analysis. $GRA15_{I}$ and $GRA15_{II}$ induced both known and novel transcriptional changes in the transfected BHK-21 cells compared with nontransfected cells. Pathway analysis revealed that $GRA15_{II}$ was mainly involved in the regulation of Tumor Necrosis Factor (TNF), NF-κB, HTLV-I infection and NOD-like receptor signaling pathways. $GRA15_{I}$ preferentially influenced the synthesis of unsaturated fatty acids in host cells. Our findings support the hypothesis that certain functions of GRA15 protein are strain-dependent; and that GRA15 modulates the expression of signaling pathways and genes with important roles in *T. gondii* pathophysiology. A greater understanding of host signaling pathways influenced by *T. gondii* effectors, would allow the development of more efficient anti-*T. gondii* therapeutic schemes, capitalizing on disrupting parasite virulence factors to advance the treatment of toxoplasmosis.

Keywords *Toxoplasma gondii* · GRA15 · host-pathogen interaction · transcriptome · signaling pathways · differential gene expression

Introduction

Toxoplasma gondii is a zoonotic protozoan parasite capable of infecting almost any nucleated cell type in most species of warm-blooded mammals (Dubremetz 1998; Liu et al. 2015). Approximately 138 unique genotypes of *T. gondii* have been identified (Su et al. 2012). Of which genotypes I, II and III are the predominant lineages in North America and Europe (Howe et al. 1995). These genotypes have remarkable differences in their virulence in mice; a single parasite of the hypervirulent genotype I *T. gondii* strain can be 100% lethal to a mouse; however, genotype II (intermediate in virulence) and genotype III (avirulent) strains had LD_{50} values of 10^3-10^4 and 10^4-10^5, respectively (Sibley et al. 1992; Howe et al. 1996; Saeij et al. 2006). Parasites of genotype I have a greater ability to breach biological barriers than those of genotype II and genotype III (Boothroyd et al. 2002; Barragan et al. 2003). Differences in strain virulence are paralleled with strain-specific differences in the parasite-derived effector proteins originating from apical secretory organelles - such as microneme proteins (MICs), rhoptry proteins (ROPs) and dense granule proteins (GRAs) (Mercier et al. 2015; Hakimi et al. 2017; Liu et al. 2017).

T. gondii secretes a large array of polymorphic effector proteins, such as MICs, ROPs and GRAs, which are crucial for host cell invasion, intracellular growth and evasion of the host immune response (Fox et al. 2011; Bougdour et al. 2013; Braun et al. 2013; Ma et al. 2014). The GRAs, in particular, have been shown to contribute to *T. gondii* pathogenesis in multiple ways (Hakimi et al. 2017). GRA7/MyD88-dependent NF-κB activation has been linked to the

activation of TRAF6 and the generation of reactive oxygen species (ROS), leading to enhanced proinflammatory responses by macrophages (Yang et al. 2015). GRA16 regulates several genes associated with cell cycle progression and the p53 tumor suppressor pathway (Bougdour et al. 2013). GRA17 and GRA23 enable *T. gondii* to obtain more nutrients by altering parasitophorous vacuole membrane (PVM) permeability (Gold et al. 2015). The dense granule protein TgIS blocks the interferon (IFN) response by recruiting the Mi-2/NuRD repressor complex to STAT1 binding sites in the promoter regions of the responsive genes. GRA24 activates MAPK signaling and regulates expression of genes encoding growth factors and/or cytokines/chemokines that support the parasite replication (Braun et al. 2013).

Of note is GRA15 in type II strains ($GRA15_{II}$), a polymorphic protein, which has been shown to be essential in the activation of NF-κB pathway via induction of TRAF6 and IκB kinase (IKK), leading to phosphorylation and degradation of IκB, and IL-1β induction via activation of host caspase-1 (Rosowski et al. 2011; Gov et al. 2013). Interestingly, *T. gondii* type II-specific induction of the immunomodulatory microRNAs, miR-146a and miR-155, was found to be independent of GRA15, which is a known activator of these microRNAs (Cannella et al. 2014), suggesting that other effectors are likely to be involved in this process. Despite significant advances in our understanding of the basic biology of *T. gondii* GRAs and the identification of 30 different GRAs (Mercier et al. 2015), the full spectrum of host signaling mechanisms modulated by GRA15 remains to be elucidated and $GRA15_{I}$-induced transcriptional responses has yet to be defined.

To further explore the biological function of *T. gondii* GRA15, we examined the transcriptomes of Baby Hamster Kidney cells (BHK-21) transfected with $GRA15_I$ or $GRA15_{II}$. We hypothesized that transcriptomic analysis of host BHK-21 cells transfected with GRA15 protein from different *T. gondii* genotypes may reveal new insights into the molecular mechanisms of genotype-specific virulence contributions of GRA15 effector to the pathogenesis of *T. gondii*. Results showed that $GRA15_I$ and $GRA15_{II}$ induced known and novel transcriptional changes in transfected BHK-21 cells. Notably, $GRA15_{II}$ was mainly involved in the regulation of TNF, NF-κB, HTLV-I infection and NOD-like receptor signaling pathways; whereas, $GRA15_I$ upregulated genes associated with the synthesis of unsaturated fatty acids, probably to meet the metabolic demands of the growing parasite within the host cells.

Materials and methods

Parasite and cell cultures

Toxoplasma gondii tachyzoites, type I GT1 strain and type II PRU strain, were maintained by weekly passage in human foreskin fibroblast (HFF) cells at 37 °C with 5% CO_2 in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 10 μg/ml gentamicin, as previously described (Wang et al. 2017). Baby Hamster Kidney cells

(BHK-21) used in the transfection experiments were purchased from the American Type Culture Collection (ATCC) and maintained in DMEM containing 5% FBS.

Construction of the recombinant plasmids

Total RNA from *T. gondii* GT1 strain was isolated using TRIzol reagent according to the manufacturer's instructions (Invitrogen, USA) in order to generate a cDNA library. The fragment of GRA15 gene from GT1 strain encoding a 584-residue (from aa52 to aa635) peptide was amplified from cDNA using RT-PCR. The GRA15 PCR primers included GRA15-forward (5'-GGGGGATCCATAATTCGGTGGCTTGGGTATCTTAC-3') and GRA15-reverse (5'-GGGGAATTCTCATGGAGTTACCGCTGATTGTGTG-3'). The PCR product was cloned into a pMD19-T vector (Takara, Dalian, China) and digested with the restriction enzymes *Bam*HI and *Eco*RI. Digested fragments were purified and ligated into a pCMV-mCherry vector at the *Eco*RI and *Bam*HI sites. The recombinant plasmid (designated pCMV-GRA15$_{I}$) was transformed into *E. coli* DH5α strain (Transgen, Beijing, China) and verified by restriction digestion. The sequence of the GRA15 coding region was confirmed by sequencing (Sangon Biotech, Shanghai, China). The recombinant plasmid pCMV-GRA15$_{II}$ containing the fragment of the GRA15$_{II}$ gene encoding a 500-residue (from aa51 to aa550) peptide was similarly constructed, with the exception that total RNA was isolated from *T. gondii* PRU strain.

Expression analysis of recombinant plasmids in BHK-21

BHK-21 cells (~80% confluent) were transfected with the recombinant plasmids (pCMV-GRA15$_{I}$ or pCMV-GRA15$_{II}$) using Lipofectamine 2000 reagent (Life Technologies, USA), and harvested 48 hr post-transfection for Western blot analysis. Cells transfected with pCMV-GRA15$_{I}$ or pCMV-GRA15$_{II}$ were separately lysed using cell lysis buffer (Beyotime, China). After pelleting the cellular debris by centrifugation, cell proteins in the supernatant were collected and separated using 12% SDS-polyacrylamide gels, followed by transfer onto a polyvinylidene fluoride (PVDF) membrane (Millipore, USA) at 15 mA for 30 min. The PVDF membrane was then incubated with anti-mCherry mouse monoclonal antibody for 2 hr, and HRP-labeled goat anti-mouse IgG antibody for 1 hr. Finally, DAB reagent (TIANGEN, China) was used to reveal peroxidase activity. The BHK-21 cells transfected with an empty pCMV-mCherry vector served as a control.

Transcriptome sequencing and read alignment

Here, the transcriptomic response of BHK-21 cells to *T. gondii* GRA15 protein was determined. We performed transcriptomic analysis of BHK-21 cells transfected with pCMV-GRA15$_{I}$, pCMV-GRA15$_{II}$ or pCMV-mCherry. Three biological replicates were examined for each condition. RNA was extracted individually from each biological replicate using TRIzol reagent (Invitrogen, USA). The quality of RNA samples was assessed on RNA

6000 Nano LabChip using Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). The concentration of RNA was determined using Nanodrop 2000 (Thermo Scientific, USA). Three micrograms of RNA sample from each biological replicate was used for the construction of transcriptome libraries with a NEBNext® Ultra™ RNA Library Prep Kit for Illumina® (NEB, USA), and the index codes were added. After filtering low quality reads and removing adapters, high quality clean reads were aligned to the golden hamster genome using SOAPaligner/SOAP2. No more than 5 mismatches in the alignment were permitted. RNA isolation, library construction, RNA sequencing and reads alignment were performed at The Novogene Bioinformatics Institute (Beijing, China).

Bioinformatics analysis

Differential expression of RNAs was identified using the "DESeq" bioconductor package in R (Anders and Huber 2010). The Benjamini-Hochberg’s approach was used for adjusting false discovery rate (FDR). We defined genes as being differentially expressed between groups if they had an FDR adjusted *P*-value (q-value) of < 0.05. GO enrichment analysis was performed, and only GO terms with a corrected *P*-value < 0.05 were considered significantly enriched. KEGG database (http://www.genome.jp/kegg/) was used for pathway analysis, with a corrected *P*-value <0.05 was set as the threshold.

Results and discussion

Toxoplasma gondii expresses effectors during different stages of infection, suggesting that these effector molecules play critical roles in the parasite's life cycle (Mercier et al. 2015; Hakimi et al. 2017; Liu et al. 2017). Indeed, growth and replication of *T. gondii* within the host cell require the production of multiple effector proteins that the parasite employs in order to establish a successful infection (Fox et al. 2011; Bougdour et al. 2013; Braun et al. 2013; Ma et al. 2014; Hakimi et al. 2017). Despite considerable research efforts, many questions remain about the level of impact that these effector proteins have on the host cell signaling pathways. To identify host factors that underpin *T. gondii* pathogenesis, we compared the transcriptomes of BHK-21 cells exposed to *T. gondii* GRA15 protein with untreated cells using a transcriptome sequencing (RNA-seq) approach. We focused on examining the effect of GRA15 protein in two *T. gondii* strains belonging to two different genotypes (type 1 GT1 and type 2 PRU) on host cell transcriptomes. Global transcriptomic analysis revealed strain-specific gene expression patterns of GRA15-transfected BHK-21 cells; and identified candidate genes for future studies into the molecular mechanisms of host-*T. gondii* interaction. Our results add to the growing body of evidence that strain-specific polymorphism in *T. gondii* effectors released into host cells during infection can induce distinct differences in the modulation of host signaling pathways (Reese et al. 2011; Niedelman et al. 2012; Hakimi et al. 2017).

Construction of plasmids pCMV-GRA15$_{I}$ and pCMV-GRA15$_{II}$

Fragments of $GRA15_I$ and $GRA15_{II}$ were successfully amplified from cDNA of *T. gondii* GT1 strain and PRU strain, respectively (Fig. 1A); and recombinant pCMV-$GRA15_I$ and pCMV-$GRA15_{II}$ plasmids were successfully constructed. The resultant plasmids were confirmed by restriction enzyme digestion (Fig. 1B). To detect the expression of transduced genes, total protein was extracted from BHK-21 cells at 48 h post transfection. Western blotting analysis showed that BHK-21 cells transfected with pCMV-$GRA15_{II}$ or pCMV-$GRA15_I$ plasmids, expressed 82 kDa $GRA15_{II}$-mCherry and 90 kDa $GRA15_I$-mCherry fusion proteins, respectively (Fig. 2, lane 1 and lane 2). Cells transfected with the empty control pCMV-mCherry vector expressed a ~30 kDa protein (Fig. 2, lane 3).

Analysis of differential gene expression

The RNA integrity number values (RIN) of all 9 RNA extracts were >8.0, indicating the high quality of samples used as input to the RNA sequencing library preparation. RNA-seq raw data were submitted to the NCBI database under the accession number PRJNA394179. Comparing mRNA libraries from pCMV-$GRA15_I$-transfected BHK-21 cells with BHK-21 cells transfected with pCMV-mCherry, identified 128 differentially expressed genes (DEGs), of which 61 were up-regulated and 67 were down-regulated (Fig. 3A). Comparing mRNA libraries from BHK-21 cells transfected with pCMV-$GRA15_{II}$ to BHK-21 cells transfected with pCMV-mCherry identified 125 DEGs, including 96 up-regulated and 29 down-regulated

mRNAs (Fig. 3B). Clustering analysis of DEGs in the two treatment conditions compared with the control is shown in Fig. 3C.

GO enrichment and KEGG analysis

Using a corrected *P*-value <0.05 as a cut-off, no GO term was found significantly enriched. However, the top GO terms enriched in BHK-21 cells transfected with pCMV-GRA15$_{I}$ were involved in glucose metabolism, amino acid biosynthesis, entry into host cell, gene transcription and metabolic processes (Fig. 4A). The top enriched GO terms of DEGs in BHK-21 cells transfected with pCMV-GRA15$_{II}$ were involved in cell growth, immune response, inflammation and metal ion transport (Fig. 4B).

KEGG enrichment analysis, based on a corrected *P*-value <0.05, indicated that DEGs in BHK-21 cells transfected with pCMV-GRA15$_{I}$ were significantly enriched in the synthesis of unsaturated fatty acids (Table 1). This indicated that the *T. gondii* GT1 strain might induce unsaturated FAs synthesis in host cells (compared to PRU strain) via GRA15$_{I}$, possibly to meet the nutritional demands of the proliferating parasite. *T. gondii* contains three FAs synthesis pathways and can scavenge unsaturated FAs from host cells (Waller et al. 1998; Seeber et al. 2003; Ramakishnan et al. 2012). Lipids are key to the survival of *T. gondii* as an obligate intracellular parasite and unsaturated FAs are essential for sustaining the parasite growth and genesis of the membrane of the newly formed parasites (Gratraud et al. 2009; Alloatti et al. 2011; Ramakrishnan et al. 2013; Ramakrishnan et al. 2015).

In agreement with data from previous studies, $GRA15_{II}$ was found to activate the host NF-κB signaling pathway (Rosowski et al. 2011; Morgado et al. 2014). $GRA15_{II}$ was shown to activate the NF-κB pathway in HFFs independent of MyD88 and TRIF, but dependent on TRAF6 and the IKK complex (Rosowski et al. 2011). Our results showed that $GRA15_{II}$ activates NF-κB pathway downstream of TRAF2, TRAF3 and TRAF5, probably via the upregulation of the mitogen-activated protein kinase kinase kinase 14 (also known as NF-kappa-B-inducing kinase [NIK]), a key molecule involved in activating canonical and noncanonical NF-κB signaling pathway (Table 1, Fig. 5). Thus, it is possible that $GRA15_{II}$ contributes to the activation of NF-κB signaling pathway using a number of molecular mechanisms.

Differential gene expression analysis showed that cells transfected with pCMV-$GRA15_{II}$ were significantly enriched in TNF, HTLV-I infection and NOD-like receptor signaling pathways (Table 1). Activation of these pathways can lead to the upregulation of several host molecules; such as NLR family pyrin domain containing 3 (Nlrp3), C-C motif chemokine 2, tumor necrosis factor and alpha-induced protein 3 (Tnfaip3), all play critical roles in immune and inflammatory responses (Ozaki et al. 2015; Hung et al. 2017). $GRA15_{II}$ alone, without involvement of the parasite, was found to induce CD40 expression in bone marrow-derived macrophages in a NF-κB-dependent manner, and to promote *T. gondii* immunity through the production of interleukin-12 (Morgado et al. 2014). The $GRA15_{II}$-associated dysregulation of these immune pathways is possibly a strategy used by the parasite to evade or exploit NF-κB

immune signaling and other host defense pathways, to proliferate and persist within their hosts.

The manipulation of NF-κB pathway by *T. gondii* remains undefined. Type 1 *T. gondii* strains can transiently block NF-κB nuclear translocation regardless of the host cell infected (Shapira et al. 2002). However, type 2 strains had the opposite effect through the activity of GRA15 (Rosowski et al. 2011). Another transcription factor, GRA7, was found to activate MAPK and NF-κB, and the proinflammatory cytokine expression in macrophages (Yang et al. 2015). It is possible that other transcription factors with yet unknown molecular mechanisms are also involved in the regulation of the NF-κB pathway. We have recently shown that $GRA15_{II}$ interacts with mouse Luzp1, which is involved in regulating host non-coding RNA genes (Liu et al. 2017). It remains to be elucidated whether or not Luzp1 plays a role in the $GRA15_{II}$-induced activation of the NF-κB signaling pathway.

GRA15 appears to play additional roles in modulating the parasite's interaction with host cells. Through activation of NF-κB pathway, $GRA15_{II}$ can induce polarization of macrophages to classically activated phenotype M1 (Jensen et al. 2011). M1 macrophages produce pro-inflammatory cytokines, such as TNF-α, IL-1β, IL-6 and IL-12, and high levels of ROS and nitric oxide (Gordon 2003), probably to limit the parasite growth, hence facilitating the establishment of chronic type II *T. gondii* infection. The proto-oncogene *c-Myc* is a key transcription factor, regulating the expression of many genes involved in critical host cellular processes; such as cell cycle progression, cell metabolism and apoptosis (Dang and Lewis 1997; Dang et al. 1999), and can be upregulated during *T. gondii* infection (Franco et al.

2014). Our data also showed that $GRA15_{II}$ induces upregulation of *c-Myc* in BHK-21 cells (additional file 3), suggesting that upregulation of *c-Myc* is a potential mechanism by which $GRA15_{II}$ inhibits apoptosis of infected host cells.

In conclusion, we determined the host cell transcriptomic changes associated with exposure to *T. gondii* GRA15 protein. We analyzed RNA-seq data from BHK-21 cells expressing $GRA15_{I}$ protein of GT1 strain, or $GRA15_{II}$ protein of PRU strain and discovered altered signaling pathways in cells transfected with each protein compared to control cells. $GRA15_{II}$ was mainly involved in the regulation of TNF, NF-κB, HTLV-I and NOD-like receptor signaling pathways. $GRA15_{I}$ induced the synthesis of host unsaturated FAs. These results provide new insight into the role of $GRA15_{II}$-NIK signaling in immune response against *T. gondii*, and confirmed the finding that $GRA15_{II}$ induces activation of host cell NF-κB signaling in a strain-specific, not a cell type-specific, manner. These previously unidentified features of the host transcriptomic response to *T. gondii* GRA15 extend our understanding of the cellular signaling pathways and effectors activated following *T. gondii*. Further investigations are required to better understand the interrelationship between synergistic effects of GRA15s on host cell regulatory networks.

Conflict of interest The authors declare that they have no competing interests.

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Figure legends:

Fig. 1 Generation of the recombinant plasmids. (A) Lane M: DNA marker. Lane 1 and lane 2 represent gel electrophoresis of the $GRA15_{I}$ fragment and $GRA15_{II}$ fragment amplified from cDNA of *T. gondii* GT1 strain and *T. gondii* PRU strain, respectively. (B) Enzymatic digestion of the recombinant plasmids. Lane M: DNA marker. Lane 1 and lane 2 represent the recombinant plasmids pCMV-mCherry-$GRA15_{I}$ and pCMV-mCherry-$GRA15_{II}$ digested with *Eco*RI and *Bam*HI, respectively.

Fig. 2 Western immunoblotting analysis of the expressed protein products by positive plasmids. Lane 1: protein of the pCMV-mCherry-$GRA15_{II}$-transfected BHK-21 cell lysate; lane 2: protein of the pCMV-mCherry-$GRA15_{I}$-transfected BHK-21 cell lysate; and lane 3: protein of the empty pCMV-N-mCherry-transfected BHK-21 cell lysate.

Fig. 3 Effects of *Toxoplasma gondii* GRA15 on host gene transcription. Volcano plots showing the differentially expressed genes (DEG) of BHK-21 cells transfected with (A) pCMV-mCherry-$GRA15_{I}$ or (B) pCMV-mCherry-$GRA15_{II}$ compared to control cells. Red: upregulated genes; green: downregulated genes; blue: non-differentially expressed genes. padj, adjusted *P*-value. (C) Heat map and hierarchical clustering showing expression values of all DEGs. Each column represents a different treatment condition and each row represents a gene.

Fig. 4 Gene Ontology (GO) enrichment analysis of differentially expressed genes (DEGs) in the transfected BHK-21 cells. (A) Top GO terms in cells transfected with pCMV-$GRA15_{I}$ were involved in glucose metabolism, amino acid biosynthesis, metabolic processes, membrane remodeling, entry into host cell, and gene transcription. (B) GO terms of DEGs in cells transfected with pCMV-$GRA15_{II}$ were involved in cell growth, immune response, inflammation, and metal ion transport.

Fig. 5 NF-κB signaling pathway. Red boxes represent up-regulated genes

Table 1 Differentially expressed genes and associated signaling pathways.

Signaling pathway	Differentially expressed genes *
Mediated by $GRA15_{II}$	
TNF	C-X-C motif chemokine 2-like, Csf1, Mmp9, C-C motif chemokine 2, Tnfaip3, Csf2, Vcam1, C-X-C motif chemokine 2-like, Icam1, NIK
NF-κB	C-X-C motif chemokine 2-like, Plau, Bcl2l1, Tnfaip3, Vcam1, C-X-C motif chemokine 2-like, Icam1, NIK
HTLV-I infection	Fosl1, Fzd4, Myc, Bcl2l1, Cdkn1a, Csf2, Vcam1, Zfp36 Icam1, NIK
NOD-like receptor	Nlrp3, C-X-C motif chemokine 2-like, C-X-C motif chemokine 2-like, Tnfaip3, C-C motif chemokine 2
Mediated by $GRA15_{I}$	
Biosynthesis of unsaturated fatty acids	acyl-CoA desaturase, acyl-CoA desaturase 2-like, acyl-CoA desaturase 1-like, acyl-CoA thioesterase 7

* All differentially expressed genes were upregulated.

Supplement materials:

Additional file 1: Gene expression profiling based on Fragments per Kilobase per Million mapped reads (FPKM) values.

Additional file 2: Gene expression analysis of RNA-seq data, showing DEGs between BHK-21 cells transfected with pCMV-$GRA15_{I}$ and control BHK-21 cells transfected with pCMV-mCherry.

Additional file 3: Gene expression analysis of RNA-seq data, showing DEGs between BHK-21 cells transfected with pCMV-$GRA15_{II}$ and control BHK-21 cells transfected with pCMV-mCherry.

Additional file 4: Gene expression analysis of RNA-seq data, showing DEGs between BHK-21 cells transfected with pCMV-$GRA15_{I}$ and BHK-21 cells transfected with pCMV-$GRA15_{II}$.