Immunobiology 222 (2017) 676–683

Contents lists available at ScienceDirect

Immunobiology

journal homepage: www.elsevier.com/locate/imbio

Inflammatory early events associated to the role of P2X7 receptor in acute murine toxoplasmosis

Gladys Corrêa[a,b,1], Carolina de Almeida Lindenberg[a,1], Aline Cristina de Abreu Moreira-Souza[a,1], Luiz Eduardo Baggio Savio[a], Christina Maeda Takiya[a], Camila Marques-da-Silva[a], Rossiane Claudia Vommaro[b,*], Robson Coutinho-Silva[a,*]

[a] Laboratório de Imunofisiologia, Instituto de Biofísica Carlos Chagas Filho, Universidade Federal do Rio de Janeiro, Brazil
[b] Laboratório de Ultraestrutura Celular Hertha Meyer, Instituto de Biofísica Carlos Chagas Filho, Universidade Federal do Rio de Janeiro, Brazil

ARTICLE INFO

Article history:
Received 10 November 2016
Received in revised form
19 December 2016
Accepted 27 December 2016
Available online 30 December 2016

Keywords:
P2X7 receptor
Toxoplasma gondii
Purinergic signaling
Inflammatory response

ABSTRACT

Activation of the purinergic P2X7 receptor by extracellular ATP (eATP) potentiates proinflammatory responses during infections by intracellular pathogens. Extracellular ATP triggers an antimicrobial response in macrophages infected with *Toxoplasma gondii in vitro*, suggesting that purinergic signaling may stimulate host defense mechanisms against toxoplasmosis. Here, we provide *in vivo* evidence in support of this hypothesis, by showing that P2X7$^{-/-}$ mice are more susceptible than P2X7$^{+/+}$ mice to acute infection by the RH strain of *T. gondii*, and that this phenomenon is associated with a deficient proinflammatory response. Four days post-infection, peritoneal washes from infected P2X7$^{-/-}$ mice had no or little increase in the levels of the proinflammatory cytokines IL-12, IL-1β, IFN-γ, and TNF-α, whose levels increased markedly in samples from infected P2X7$^{+/+}$ mice. Infected P2X7$^{-/-}$ mice displayed an increase in organ weight and histological alterations in some of the 'shock organs' in toxoplasmosis – the liver, spleen and mesenteric lymph nodes. The liver of infected P2X7$^{-/-}$ mice had smaller granulomas, but increased parasite load/granuloma. Our results confirm that the P2X7 receptor is involved in containing *T. gondii* spread *in vivo*, by stimulating inflammation.

1. Introduction

Toxoplasma gondii, the etiological agent of human toxoplasmosis, is an obligate intracellular parasite belonging to the phylum Apicomplexa, which includes *Plasmodium* spp. and other parasitic protozoa with specialized organelles in the apical region (Black and Boothroyd, 2000). *T. gondii* infection control depends on genetic characteristics and the immunological status of the host. During acute toxoplasmosis, rapid proliferation of tachyzoite forms of the parasite is contained by a vigorous INF-γ dependent cellular immune response (Mordue et al., 2001). Tachyzoites stimulate macrophages and dendritic cells to produce Interleukin 12 (IL-12) (Liu et al., 2006), triggering activation and an initial interferon-γ (IFN-γ) production by natural killer (NK) and T cells (Hunter et al., 1995). This response is crucial for the resistance against toxoplasmosis (Denkers and Gazzinelli, 1998). In addition, dendritic cells (Reis e Sousa et al., 1997), neutrophils (Bliss et al., 1999) and macrophages (Scharton-Kersten et al., 1996) respond directly to parasite antigens, producing IL-12 and the tumor necrosis factor (TNF-α), which has a role in the resistance to both acute (Johnson, 1992) and chronic (Yap et al., 1998) *T. gondii* infections. IFN-γ and TNF-α act synergically to stimulate tachyzoite killing by infected macrophages, via the production of microbicidal free radicals and nitric oxide (NO) (Denkers and Gazzinelli, 1998). T CD8$^+$ cells are the main effector T cells in the protection against *T. gondii*, while T CD4$^+$ cells have a synergic role potentiating the effect of the T CD8$^+$ cells (Parker et al., 1991). Both mouse and human T CD8$^+$ cells secrete IFN-γ and are cytotoxic to infected cells *in vitro* (Montoya et al., 1996). T CD4$^+$ cells (Th1) produce IFN-γ and interleukin-2 (IL-2), which up-modulate NK and T cell cytotoxicity towards target cells infected with *T. gondii* (Mosmann et al., 2005).

P2X receptors are ligand-gated ion channels that respond to extracellular ATP (eATP), and can be divided into seven subtypes

* Corresponding authors at: Instituto de Biofísica Carlos Chagas Filho – UFRJ, Centro de Ciências da Saúde, Bloco G. Av. Carlos Chagas Filho, 373. Cidade Universitária, Ilha do Fundão, Rio de Janeiro, RJ, 21941–902, Brazil.
E-mail addresses: vommaro@biof.ufrj.br (R.C. Vommaro), rcsilva@biof.ufrj.br, coutinhosilva.robson@gmail.com (R. Coutinho-Silva).
[1] These authors contributed equally with this work.

http://dx.doi.org/10.1016/j.imbio.2016.12.007
0171-2985/

(P2X1–7) (Ralevic and Burnstock, 1998). P2X7 receptor expression has been detected in majority of hematopoietic cells (Burnstock and Boeynaems, 2014). Importantly, the P2X7 receptor is overexpressed during inflammation (Lister et al., 2007; Moncao-Ribeiro et al., 2011; Welter-Stahl et al., 2009) and is deeply involved in inflammatory responses, since its activation induces the release of IL-1β (Ferrari et al., 1997), and TNF-α (Hide et al., 2000) by a variety of cells types, including microglia, macrophages, monocytes, dendritic cells, and fibroblasts (Idzko et al., 2014; Coutinho-Silva et al., 1999; Solini et al., 1999). Several pathological conditions that involve inflammation are associated with increased P2X7 receptor expression (Idzko et al., 2014), and both inflammatory and neuropathic pain are completely absent in P2X7$^{-/-}$ mice, where the secretion of proinflammatory cytokines (*e.g.* IL-1β) is decreased (Labasi et al., 2002; Moncao-Ribeiro et al., 2014).

During infection by the intracellular parasites *Mycobacterium tuberculosis, Chlamydia* sp. and *Leishmania amazonensis*, eATP released by ruptured infected cells contributes to local inflammation and modulates parasite intracellular survival via the activation of P2X7 receptor (Coutinho-Silva and Ojcius, 2012). In previous studies our group and others showed that *T. gondii* elimination by infected macrophages *in vitro* is induced by eATP (Lees et al., 2010; Correa et al., 2010), in a process associated with the production of reactive oxygen species (ROS) (Correa et al., 2010). Recently, it was shown that P2X7 knockout mice are acutely susceptible to toxoplasmic ileitis (Miller et al., 2015). These data suggested that P2X receptors – particularly the P2X7 subtype abundant in immune cells – contribute to *T. gondii* infection control. However, more *in vivo* studies are required to confirm if the P2X7 receptor is also important in the control of toxoplasmosis disease.

Here, we analyzed the susceptibility of P2X7 knockout mice to acute toxoplasmosis, in order to evaluate the role of the P2X7 receptor during *T. gondii* infection *in vivo*. We focused our analysis on the 'shock organs' (*i.e.*, liver, spleen, lymph nodes), which are particularly affected during acute disease.

2. Materials and methods

2.1. Animals

All experiments were performed using female and male mice (up to 12 weeks old) from one of the following strains: wild type C57BL/6 (P2X7$^{+/+}$) or P2X7 receptor knockout (P2X7$^{-/-}$) in the C57BL/6 genetic background, kindly provided by Dr. James Mobley (PGRD, Pfizer Inc., Groton, CT, USA), or obtained from The Jackson Laboratory (USA); and CF1 female and male mice (4–6 weeks old) used for *T. gondii* maintenance. The animals were kept at 22 °C, respecting their circadian rhythm.All experimental protocols were approved by the ethical committee of the Federal University of Rio de Janeiro (Comissão de Avaliação do Uso de Animais em Pesquisa – CAUAP) from the Instituto de Biofísica Carlos Chagas Filho (IBCCF, UFRJ, RJ, Brazil), as described in documents IBCCF 039 for animal handling and 99 for *Toxoplasma gondii* manipulation.

2.2. Parasites

Tachyzoites of *Toxoplasma gondii* RH strain were maintained in CF1 mice as previously described (Martins-Duarte et al., 2008). For experimental infections, tachyzoites were harvested from the peritoneal cavity of infected CF1 mice, by washing the cavity with 8 mL of PBS and recovering parasites by centrifugation (1000*g*, for 10 min). Recovered tachyzoites were counted in a hemocytometer.

2.3. T. gondii *infection and mouse survival curve*

Wild Type and knockout, 8-week-old mice (≥5 mice/group) were infected intraperitoneally with 10^2 *T. gondii* tachyzoites, in 100 μL of DMEM, and their survival was monitored for 15 days.

2.4. Peritoneal lavage fluid

Four days post-infection, the peritoneal cavity of mice was washed with cold PBS and the peritoneal wash fluid was centrifuged for 8 min at 250*g*. The supernatant was kept at −20 °C, for cytokine analysis (see "Cytokine quantification").

2.5. Organ weighing and histology

At 4 days post-infection, the organs from infected mice were removed, weighed, and then all but the livers were fixed by immersion in 10% formaldehyde buffered solution for 48 h (the solution was replaced for fresh fixative after 24 h of fixation). Livers were first fixed in Gendre solution for 4 h, and then immersed in formaldehyde as mentioned above. After fixation, all organs were dehydrated in ethanol and xylol, and then embedded in paraffin. Paraffin sections (5-μm thick) were deparaffinized and stained with hematoxylin-eosin (HE). Morphometric analysis of the perimeter of liver granulomas was performed from images of HE-stained liver sections, using the program ImageProPlus 4.0.

2.6. Cytokine quantification

The supernatant of peritoneal wash samples (see "Peritoneal lavage fluid") was used for cytokine analysis. For ELISA assays, 100 μL aliquots of supernatants were added to wells of 96-well plates (BD-Biosciences – USA), and the levels of the inflammatory cytokines INF-γ, IL-1β, IL-12, TNF-α, and IL-10 were estimated using the cytokine dosage kit according to manufacturer's protocol (Peprotech Inc., USA).

2.7. Enzymatic assay

Four days post-infection, mice were sacrificed in a CO_2 chamber and cardiac puncture was performed using a 1 mL heparin-syringe. Blood samples were kept at 4 °C overnight, to precipitate the red blood cells, and then 20 μL of serum were collected from each sample. Serum levels of ALT (alanine aminotransferase) were detected using the Transaminase ALT kinetics kit, according to the manufacturer's instructions (Bioclin, Brazil).

2.8. DNA extraction and reverse transcription-quantitative real-time PCR (RT- qPCR)

Total liver DNA was isolated using the DNeasy® Blood & Tissue Kit reagent (Qiagen), according to the manufacturer's instructions. Total DNA was quantified using a ND-1000 spectrophotometer (NanoDrop). Reactions were carried out in a final volume of 10 μL, using 700 ng of DNA and 600 nM each of reverse and forward primers. The following primers were used for qRT-PCR: for B1 *T. gondii* gene, 5′- GGAACTGCATCCGTTCATGAG-3′ (forward) and 5′- TCTTTAAAGCGTTCGTGGTC-3′ (reverse); for murine *Actb*, 5′-TATGCCAACACAGTGCTGTCTGG-3′ (forward) and 5′-TACTCCTGCTTGCTGATCCACAT-3′ (reverse). Reactions were performed in a 7500 Fast Real-Time System (Applied Biosystems). Relative gene amount were determined using the Sequence Detection Software v.2.0.5 (Applied Biosystems). B1 *T. gondii* gene to *Actb* relative amount was calculated using the comparative cycle thresh-