Immunobiology 224 (2019) 50–59

Contents lists available at ScienceDirect

Immunobiology

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

P2X7 receptor-mediated leukocyte recruitment and *Porphyromonas gingivalis* clearance requires IL-1β production and autocrine IL-1 receptor activation

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ARTICLE INFO

Keywords:
P2X7 receptor
IL-1 receptor
Porphyromonas gingivalis
IL-1β
Inflammasome

ABSTRACT

The Gram-negative bacterium *Porphyromonas gingivalis* is strongly associated with periodontitis. We previously demonstrated that P2X7 receptor activation by extracellular ATP (eATP) triggers elimination of intracellular pathogens, such as *Leishmania amazonensis, Toxoplasma gondii* and *Chlamydia trachomatis*. We also showed that eATP-induced IL-1β secretion *via* the P2X7 receptor is impaired by *P. gingivalis* fimbriae. Furthermore, enhanced P2X7 receptor expression was detected in the maxilla of *P. gingivalis*-orally infected mice as well as in human periodontitis patients. Here, we examined the effect of P2X7-, caspase-1/11- and IL-1 receptor-mediated responses during *P. gingivalis* infection. P2X7 receptor played a large role in controlling *P. gingivalis* infection and *P. gingivalis*-induced recruitment of inflammatory cells, especially neutrophils. In addition, IL-1β secretion was detected at different time points only when P2X7 receptor was expressed and in the presence of eATP treatment *ex vivo*. Activation of P2X7 receptor and IL-1 receptor by eATP and IL-1β, respectively, promoted *P. gingivalis* elimination in macrophages. Interestingly, eATP-induced *P. gingivalis* killing was inhibited by the IL-1 receptor antagonist (IL-1RA), consistent with autocrine activation of the IL-1 receptor for *P. gingivalis* elimination. *In vivo*, caspase-1/11 and IL-1 receptor were also required for bacterial clearance, leukocyte recruitment and IL-1β production after *P. gingivalis* infection. Our data demonstrate that the P2X7-IL-1 receptor axis activation is required for effective innate immune responses against *P. gingivalis* infection.

1. Introduction

Porphyromonas gingivalis (*P. gingivalis*) is a Gram-negative, asaccharoly-tic and anaerobic oral bacterium that is normally detected in 10–25% of healthy subjects and 79–90% of subjects with periodontal disease (Igboin et al., 2009). Periodontitis is a chronic inflammatory disease induced by pathogenic biofilm formation associated with the periodontium, and the progression of this disease affects the tooth-supporting structures leading to alveolar bone resorption and tooth loss (Hajishengallis, 2014, 2015). *P. gingivalis* is considered a "keystone pathogen" in the etiology of periodontitis because, along with host susceptibility factors (such as host genotype, stress, diet, smoking), the microorganism can promote dysbiosis in the oral microbiota, leading to chronic severe forms of periodontitis (Hajishengallis et al., 2012).

P. gingivalis can be recognized by pattern recognition receptors (PRRs) of the innate immune system *via* the conserved molecular products of the bacteria also known as pathogen-associated molecular patterns (PAMPs). After this recognition, intracellular signaling leads to activation of pro-inflammatory transcription factors such as NF-κB and AP-1, which promote chemokine and cytokine production (Gmiterek et al., 2016). Previous studies had shown high levels of the pro-inflammatory cytokines interleukin (IL)-1β and tumor necrosis factor

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https://doi.org/10.1016/j.imbio.2018.10.008
Received 4 July 2018; Received in revised form 28 October 2018; Accepted 30 October 2018
Available online 02 November 2018

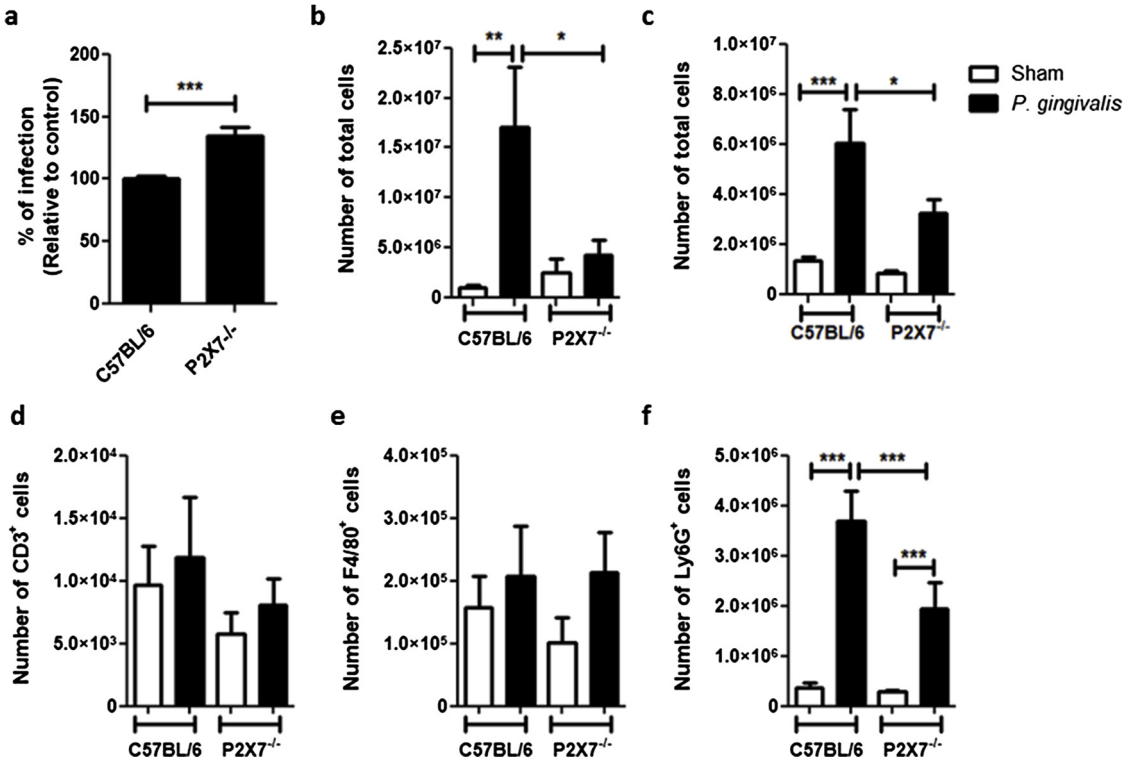

Fig. 1. P2X7 receptor is essential for bacterial elimination and inflammatory cell migration during *P. gingivalis* infection *in vivo*. C57BL/6 and P2X7$^{-/-}$ mice were infected with *P. gingivalis* using the air pouch model and bacterial load or recruited cells were analyzed after 4 h or 24 h. **(a)** Infected mice had exudates collected after 4 h of infection for bacterial quantitation. Graph represents mean ± SEM of the bacterial load normalized to the control group C57BL/6 in percentage. **(b and c)** Infected mice had their exudates collected and the total cells quantified after 4 h **(b)** or 24 h **(c)** of infection. Graphs represent mean ± SEM of absolute total cell numbers recovered by mouse. **(d – f)** The exudates of 24 h-infected mice were analyzed by flow cytometry and absolute numbers of lymphocytes (CD3$^+$), macrophages (F4/80$^+$) or neutrophils (Ly6G$^+$) were enumerated as shown in graphs **(d)**, **(e)** and **(f)**, respectively. Figures show mean ± SEM of absolute total cell numbers recovered per mice. **(a and b)** n = 7 mice per group; **(c)** n = 8–11 mice per group; **(d, e and f)** n = 5–8 mice per group. Data pooled from two independent experiments. Asterisks indicate significant differences. *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001.

(TNF)-α in gingival tissue and gingival crevicular fluid from subjects with chronic periodontitis, compared with healthy subjects (Matsuki et al., 1992; Prabhu et al., 1996; Preiss and Meyle, 1994). Importantly, IL-1β requires activation of the inflammasome complex in order to be secreted (Broz and Dixit, 2016).

Inflammasomes are multi-protein cytoplasmic platforms assembled in the host cell in response to infection or cellular stress, leading to a type of cell death called pyroptosis and/or maturation and secretion of pro-inflammatory cytokines, such as IL-1β and IL-18 (Lamkanfi and Dixit, 2014; Martinon et al., 2002; Schroder and Tschopp, 2010; Giuliani et al., 2017; Luna-Gomes et al., 2015). The most extensively studied inflammasome is the NLRP3 inflammasome, which typically requires two signals: (1) a PAMP, such as lipopolysaccharide (LPS), leading to transcription of NF-κB and upregulation of genes encoding pro-inflammatory cytokines, chemokines, and proteins associated with the inflammasome; and (2) a danger signal, such as extracellular ATP (eATP), which induces inflammasome activation and pro-caspase-1 cleavage into active caspase-1 (Coutinho-Silva and Ojcius, 2012; Rathinam and Fitzgerald, 2016). Caspase-1 in turn cleaves pro-IL-1β and pro-IL-18 into their bioactive forms IL-1β and IL-18, which can be released (Giuliani et al., 2017). The NLRP3 inflammasome is also activated by *P. gingivalis* and eATP in gingival epithelial cells (Yilmaz et al., 2010), and murine (Morandini et al., 2014a) and human macrophages (Park et al., 2014).

eATP is one of the most studied danger signals and is recognized by the purinergic nucleotide receptor P2X7 (Mariathasan et al., 2006; Giuliani et al., 2017; Morandini et al., 2014b). The P2X7 receptor is a low affinity, ATP-gated plasma membrane ion channel that can form a non-selective pore permeable to molecules up to 900 Da in size (Morandini et al., 2014b; Coutinho-Silva and Persechini, 1997, 1997). P2X7 receptor is widely distributed in all tissues, with the highest expression being in immune cells, especially of the myeloid lineage (Burnstock and Knight, 2004; Morandini et al., 2014b). We and others have reported that P2X7 receptor activation leads to elimination of different intracellular pathogens such as *Leishmania amazonensis* (Chaves et al., 2014), *Chlamydia trachomatis* (Coutinho-Silva et al., 2003), *Mycobacterium tuberculosis* (Kusner and Adams, 2000; Placido et al., 2006; Santos et al., 2013), and *Toxoplasma gondii* (Correa et al., 2010; Almeida-da-Silva et al., 2017; Correa et al., 2017). Even though P2X7 receptor activation has been examined in different models of intracellular infection, it was never studied with regards to its role in controlling *P. gingivalis* infection *in vivo*.

Our group previously reported that *P. gingivalis* fimbriae dampen P2X7 receptor-dependent IL-1β secretion in murine macrophages *ex vivo* (Morandini et al., 2014a). P2X7 receptor is also relevant for infection *in vivo*, since it is upregulated in the maxilla of *P. gingivalis*-infected mice and its deficiency resulted in lower levels of the pro-inflammatory cytokines IFN-γ and IL-17 in draining lymph node cells in infected mice (Ramos-Junior et al., 2015). Here, we investigated whether P2X7 receptor, caspase-1/11 and the IL-1 receptor are involved in recruitment of inflammatory cells and IL-1β production during *P. gingivalis* infection, and whether they play a role in bacterial clearance.

2. Results

2.1. P2X7 receptor is required for bacterial elimination and leukocyte recruitment after P. gingivalis infection in vivo

A large body of evidence shows that P2X7 receptor is important for controlling intracellular infection by different pathogens (Almeida-da-Silva et al., 2017, 2016; Chaves et al., 2014; Morandini et al., 2014a; Correa et al., 2010; Kusner and Adams, 2000; Coutinho-Silva et al., 2003), but the role of P2X7 receptor in control of *P. gingivalis* infection has never been explored *in vivo*. We therefore infected wild-type C57BL/6 or P2X7$^{-/-}$ mice using the air pouch model. Significantly higher numbers of bacteria were recovered from P2X7$^{-/-}$ mice than the wild-type control group (Fig. 1a), suggesting that P2X7 receptor is involved in controlling *P. gingivalis* infection *in vivo*.

We next determined if P2X7 receptor affects migration of inflammatory cells to the site of infection, infecting C57BL/6 or P2X7$^{-/-}$ mice with *P. gingivalis* and analyzing the exudates after 4 h (Fig. 1b) or 24 h (Fig. 1c) of infection. *P. gingivalis* induced robust recruitment of inflammatory cells to the site of infection in wild-type mice (Figs. 1b and c); while the number of cells was significantly reduced in infected P2X7$^{-/-}$ mice. The cells in the exudate after *P. gingivalis* infection were quantified for CD3^{+} cells (lymphocytes), F4/80^{+} cells (macrophages) and Ly-6G^{+} cells (neutrophils). As shown in Figures 1d - f, the main inflammatory cells recruited to the site of infection were neutrophils (Fig. 1f), followed by fewer macrophages (Fig. 1e) and lymphocytes (Fig. 1d). Significantly fewer neutrophils were found in exudates from P2X7$^{-/-}$ mice compared with infected wild-type mice (Fig. 1f). Taken together, these results suggest that P2X7 receptor is required for bacterial elimination and inflammatory cell recruitment to the site of infection during *P. gingivalis* infection.

2.2. P2X7 receptor activation is essential for eATP-induced IL-1β secretion during P. gingivalis infection in macrophages

We next analyzed the production and secretion of IL-1β in bone-marrow derived macrophages (BMDMs) derived from wild-type or P2X7$^{-/-}$ mice infected with *P. gingivalis* and treated with or without 5 mM eATP during the last 30 min of infection. We measured IL-1β secretion in BMDMs infected with *P. gingivalis* for 6 h (Fig. 2a), 18 h (Fig. 2 b) and 24 h (Fig. 2c). *P. gingivalis* infection or eATP treatment by themselves did not induce IL-1β secretion in BMDMs, as shown in Figs. 2 a, b and c; however, infected and eATP-treated cells secreted high levels of IL-1β after 6 h, 18 h and 24 h of infection. Therefore, BMDMs require *P. gingivalis* and eATP-stimulation to secrete IL-1β at different time points. Significantly, unlike IL-1β, a pro-inflammatory cytokine that does not require the inflammasome, such as TNF-α, was not affected by the lack of P2X7 receptor or the presence of eATP (Supplementary Fig. 1).

There was essentially no IL-1β release when BMDMs derived from P2X7$^{-/-}$ mice were infected and stimulated with *P. gingivalis* and treated with eATP (Fig. 2a–c). Our data imply that P2X7receptor ligation of eATP is crucial for IL-1β secretion from *P. gingivalis*-infected BMDMs.

We next examined pro-IL-1β production and IL-1β secretion in BMDMs derived from wild-type or P2X7$^{-/-}$ mice infected or not with *P. gingivalis* for 6 h (Fig. 2d and e). Cellular extracts levels of pro-IL1β were only detected after *P. gingivalis* infection (Fig. 2d and e). The lack of P2X7 receptor did not change pro-IL-1β production induced by *P. gingivalis* in cellular extracts (Figs. 2d and e, superior panel). Densitometric analysis confirmed that there was no difference in the pro-IL-1β levels in the cellular extracts (Fig. 2f). In addition, we detected IL-1β in the supernatants of *P. gingivalis*-infected and eATP-treated BMDMs derived from wild-type mice but not from P2X7$^{-/-}$ mice (Figs. 2d and e, medium panel), as expected. These results confirmed that *P. gingivalis* alone can promote pro-IL-1β production (acting as the first signal of the inflammasome) but cannot induce mature IL-1β secretion *in vitro*. Although P2X7 receptor is dispensable for pro-IL-1β production during *P. gingivalis* infection *in vitro,* purinergic receptor activation by eATP is crucial for IL-1β secretion.

2.3. eATP and IL-1β inhibit P. gingivalis infection in macrophages through P2X7 and the IL-1 receptor

To determine if P2X7 receptor could affect the ability of *P. gingivalis* to invade BMDMs, colony-forming units (CFUs) were measured after 2 h of *P. gingivalis* infection in wild-type or P2X7$^{-/-}$ BMDMs. Similar levels of bacteria were recovered from both BMDMs (Fig. 3a), indicating that P2X7 receptor does not affect *P. gingivalis* invasion in macrophages.

We and others have previously reported that P2X7 receptor activation by eATP eliminates intracellular parasites (Chaves et al., 2014; Almeida-da-Silva et al., 2017; Coutinho-Silva et al., 2003; Kusner and Adams, 2000). To evaluate whether eATP may affect *P. gingivalis* survival in macrophages, infected PMA-primed THP-1 cells were treated with 3 mM eATP or control buffer (Figs. 3b and c), or pretreated with the P2X7 receptor antagonist, A740003 (Fig. 3c). After 24 h of infection, eATP treatment led to a decrease in bacterial infection, as measured by CFU quantification (Fig. 3b) and qPCR (as reflected by *P. gingivalis* DNA - Fig. 3c). The P2X7 receptor antagonist completely inhibited the eATP-induced bacterial killing, suggesting eATP mediates intracellular *P. gingivalis* killing through P2X7 receptor activation. Since eATP leads to IL-1β release from *P. gingivalis*-infected cells (Fig. 2a, b and c), we next examined if IL-1β cytokine could affect bacterial survival in macrophages. To address this issue, infected BMDMs (Fig. 3d) and PMA-primed THP-1 cells (Fig. 3e) were treated with 100 pg/ml IL-1β or control buffer, or pretreated with the IL-1 receptor antagonist, IL-1RA (Fig. 3e). After 24 h of infection, recombinant IL-1β treatment significantly decreased *P. gingivalis* infection as measured in BMDMs (Fig. 3d) or PMA-primed THP-1 cells (Fig. 3e) by qPCR. IL-1RA pretreatment inhibited IL-1β-induced bacterial killing, demonstrating that IL-1β exerts its function through activation of its receptor (Fig. 3e). Additionally, IL-1RA inhibited ATP-induced *P. gingivalis* killing in macrophages, demonstrating the link between ATP-induced IL-1β production and IL-1β-mediated bacterial elimination. Together, these results demonstrate that eATP induces IL-1β release by *P. gingivalis*-infected macrophages and that this cytokine subsequently decreases *P. gingivalis* infection through an autocrine mechanism involving the IL-1 receptor.

2.4. P2X7 receptor-, caspase-1/11- and IL-1 receptor are involved in P. gingivalis-induced IL-1β production in vivo

Because IL-1β production after *P. gingivalis* infection *in vitro* was required for bacterial elimination, we measured IL-1β levels after *P. gingivalis* infection *in vivo* in wild-type, P2X7$^{-/-}$, caspase-1/11$^{-/-}$ and IL-1R$^{-/-}$ mice. A significant increase in IL-1β levels was observed after *P. gingivalis* infection in wild-type mice compared with uninfected sham controls after 4 h (Fig. 4a, c and d) or 24 h of infection (Fig. 4b), as well as in infected P2X7$^{-/-}$ mice compared with uninfected sham controls after 4 h (Fig. 4a). Furthermore, IL-1β levels in infected P2X7$^{-/-}$ (Fig. 4a and b) and Caspase-1/11$^{-/-}$ (Fig. 4c) mice were significantly lower than in infected wild-type mice. Interestingly, IL-1β levels were also lower in IL-1R$^{-/-}$ mice (Fig. 4d), suggesting that IL-1β stimulates its own synthesis in an autocrine signaling loop, as previously observed for other cells (Brown et al., 2013). These results show that *P. gingivalis* promotes IL-1β production after infection *in vivo* and the cytokine production is impaired in the absence of P2X7 receptor, caspase-1/11 or the IL-1 receptor.

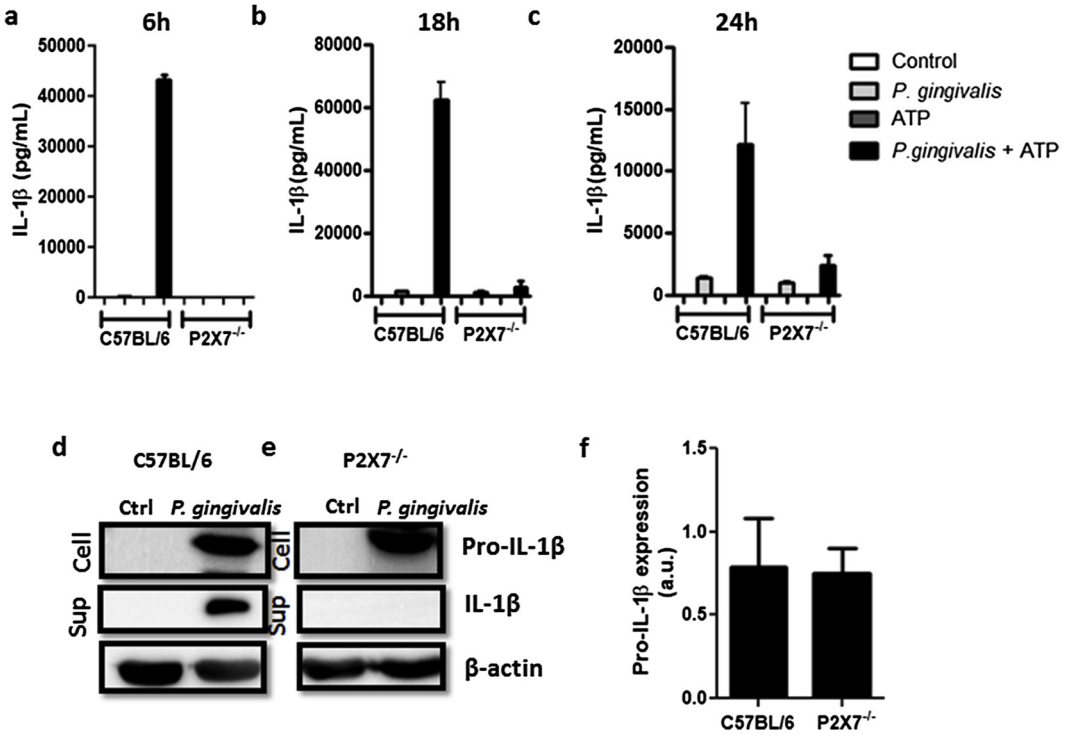

Fig. 2. P2X7 receptor is essential for eATP-induced IL-1β release, but not for pro-IL-1β production after *P. gingivalis* infection in macrophages. BMDMs derived from C57BL/6 or P2X7$^{-/-}$ mice were infected with *P. gingivalis* for 6 h, 18 h or 24 h and treated with or without 5 mM eATP during the last 30 min of infection. **(a – c)** show IL-1β secretion after 6 h **(a)**, 18 h **(b)** or 24 h **(c)** of infection and eATP treatment. **(a – c)** graphs show mean ± SEM from duplicates of the IL-1β cytokine, in pg/ml **(d and e)** are representative western blot figures showing pro-IL-1β and mature IL-1β production in BMDMs derived from C57BL/6 **(d)** or P2X7$^{-/-}$ **(e)** after *P. gingivalis* infection for 6 h without eATP treatment in the cellular extracts (cell) and with eATP treatment in the supernatants (Sup). β-actin was used as loading control. **(f)** shows densitometric analysis (mean ± SEM) of pro-IL-1β expression in arbitrary units. Data is representative of 2 or 3 independent experiments.

2.5. IL-1 receptor signaling and inflammasome activation are essential for P. gingivalis elimination in vivo

Since P2X7 receptor and caspase-1/11 are involved in activation of the inflammasome complex and IL-1β secretion, which eliminates *P. gingivalis in vitro*, we next examined if signaling by the IL-1 receptor and caspase-1/11 are required for elimination of *P. gingivalis in vivo*. Thus, we infected wild-type, IL-1R$^{-/-}$ and Caspase-1/11$^{-/-}$ mice using the air pouch model and quantified *P. gingivalis* infection and cell recruitment. Significantly higher numbers of bacteria were found in infected IL-1R$^{-/-}$ (Fig. 5a) and infected Caspase-1/11$^{-/-}$ (Fig. 5b) mice, compared with infected wild-type controls, suggesting that IL-1R signaling and inflammasome activation are important for controling *P. gingivalis* infection *in vivo* (Fig. 5a and b). *P. gingivalis* was not detected in uninfected mice (data not shown). Consistent with the data in Fig. 1, wild-type mice infected with *P. gingivalis in vivo* showed significantly higher levels of recruited inflammatory cells (Fig. 5c and d), compared with wild-type controls. However, IL-1R$^{-/-}$ mice infected with *P. gingivalis* demonstrated significantly lower levels of recruited inflammatory cells (Fig. 5c), suggesting that IL-1 receptor signaling contributes to the innate immune response against *P. gingivalis* infection *in vivo*. On the other hand, caspase-1/11$^{-/-}$ infected mice did not show significant differences in leukocyte recruitment compared with infected wild-type controls (Fig. 5d). Finally, to confirm the role of the IL-1 receptor in the control of *P. gingivalis* infection *in vivo*, infected mice were treated with or without recombinant IL-1β and the bacterial load was measured. IL-1β treatment *in vivo* significantly decreased *P. gingivalis* infection in wild-type mice compared with the PBS-treated control (Fig. 5e), suggesting that P2X7 receptor-mediated IL-1β secretion contributes to bacterial elimination *in vivo*. Importantly, *P. gingivalis* levels after IL-1β treatment also decreased in P2X7$^{-/-}$ mice but to a lesser extent than in infected IL-1β-treated wild-type mice (Fig. 5e). This reinforces the view that P2X7 receptor-dependent IL-1β production contributes to bacterial elimination.

3. Discussion

Activation of P2X has been reported over the years to promote bacterial elimination (Coutinho-Silva and Ojcius, 2012; Almeida-da-Silva et al., 2016). The P2X7 receptor was already reported to control infections *in vivo* and *in vitro* by intracellular pathogens such as *L. amazonensis* (Chaves et al., 2014; Figliuolo et al., 2017; Thorstenberg et al., 2018), *M. tuberculosis*(Kusner and Adams, 2000; Placido et al., 2006; Santos et al., 2013), *C. trachomatis* (Coutinho-Silva et al., 2003) and *T. gondii* (Lees et al., 2010; Almeida-da-Silva et al., 2017; Correa et al., 2010). The mechanisms described so far for P2X7 receptor-mediated elimination of intracellular infection are: ROS/ NO production, induction of host cell death, phagosome and lysosome fusion, and inflammasome activation inducing IL-1β secretion (Almeida-da-Silva et al., 2016; Coutinho-Silva et al., 2007). In the present work, we show that eATP treatment promoted killing of intracellular *P. gingivalis* in infected cells. We showed that eATP induces IL-1β secretion through P2X7 receptor in infected macrophages, and that this cytokine binds to its receptor, leading to elimination of *P. gingivalis* (Fig. 6). This mechanism of killing of intracellular pathogen is similar to a recent study on *T. gondii* infection in macrophages (Almeida-da-Silva et al., 2017), in which eATP treatment led to inflammasome activation and secretion of IL-1β, which in turn inhibited parasite survival through ROS production (Almeida-da-Silva et al., 2017).

We also showed IL-1 receptor signaling and the inflammasome (using caspase-1/11$^{-/-}$ mice) were important for bacterial control. The main function of the cytokines from the IL-1 family is to control

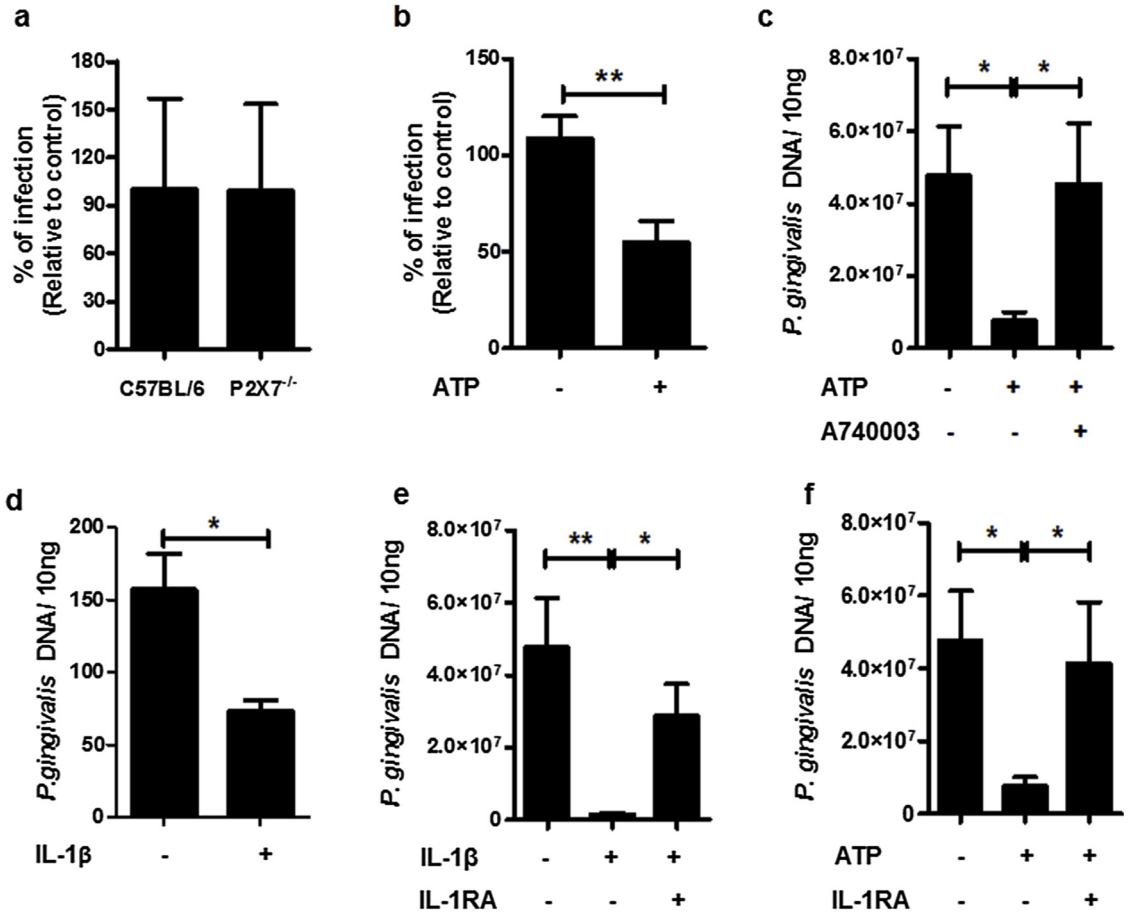

Fig. 3. P2X7-IL-1 receptor axis activation eliminates *P. gingivalis* infection in macrophages. Macrophages were infected with *P. gingivalis* for 24 h and treated with or without the agonists 3 mM eATP or 100 pg/ml recombinant IL-1β; or the antagonists 50 nM A740003 or 10 μg/ml IL-1RA, as indicated. **(a)** BMDMs derived from C57BL/6 or P2X7$^{-/-}$ mice were infected with *P. gingivalis*, and after 2 h of infection CFU were enumerated. Graph shows mean ± SEM of bacterial load normalized in percentage to the C57BL/6 group, data represents the average from 2 independent experiments. **(b)** PMA-treated THP-1 cells were infected and treated with or without 3 mM eATP after 2 h of infection and samples were collected for CFU counts after 24 h of infection. Graph represents mean ± SEM of bacterial load normalized in percentage to the untreated control group. The result shows a pool of 4 independent experiments. (**c, e and f**) PMA-treated THP-1 cells were infected with *P. gingivalis* for 2 h, treated with A740003 or IL-1RA for 40 min, and with ATP or IL-1β for 30 min, as indicated. *P. gingivalis* DNA was quantified after 24 h of infection. Graphs show mean ± SEM of *P. gingivalis* DNA per 10 ng of total extracted DNA. The result represents a pool of 3 independent experiments. **(d)** BMDMs were infected and treated with or without 100 pg/ml. IL-1β after 6 h of infection and *P. gingivalis* DNA were quantified after 24 h of infection. Graph shows mean ± SEM of *P. gingivalis* DNA per 10 ng of total extracted DNA. The result is from a pool of 2 independent experiments. Asterisks indicate significant differences. **$p \leq 0.01$, *$p \leq 0.05$.

pro-inflammatory reactions in response to tissue injury by PAMPs and/or DAMPs (Weber et al., 2010; Dinarello, 2009). As a matter of fact, IL-1 receptor signaling has been demonstrated to be important for controlling infection by the intracellular parasite *Leishmania* (Lima-Junior et al., 2013), the fungus *Aspergillus fumigatus* (Caffrey et al., 2015) and the bacterium *Mycobacterium tuberculosis* (Mayer-Barber et al., 2010) in mouse models. Additionally, several studies have also demonstrated the role of the inflammasome activation for recognition and the host control of different infections, such as *Trypanosoma cruzi*, *T. gondii*, *Plasmodium* spp., and *Leishmania* spp, *Bacillus anthracis*, *A. fulmigatus* and *Legionella pneumophila* (Lamkanfi and Dixit, 2011; Zamboni and Lima-Junior, 2015). Our results in *P. gingivalis* model of infection *in vitro* and *in vivo* are in favor with previous reports and confirm the important role for the IL-1 receptor signaling and inflammasome activation for microbial elimination.

Mice lacking P2X7 receptor, caspase-1/11, or the IL-1 receptor showed higher susceptibility to *P. gingivalis* infection *in vivo*, suggesting that these molecules are involved in bacterial clearance. However, caspase-1/11$^{-/-}$ mice showed lower susceptibility compared with IL-1R$^{-/-}$ mice (Fig. 5 a and b). This difference could be explained by the fact that mice lacking caspase-1/11 recruited more leukocytes compared with mice lacking IL-1R. Thus, while IL-1R$^{-/-}$ mice exhibited impaired leukocyte recruitment and IL-1β production, caspase-1/11$^{-/-}$ mice had impaired IL-1β production but could still recruit inflammatory cells to the site of infection. Therefore, we suggest that both recruited leukocytes and IL-1β produced after infection can contribute to *P. gingivalis* killing *in vivo*.

In the present work, we demonstrated *P. gingivalis* induces migration of neutrophils to the site of infection using the air pouch model of infection, corroborating previous studies on *P. gingivalis* infection in a chamber model in mice (Maekawa et al., 2014). The absence of P2X7 receptor in mice led to significantly reduced cell recruitment (total number of cells and neutrophil numbers) in our *P. gingivalis* model of infection, consistent with data from other inflammatory mouse models such as dermatitis (da Silva et al., 2013) and sepsis (Santana et al., 2015). Moreover, we showed signaling through the IL-1 receptor, but not caspase-1/11, also participates in leukocyte recruitment to the site of *P. gingivalis* infection. This result is consistent with previous studies showing that IL-1 receptor signaling participates in leukocyte recruitment to the site of infection in different mouse models of inflammation (Rider et al., 2011; Caffrey et al., 2015). Our data show for the first time the importance of P2X7 and the IL-1 receptor for leukocyte recruitment

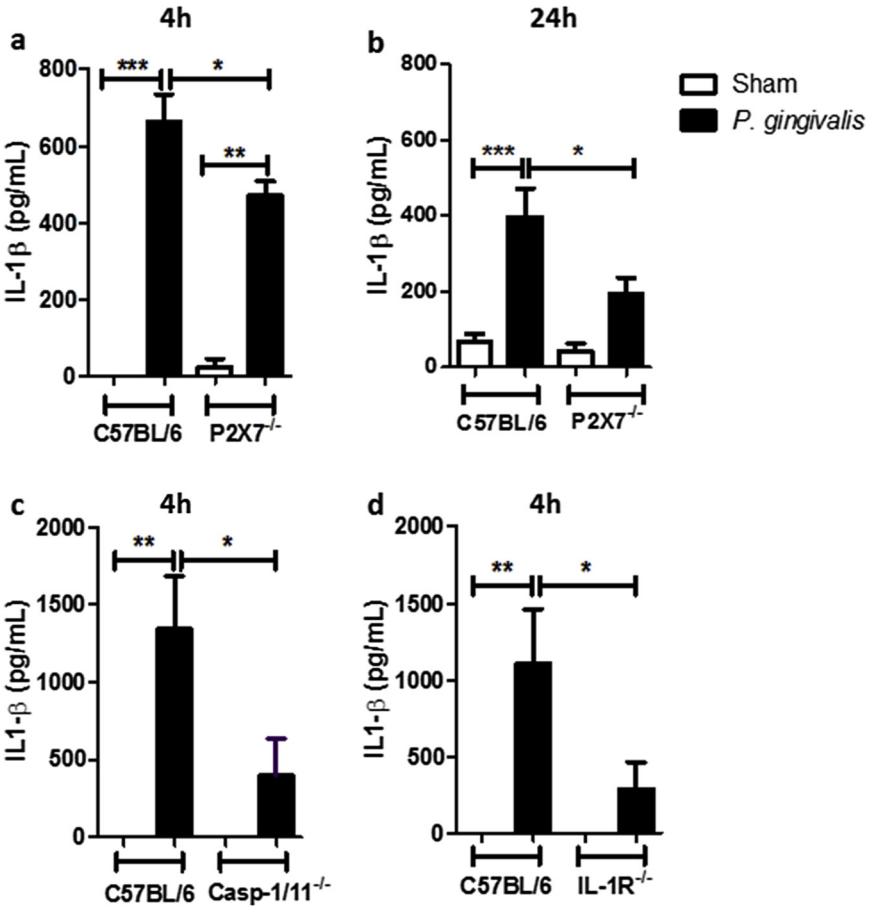

Fig. 4. P2X7 receptor, caspase-1/11 and IL-1 receptor are required for *P. gingivalis*-induced IL-1β production *in vivo*. C57BL/6, P2X7$^{-/-}$ **(a and b)**, Caspase-1/11$^{-/-}$ **(c)** and IL-1R$^{-/-}$ **(d)** mice were infected with *P. gingivalis* and the exudates were analyzed for IL-1β production after 4 h **(a, c and d)** or 24 h **(b)** of infection. Graphs show mean ± SEM of IL-1β, pg/mL, collected from mice. Data were pooled from two independent experiments, n = 5–11 mice per group.

to the inflammatory site during *P. gingivalis* infection. In fact, during periodontal disease, neutrophils play a large role in bacterial elimination and host tissue damage (Sochalska and Potempa, 2017).

Our *in vivo* experiments confirmed the role of P2X7 receptor in the production of IL-1β, since *P. gingivalis*-infected P2X7$^{-/-}$ mice produced significantly lower levels of this cytokine than infected wild-type mice. Similar results showing lower levels of pro-inflammatory cytokine production were observed with P2X7$^{-/-}$ mice in the mouse inflammatory model of sepsis (Santana et al., 2015). Furthermore, we showed that the IL-1 receptor contributes to IL-1β production during *P. gingivalis* infection *in vivo*, consistent with previous reports that IL-1 receptor signaling leads to activation of NF-κB, which induces the expression of canonical IL-1 target genes, such as IL-6, IL-8, IL-1α and IL-1β (Weber et al., 2010;Acuner Ozbabacan et al., 2014). Therefore, our data using IL-1R$^{-/-}$ mice suggest that IL-1β exerts its effects in both an autocrine and paracrine manner.

Interestingly, our results show a low level of IL-1β production even in the absence of caspase-1/11 in infected mice. This small production could be due to the involvement of alternative caspases such as caspase-8 (Maelfait et al., 2008) and confirms studies showing that infected mice can still produce low levels of IL-1β even in the absence of caspase-1/11 (Mayer-Barber et al., 2010).

Our experiments using knock-out mice were repeated using THP-1 cells to extrapolate our findings from murine to human cells. Our results agree with previous studies showing no difference between murine and human macrophages in their response to inflammasome activation (Fleetwood et al., 2017; Smalley et al., 2016; Nomura et al., 2015; Pazar et al., 2011). Furthermore, we show that IL-1β treatment led to a decrease in *P. gingivalis* survival in either murine or human macrophages (BMDM or THP-1 cells).

In summary, in the present study we show that the activation of P2X7 receptor, the inflammasome and the IL-1 receptor play a large role in the immune response against *P. gingivalis* infection, including leukocyte recruitment to the site of infection and P2X7 receptor-dependent IL-1β cytokine secretion, and that this cytokine plays a critical role in elimination of bacterial infection *in vitro* and *in vivo*. Our results also suggest that P2X7 and the IL-1 receptor should be considered as potential therapeutic targets for controlling *P. gingivalis* infection.

4. Material and methods

4.1. Mice

Male C57BL/6, P2X7 receptor deficient mice (P2X7$^{-/-}$ mice, originally from Jackson Laboratory, Bar harbor, MN, USA), Caspase-1/11 deficient mice (Casp-1/11$^{-/-}$) and IL-1 receptor 1 deficient mice (IL-1R$^{-/-}$ mice), were bred and maintained at the Animal Facilities of the Federal University of Rio de Janeiro. This study was approved by and all methods were performed in accordance with guidelines and regulations of the Ethics Committee of the Biophysics Institute Carlos Chagas Filho (CEUA- UFRJ, n° IBCCF154 and 076/15).

4.2. Bacteria

P. gingivalis (strain ATCC 33277®) was grown anaerobically at 37 °C for approximately 7 days in brain and heart infusion (BHI - Sigma-Aldrich) agar-plates supplemented with defibrinated blood (5%; Biocampo, SP, Brazil), yeast extract (0.5%; Sigma-Aldrich), L-cysteine (0.5 mg/ml; Sigma-Aldrich), menadione (1 μg/ml; Sigma-Aldrich) and hemin (10 μg/ml; Sigma-Aldrich) as previously described (Morandini et al., 2014a; Ramos-Junior et al., 2015). For experiments, plated-bacteria were inoculated in supplemented BHI broth for 48 h and grown

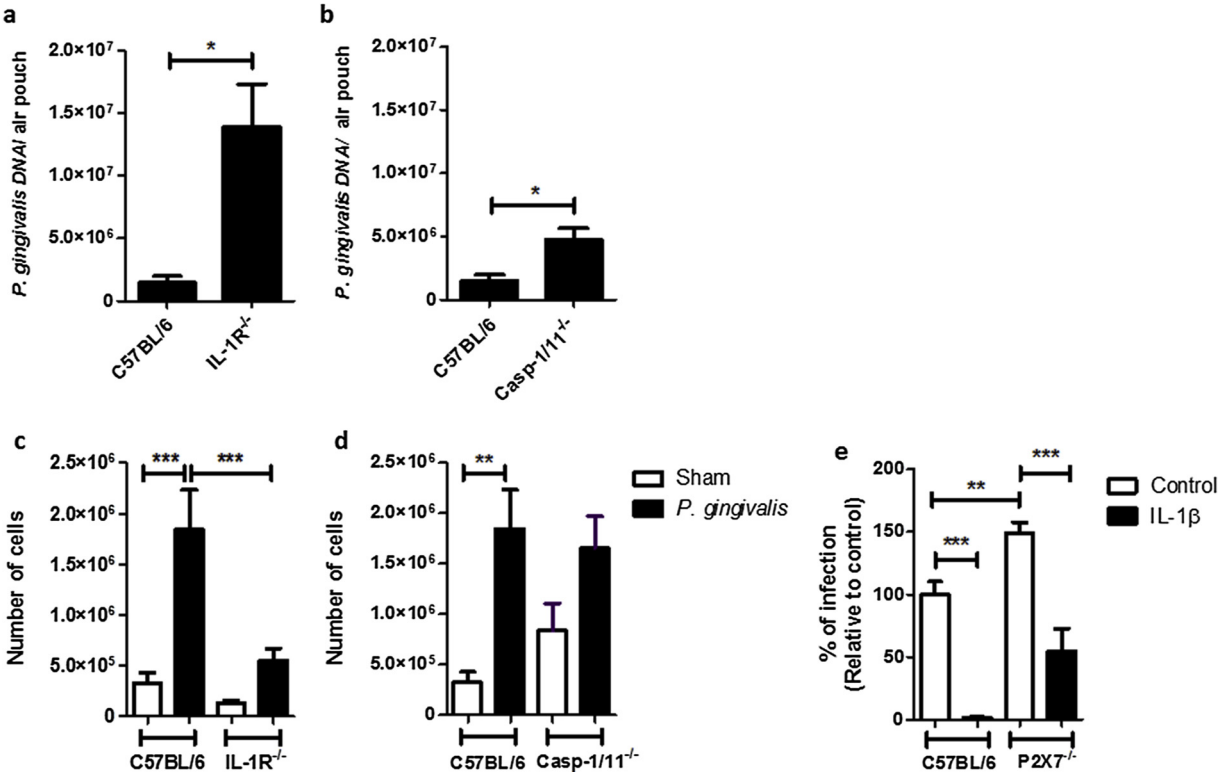

Fig. 5. IL-1 receptor signaling and caspase-1/11 eliminates *P. gingivalis* infection *in vivo*. C57BL/6, IL-1R$^{-/-}$ **(a and c)**, Caspase-1/11$^{-/-}$ **(b and d)** and P2X7$^{-/-}$ **(e)** mice were infected with *P. gingivalis,* treated with or without 500 ng/kg of recombinant IL-1β after 1 h of infection and the bacterial load and number of recruited cells were analyzed after 4 h of infection, as indicated. **(a and b)** Exudates were collected from infected mice for bacterial quantitation. The graph represents the mean ± SEM of the amount of *P. gingivalis* DNA detected from the total DNA of 10^6 cells collected from infected mice; n = 3 mice per group. **(c and d)** Infected mice had their exudates collected and the total cells quantified. The graph represents the mean ± SEM of the absolute total cell numbers recovered per mouse. Data pooled from two independent experiments, n = 5–6 mice per group. **(e)** Graph represents mean ± SEM of the bacterial load normalized in percentage, compared with the untreated control group. n = 3–10 mice per group. ***p < 0.001, **p < 0.01, *p < 0.05.

anaerobically (Ramos-Junior et al., 2015). Bacteria were collected at the log phase and quantified by absorbance in 550 nm.

4.3. Air pouch model

The air pouch protocol was performed as previously described (Elliott et al., 2009; Kadl et al., 2009; Herrera et al., 2015) to analyze cell migration and bacterial load after *P. gingivalis* infection. Briefly, 8–12 week-old C57BL/6, Casp-1/11$^{-/-}$, IL-1R$^{-/-}$ or P2X7$^{-/-}$mice were anesthetized with isoflurane (100%) and air pouches were raised by subcutaneous injection of 5 ml of sterile air on day 0, and a reinforcement of 3 ml of sterile air on day 3. On Day 7, each mouse had the air pouches infected with 10^9 *P. gingivalis* in 1 ml of sterile PBS. For cell recruitment and cytokine analysis, mice were sacrificed 4 h or 24 h after infection and individual air pouches were lavaged with 1 ml of sterile PBS. Total cells were quantified by light microscopy and the data was corrected as cells collected per mice. Where indicated, some volume was separated for *P. gingivalis* or cytokine quantification in duplicates.

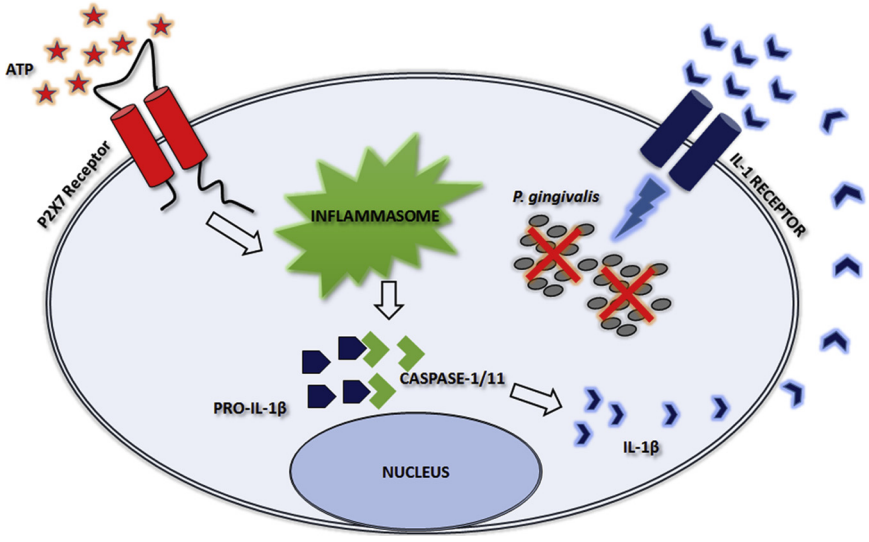

Fig. 6. Proposed mechanism for *P. gingivalis* clearance mediated by activation of the P2X7-IL-1R axis. *P. gingivalis* infection promotes pro-IL-1β production and binding of eATP to P2X7 receptor, leading to inflammasome activation, which activates caspase-1/11.This protease, in turn, cleaves pro-IL-1β into its mature form IL-1β, which can be released from infected cells and bind to the IL-1 receptor. This in turn results in bacterial clearance.

Then, the exudates were centrifuged at 200 x g, 4 °C, 3 min and the cells ressuspended in 200 µl of PBS for flow cytometry analysis.

4.4. P. gingivalis quantification in vivo

Quantification of *P. gingivalis* infection *in vivo* was as previously described (Herrera et al., 2015). In summary, C57BL/6 or P2X7$^{-/-}$ mice were infected using the air pouch model and had the air pouches treated with or without 500 ng/kg of recombinant IL-1β (R&D Systems) after 1 h of infection. Mice were sacrificed after 4 h of infection and the air pouches of each mouse was lavaged with 1 ml sterile PBS. Right after collecting the samples, 500 µl of the collected exudates were inoculated in 10 ml of BHI-supplement broth and incubated at 37 °C, anaerobically. Bacterial concentration was measured spectrophotometrically (O.D. 550 nm). For experiments with IL-1R$^{-/-}$ and Casp-1/11$^{-/-}$ mice, the *P. gingivalis* load was measured by quantifying bacterial DNA as described for the *in vitro* experiments, as previously described (Hajishengallis et al., 2011).

4.5. Cell characterization by flow cytometry

Exudates from C57BL/6 or P2X7$^{-/-}$ mice infected with *P. gingivalis* with the air pouch model were collected and the cells were immunostained for flow cytometry analysis. 5×10^5 cells were blocked using 5 µg/ml CD16/32 mAb (clone 93) for 30 min on ice, and stained with 1 µg/ml of the following monoclonal antibodies: anti-CD3 PE (clone 145-2C11, from eBioscience), anti-F4/80 Alexa Fluor® 450 (clone BM8, from eBioscience) or anti-Ly-6 G Alexa Fluor® 647 (clone 1A8, fromBiolegend). Cells were analyzed in a FACScalibur flow cytometer (BD Biosciences) by evaluating 30,000 events/ sample of viable cells identified according to size and complexity parameters. Flow cytometry data were analyzed using Summit 2.3 software and presented as percentage of positive events. Percentage data were multiplied by the total amount of cells of the corresponding sample and then divided by 100. The results were then expressed as total number of cells.

4.6. Bone-marrow-derived-macrophages and cell culture

Bone-marrow-derived-macrophages (BMDMs) were generated using L929 cell conditioned media (LCCM) as a source of macrophage colony-stimulating factor (M-CSF), as previously described (Marim et al., 2010; Morandini et al., 2014a; Almeida-da-Silva et al., 2017). Briefly, fresh bone marrow from C57BL/6 or P2X7$^{-/-}$ mice were obtained and 5×10^6 cells were incubated in 90 x 15 mm sterile culture dishes at 37 °C, 5% CO_2 in 10 ml of RPMI 20% FBS, 30% LCCM, 100 U/ml penicillin, 100 µg/ml streptomycin and 1 mM sodium pyruvate. On day 3, 10 ml of the same media were added to these cells. On day 7, cells were washed and collected with 10 ml of cold sterile PBS, centrifuged at 200xg for 5 min and resuspended in RPMI containing 10% FBS, 5% LCCM, 100 U/ml penicillin, 100 µg/ml streptomycin and 1 mM sodium pyruvate. Cells were plated at least 18 h before the experiment. At the time of the experiment, cells were washed three times with prewarmed, sterile PBS. Macrophage differentiation was previously confirmed by flow cytometry showing > 95% purity (Morandini et al., 2014a). In all experiments, BMDMs were infected with *P. gingivalis* at a multiplicity of infection (MOI) of 100 for 6 h, 18 h or 24 h, as specified in the figure legends.

4.7. ELISA

IL-1β or TNF-α levels were quantified in supernatants from 10^6 BMDMs/ml infected with *P. gingivalis* for 6 h, 18 h or 24 h (see figure legends) treated with or without 5 mM eATP (Sigma-Aldrich) during the last 30 min of infection. IL-1β and TNF-α measurement were performed according to the manufacturer's instructions (R&D Systems), in duplicates.

4.8. Western blotting

Briefly, 2×10^6 BMDMs derived from C57BL/6 or P2X7$^{-/-}$ mice were plated and infected with *P. gingivalis* for 6 h and treated with or without5 mMeATP for the last 30 min of infection. Cellular extracts and supernatants were collected for analysis. Cells were resuspended and collected in lysis buffer (Sigma-Aldrich) containing protease inhibitor (1%; Sigma-Aldrich). The protein concentration was obtained using the Bradford method (Bio-Rad Kit), and 30 µg of protein was subjected to 12% SDS-PAGE gel electrophoresis. The protein was transferred to immobilion polyvinylidene difluoride membranes (Millipore) by electroblotting (Bio-Rad), blocked in 5% dried milk solution diluted in Tris-buffered saline (TBS) containing 0.01% Tween 20, incubated with anti-IL-1β (Sigma-Aldrich) antibody at a dilution of 1:1000 (Sigma-Aldrich), and treated with a horseradish peroxidase (HRP)-conjugated secondary antibody at 1:5000 (Sigma-Aldrich). The same membranes were stripped and re-probed with antibodies against β-actin as a loading control. Supernatant proteins (300 µl of each sample) were concentrated by 10% trichloroacetic acid (TCA). Protein bands were detected using WestPico Super Signal chemiluminescent substrate (Thermo Scientific) and visualized in ImageQuant 2000 equipment (General Eletronics). Densitometric analyses were performed using ImageJ software.

4.9. P. gingivalis quantification in vitro

Antibiotic protection assay was performed as previously described (Wang et al., 2007) to quantify bacterial invasion (after 2 h of infection) or survival (after 24 h of infection). 2×10^6BMDMs derived from C57BL/6 or P2X7$^{-/-}$ were infected with *P. gingivalis* and incubated at 37 °C for 1 h. BMDMs were washed three times with pre-warmed sterile PBS and incubated at 37 °C for 1 h with 1 ml of RPMI 1% FBS, 1 mM sodium pyruvate, gentamicin (300 µg/ml; Sigma-Aldrich) and metronidazole (200 µg/ml; Fluka). To evaluate bacterial invasion in BMDMs, supernatants were discarded, and the cells were lysed with 1 ml of sterile distilled water for 20 min after 2 h of infection. Lysates were incubated in agar plates anaerobically for 10 days at 37 °C before quantification of colony forming units (CFU). To evaluate bacterial survival, 2×10^6 THP-1 cells were primed with 50 nM PMA overnight as previously described (Park et al., 2014). PMA-primed THP-1 cells were infected, treated with antibiotics and then treated with 3 mM eATP for 30 min. After 24 h of infection, PMA-primed THP-1 cells were then lysed and the protocol for quantification followed the procedure described above.

Where indicated, *P. gingivalis* DNA was detected by quantitative polymerase chain reaction (qPCR) to estimate bacterial survival after treatment with or without 100 pg/ml of recombinant IL-1β (R&D Systems). 2×10^6 BMDMs were infected for 2 h, treated with antibiotics and then treated with or without 50 nM A740003 (P2X7 receptor antagonist, Tocris) or with or without 10 µg/ml IL-1RA (human IL-1 receptor antagonist, Sigma-Aldrich) for 40 min. After this, cells were treated with or without recombinant IL-1β or 3 mM ATP for 30 min. After 24 h of infection, total DNA was extracted from infected BMDMs using TRIzol (Life Technologies) and quantified using NanoDrop (Thermo Scientific). The qPCR was performed using 10 ng of total DNA extracted per sample with SYBR-green fluorescence quantification system and the PCR cycling parameters were 95 °C (10 min) and then 40 cycles of 95 °C (30 s) and 60 °C (1 min). The specific primers for *P. gingivalis* were: *ISPg1* forward5′-CGCAGACGACAGAGAAGACA-3′, *ISPg1* reverse 5′-ACGGACAACCTGTTTTTGATAATCCT-3′ (McIntosh and Hajishengallis, 2012). *Gapdh* was used as a loading control: *Gapdh* forward, 5′-GGTCATCCCAGAGCTGAACG; *Gapdh* reverse, 5′-TTGCTGTTGAAGTCGCACGA. qPCR was performed in the Step-One Plus (Applied Biosystems, Life Technologies) using the Relative Standard-Curve method with the samples being interpolated in a *P. gingivalis* DNA curve.

4.10. Statistical analysis

For comparisons of two groups, two-tailed *t*-test was used. For multiple comparisons, one-way ANOVA with Bonferroni's post-test was performed. The statistical analysis was performed with Graph Pad Prism v. 5 software. Statistical differences were shown as asterisks, where: ***$p < 0.001$, **$p < 0.01$, *$p < 0.05$.

Conflict of interests

The authors declare there is no conflict of interests.

Contributions

CLCAS designed, performed the experiments, analyzed the results, and wrote the manuscript. ESRJ and ACM designed, performed *in vitro* experiments, discussed the data and revised the manuscript. GCR, YM, AT, KQ and LEBS performed the experiments and revised the manuscript. JS, DMO and MB analyzed the results and revised the manuscript. RCS designed the experiments, analyzed the results, and revised the manuscript.

Acknowledgements

The authors would like to thank Pryscilla Braga and Kelliane Dias da Silva for technical assistance. This work was supported by funds from Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq, Brazil— 311362/2014-1, 448152/2014-2), Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES, Brazil; Program Science Without Borders, grant no. 038/2012), and Programa de Núcleos de Excelência (PRONEX) from the Fundação de Amparo à Pesquisa do Estado do Rio de Janeiro (FAPERJ, Brazil—E-26/010.002985/2014). Cássio Luiz Coutinho Almeida-da-Silva received PhD fellowship from CNPq (141715/2014-6) and FAPERJ (200417/2016; 200092/2016) agencies.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.imbio.2018.10.008.

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