Parasitol Res (2008) 103:829–838
DOI 10.1007/s00436-008-1063-8

ORIGINAL PAPER

$P2X_7$ modulatory web in *Trypanosoma cruzi* infection

C. M. Cascabulho · R. F. S. Menna-Barreto · R. Coutinho-Silva · P. M. Persechini · A. Henriques-Pons

Received: 4 March 2008 / Accepted: 26 May 2008 / Published online: 8 July 2008

Abstract $P2X_7$ is a member of the purinergic receptors family, with extracellular adenosine triphosphate (ATP) as the main agonist, promoting cations influx and membrane permeabilization that can lead to cell death. We previously proposed that extracellular ATP is involved in thymus atrophy induced by *Trypanosoma cruzi* infection through the induction of $CD4^+/CD8^+$ double-positive cell death and that $P2X_7$ could be involved in this process. To further elucidate this possibility raised by in vitro assays, in this study, we used $P2X_7^{-/-}$ mice and observed no difference in thymus atrophy or parasitemia when compared to C57Bl/6. We then decided to investigate other aspects of purinergic receptor interplay that could be better evidenced by the infection and observed that (1) thymocytes from infected and noninfected C57Bl/6 mice express $P2X_4$ and $P2X_7$ receptors (Western blotting), but ATP-induced membrane permeabilization only occurs in thymocytes from infected mice; (2) peritoneal macrophages from noninfected C57Bl/6 mice ($P2X_4^+$ and $P2X_7^+$) are permeabilized by ATP. Although macrophages from infected C57Bl/6 mice are $P2X_7^-$ but $P2X_4^+$, they are resistant to ATP, either through permeabilization or Ca^{++} influx (fluorimetry); (3) using noninfected $P2X_7^{-/-}$ mice, C57Bl/6 infected mice, and different agonistic stimuli, we observed interesting cross-talks among P2X and P2Y receptors (flow cytometry).

C. M. Cascabulho · R. F. S. Menna-Barreto · A. Henriques-Pons (✉)
Laboratório de Biologia Celular, Fundação Oswaldo Cruz,
Instituto Oswaldo Cruz, FIOCRUZ,
Manguinhos,
Rio de Janeiro, Brazil 21045-900
e-mail: andreah@ioc.fiocruz.br

R. Coutinho-Silva · P. M. Persechini
Laboratório de Imunobiofísica,
Instituto de Biofísica Carlos Chagas Filho,
Universidade Federal do Rio de Janeiro,
Rio de Janeiro, Brazil

Introduction

Chagas' disease is caused by the protozoan parasite *Trypanosoma cruzi* and has a widespread distribution in South America. Transmission to humans occurs primarily through blood-sucking reduvid bugs, but it may also occur, for example, through blood transfusion, organs transplant, and transplacentary infection (Moncayo 2003; WHO 2004). The disease is characterized by an initial acute phase, and it is generally accepted that patients with more severe acute infection may develop a more aggressive chronic phase (Coura 2007; Higuchi et al. 2003). A number of alterations are imposed by *T. cruzi* infection, and many relating to the immune system have been reported, such as polyclonal activation of B and T cells (Minoprio et al. 1989), splenomegaly, and Fas-dependent activation induced T cell death (AICD) of $CD4^+$ T cells (Lopes et al. 1999). Moreover, phagocytosis of apoptotic cells by macrophages leads to declined proinflammatory cytokine expression and blockage of NO production by these cells, thus favoring the infection (Freire-de-Lima et al. 2000). Structural and functional alterations of the thymus are also observed in the acute phase of the pathology, with a severe atrophy of the organ characterized by $CD4^+/CD8^+$ double-positive (DP) cell loss (Leite-de-Moraes et al. 1992). Additionally, the thymic microenvironment is greatly altered, as shown by the increased production of extracellular matrix components (Cotta-de-Almeida et al. 2003), decreased epithelial thymic nurse cell (TNC) viability, and decreased number of thymocytes per TNC (Cotta-de-Almeida et al. 1997).

In the case of thymus atrophy, a potential role for *trans*-sialidase, a virulence factor shed by *T. cruzi*, has been reported. This enzyme induces apoptosis in TNC complexes, where immature DP cells undergo selection (Mucci et al. 2002). However, cellular death triggered by this pathway is observed only in males, suggesting the requirement of androgen hormones in this process (Mucci et al. 2005). Our group has published that the $P2X_7$ receptor may also be involved in thymus atrophy induced by the acute phase of the infection. This receptor is a member of the purinergic receptor family, and its activation renders cell membranes permeable to molecules up to 900 Da in macrophages and 400 Da in lymphocytes, leading to cell death. Our previous results were obtained by in vitro assays and showed a positive correlation between thymus involution and extracellular adenosine triphosphate (ATP_e)-induced pore opening with uptake of large solutes in thymocytes. DP cells from noninfected mice were refractory to ATP_e-induced permeabilization or death, while DP thymocytes from *T. cruzi*-infected mice were susceptible to both. In addition, thymuses restored age-matched numbers of cells and structural architecture during the chronic phase, recovering low responsiveness to ATP_e in vitro (Mantuano-Barradas et al. 2003).

ATP_e plays a variety of roles by interacting with P2 receptors, such as: the G protein-coupled P2Y receptors and the ligand-gated cation channel P2X receptors (Ralevic and Burnstock 1998). Besides ATP_e, other agonists, such as adenosine diphosphate (ADP), uridine diphosphate (UDP), and uridine triphosphate (UTP) can activate some of the P2 receptors.

A complex network of intermolecular interactions within the family of purinergic receptors, called a "combinatorial receptor web," has been suggested (Volonte et al. 2006). This set of interactions is possible due to: (1) similar molecular structures, allowing a certain agonist to interact with one or more receptors, altering their final responses; (2) the fact that P2X and P2Y subtypes can form homomers and heteromers, increasing the diversity in agonist and antagonist selectivity (Guo et al. 2007; Surprenant et al. 2000); and (3) cross-talk mechanisms among purinergic receptors and other families of receptors. Platelets, for example, share a reciprocal cross-talk between $P2Y_{12}$ and $P2Y_1$ (Hardy et al. 2004), and retinal pericyte cells couple P2Y and $P2X_7$ activity (Sugiyama et al. 2005). P2 receptors can also interact with Cys-loop channels for acetylcholine, γ-amino butyric acid, glycine, and serotonin, glutamate-gated channels (kainite, alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid, and *N*-methyl-D-aspartic acid), and opioid receptors (Volonte et al. 2006).

Other effects of $P2X_7$ were proposed including the release of interleukin 1β from macrophages (Ferrari et al. 2006), CD62-L shedding (Gu et al. 1998), and maturation of T cells (Tsukimoto et al. 2006). Furthermore, innumerous loss-of-function polymorphisms (Adriouch et al. 2002; Shemon et al. 2006) and one that results in gain-of-function have been identified (Cabrini et al. 2005).

The activation of $P2X_4$ receptor has also been reported to induce membrane permeability to cationic molecules (Virginio et al. 1999). However, in contrast to $P2X_7$, few studies address its physiological properties and functional roles. It was only recently shown that $P2X_7$ and $P2X_4$ can be structurally and functionally associated on the cell surface, and this notion may unveil new approaches to target pharmacological studies (Guo et al. 2007; Dubyak 2007).

In the present study, we used $P2X_7$-defficient $\left(P2X_7^{-/-}\right)$ mice to evaluate the role played by this receptor in in vivo thymic atrophy induced by *T. cruzi* infection. Although these experiments indicate that the receptor is not responsible for thymic involution, using $P2X_7^{-/-}$ mice, we observed important interactions between P2X and P2Y receptors. These data further illustrate the complex modulatory web set up by purinergic receptors and that additional interactions can be imposed by pathological situations.

Materials and methods

Animals Seven-week-old male C57Bl/6 $\left(P2X_7^{+/+}\right)$ mice were purchased from the Breeding Laboratory Animal Center at Fundação Oswaldo Cruz, and $P2X_7^{-/-}$ mice (C57Bl/6 background), derived from Pfizer (Groton, CT, USA) and generated by Solle et al. (2001), were kindly supplied by Dr. A Gabel and bred at the Transgenic Mice Laboratory at the Biophysics Institute Carlos Chagas Filho at the Federal University do Rio de Janeiro. Mice were housed for 7–10 days in the Laboratory of Cellular Biology, Division of Animal Experimentation, under environmental factors and sanitation conforming to the guide for the Care and Use of Laboratory Animals (DHEW publication no. [NIH] 80-23). This project was approved by the Fiocruz Committee of Ethics in Research (0308-06), according to resolution 196/96 of the National Health Council of Brazilian Ministry of Health. Experiments were carried out using C57Bl/6 mice, except when indicated as $P2X_7^{-/-}$.

Parasites and infection Parasites were obtained from infected Swiss–Webster mice and isolated as previously described (Araújo-Jorge et al. 1989). Mice were intraperitoneally injected with 1×10^4 blood trypomastigote forms of *T. cruzi* Y strain in 200 μl of phosphate-buffered saline (PBS). Age-matched noninfected (control) mice received 200 μl of PBS and were treated under the same conditions. Individual parasitemia was scored in 5 μl of blood collected from tail snips.

Cell isolation Thymocytes were isolated by mechanical dissociation, and peritoneal cells were harvested by injection of ice-cold medium RPMI 1640 (Sigma Chemical, St. Louis, MO, USA). Cells were washed, homogenized in RPMI/4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) 10 mM (Sigma) pH 7.4, and maintained in ice until use. Samples were collected on days 11 or 14 post infection (dpi), as described in the text.

Permeabilization assay Peritoneal cells (a population with high susceptibility to ATP_e used as a positive control) and thymocytes were prewarmed for 5 min in RPMI 1640/HEPES 10 mM at 37°C and then incubated for 10 min in the presence or absence of 10 μM, 100 μM, 1 mM, or 5 mM of ATP_e, adenosine monophosphate (AMP), adenosine, UTP, or ATP plus UTP (all purchased from Sigma). In some cases, cells were preincubated for 5 min in 100 μM $ZnCL_2$, 50 μM $CuCl_2$, 5 mM UTP, or 35 μM before Brilliant Blue G Coomassie (BBG) before exposure to ATP_e. To evaluate agonist-induced cell permeabilization, one of the following membrane-impermeant deoxyribonucleic acid-staining fluorescent dyes was added during the last 5 min of incubation, as mentioned in the text: propidium iodide (Sigma) 2.5 μM or TO-PRO-3 (Molecular Probes, Eugene, OR, USA) 1 μM. Samples were analyzed using a FACScalibur flow cytometer (Becton & Dickinson, San Jose, CA, USA).

Permeabilization of each subpopulation of thymocytes was achieved by labeling the cells for 30 min in ice with phycoerythrin (PE)-conjugated anti-CD4 and fluorescein isothiocyanate (FITC)-conjugated anti-CD8 mAb (SouthernBiotech, Birminghan, AL, USA) before exposure to the agonist. Using these fluorochromes, permeabilization assays were carried out using TO-PRO-3. In most experiments, we used total peritoneal cells, except in permeabilization assays where macrophages were gated by a combination of forward scatter × side scatter and MAC-1^+ labeling. Flow cytometry data were analyzed using CellQuest software version 3.2 (Becton & Dickinson).

Cell sorting DP thymocytes were incubated with bead-conjugated anti-CD8 (Miltenyi Biotec, Auburn, CA, USA) at 4°C for 15 min, centrifuged (25×*g*/10 min), homogenized in 500 μl of PBS, and applied to a MiniMACS column (Miltenyi Biotec) in a magnetic field. Sort purity of $CD8^+$ cells was determined by labeling the cells with anti-CD8 FITC and anti-CD4 PE mAb for flow cytometry analysis. We used samples of at least 95% enrichment of DP cells.

Intracellular calcium measurements Freshly collected intraperitoneal cells or enriched DP thymocytes were loaded with Fura 2-AM (Molecular Probes) 5 μM for 45 min at room temperature in RPMI-1640/HEPES 20 mM/probenecid 2.5 mM (Sigma). Cells were then left to decant for 10 min at room temperature on glass coverslips coated with poly-L-lysin 0.001% and placed in a three-compartment superfusion chamber. The central chamber containing cells was continuously perfused at a rate of 1 ml/min with RPMI-1640/HEPES 10 mM at 37°C until stabilization of background signal. Intracellular calcium was monitored using a fluorescence photometer (Photon Technology, Princeton, NJ, USA) throughout perfusion, initially with medium only and then with medium containing ATP_e 5 mM. All traces recorded were representative of 30–60 cells preincubated or not with BBG. Saponin 0.01% was used to control Fura-2 loading.

Western blotting Intraperitoneal cells or thymocytes were lysed with extraction buffer (Tris–HCl 50 mM, NP-40 1%, leupeptin 1 mM, phenylmethylsulfonyl fluoride (PMSF) 100 mM, pepstatin A 1 mM, ethylenediamine tetraacetic acid (EDTA) 100 mM); (Sigma), and total proteins (50 μg) were resolved using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) 12%. Proteins on nitrocellulose membranes were incubated with anti-$P2X_7$ Ab (Alomone, Jerusalem, Israel) for 2 h, rinsed in blocking buffer, and incubated with alkaline phosphatase-conjugated anti-rabbit IgG (SouthernBiotech) for 1 h. The detection was performed using 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium solution (BCIP/NBT) (SouthernBiotech). Thymocytes from $P2X_7^{-/-}$ mice were used as negative controls.

Transmission electron microscopy DP-enriched thymocytes were collected from noninfected mice, washed in cold PBS, and fixed using paraformoldehyde 4% and glutaraldehyde 0.1% in 0.1 M of Na-cacodylate buffer (pH 7.2) plus 0.01% of saponine at 4°C for 30 min. Cells were washed in washing buffer (PBS containing bovine serum albumin 4% and saponin 0.05%) at 20°C and incubated with anti-$P2X_7$ Ab diluted in washing buffer for 1 h. After washing, samples were incubated with gold-conjugated goat anti-rabbit mAb (Sigma), washed in washing buffer, followed by saponine-free washing buffer and finally Na-cacodylate buffer 0.1 M. Samples were fixed with glutaraldehyde 2.5% in Na-cacodylate buffer 0.1 M (pH 7.2) at room temperature for 40 min and postfixed with a solution of OsO_4 1%. Cells were dehydrated in an ascending acetone series and embedded in PolyBed 812 resin. Ultrathin sections were stained with uranyl acetate and lead citrate and examined in a Zeiss EM10C (Zeiss, Germany) transmission electron microscope.

Statistical analysis Using the software SPSS version 8.0, Student's *t* test was used to compare two sets of data. *p* values are indicated in figure legends.

Results

Given that macrophages are important host cells for *T. cruzi* life cycle and express high levels of $P2X_7$, we evaluated blood parasitemia as a basic parasitological parameter in $P2X_7^{+/+}$ and $P2X_7^{-/-}$ mice. Using a *T. cruzi* strain that induces an early acute peak, we observed similar results in both groups of mice (Fig. 1a). Thymus atrophy was also similar in infected $P2X_7^{+/+}$ or $P2X_7^{-/-}$ mice (Fig. 1b). In addition, we observed that noninfected $P2X_7^{-/-}$ mice maintained normal distribution of thymocyte subpopulations, when compared to $P2X_7^{+/+}$ (Fig. 1c,d). Importantly, the DP subpopulation was equally affected in both groups of infected mice (Fig. 1e,f). Therefore, contrary to what we might expect based on our previous in vitro experiments (Mantuano-Barradas et al. 2003), these in vivo results indicate that $P2X_7$ is not a central molecule in thymus atrophy induced by *T. cruzi* infection. Therefore, we decided to investigate whether other aspects of P2 receptors were altered by the infection.

Western blotting analyses were performed on dpi 11, and we found $P2X_7$ labeling in total thymocytes from noninfected (Fig. 1g arrowhead—lane 1) and infected mice (Fig. 1g—lane 2), as well as in peritoneal cells from noninfected mice (Fig. 1g—lane 3). However, peritoneal cells from infected mice did not express the receptor (Fig. 1g—lane 4) as well as negative control (Fig. 1g—lane 5). The same results were obtained using DP-enriched thymocytes (data not shown).

Since thymocytes from noninfected mice expressed $P2X_7$ but were resistant to ATP_e-induced permeabilization, we used electron microscopy to determine the receptor

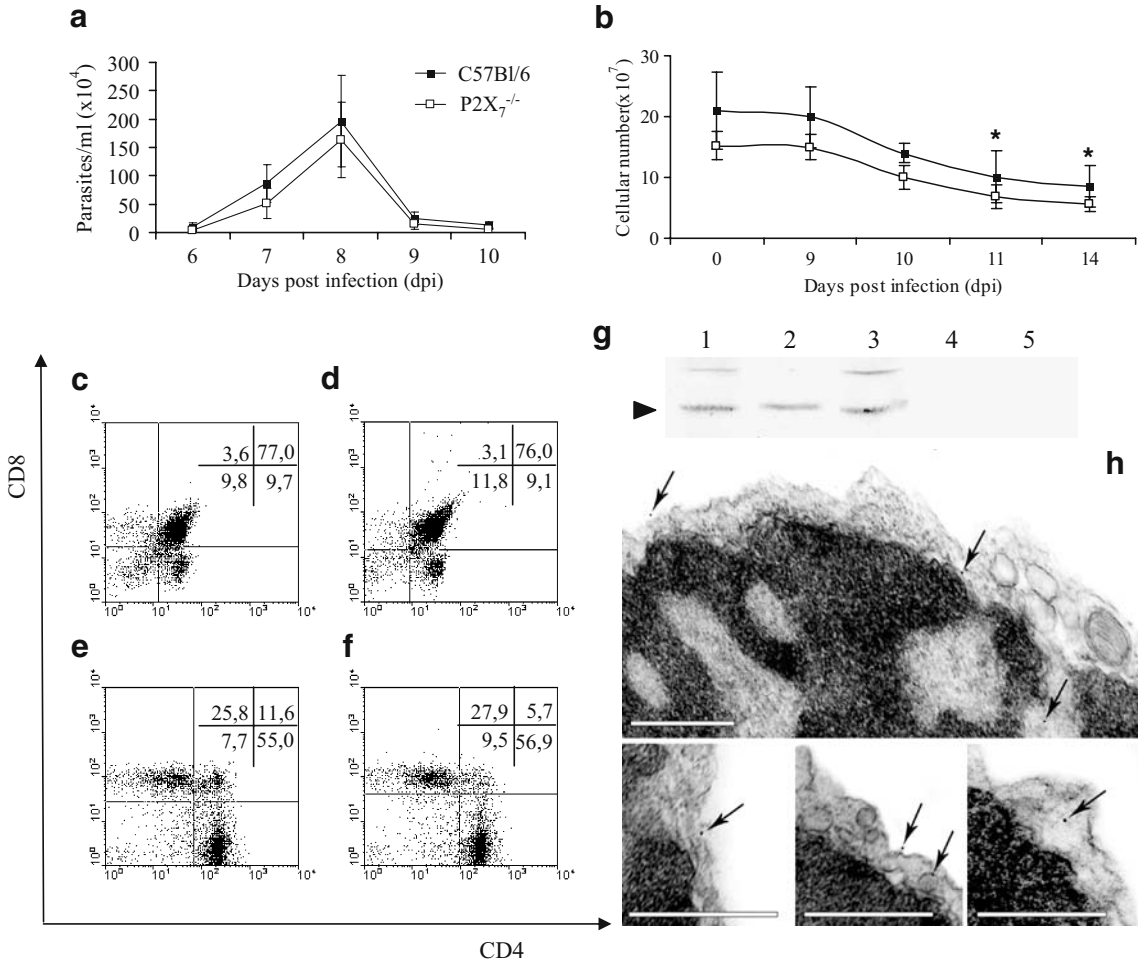

Fig. 1 Thymus atrophy and $P2X_7$. Blood parasitemia (**a**) and total thymocytes (**b**) were counted at indicated time points. Thymocyte subpopulations were labeled with anti-CD4 and anti-CD8, and *quadrant numbers* indicate individual percentages from: noninfected C57Bl/6 (**c**) or $P2X_7^{+/+}$ (**d**); infected (dpi 14) C57Bl/6 (**e**) or $P2X_7^{-/-}$ (**f**) mice. Cellular extracts of: thymocytes from noninfected (**g**—*lane 1*) and infected (*2*), peritoneal cells from noninfected (*3*) and infected (*4*), all from C57Bl/6 mice and thymocytes from noninfected $P2X_7^{+/+}$ mice (*5*, negative control) were used in Western blotting. *Arrowhead* indicates 68 kDa. Results are representative of five independent experiments with ten mice per experiment. Ultrastructural immunocytochemical labeling indicated $P2X_7$ labeling (*arrows* in **h**). *Bar*= 10 μm. *Asterisk* means $p \leq 0.05$ when compared to noninfected mice

localization, as it can be sequestered within cytoplasmic granules. We observed $P2X_7$ labeling in chromatin, nuclear membrane, cytoplasmic vesicles, and plasma membrane (Fig. 1h), suggesting that the resistance to permeabilization is not due to the lack of $P2X_7$ expression on the cell surface.

To further investigate the modulation of P2 receptors during the infection, we investigated ATP_e-triggered Ca^{++} influx. In peritoneal cells from noninfected mice, ATP_e induced a biphasic Ca^{++} response, consisting of a rapid transient signal, peaking within 20–30 s and followed by a plateau, compatible with a permeabilization response (Fig. 2a). Importantly, the second component of the signal (plateau) was blocked by BBG, an antagonist of $P2X_7$ (Eschke et al. 2002; Fig. 2b). When peritoneal cells from infected mice (dpi 11) were treated with ATP_e (Fig. 2c), neither component of the response was observed. This result is in agreement with our data showing that the infection downregulates the expression of the $P2X_7$ receptor in macrophages and suggests that besides $P2X_7$, other P2 receptors are absent or not functional in these cells.

DP thymocytes from noninfected mice showed only the rapid component of the signal and then declined to basal levels after 100 s (Fig. 3a). Pretreatment with BBG did not block this signal (Fig. 3b), further suggesting that although expressed on the membrane, $P2X_7$ receptor is functionally downregulated in these cells. On the other hand, DP thymocytes from infected mice showed a continuous response induced by the agonist, reaching a plateau after 100 s (Fig. 3c). After pretreatment with BBG, we observed only the rapid peak within 30–40 s, declining to basal levels in 80–90 s (Fig. 3d). In conclusion, we observed that the infection induces a gain of function of $P2X_7$ in thymocytes but a downregulation of P2 receptors in peritoneal cells.

As there was no response induced by ATP_e in noninfected thymocytes and P2X receptors may share extracellular domains and agonists due to heteromerization, we evaluated whether other agonists could trigger cellular permeabilization. However, we still observed no cellular response using adenosine, AMP, or UTP (data not shown).

Since ATP_e-induced permeabilization can also be mediated by $P2X_2$ and $P2X_4$ (Virginio et al. 1999), we evaluated whether $P2X_7$ modulations could affect other purinegic receptors (cross-talks). We observed that the preincubation with $ZnCl_2$, a condition that favors $P2X_4$ activity (Coddou et al. 2003), rendered peritoneal cells from noninfected C57Bl/6 mice more susceptible to permeabilization with lower concentrations of ATP_e (100 μM), when compared to ATP_e only (1 mM; Fig. 4a). In addition, incubation with $CuCl_2$, a condition that prevents $P2X_4$ activity (Coddou et al. 2003), induced significant permeabilization only with 5 mM of ATP_e, and there was no response in the presence of BBG. All incubations using $P2X_7^{-/-}$ cells induced no response, even in the presence of $ZnCl_2$, where we

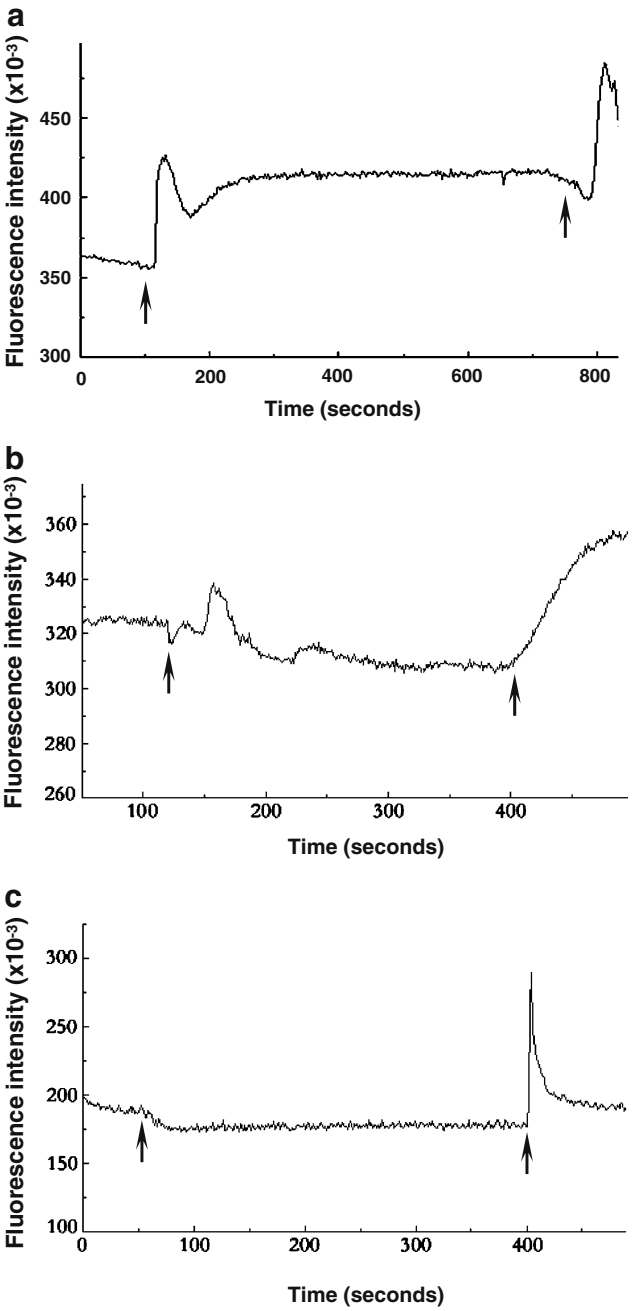

Fig. 2 Calcium influx of peritoneal cells. Calcium influx of Fura-2-loaded C57Bl/6 peritoneal cells was evaluated in: noninfected mice/untreated cells (**a**) or BBG-treated (**b**) and infected mice/untreated cells (**c**). ATP_e 5 mM was added (*first arrow*), and saponin was used as a positive control (*second arrow*). Results are representative of four independent experiments with eight mice per experiment (30 to 60 cells were recorded per microscopic field per mouse)

expected to observe membrane permeabilization through $P2X_4$ (Fig. 4a). Using peritoneal cells from infected (Fig. 4b) and DP thymocytes from noninfected mice (Fig. 4c), we observed no response under any condition tested. Thymocytes from infected mice were permeabilized in the presence of ATP_e or $ZnCl_2/ATP_e$ (Fig. 4d), and once

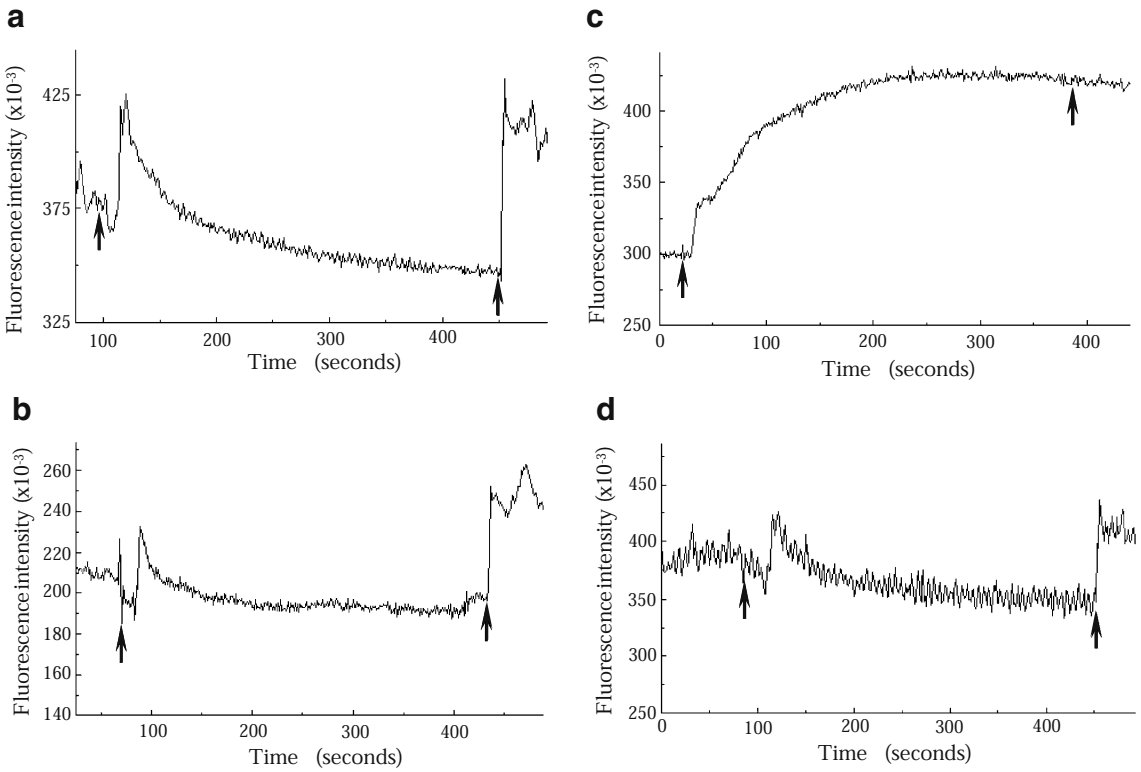

Fig. 3 Calcium influx of thymocytes. Calcium influx of Fura-2-loaded C57Bl/6 thymocytes was evaluated in: noninfected mice/untreated cells (**a**) or BBG-treated (**b**), infected mice/untreated cells (**c**) or BBG-treated (**d**). ATP_e 5 mM was added (*first arrow*), and saponin was used as a positive control (*second arrow*). Results are representative of four independent experiments with eight mice per experiment (30 to 60 cells were recorded per microscopic field per mouse)

more, there was no permeabilization of DP thymocytes from infected $P2X_7^{+/+}$ mice preincubated with $ZnCl_2$, suggesting $P2X_4$ functional downregulation also in this cell type.

Since previous data from literature (Sugiyama et al. 2005) and from our laboratory indicated a possible cross-interaction between $P2X_7$ and P2Y, we evaluated if pre- and/or coincubation of UTP with ATP_e could modulate the permeabilization response. We observed that pre-exposure to UTP blocked ATP_e-induced pore opening only in peritoneal cells from noninfected mice (Figs. 5a,b). Regarding DP thymocytes, this effect was observed only in cells from infected mice (Figs. 5c,d). Taken together, these data indicate a highly complex group of functional interactions not only involving P2X receptors but also P2X and P2Y receptors.

Discussion

Our previous in vitro data indicated a role for ATP_e-induced cell death in thymus atrophy induced by *T. cruzi* infection, raising the possibility that the $P2X_7$ receptor is involved in this phenomenon (Mantuano-Barradas et al. 2003). However, in vivo assays using $P2X_7^{-/-}$ mice indicated that the receptor is not a central molecule in the process. On the other hand, knockout mice may not be an appropriate tool to evaluate this possibility, since alternative pathways may take place and substitute the deleted molecule or interdependent intracellular pathways of signal transduction could be masking the relevance of $P2X_7$ in the atrophy (cross-talk). Therefore, we suggest that this interdependence among P2 receptors may make purinergic knockout mice a two-edged sword, and we should be aware of this possibility. Accordingly, in this work we observed important interactions and modulations concerning not only $P2X_7$ function and expression but also $P2X_4$ and P2Y. These data illustrate the complexity of roles played by members of P2 purinergic receptors family and the wide range of possible immune modulatory interplays.

Our previous data using C57Bl/6 mice showed that DP thymocytes from noninfected mice are resistant to ATP_e-induced permeabilization and cell death (Mantuano-Barradas et al. 2003). In the present work, we extended these observations by demonstrating that although the $P2X_7$ receptor is expressed on the plasma membrane of these cells, ATP_e did not induce Ca^{++} influx compatible to membrane permeabilization. One possibility could be that the receptor and the component(s) of the pore itself are

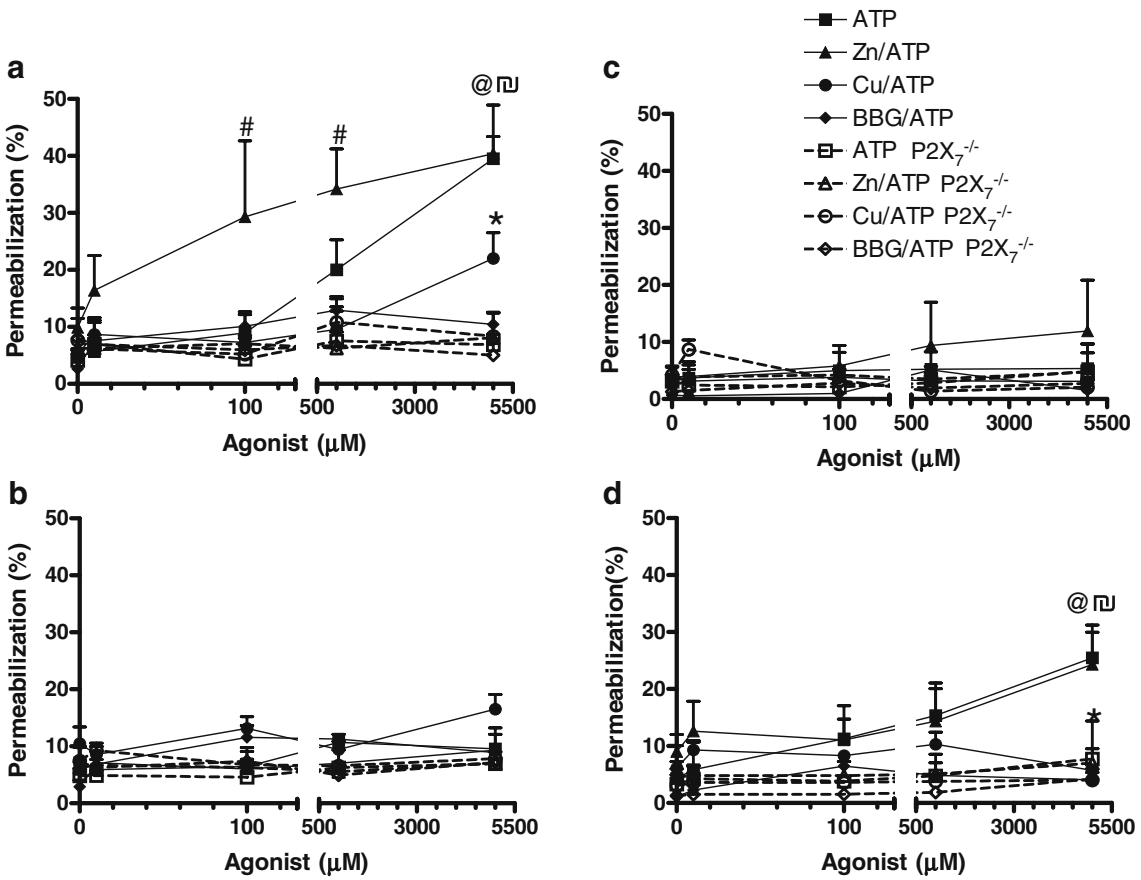

Fig. 4 $P2X_7$ and $P2X_4$ interaction. Peritoneal macrophages from noninfected (**a**) or infected mice (**b**) or thymocytes from noninfected (**c**) or infected mice (**d**) were collected from C57Bl/6 and $P2X_7^{-/-}$ mice (as indicated) and preincubated or not with $ZnCl_2$, $CuCl_2$, or BBG. ATP was then added in indicated concentrations (μM), and propidium iodide incorporation was measured by flow cytometry. Results are representative of six independent experiments with ten mice per experiment. @ means $p<0.05$ when comparing Zn/ATP 5 mM with Zn/ATP 10 μM; # when comparing Zn/ATP 100 μM and 1 mM with other parameters at the same concentration; * when comparing Cu/ATP 5 mM with ATP 5 mM; ₪ when comparing Zn/ATP 5 mM with Cu/ATP 5 mM

different entities (Coutinho-Silva and Persechini 1997) and, although the receptor is present in DP cells, the molecules that compose the pore are missing. It has recently been proposed that pannexin hemichannels participate in the pore structure (Pelegrin and Surprenant 2006), and it could be possible that this molecule was not being expressed or even not coupled to the signaling cascade of permeabilization or death before infection. It would be interesting to examine the expression of this molecule in thymocytes.

During the phase of thymus atrophy induced by *T. cruzi* infection, DP thymocytes become responsive to ATP_e-induced permeabilization and Ca^{++} signaling through $P2X_7$ activity. This could be due to cellular phenotypic modulation and induction of pannexin expression, for example, completing the panel of required molecules to induce $P2X_7$ response. It could also be possible that the infection, through cytokines for example, induced the coupling of signaling machinery to pre-existing surface molecules.

A different type of P2 receptors modulation was observed in peritoneal cells. We observed that these cells from infected mice downregulate the expression of $P2X_7$, making them no longer susceptible to ATP_e. Similarly, it has been shown that J774 cells infected with *Chlamydia* also reduce ATP_e-induced cell membrane permeabilization, but sustain Ca^{++} influx induced by ATP_e (Coutinho-Silva et al. 2001).

Although it has been reported that interferon-γ and tumor necrosis factor-α upregulate the expression of $P2X_7$ in monocytes (Humphreys and Dubyak 1998) and these cytokines are abundant in the acute phase of the infection, other stimuli certainly took place, abolishing the expression of this receptor in peritoneal cells. Since there was no detectable Ca^{++} signal triggered by ATP_e in these peritoneal cells from infected mice, other P2 receptors are also probably not functional or expressed at very low levels. At least in the case of $P2X_4$, Western blotting assays indicated that these cells express the receptor (data not shown). However, in this

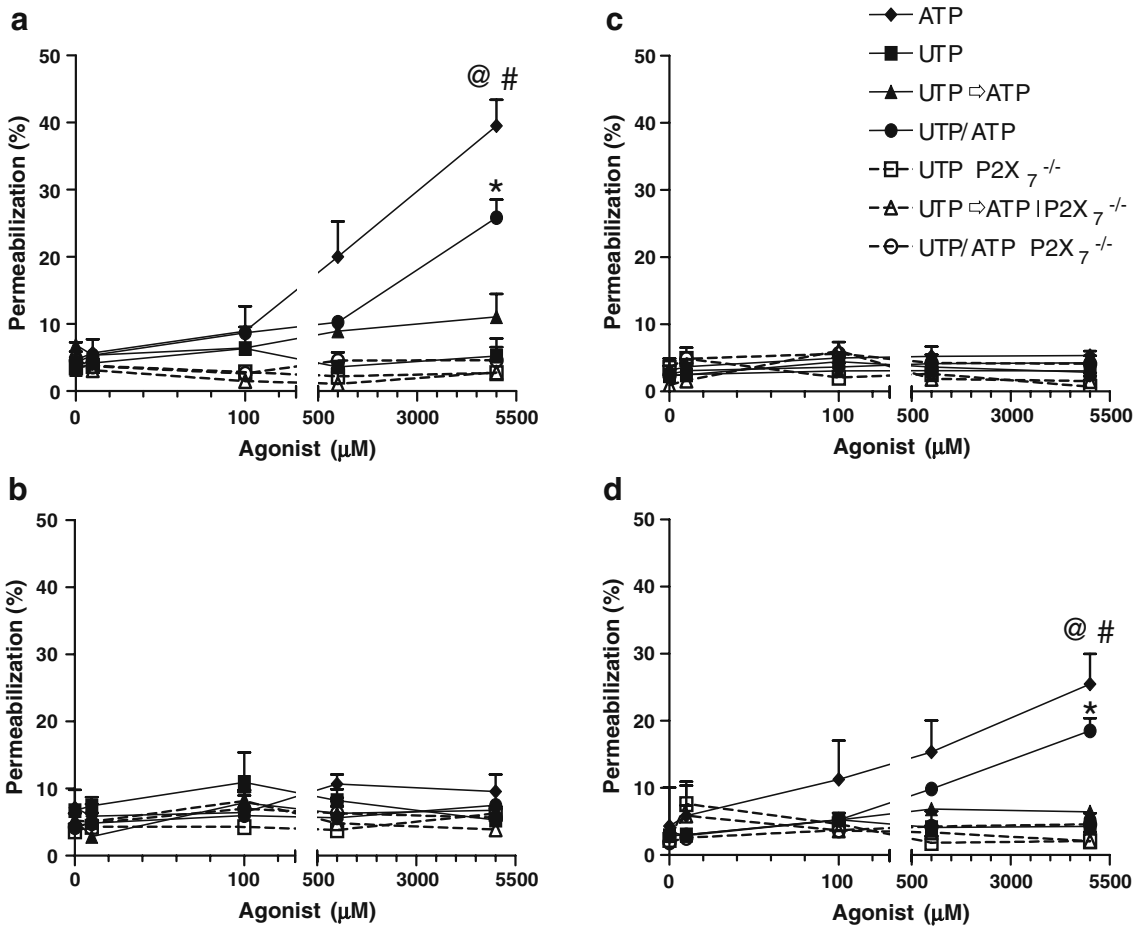

Fig. 5 $P2X_7$ and P2Y interaction. Peritoneal macrophages from noninfected (**a**) or infected mice (**b**) or thymocytes from noninfected (**c**) or infected mice (**d**) were collected from C57Bl/6 and $P2X_7^{-/-}$ mice (as indicated) and were incubated, preincubated (*UTP⇒ATP*), or coincubated with UTP (*UTP/ATP*) (5 mM). ATP_e was then added in indicated concentrations (μM), and propidium iodide incorporation was measured by flow cytometry. Results are representative of six independent experiments with ten mice per experiment. @ means $p<0.05$ when comparing ATP 5 mM with UTP⇒ATP 5 mM; # when comparing ATP 5 mM with UTP/ATP 5 mM; * when comparing UTP/ATP 5 mM with UTP⇒ATP 5 mM

case, due to the absence of $P2X_7$, the $P2X_4$, for example, can mostly be sequestered in intracellular vesicles, not exposed to agonistic stimulation (Guo et al. 2007).

Our present data indicate normal expression and function of $P2X_4$ and $P2X_7$ in peritoneal cells from noninfected mice. However, our findings with cells from noninfected $P2X7^{-/-}$ mice evidenced an interesting cross-talk between both receptors, with no permeabilization even in the presence of $ZnCl_2$, which indicates no $P2X_4$ response. In addition, the preincubation of macrophages from noninfected C57Bl/6 mice with BBG (a blocker of $P2X_7$ activity) showed no $P2X_7$ or $P2X_4$ activity after incubation with ATP_e. These results were reproduced using thymocytes from infected mice, indicating a phenomenon conserved in cells from myeloid and lymphoid lineages. These data are in agreement with the literature that shows both receptors forming heteromeric structures that ultimately affect their final function (Dubyak 2007; Guo et al. 2007).

Although we had no indication of the possible relevance of P2Y in thymus atrophy, our present data concerning P2X cross-talks, previous results from our laboratory, and related literature (Sugiyama et al. 2005) motivated the investigation of $P2Y/P2X_7$ interaction. To this regard, we observed that preincubation with 5 mM of UTP blocked ATP_e-dependent permeabilization both in peritoneal cells from noninfected mice and DP thymocytes from infected mice. Similar results have been obtained with retinal pericyte cells, where the incubation of isolated cells with 30 μM of UTP for 30 min before exposure to 100 μM of benzoylbenzoyl-ATP blocks $P2X_7$-dependent permeabilization (Sugiyama et al. 2005)

We thus consider that additional experiments based, for example, on electrophysiology and pharmacological approaches are necessary to re-evaluate $P2X_7$ receptor relevance in thymus atrophy induced by *T. cruzi* infection in vivo. Approaches other than those using knockout mice should be adopted, since compensatory mechanisms may

take control and mask primary physiological roles of individual purinergic receptors during the atrophy. However, these mice, together with the infection, revealed interesting interactions that can help us to understand the role of P2 receptors in immunomodulations of protozoan infections.

Acknowledgments This work was supported by Fiocruz, Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) and PRONEX—Fundação de Amparo à Pesquisa do Rio de Janeiro (FAPERJ). We are grateful to Dr. James Mobley (PGRD, Pfizer, Groton, CT, USA) for kindly providing the $P2X7R^{-/-}$ mice. We thank Dr. Luis Anastácio Alves for invaluable assistance and Mr. Marcos Meuser Batista for excellent technical assistance. We declare that the experiments comply with the current laws of the country, as stated in "Materials and methods."

References

Adriouch S, Dox C, Welge V, Seman M, Koch-Nolte F, Haag F (2002) Cutting edge: a natural P451L mutation in the cytoplasmic domain impairs the function of the mouse $P2X_7$ receptor. J Immunol 169:4108–4112

Araujo-Jorge TC, Sampaio EP, De Souza W, Meirelles N (1989) *Trypanosoma cruzi*: the effect of variations in experimental conditions on the levels of macrophage infection in vitro. Parasitol Res 75:257–263

Cabrini G, Falzoni S, Forchap SL, Pellegatti P, Balboni A, Agostini P, Cuneo A, Castoldi G, Baricordi OR, Di Virgilio F (2005) A His-155 to Tyr polymorphism confers gain-of-function to the human $P2X_7$ receptor of human leukemic lymphocytes. J Immunol 175:82–89

Coddou C, Morales B, Huidobro-Toro JP (2003) Neuromodulator role of zinc and copper during prolonged ATP applications to $P2X_4$ purinoceptors. Eur J Pharmacol 472:49–56

Cotta-de-Almeida V, Bertho AL, Villa-Verde DM, Savino W (1997) Phenotypic and functional alterations of thymic nurse cells following acute *Trypanosoma cruzi* infection. Clin Immunol Immunopathol 82:125–132

Cotta-de-Almeida V, Bonomo A, Mendes-da-Cruz DA, Riederer I, De Meis J, Lima-Quaresma KR, Vieira-de-Abreu A, Villa-Verde DM, Savino W (2003) *Trypanosoma cruzi* infection modulates intrathymic contents of extracellular matrix ligands and receptors and alters thymocyte migration. Eur J Immunol 33:2439–2448

Coura JR (2007) Chagas disease: what is known and what is needed-a background article. Mem Inst Oswaldo Cruz 102(Suppl 1):113–122

Coutinho-Silva R, Persechini PM (1997) P2Z purinoceptor-associated pores induced by extracellular ATP in macrophages and J774 cells. Am J Physiol 273:C1793–C1800

Coutinho-Silva R, Perfettini JL, Persechini PM, Dautry-Varsat A, Ojcius DM (2001) Modulation of P2Z/P2X(7) receptor activity in macrophages infected with *Chlamydia psittaci*. Am J Physiol 280:C81–C89

Dubyak GR (2007) Go it alone no more-$P2X_7$ joins the society of heteromeric ATP-gated receptor channels. Mol Pharmacol 72:1402–1405

Eschke D, Wust M, Hauschildt S, Nieber K (2002) Pharmacological characterization of the P2X(7) receptor on human macrophages using the patch-clamp technique. Naunyn-Schmiedeberg's Arch Pharmacol 365:168–171

Ferrari D, Pizzirani C, Adinolfi E, Lemoli RM, Curti A, Idzko M, Panther E, Di Virgilio F (2006) The $P2X_7$ receptor: a key player in IL-1 processing and release. J Immunol 176:3877–3883

Freire-de-Lima CG, Nascimento DO, Soares MB, Bozza PT, Castro-Faria-Neto HC, de Mello FG, DosReis GA, Lopes MF (2000) Uptake of apoptotic cells drives the growth of a pathogenic trypanosome in macrophages. Nature 403:199–203

Gu B, Bendall LJ, Wiley JS (1998) Adenosine triphosphate-induced shedding of CD23 and L-selectin (CD62L) from lymphocytes is mediated by the same receptor but different metalloproteases. Blood 92:946–951

Guo C, Masin M, Qureshi OS, Murrell-Lagnado RD (2007) Evidence for functional $P2X_4/P2X_7$ heteromeric receptors. Mol Pharmacol 72:1447–1456

Hardy AR, Jones ML, Mundell SJ, Poole AW (2004) Reciprocal cross-talk between P2Y1 and P2Y12 receptors at the level of calcium signaling in human platelets. Blood 104:1745–1752

Higuchi ML, Benvenuti LA, Martins Reis M, Metzger M (2003) Pathophysiology of the heart in Chagas' disease: current status and new developments. Cardiovasc Res 60(1):96–107

Humphreys BD, Dubyak GR (1998) Modulation of P2X7 nucleotide receptor expression by pro- and anti-inflammatory stimuli in THP-1 monocytes. J Leukoc Biol 64:265–273

Leite-de-Moraes MC, Hontebeyrie-Joskowicz M, Dardenne M, Savino W (1992) Modulation of thymocyte subsets during acute and chronic phases of experimental *Trypanosoma cruzi* infection. Immunology 77:95–98

Lopes MF, Nunes MP, Henriques-Pons A, Giese N, Morse HC, Davidson WF, Araujo-Jorge TC, DosReis GA (1999) Increased susceptibility of Fas ligand-deficient gld mice to *Trypanosoma cruzi* infection due to a Th2-biased host immune response. Eur J Immunol 29:81–89

Mantuano-Barradas M, Henriques-Pons A, Araujo-Jorge TC, Di Virgilio F, Coutinho-Silva R, Persechini PM (2003) Extracellular ATP induces cell death in $CD4^+/CD8^+$ double-positive thymocytes in mice infected with *Trypanosoma cruzi*. Microbes Infect 5:1363–1371

Minoprio P, Itohara S, Heusser C, Tonegawa S, Coutinho A (1989) Immunobiology of murine *Trypanosoma cruzi* infection: the predominance of parasite-nonspecific responses and the activation of TCRI T cells. Immunol Rev 112:183–207

Moncayo A (2003) Chagas disease: current epidemiological trends after the interruption of vectorial and transfusional transmission in the Southern Cone countries. Mem Inst Oswaldo Cruz 98(5):577–591

Mucci J, Hidalgo A, Mocetti E, Argibay PF, Leguizamon MS, Campetella O (2002) Thymocyte depletion in *Trypanosoma cruzi* infection is mediated by trans-sialidase-induced apoptosis on nurse cells complex. Proc Natl Acad Sci USA 99:3896–3901

Mucci J, Mocetti E, Leguizamon MS, Campetella O (2005) A sexual dimorphism in intrathymic sialylation survey is revealed by the trans-sialidase from *Trypanosoma cruzi*. J Immunol 174:4545–4550

Pelegrin P, Surprenant A (2006) Pannexin-1 mediates large pore formation and interleukin-1beta release by the ATP-gated $P2X_7$ receptor. EMBO J 25:5071–5082

Ralevic V, Burnstock G (1998) Receptors for purines and pyrimidines. Pharmacol Rev 50:413–492

Shemon AN, Sluyter R, Fernando SL, Clarke AL, Dao-Ung LP, Skarratt KK, Saunders BM, Tan KS, Gu BJ, Fuller SJ, Britton WJ, Petrou S, Wiley JS (2006) A Thr357 to Ser polymorphism in homozygous and compound heterozygous subjects causes absent or reduced $P2X_7$ function and impairs ATP-induced mycobacterial killing by macrophages. J Biol Chem 281:2079–2086

Solle M, Labasi J, Perregaux DG, Stam E, Petrushova N, Koller BH, Griffiths RJ, Gabel CA (2001) Altered cytokine production in mice lacking P2X(7) receptors. J Biol Chem 276:125–132

Sugiyama T, Kawamura H, Yamanishi S, Kobayashi M, Katsumura K, Puro DG (2005) Regulation of P2X7-induced pore formation and cell death in pericyte-containing retinal microvessels. Am J Physiol 288:C568–576

Surprenant A, Schneider DA, Wilson HL, Galligan JJ, North RA (2000) Functional properties of heteromeric P2X(1/5) receptors expressed in HEK cells and excitatory junction potentials in guinea-pig submucosal arterioles. J Auton Nerv Syst 81:249–263

Tsukimoto M, Maehata M, Harada H, Ikari A, Takagi K, Degawa M (2006) P2X7 receptor-dependent cell death is modulated during murine T cell maturation and mediated by dual signaling pathways. J Immunol 177:2842–2850

Virginio C, MacKenzie A, Rassendren FA, North RA, Surprenant A (1999) Pore dilation of neuronal P2X receptor channels. Nat Neurosci 2:315–321

Volonte C, Amadio S, D'Ambrosi N, Colpi M, Burnstock G (2006) P2 receptor web: complexity and fine-tuning. Pharmacol Ther 112:264–280

WHO (2004) Online newsletter: TDRNews. Available at: www.who.int/tdr/publications/tdrnews/default.htm. Access: May 2007