ME49 stain Toxoplasma gondii enhances macrophage apoptosis and induces macrophage inflammatory gene expression

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Abstract

Toxoplasma Gondii is an obligate intracellular parasite that can invade and replicate in almost all nucleated cells of warm-blooded animals. Previous studies have shown that Macrophages are necessary for host protection to Toxoplasma, but the related mechanisms of T. gondii interaction with macrophages still elusive. To explore the cell survival ability and the ability of macrophages to change between polarization states, we conducted a series of experiments using in vitro peritoneal macrophages. In this study, ME49 T. gondii was used to co-culture with mice peritoneal macrophage, and our data showed that T.gondii could invade mouse peritoneal macrophage rapidly. Meantime, Toxoplasma also could induce peritoneal macrophage apoptosis after 36h infection compare with control group. In addition, macrophage polarization was dynamically altered into M1 macrophage after T.gondii infection, while M2 polarization maker Arg-1 expression also increased. The present results are helpful for understanding the underlying mechanisms in the abnormal outcome of macrophage polarization cause by T. gondii in vitro or in vivo.

Keywords

Toxoplasma Gondii, Apoptosis, Macrophage polarization.

1. Introduction

Toxoplasma Gondii is an obligate intracellular parasite that can invade and replicate in almost all nucleated cells of warm-blooded animals[1]. It has a world-wide geographic distribution and is known to infect many species of birds and mammals, including approximately one third of humans[2]. Following oral infection, Toxoplasma initially crosses the intestinal epithelium, disseminates into the tissues and traverses biological barriers such as the placenta and the blood–brain barrier to reach immunologically privileged sites.

Toxoplasma infection can manifest with acute symptoms or be chronically contained in thetissues, e.g. in the developing fetus, the retina and the CNS[3]. It is in these loci that the parasite causes the most severe pathology: disseminated congenital infections, severe neurological complications in immune-compromised individuals and ocular pathology in otherwise healthy individuals[4]. Thus, the pathogenesis of toxoplasmosis is linked to the passage of this obligate intracellular protozoan across normally restrictive physiological barrier.

Toxoplasma is a highly successful parasite that establishes a life-long chronic infection. Todo this, it must carefully regulate immune activation and host cell effector mechanisms. Inearly infection stage, T. gondii infect would modulate the host's Th1 response, which culminates in the production of IFN-g[5], the main mediator of resistance against Toxoplasma. Th1 type cytokines (e.g., IFN-g) synergize with pattern recognition receptors on macrophages to signal for the classical activation (or M1) of macrophages, which exert antimicrobial functions against intracellular pathogens and require the activity of NF-κB, IRF, and C/EBPδ transcription factors[6,7]. Macrophages are necessary for host

protection to Toxoplasma[8],and the IL-12 response by infected macrophages is greatly influenced by the strain type[9]. The molecular mechanism underlying parasite strain differences in proinflammatory cytokine induction by macrophages has not been resolved.

In contrast, alternatively activated macrophages (or M2) develop in a Th2 cytokine environment (IL-4, IL-13) and are inhibited by Th1 type cytokines[10]. M2 macrophages secrete anti-inflammatory molecules that can down-regulate Th1 immune processes and are important in the immune response against worm infections. M2 activation is promoted by STAT6 and PPARγ transcription factors[11].

Thus, the ability of Toxoplasma to induce specific macrophage activation states could have immediate consequences on virulence, local parasite burden, and inflammatory-related pathologies. In this paper, we try to reveal the macrophage's viability and immune response during the early stage of T. gondii infection in vitro.

2. Materials and Methods

2.1 Mice, Cells and Parasites

Six to ten week old female C57BL/6J mice was used in all experiments. All mice were maintained in specific pathogen-free conditions in accordance with institutional and federal regulations. Six to ten week old female C57BL/6J mice was peritoneal injected with 0.1ml 3% Thioglycolate for 3-6 days before harvest peritoneal macrophages. Then, thioglycolate-elicited peritoneal macrophages were obtained from C57BL/6 mice by peritoneal cold-PBS washing. Macrophages were infected with T. gondii（Moi=4）, and medium alone (RPMI 1640 (Sigma)) was used as control.

Isogenic green fluorescent protein (GFP)-expressing typeII ME49 T. gondii strain was used in all infection experiments. ME49 T. gondii was maintain in human foreskin fibroblas(HFF) cell with DMEM medium(Life Technologies) ,and supplemented with 10% fetal bovine serum (FBS; Omega Scientific) and antibiotics. Parasites were obtained by scraping T-25 flasks containing heavily vacuolated HFFs and sequentially syringe lysing the suspension through 25-gauge and 27-gauge needles. The released parasites were pelleted by spinning at 572g for 7min, washed, and counted in phosphate-buffered saline (PBS).

2.2 Flow cytometric analysis

Differently infected time-point and control (media) groups of Peritoneal exudatecells(PECs) were obtained from 6 well-plates. The collected PECs were washed twice with ice-cold PBS and passed twice through a 70μm cell strainer to prevent clogging of the FACS machine. Cells were resuspended in FACS buffer (1% FBS in PBS) at a concentration of 1-3x10^7 cells/ml on ice. A flow cytometer (BD Verse) was adopted to measure the PECs infection rate through measure the GFP+ cells.

2.3 Fluorescence microscope imaging

Mouse peritoneal macrophage cells were plated at 3x106 cells per well (6 well-plate) infected (Moi=4) for various time（0h,4h,8h,12h,24h,36h). Before microscope imaging, cells were washed twice by ice-cold PBS and fixed with 3% formaldehyde for 15min. Then, remove the formaldehyde and washing again by ice-cold PBS. Fluorescence microscope imaging of the interaction between macrophage and ME49 T. gongdii was performed using the LEICA TCS SP8 scan system (LEICA).

2.4 Cell apoptosis analysis

Double staining for AnnexinV-APC and 7-aminoactinomycinD (7-AAD) was applied to measure the apoptotic rate[12]， The operations were as: 2.0 ×105 cells were seeded in 6-well plates for 12h for cell attachment and were then incubated for 12h, 24h and 36h with T. gondii. Then each group was washed up through PBS and collecting cells with cold PBS.After they have been mixed thoroughly, the mixture was centrifuged for 5 minutes at the speed of 1000 r/min. After the superannuates is removed, PBS suspension cells were added 1000 r/min. After the superannuate is removed, PBS suspension cells were added. Then, the cell concentration was adjusted to 2 ×106/ml. 0.3ml of the

cell suspension above was chosen and spun for 5 minutes at the speed of 3000r/min. When the supernatant is removed, 0.2 ml of binding buffer suspension cells and 5μL of fluorescent labeling AnnexinV-APC reagent were added and mixed thoroughly. This mixture was cultured for 10 minutes at room temperature without exposure to light. Then, 5μL of 7-AAD was added and mixed thoroughly, followed by a 5-min culture at the temperature in a dark place. Finally, a flow cytometer (BD Verse) was adopted to measure the apoptosis rate. The software Flowjo-V10 was applied to conduct data analysis.

2.5 RNA extraction and cDNA synthesis

Primary mice peritoneal macrophages were infected with T. gondii. Macrophages were harvested by lysis in Trizol (Invitrogen, Carlsbad, California) and stored at-80℃. Total RNA was purified by chloroform extraction. Samples were centrifuged at 12,000 g for 15 min at 4 C. The aqueous layer was precipitated by addition to isopropanol followed by incubation at room temperature for 10 min and centrifugation at 12,000 g for 10 min at 4 C. RNA pellets were washed with 70% ethanol, followed by centrifugation for 10 min at 12,000 g and dissolving in DEPC-treated water. RNA solutions were treated with DNase to remove genomic DNA using the DNA-freee kit (Ambion, Austin, Texas) according to the manufacturer's instructions. RNA content was determined using nanodrop spectrophotometry (Thermo Scientific, Wilmington, Delaware). Two micrograms total RNA were used to synthesize the first-strand cDNA by a reverse transcription system, according to the manufacturer's instructions, using Prime-Script™ RT reagent Kit（Takara）.

2.6 Real-time PCR

Quantitative real-time RT-PCR was performed on an ABI7500 real-time PCR system (Applied Biosystems). The relative gene expression was determined by SDS 2.05 real-time detection system software with an adaptive baseline to determine the threshold cycle(Ct). The mRNA fold induction values were calculated by the following equation: ΔCt=Ct(target)-Ct(Hprt) ,ΔΔCt= (ΔCt(infected) – ΔCt (control) , mRNA fold change = 0.5^(ΔΔCt). Experiments were performed in triplicate, and data were expressed as mean±SD. The PCR primers for targeted genes amplification in the study are as follows:

Hprt,forward:5'-TCATTATGCCGAGGATTTG;reverse:5'-GCCTCCCATCTCCTCAT;TNFa,forward:5'-GCCTCCCATCTCCTTCAT;reverse:GAGGACCTGGGAGTAGATGAG;IL6,forward:TAGTCCTTCCTACCCCAATTTCC;reverse:TTGGTCCTTAGCCACTCCTTC;IL10,forward: GCTCTTACTGACTGGCATGAG; Arg1, forward: CAGTCTGGCAGTTGGAAGC; reverse: GGTTGTCAGGGGAGTGTTG; iNOS-II, forward: ATGGCAACATCAGGTCGG; reverse: GCACAA CTGGGTGAACTCC; IL12, forward: AGCA CTCCCC ATTCCTA CTT;reverse: ACGCACCTTTCTGGTTACAC.

2.7 Statistical Analysis

Data are represented as mean ± SD. Statistical analysis was carried out by using Microsoft Excel. Statistical significance was analyzed by unpaired two-tails Student t test.

3. Result

3.1 ME49 stain T. Gondii could invade macrophage rapidly

Most Toxoplasma strains isolated in differential area belong to just three distinct clonal lineages, the type I, II, and III strains. These strains differ in virulence in mice and likely cause different sequelae in humans [13]. Stain II Toxoplasma is a common stain in previous research because of its moderate lethality. To investigate whether avirulent stain II Toxoplasma (ME49) could invade macrophage in early stage, the ME49 Toxoplasma(Moi=4)was used to co-culture with mice peritoneal macrophage for different infect time (4h,8h,12h,24h,36h)(Figure.1). In our experiment, we found that ME49 strain Toxoplasma could invade mouse peritoneal macrophage in early stage(macrophage GFP-positive rate have been reached a stable rate after 4 hours infection(Figure.1A&1B). Furthermore, Fluorescence

microscope imaging also showed that T. gondii(ME49 strain)could invade mouse peritoneal macrophage rapidly(Figure.1C). Otherwise, from Fluorescence microscope imaging data we also found that the same macrophage can be infected more than one T. gondii.

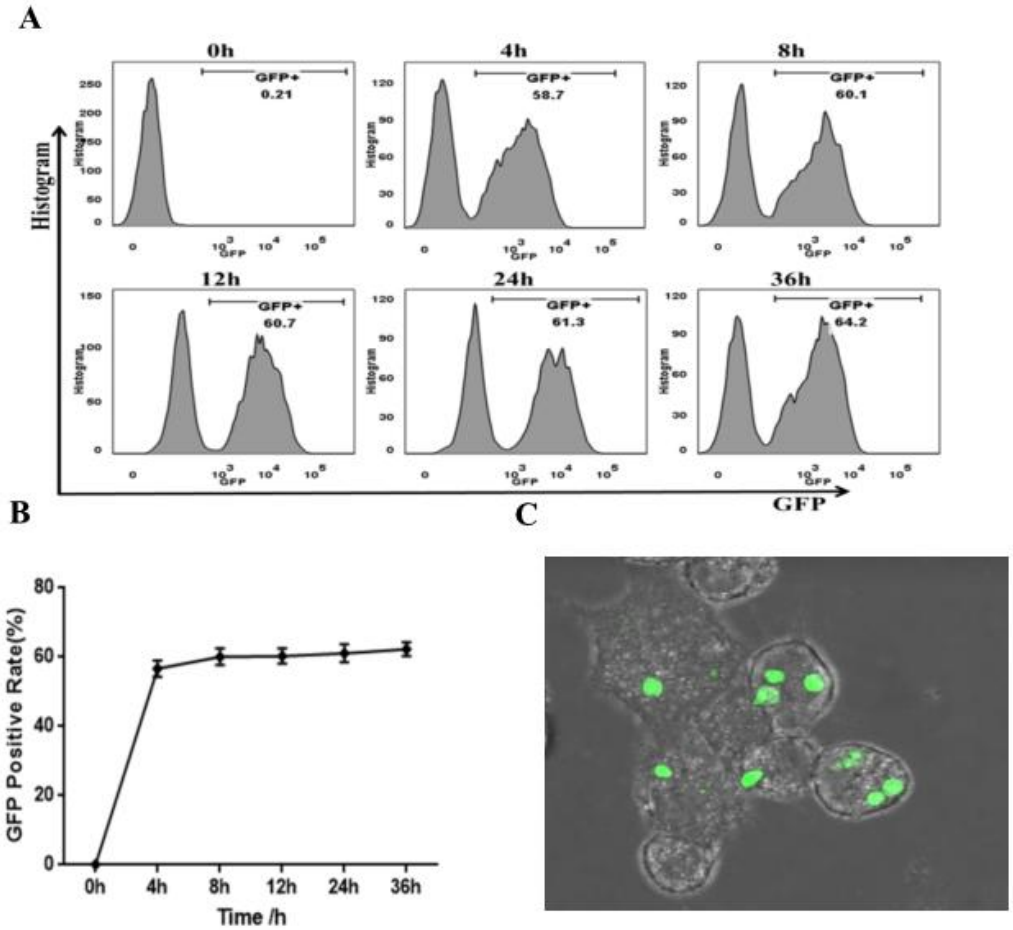

Figure.1 Toxoplasma Gondii infected mouse peritoneal macrophage.
(A).T. Gondii infect peritoneal macrophage with different infected time (4h,8h,12h,24h,36h); (B).Bar graphs depict the percentage of GFP-positively(n=3); (C). Mouse peritoneal macrophage was infect with T. Gondii (Moi=4, 24h) and stained with GFP Fluorescence microscope imaging.

3.2 Toxoplasma Gondii infection induced macrophage apoptosis in vitro

Previous study had shown that Toxoplasma infection can cause acute symptoms, such as disseminated congenital infections, severe neurological complications in immune-compromised individuals and ocular pathology in otherwise healthy individuals.Thus,we want to examine the macrophage's viability during the Toxoplasma infection; we quantified the apoptosis by phosphatidylserine externalization using the Annexin-V assay in mice peritoneal macrophage in a time kinetics based experiment. Results revealed that Toxoplasma could induce mice peritoneal macrophage apoptosis after 36h infection compare with control group (Figure.2&Table.1). Meanwhile, both the early and later stage of apoptosis rates were higher than the control group (Table.1); While, there was no difference between the control group and the infection group after 24h infection (Figure.2&Table.1).

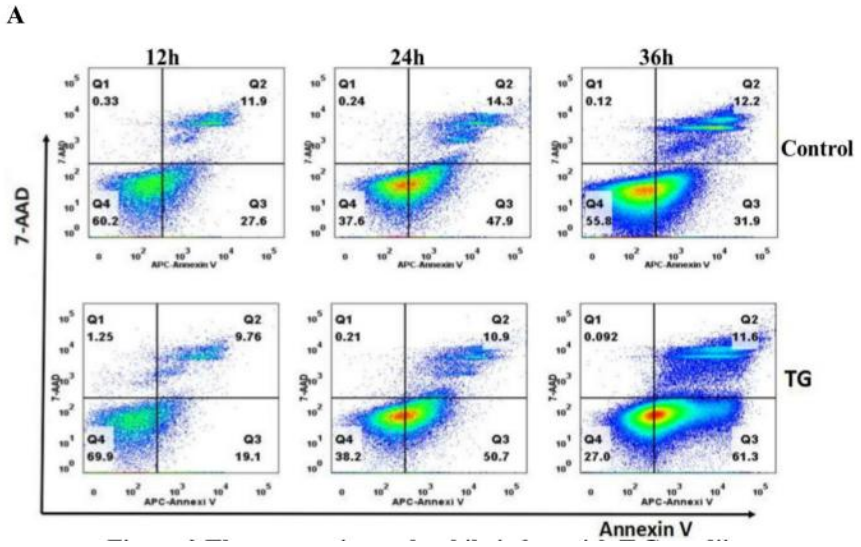

Figure.2 The apoptosis result while infect with T.Gongdii

(A).T. Gondii infected peritoneal macrophage with different infection time (12h, 24h, 36h, Moi=4)

Table.1 The apoptosis result while infect with T.Gongdii

Group(%)	12h		24h		36h	
	$APC^+/7AAD^-$	$APC^+/7AAD^+$	$APC^+/7AAD^-$	$APC^+/7AAD^+$	$APC^+/7AAD^-$	$APC^+/7AAD^+$
Control	27.6±2.31	39.5±1.64	47.9±2.81	62.2±3.72	31.9±1.48	44.1±2.57
TG	19.1±3.15	28.86±2.37*	50.7±1.75	61.6±3.49	61.3±2.44**	72.9±4.51**

*P<0.05, ** P<0.01, versus control

3.3 Macrophage polarization is dynamically altered during infection withT. Gondii in vitro

To explore the ability of macrophages to change between polarization states when suffer with T. gondii infection, we conducted a series of experiments using peritoneal macrophages which infect with ME49 stain T. gondii (Moi=4) in different infection time point(0h,12h,24h,48h). The mRNA expression of the primary M1 polarizationmarkers: iNOS(Figure.3A),TNFa (Figure.3B), IL12 (Figure.3C), IL6 (Figure.3D) and M2 polarization markers: Arg-1 was evaluated in adherence-enriched from T. gondii infected mice peritoneal macrophages. At early stage of T. gondii infection, the peritoneal macrophages were strongly M1 polarized, as they expressed high levels mRNA (Figure.3A-D) compared with control groups. Surprisingly, Arg-1, which was the M2 polarization marker, expression also increased (Figure.3E).

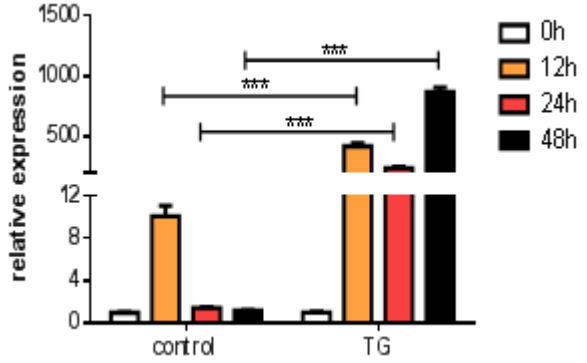

Figure.3 Macrophage polarization is dynamically altered during infection with T. Gondii in vitro

Mice peritoneal macrophages were respectively exposed to T.gondii (Moi=4) for 0h,12h,24h and 48h**.** The RT-PCR were then performed. (A-D). The expression of M1 polarization markers (iNOS,TNFa,IL12and IL6) were detected between control group and T.gondii infected group.;(E)the M2 polarization markers Arg-1 was detected in different infection point. The results represent the mean ±SD of three experiments. **P<0.01, ***P<0.001 versus control.

4. Discussion and conclusion

Macrophages are important effector cells for the control and killing of intracellular T. gondii, and also serve as long-term host cells for the replication and survival of the parasite [14]. The ability of Toxoplasma to induce specific macrophage activations could be associated with consequences on virulence, local parasite burden and inflammatory-related pathology [15]. Here, our study was aimed to explore whether the macrophage's viavility would be affected and whether the M1 and M2 macrophages could be activated by ME49 stain T. gondii.

Our experiments demonstrated that ME49 strain T.gondii infected peritoneal macrophages were preferentially biased toward the M1 activation and secreted M1 cytokines (Figure3A-D). The M1 macrophage subpopulation mediates defense of the host from a variety of pathogens and is believed to participate in various chronic inflammatory and autoimmune diseases. Mediators produced by M1, such as iNOS, TNFa, IL12and IL6(Figure.3A-D) are decisive in influencing the polarization of Th1 and Th17 cells, which further drive inflammatory responses forward. ME49 strains infected peritoneal macrophages resemble aspects of M1 activation. In addition, in T.gondii infected group, high expression of TNFa and nitric oxide (NO) in the supernatant and increased peritoneal macrophage apoptosis index in the ME49 T.gondii challenged group were observed (Figure2A &Table.1). Some evidence suggests that TNF-α could contribute to cell apoptosis[16]. These data strongly suggested that ME49 T.gondii-triggered peritoneal macrophages apoptosis might have mainly resulted from M1-secreted soluble effectors.

M2 macrophages can amplify Th2 responses and antagonize M1 macrophage responses[17]. Surprisingly, we also found that Arg-1 was highly expression in peritoneal macrophage during the ME49 T.gondii infection (Figure.3E). There have some evidence suggests that through induction of the arginase enzyme (Arg), M2 alters its L-Arginine metabolism. L-Arginine and the arginase catabolite ornithine can be scavenged by parasites to generate ATP[18] and assist their replication[19].

The present results are helpful for understanding the underlying mechanisms in the abnormal outcome of macrophage polarization cause by T.gondii in vitro or in vivo.

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