Immunobiology 218 (2013) 1104–1112

Contents lists available at SciVerse ScienceDirect

Immunobiology

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

Implication of purinergic P2X7 receptor in *M. tuberculosis* infection and host interaction mechanisms: A mouse model study

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ARTICLE INFO

Article history:
Received 29 March 2012
Received in revised form 6 February 2013
Accepted 8 March 2013
Available online 16 March 2013

Keywords:
ATP
Mouse
Mycobacterium tuberculosis
P2X7 receptor
RAW 264.7

ABSTRACT

In the present study, we analyzed the role of purinergic P2X7 receptor in *Mycobacterium tuberculosis* infection and host interaction mechanisms *in vitro* and *in vivo*. For experimental procedures, a macrophage murine cell line RAW 264.7, and male Swiss, wild-type C57BL/6 and P2X7 receptor knockout ($P2X7R^{-/-}$) mice were used throughout this study. We have demonstrated that treatment of RAW 264.7 cells with ATP (3 and 5 mM) resulted in a statistically significant reduction of *M. tuberculosis*-colony-forming units. The purinergic P2X7 receptor expression was found significantly augmented in the lungs of mice infected with *M. tuberculosis* H37Rv. Infected wild-type mice showed a marked increase in the spleen weight, in comparison to non-infected animals. Furthermore, *M. tuberculosis*-infected $P2X7R^{-/-}$ mice showed an increase of *M. tuberculosis* burden in lung tissue, when compared to infected wild-type mice. In $P2X7R^{-/-}$ spleens, we observed a significant decrease in the populations of Treg ($CD4^+Foxp3^+$), T cells ($CD4^+$, $CD8^+CD25^+$ and $CD4^+CD25^+$), dendritic cells ($CD11c^+$) and $B220^+$ cells. However, a significant increase in $CD11b^+$ cells was observed in $P2X7R^{-/-}$ mice, when compared to wild-type animals. In the lungs, $P2X7R^{-/-}$ *M. tuberculosis*-infected mice exhibited pulmonary infiltrates containing an increase of Treg cells ($CD4^+Foxp3^+$), T cells ($CD4^+$ and $CD8^+$) and a decrease in the $B220^+$ cells, when compared with wild-type *M. tuberculosis*-infected mice. The findings observed in the present study provide novel evidence on the role of P2X7 receptors in the pathogenesis of tuberculosis.

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Introduction

Tuberculosis (TB) is a worldwide public health priority and remains the leading cause of mortality due to a single bacterial pathogen, *Mycobacterium tuberculosis*. The World Health Organization (WHO) has estimated 8.8 million incident cases of TB, and approximately 1.4 million people died of TB in 2010 (World Health Organization 2011). *M. tuberculosis* has been identified as the most successful pathogen of all times, because it remains silent and latent within the host, being able to escape from the immune defenses (Enserink 2001; Wickelgren 2000). By arresting phagosomal maturation (Malik et al. 2000; Pethe et al. 2004), it persists dormant until the host defenses are down, as seen in human immunodeficiency virus (HIV) infection. The growing rate of HIV–TB co-infection (Fatkenheuer et al. 1999; World Health Organization 2010, 2011), the emergence of multidrug-resistant (MDR), extensively drug-resistant (XDR), and more recently, totally drug-resistant (TDR) strains of *M. tuberculosis* (World Health Organization 2010, 2011), increased the need for identifying different therapeutic options to treat TB, as well as to better understand the pathophysiology of this insidious disease.

Abbreviations: APC, antigen-presenting cell; ATP, adenosine-5′-triphosphate; BCG, bacille Calmette-Guerin; CFU, colony-forming unit; FBS, fetal bovine serum; HIV, human immunodeficiency virus; MDR, multidrug-resistant; OADC, oleic acid, albumin, dextrose, catalase; PBS, phosphate buffered saline; SDS, sodium dodecyl sulfate; TB, tuberculosis; TDR, totally drug-resistant; Treg, regulatory T cell; WHO, World Health Organization; XDR, extensively drug-resistant.

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0171-2985/$ – see front matter © 2013 Elsevier GmbH. All rights reserved.
http://dx.doi.org/10.1016/j.imbio.2013.03.003

Extracellular purines, including ATP, display a series of roles in several physiological processes, such as vascular tonus, pain sensation, neurotransmission, cell proliferation, differentiation, and cell death (Burnstock 2006). The purinergic receptors mediating these effects are classified into P1 (A_1, A_{2A}, A_{2B}, and A_3), which are activated by nucleosides, and P2 receptors that respond to nucleotides. These receptors are further subdivided into metabotropic P2Y ($P2Y_{1,2,4,6,11–14}$) and ionotropic P2X subtypes (P2X1–7) (Burnstock 2006).

The P2X7 receptor subtype presents a series of particularities. First, its activation requires high concentrations of ATP (up to 300 μM), while the other P2X receptors display a very high sensitivity for ATP. Importantly, P2X7 receptor is highly expressed in immune and inflammatory cells, throughout the central or peripheral nervous systems (Burnstock 2011; Donnelly-Roberts and Jarvis 2007; Ferrari et al. 1997).

P2X7 receptor activation results in a rapid and reversible opening of channels that are permeable to Na^+, K^+ and Ca^{2+} (Donnelly-Roberts and Jarvis 2007). This receptor also serves as a regulator of inflammation, and it is involved in the production of pro-inflammatory cytokines such as TNF-α and IL-1 β, leading to the induction of cyclooxygenase-2 (COX-2), metalloproteinases, inducible nitric oxide synthase (iNOS) and production of superoxide anion (Donnelly-Roberts and Jarvis 2007; Labasi et al. 2002). This receptor has been involved in the activation of peripheral macrophages and glia, neutrophil infiltration, and prostaglandin production (Burnstock 2006; Di Virgilio 2007; Donnelly-Roberts and Jarvis 2007; Goncalves et al. 2006; Labasi et al. 2002). Recent studies have demonstrated the influence of P2X7 receptor in a wide range of experimental models of disease, such as depression, epilepsy, Parkinson's disease, arthritis, cancer, hemorrhagic cystitis, and chronic pain, by using either selective P2X7 receptor antagonists or knockout (KO) mice to P2X7 receptors (Basso et al. 2009; Chessell et al. 2005; Donnelly-Roberts and Jarvis 2007; Honore et al. 2006; Li et al. 2006; Marcellino et al. 2010; Martins et al. 2012; Teixeira et al. 2010).

It was shown that absence of P2X7 receptor make epithelial cells less apoptotic, suggesting that its activation might be involved in the regulation of apoptosis (Goncalves et al. 2006). In this context, it has been proposed that prolonged exposure of P2X7 receptor to agonists leads a formation of a cytolytic pore in the cell membrane, allowing the entry of larger particles up to 900 Da, and consequent cell death (Burnstock 2006; Donnelly-Roberts and Jarvis 2007). Noteworthy, ATP was found to induce both cell death and killing of intracellular mycobacteria within BCG-infected human macrophages (Lammas et al. 1997; Molloy et al. 1994). The cytotoxic and mycobactericidal effects of ATP have been shown to be mediated by the specific interaction of ATP with macrophage surface P2X7 receptors (Lammas et al. 1997).

Recent studies have demonstrated that the polymorphism 1513A→C of P2X7 receptor gene causes an amino acid change from glutamic acid at amino acid position 496 to an alanine in the C-terminus (Gu et al. 2001), impairing multiple P2X7 receptor functions, including cation fluxes in a variety of cells, the release of IL-1β, IL-18, and matrix metalloproteinase (MMP)-9 from macrophages, or shedding of CD23 and CD62L from lymphocytes (Gu et al. 2001; Saunders et al. 2003; Sluyter et al. 2004a,b; Sluyter and Wiley 2002). This polymorphism is associated with impaired ability to kill *Mycobacterium bovis* BCG *via* ATP *in vitro* (Fernando et al. 2005; Saunders et al. 2003), and increased susceptibility to extra pulmonary TB (Fernando et al. 2007).

In the present study, we analyzed the mycobactericidal activity of ATP in *M. tuberculosis*-infected murine macrophages, as well as the *in vivo* expression of P2X7 receptors in a murine model of *M. tuberculosis*-infection. We have also assessed, for the first time, the effects of P2X7 receptor gene deletion on splenomegaly and colony-forming unit (CFU) counts in lungs and spleens of *M. tuberculosis*-infected mice. Attempts have also been made to evaluate the immune profile of P2X7 receptor KO mice infected with the laboratorial *M. tuberculosis* H37Rv strain, by means of an extensive flow cytometry analysis.

Materials and methods

Murine cell line culture and macrophage infection

The macrophage murine cell line RAW 264.7 (obtained from Banco de Células do Rio de Janeiro – BCRJ) was cultured in RPMI-1640 (Gibco) supplemented with 10% heat inactivated fetal bovine serum (FBS) and 1% penicillin–streptomycin at 37 °C with 5% CO_2. For infection of macrophages, we used the method previously described by Mascarello et al. (2010) with some modifications. Briefly, *M. tuberculosis* H37Rv strain was grown in Middlebrook 7H9 (Difco) containing 10% oleic acid/albumin/dextrose/catalase (OADC) enrichment at 37 °C. RAW 264.7 cells were seeded in 12-well tissue culture plates (Corning) at 1×10^5 cells per well in RPMI-1640 medium (supplemented with 10% FBS) for 24 h at 37 °C with 5% CO_2. The cells were then washed three times with RPMI-1640 to remove non-adherent cells. Infection of RAW 264.7 cells was performed at a multiplicity of infection (MOI) of 10:1 (bacteria/macrophage) for 3 h at 37 °C with 5% CO_2. Infected RAW cells were washed three times with RPMI-1640 to remove extracellular bacteria and replaced with 1 mL fresh RPMI (supplemented with 10% FBS), with ATP in concentrations of 3 mM and 5 mM. This was defined as time 0 h. At 2 h and 20 h the infected macrophages were lysed with 0.025% SDS, and the number of viable bacteria was determined by plating serial dilutions of each well on Middlebrook 7H10 Agar supplement with 10% OADC. The plates were incubated at 37 °C for 21 days before counting procedures. All experiments were performed in triplicates and the results are expressed in CFU per well.

Determination of minimum inhibitory concentration (MIC) of ATP against M. tuberculosis H37Rv strain

MIC values for isoniazid (control drug) and ATP (test) were determined by resazurin microtiter assay (REMA) (Palomino et al. 2002). Isoniazid and ATP growth inhibition activities were tested against the *M. tuberculosis* H37Rv strain. Mycobacteria was cultivated in Middlebrook 7H9 (Difco) liquid medium supplemented with 10% (v/v) OADC (Becton Dickinson) and 0.05% (m/v) Tween 80 (Sigma). Culture was initially grown at 37 °C up to an optical density at 600 nm (OD600) of 0.4. It was then diluted in Middlebrook 7H9 to achieve an OD600 of 0.006, and 100 μL of *M. tuberculosis* inoculum was added to each well on the microplate, containing 100 μL of compound or Middlebrook 7H9 (control inoculum) reaching an OD600 of 0.003. Final drug concentration ranged from 0.0078 to 4 $\mu g\ mL^{-1}$ for isoniazid and 0.019 to 10 mM for ATP containing 2% of dimethyl sulfoxide (DMSO). Plates were incubated for 7 days at 37 °C. Sixty microliters of 0.01% resazurin (Sigma Chem. Co.) solution was added to each well; plates were re-incubated for additional 2 days. A change in color from blue to pink indicated the growth of bacteria, and the MIC was read as the minimum drug concentration that prevented the color change in resazurin solution. The experiment was carried out in duplicate.

Animals

Male Swiss, wild-type C57BL/6 (wild-type) and P2X7 receptor KO ($P2X7R^{-/-}$) mice (25–30 g) were used throughout this study. Swiss and C57BL/6 mice were obtained from Universidade Federal de Pelotas (UFPEL; Pelotas, RS, Brazil), and $P2X7R^{-/-}$ mice were

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