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Inflammasome gene expression is associated with immunopathology in human localized cutaneous leishmaniasis

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ABSTRACT

Localized cutaneous leishmaniasis (LCL) can ultimately progress to chronic ulcerated lesions with strong local inflammatory reactions. The functional role of certain inflammasomes in mediating inflammation caused by *Leishmania braziliensis* needs to be addressed. By combining PCR-array, quantitative real-time PCR and immunohistochemical analysis, we identified inflammasome genes, such as IL-1β, NLRP3, NLRP1, NLRC5, AIM2 and P2RX7, that were upregulated in LCL patients. Temporal gene expression studies showed that the early phase of LCL displayed increased NLRP3 and reduced AIM2 and NLRP1 expression, while the late stages showed increased AIM2 and NLRP1 and lower NLRP3 expression. Our findings also showed that AIM2, NLRP1, and P2RX7 promoted susceptibility to experimental *L. braziliensis* infection. These results highlight the importance of inflammasome machinery in human LCL and suggest that inflammasome machinery plays a role in the acute and chronic phases of the disease.

1. Introduction

American tegumentary leishmaniasis (ATL) is a major health concern in South America, particularly in Brazil, which has an annual incidence of 26,000 cases [1]. The main clinical forms of ATL are localized, diffuse and mucocutaneous, which are associated with the severity of the host immune response [2–5]. ATL, caused by *Leishmania* (*Viannia*) *braziliensis* (Vianna, 1911), is characterized by chronicity, latency and metastatic tendencies, resulting in severe mucosal destruction [6]. Localized cutaneous leishmaniasis (LCL), the most common form of ATL, develops as a single lesion at the site of a sand fly bite, which ultimately progresses to ulcerated lesions [7]. Typically, an LCL lesion exhibits a strong cellular response with IFN-γ, TNF-α, IL-17 and IL-10 production [8,9], as well as chemokines such as CCL11, CXCL9 and CXCL10 [10]. A recent genetic profiling study on LCL lesions proposed a hypothetical pathway leading to IL-1β production and lesion development [11]. The IL-1β production by murine monocytes was the result of inflammasome activation during infection [12–14]. Inflammasomes are multimeric complexes of proteins composed of a sensor, an adaptor and an inflammatory caspase that upon assembly facilitate the induction of inflammatory cytokines, such as IL-1β and IL-18 [15]. Both IL-1β and the inflammasome-related genes AIM2, NLRP3, CASP5 and CASP1 have been shown to be highly expressed at the mRNA level in localized lesions of *L. braziliensis* patients [11,16]. IL-1β was associated with disease severity in *Leishmania* (*Leishmania*) *mexicana*-infected patients [17], and the NLRP3 inflammasome was shown to be indispensable for the high levels of IL-1β present within lesions of LCL patients [18]. However, other inflammasome platforms could also participate in IL-1β production and disease severity in *L. braziliensis*-infected patients. In this study, we aimed to identify key inflammasome genes in localized lesions of *L. braziliensis* infected patients and evaluate the relationship between these genes and clinical characteristics of LCL,

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such as the duration of disease. Furthermore, we also aimed to investigate the role of such genes in functional assays using a murine model of *L. braziliensis* infection. Our results suggest the involvement of the inflammasome machinery in the host pathology in LCL, which could be crucial for understanding the uncontrolled tissue destruction and granuloma formation prevalent at the site of infection.

2. Methods

2.1. Ethics statement

All healthy subjects and patients were included after signing an informed consent form. The present project was in line with the Declaration of Helsinki 1964, as revised in 2013. The study was approved by the Ethics Committee of the Faculty of Medicine – Universidade de Brasília (University of Brasilia), Brazil (35611714.7.1001.5558) and by the Ethics Committee of the University Hospital of the Ribeirão Preto Medical School, University of São Paulo, Brazil (35611714.7.2001.5440).

2.2. Patients with LCL and uninfected controls

Skin biopsies were collected from LCL patients and from consenting healthy control subjects who had previously undergone plastic surgery (diagnostic biopsy and reparative surgery, respectively). The group of LCL patients consisted of 21 individuals with active LCL (Supplementary Table S1), all of whom exhibited clinical and laboratory features of the disease. These LCL patients were admitted either to the University Hospital of the Ribeirão Preto Medical School, University of São Paulo, Brazil, or to the University Hospital of Brasília, Brasília, Brazil. Clinical diagnosis of LCL was made in patients with a typical LCL lesion, and it was confirmed by a positive PCR for *Leishmania* spp. In addition, PCR followed by restriction enzyme analysis confirmed the presence of *L. Viannia* as described elsewhere [6]. A third step, consisting of a Taqman® based reaction, was performed using a hydrolysis probe targeting a specific sequence of the kDNA from *Leishmania Viannia braziliensis*, 5′-TGCTATAAAATCGTACCACCCGACA-3′; 5′ GAA CGGGGTTTCTGTATGCCATTT-3′ and FAM TTGCAGAACGCCCCTACC CAGAGGC-TAMRA [19,20]. The group of healthy subjects consisted of 6 healthy subjects who tested negative for the Montenegro skin test (MST).

2.3. Animals and parasites

For experimental infection studies, 5–8 female mice (6–8 weeks old) were used for each group. Wild type (C57BL/6), *Nlrp1*$^{-/-}$, *P2rx7*$^{-/-}$, *Aim2*$^{-/-}$ and *IL-1R*$^{-/-}$ mice were obtained from the Isogenic Breeding Unit at Ribeirão Preto Medical School, University of São Paulo, Ribeirão Preto, Brazil. The mice were bred and maintained under specific pathogen-free conditions and provided with clean food and water ad libitum in the animal housing facility of the Department of Biochemistry and Immunology, University of São Paulo, Brazil, according to the recommendations of the Commission on Ethics in Animal Experiments (protocol 046/2012).

The *L. braziliensis* strain WHO-MHOM/BR/75/M2903 used for infection studies was maintained *in vitro* in Schneider's insect medium (Sigma-Aldrich, St. Louis, Missouri) with 20% heat-inactivated fetal bovine serum, 5% penicillin/streptomycin (Sigma-Aldrich) and 2% male human urine at 25 °C. Experiments were performed with promastigotes in the stationary phase.

2.4. Animal infection

Wild-type C57BL/6, *Nlrp1*$^{-/-}$, *P2rx7*$^{-/-}$, *Aim2*$^{-/-}$ and *IL-1R*$^{-/-}$ mice were intradermally injected into their left ear with 10^6 stationary phase *L. braziliensis* promastigotes in a 10 µl volume adjusted with PBS. The ear lesions were measured weekly with a dial gauge caliper and compared to the thickness of the uninfected contralateral ear. The parasite burdens were determined at 5 weeks post infection in the ear as described previously [21].

2.5. RNA isolation and real-time polymerase chain reaction (RT-PCR)

RNA obtained from all the patient biopsies and THP1 cells was extracted using Trizol reagent (Invitrogen) and SV Total RNA Isolation System Kit (Promega, Fitchburg, Wisconsin) according to the manufacturer's instructions. Complementary DNA was synthesized using 500 ng of RNA through a reverse transcription reaction using the SuperScript III kit (Invitrogen, Carlsbad, California). RT-PCR was performed in SYBR Green MasterMix-based reactions in 96-well plates using StepOnePlus Real-Time PCR systems (Applied Biosystems, Warrington, United Kingdom), and the expression of each gene was normalized to GAPDH or B2M expression in the same samples. The relative expression of each gene in samples from patients with LCL was calculated in relation to that observed in healthy skin samples. Gene expression was calculated using the equation $2^{-\Delta\Delta Ct}$ as shown elsewhere [8]. The primers used in this study are shown in Supplementary Table S2.

2.6. Inflammasome PCR array

Skin biopsies from four healthy controls and four LCL patients were randomly selected, and the total RNA was purified using an RNeasy microarray mini kit (Qiagen, Germany) according to the manufacturer's instructions. The RT2 first-strand kit was used to convert the RNA into cDNA (Qiagen, Germany). SYBR Green qPCR Master mix and a 96-well plate precoated with preamplified gene-specific cDNA targets were used, both of which were provided by the manufacturer (SABiosciences). A StepOnePlus PCR system was used for the RT-PCR array. The data were uploaded onto the manufacturer's website (Qiagen, Germany) for analysis.

2.7. Immunohistochemistry (IHC)

Biopsy specimens from lesion borders of patients with LCL were frozen in optimal-cutting-temperature compound (Sakura Finetek, Torrance, CA), sectioned (5 µm) in Cryostat HM 525 (Microm-Zeiss, Germany) and fixed in ice-cold acetone before incubation with anti-human IL-1β (1:100), IL-18 (1:100), Caspase 1 (1:50), Caspase 5 (1:100), ASC (1:100) and NLRP3 (1:100) MEFV (1:100) and AIM2 (1:100) antibodies (Abcam, Cambridge, MA; Santa Cruz Biotechnologies, Santa Cruz, CA). The HRP-polymerization biotin-free method (Mach1 Detection Kit, Biocare Medical, USA), followed by incubation with Betazoid DAB (Biocare Medical, USA), was used for detecting cytokine-producing cells. The slides were counterstained with Harris's Hematoxylin (Millipore, USA), dried, and mounted with Permout (Millipore, USA). Analyses were performed by two independent observers to avoid intraobserver bias. Photomicrographs (x40) were obtained in the AxioVision Imaging System by light microscope A1 (Zeiss, Germany).

2.8. Statistical analyses

Statistical analyses were performed using GraphPad Prism 5.0 software (GraphPad Software, San Diego, CA). The tests used were as follows: 2-tailed nonparametric Mann–Whitney test and 2-tailed Wilcoxon matched-pairs signed rank test. For the mouse studies, one-way or two-way ANOVA was used. For each analysis, $p < 0.05$ was considered statistically significant.

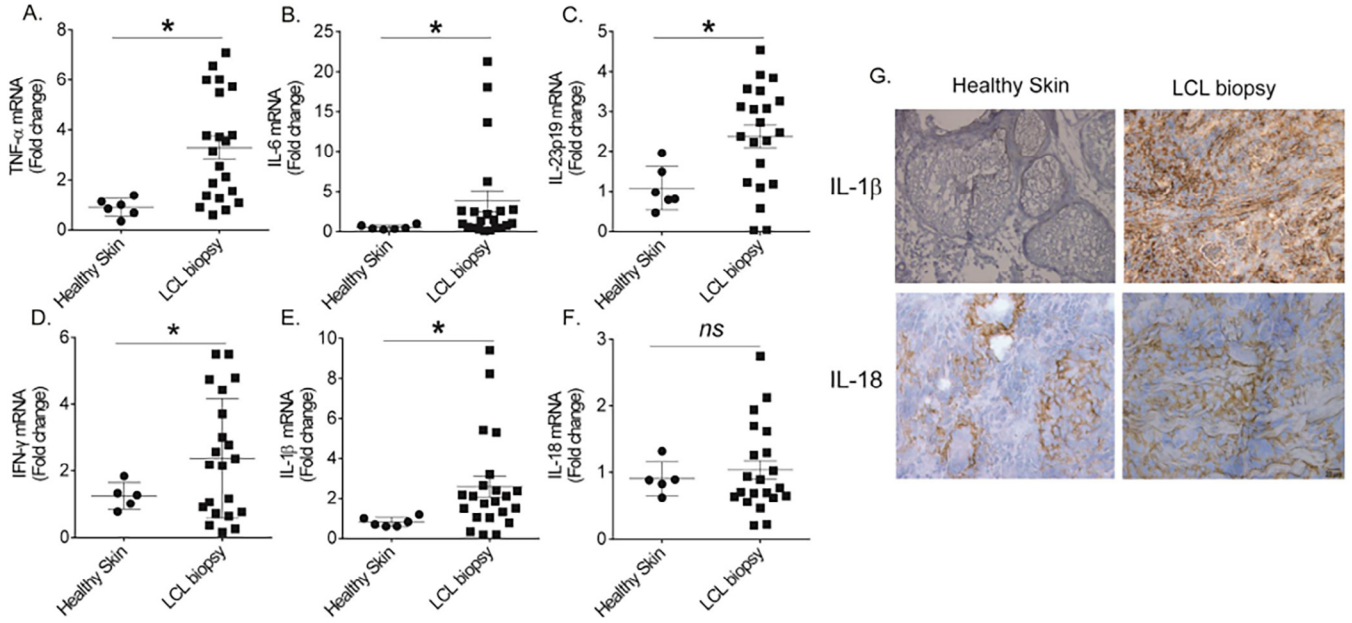


Fig. 1. Inflammatory nature of LCL biopsies. RT-PCR for inflammatory cytokines was performed from RNA obtained from the biopsies of LCL patients (n = 21) and healthy controls (n = 6). Bar graphs indicate the fold change increase or decrease in mRNA expression of TNF-α, IL-6, IL-23p19, IFN-γ, IL-1β and IL-18 ($^{*}P < 0.05$) compared to healthy controls (A-F). Immunohistochemical staining was performed for IL-1β (G) and IL-18 (H) on LCL patients (n = 6) and healthy skin (n = 6) biopsies as described in the Materials and Methods. Pictures shown are from a single LCL patient and healthy skin control, and they are representative of 6 LCL patients and 6 healthy skin controls. Original magnification 400X (G).

3. Results

3.1. Profile of inflammatory response at the site of infection

First, we found that mRNA expression of the inflammatory cytokines TNF-α, IL-6, IL-23p19, IFN-γ, and IL-1β at the site of infection of *L. braziliensis*-infected patients (n = 21) and healthy subjects (n = 6) was significantly increased (Fig. 1A-E). However, IL-18 showed comparable basal mRNA expression levels in both infected and healthy skin (Fig. 1F). Importantly, the immunohistochemical studies showed a strong positive reaction for IL-1β in the dermis of infected skin lesions, confirming the presence of high levels of IL-1β, which was apparently absent in healthy skin (Fig. 1G). Furthermore, immunohistochemical staining showed a moderate reaction for IL-18 in the dermis of both infected lesions and healthy skin. Taken together, these results suggest that inflammatory cytokines, including IL-1β, were increased at the site of infection in LCL patients.

3.2. Array-based profiling of inflammasome-specific genes

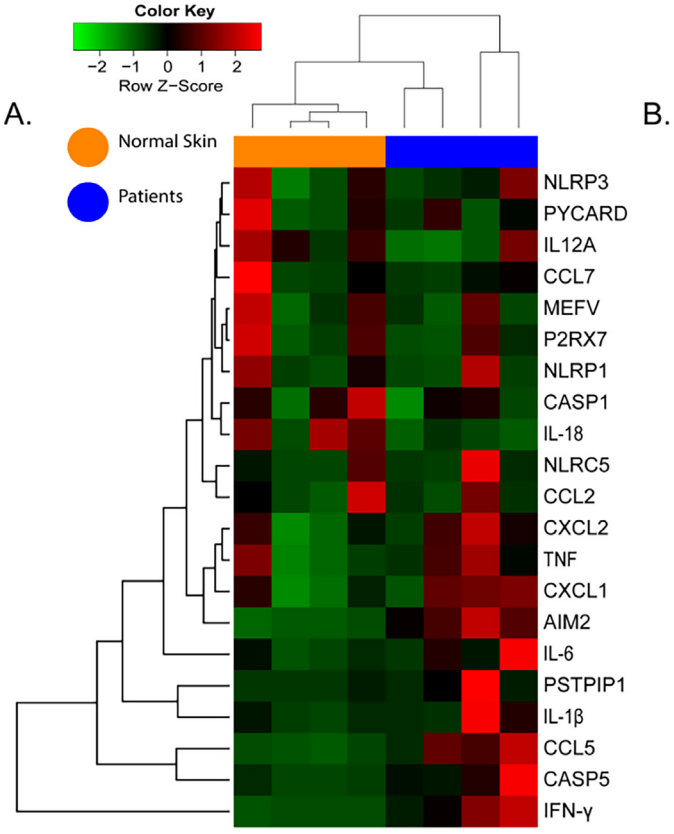
As IL-1β is released via the inflammasome activation pathway, we performed an inflammasome-specific PCR array in 4 controls and 4 LCL patients to identify key genes that regulate this pathway. PCR array analysis showed increased expression of inflammasome genes such as AIM2, MEFV, PSTPIP1, P2RX7, NLRP3, NLRC5, NLRP1, PYCARD (ASC), CASP5 and CASP1 in LCL patients at the site of infection (Fig. 2A). Inflammatory cytokine genes such as IL-6 (35.94-fold change) and IFN-y (33.84-fold change) were also expressed in high levels in LCL lesions. Among the inflammasome genes that showed a 4-fold or higher change in gene expression were AIM2 (27-fold change), CASP5 (15.89-fold change), MEFV (9.43-fold change), PSTPIP1 (9.85-fold change), NLRP1 (4.75-fold change) and NLRP3 (4.08-fold change) (Fig. 2B). These data clearly indicate the upregulation of inflammasome genes in the lesion site of LCL patients.

3.3. Expression of inflammasome-specific genes at the site of infection

We next performed RT-PCR analysis to validate our earlier array results. We observed elevated levels of the inflammasome sensors NLRC5 ($p < 0.0001$), NLRP1 ($p = 0.008$), AIM2 ($p = 0.0007$) and P2RX7 ($p = 0.0098$) in the lesions from *L. braziliensis*-infected patients (Fig. 3B-C and H-I). We also observed high expression of the inflammasome adaptor molecule ASC ($p = 0.0095$), an ASC-associated PYRIN, MEFV ($p = 0.0094$) and a PYRIN-interacting adaptor protein, PSTPIP1 ($p = 0.0002$), in lesions from *L. braziliensis*-infected patients (Fig. 3A, J and G). There were also increased mRNA levels of caspase-1 ($p = 0.0667$) and caspase-5 ($p < 0.0001$) in infected patients compared to healthy controls (Fig. 3E and F). Among all the inflammasome genes, AIM2 appeared to have the highest expression (8000-fold increase) in LCL lesions compared to healthy skin. We also evaluated the important mediators of the inflammasome signaling pathway at the protein level by immunohistochemistry and observed a strong reaction for caspase 1, NLRP3, ASC, AIM2, caspase 5 and MEFV in the dermis of the patient skin biopsy compared to the dermis of healthy skin (Fig. 3K). Thus, these results indicate the upregulation of the inflammasome machinery and possibly its activation at the site of infection.

3.4. Increased expression of inflammasome-specific genes in late lesions of LCL patients

For didactic purposes and to better demonstrate the clinical relevance of these inflammasome genes in disease progression, we divided the LCL patients into the early (0–40 days) and late (60–3600 days) phases of infection based on a bimodal distribution of illness duration in the present population. We observed increased expression of AIM2 ($p = 0.0147$), caspase-5 ($p = 0.0031$), caspase-1 ($p = 0.0031$), ASC ($p = 0.0185$), NLRC5 ($p = 0.0021$), NLRP1 ($p = 0.0349$), P2RX7 ($p = 0.0082$), MEFV ($p = 0.0057$) and PSTPIP1 ($p = 0.0095$) in the late phase of infection (Fig. 4). Although there was a slight increase in the expression of IL-1β in patients with late lesions compared to patients with early lesions, it was not significant



Genes	Fold Change
IL-6	35.94
IFN-γ	33.84
AIM2	27
CASP5	15.89
CXCL1	15.85
CCL5	14.55
PSTPIP1	9.85
MEFV	9.43
IL-1β	9.1
CCL2	7.37
TNF	6.81
CCL7	6.5
NLRP1	4.75
CXCL2	4.1
NLRP3	4.08
NLRC5	3.98
P2RX7	3.42
PYCARD	1.99
CASP1	1.98
IL-12A	1.52
IL-18	0.62

Fig. 2. Inflammasome-specific gene profiling of LCL lesions. Gene expression profile of punch biopsies from infected patients (n = 4) and healthy controls (n = 4). Heatmap represents the relative fold changes of inflammasome-specific genes normalized to Ribosomal Protein large, P0 (RPLP0). The fold change value is referring to the averages of all 4 patients compared to healthy controls (A). The table shows the inflammasome/inflammation-specific genes and their corresponding fold change based on Fig. 2A (B).

(p = 0.7718). Similarly, NLRP3 expression was also not significant (p = 0.4331). Thus, these data suggest that IL-1β and the NLRP3 inflammasome can be active in both the early and late stages of the disease, whereas AIM2, NLRP1, NLRC5 and other components of the inflammasome machinery were active mainly in the chronic phase of the infection.

3.5. AIM2, P2RX7 and NLRP1 mediate susceptibility to experimental *L. braziliensis* infection

After identifying inflammasome genes involved in human LCL, we evaluated their functional role in disease pathogenesis in a murine model of *L. braziliensis* infection. Therefore, to determine whether AIM2, P2RX7, NLRP1 and IL-1R are associated with parasite control in *L. braziliensis* infection, we challenged WT, *Aim2*$^{-/-}$, *P2rx7*$^{-/-}$, *Nlrp1*$^{-/-}$ and *Il-1R*$^{-/-}$ mice with *L. braziliensis* and assessed the parasite burden after 5 weeks post infection (wpi). We observed that *Aim2*$^{-/-}$, *Nlrp1*$^{-/-}$, *Il-1R*$^{-/-}$ and *P2rx7*$^{-/-}$ mice exhibited smaller lesions from 3 to 5 wpi compared to those of WT mice (Fig. 5A and B), along with a decrease in the number of parasites at the site of infection (Fig. 5C). AIM2, the most highly expressed gene in human LCL biopsies, seems to positively regulate disease pathogenesis in experimental *L. braziliensis* infection, as observed by the protective response and effective control of parasites. NLRP1 inflammasome- and IL-1R-deficient mice also displayed small lesions along with a significant reduction in parasite load compared to WT mice (Fig. 5A-C). Purinergic receptor, P2RX7 knockout mice showed a very small lesion and were able to mediate efficient clearance of parasites. Therefore, these data indicate that these inflammasome-related molecules in mice mediate susceptibility to *L. braziliensis* infection.

4. Discussion

Here, we describe the involvement of inflammasome platforms in *L. braziliensis*-induced lesions. In contrast to IL-18, we showed that IL-1β is highly expressed both at the protein and mRNA levels in LCL biopsies. However, previous functional studies with IL-18$^{-/-}$ mice in a C57BL/6 background showed that IL-18 is critical in mediating susceptibility to *L. amazonensis* [22]. Similarly, IL-18 promotes *L. mexicana* infection in BALB/c mice via a Th2-mediated immune response [23]. Likewise, studies with *L. major* demonstrated that IL-18-deficient mice in a BALB/c background were more resistant to infection than WT mice, whereas DBA/1 IL-18$^{-/-}$ mice were markedly more susceptible than their WT littermates [24]. Therefore, although the role of IL-18 is dependent on the mouse background, it appears to be unimportant in human lesions. In a clinical setting, we showed increased expression of key inflammasome machinery, such as AIM2, NLRP3, ASC, CASP1, CASP5, PSTPIP1, MEFV, NLRP1, NLRC5 and P2RX7, in human LCL biopsies. These results suggest that there was possible recruitment of multiple inflammasome platforms, represented by AIM2, NLRP3 and NLRP1, that might function through both caspase-1-dependent canonical and caspase-5-dependent noncanonical pathways at the site of *Leishmania* infection and could be associated with the lesion severity as evidenced by high expression of IL-1β. The majority of the studies with *Leishmania*-mediated regulation of inflammasomes has been performed in a murine model of leishmaniasis and has shown activation of the NLRP3-ASC-Caspase1 inflammasome by *Leishmania* species [13,15,25,26]. In humans, a recent study showed that cultured *L. braziliensis*-infected skin biopsies had elevated levels of IL-1β that were dependent on the NLRP3 inflammasome [18]. We also demonstrated that AIM2 was highly expressed in *L. braziliensis*-infected patients. Moreover, we observed a strong reaction for AIM2 by immunohistochemistry, confirming that it was also expressed at the protein level in infected LCL patients. Classically, AIM2 is induced by cytosolic DNA [27] and by inflammatory cytokines, such as IFN-γ [28]. Upon activation, AIM2 interacts with ASC and Caspase1, triggering the catalytic cleavage of pro-IL-1β to active IL-1β [29]. Moreover, *L. braziliensis* infection in human THP-1 macrophages triggers significant mRNA expression of AIM2 (see

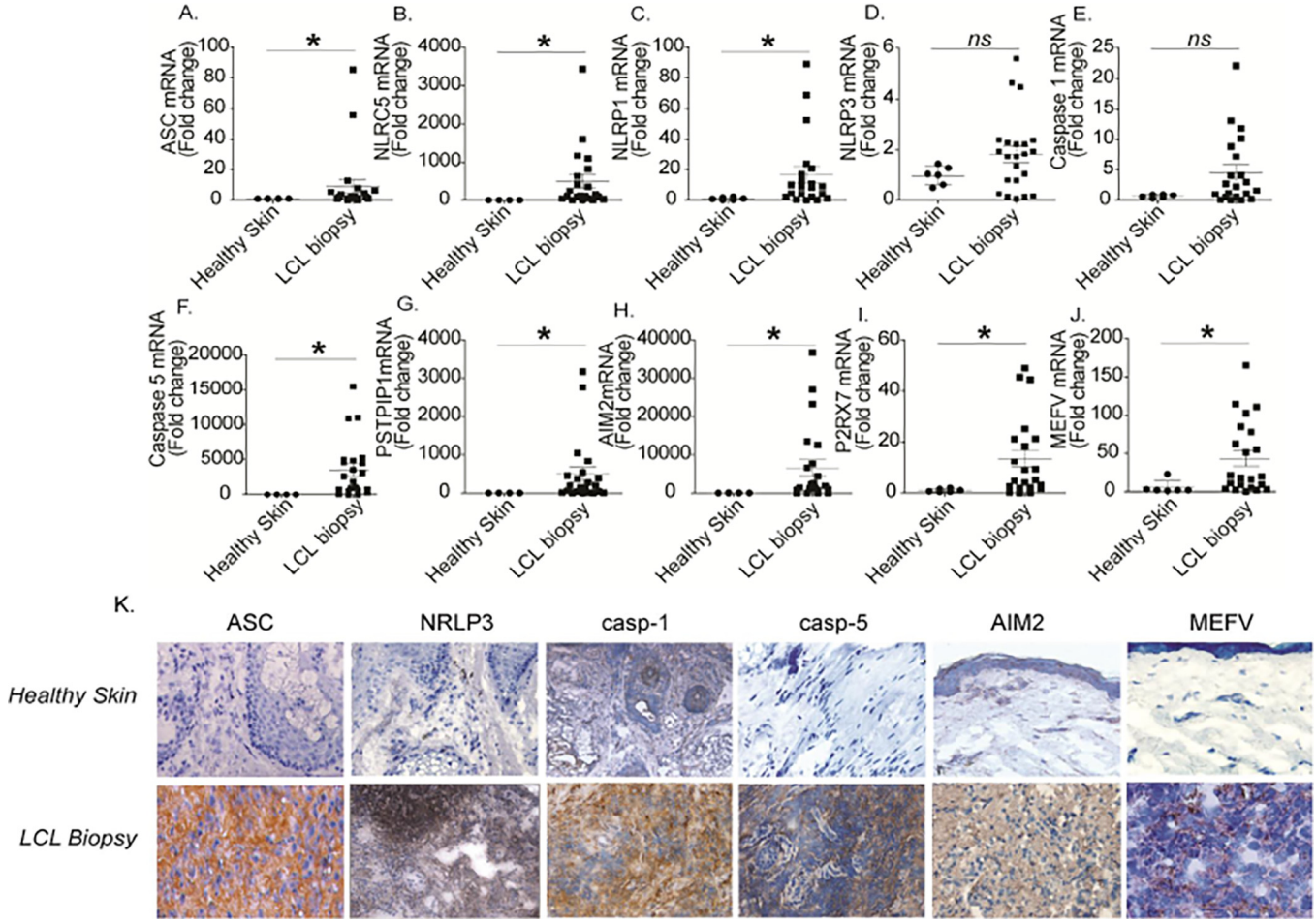


Fig. 3. *Leishmania braziliensis* infection upregulates inflammasome-specific genes in infected patients. RT-PCR for inflammasome-specific genes was performed from RNA obtained from biopsies of *L. braziliensis* infected LCL patients (n = 21) and healthy controls (n = 6). Bar graphs indicate the fold change increase or decrease in mRNA expression of ASC, NLRC5, NLRP1, NLRP3, Caspase 1, Caspase 5, PSTPIP1, AIM2, P2RX7 and MEFV ($^{*}P < 0.05$) compared to healthy controls (A-J). Immunohistochemical staining was performed for ASC, NLRP3, Caspase 1, Caspase 5, AIM2 and MEFV on LCL patients and healthy skin biopsies. The images are from a single LCL patient and healthy skin control, and they are representative of 6 LCL patients and 6 healthy skin. Original magnification 400X (K).

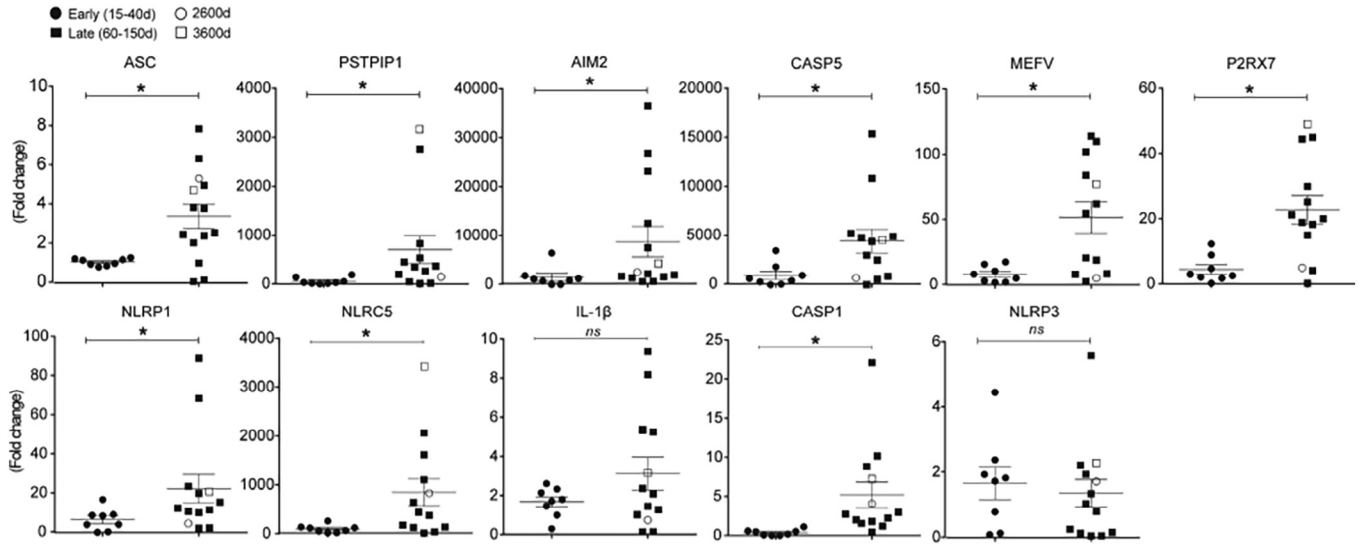


Fig. 4. Early and late expression of inflammasome-specific genes in active LCL patients. The biopsies used were obtained from LCL patients who were at 15–3600 days of symptoms (Supplementary Table S1). The LCL patients were divided into 2 groups: Black circles represent early (15–40 days) and black squares late (60–150 days) lesions. Empty circles represent 2600 days and empty squares 3600 days of disease. The graph indicates the fold change expression of the inflammasome-specific genes AIM2, Caspase 5, Caspase1, ASC, NLRC5, NLRP1, P2RX7, MEFV, PSTPIP1, IL-1β, NLRP3 in early and late lesions ($^{*}p < 0.05$) compared to healthy controls.

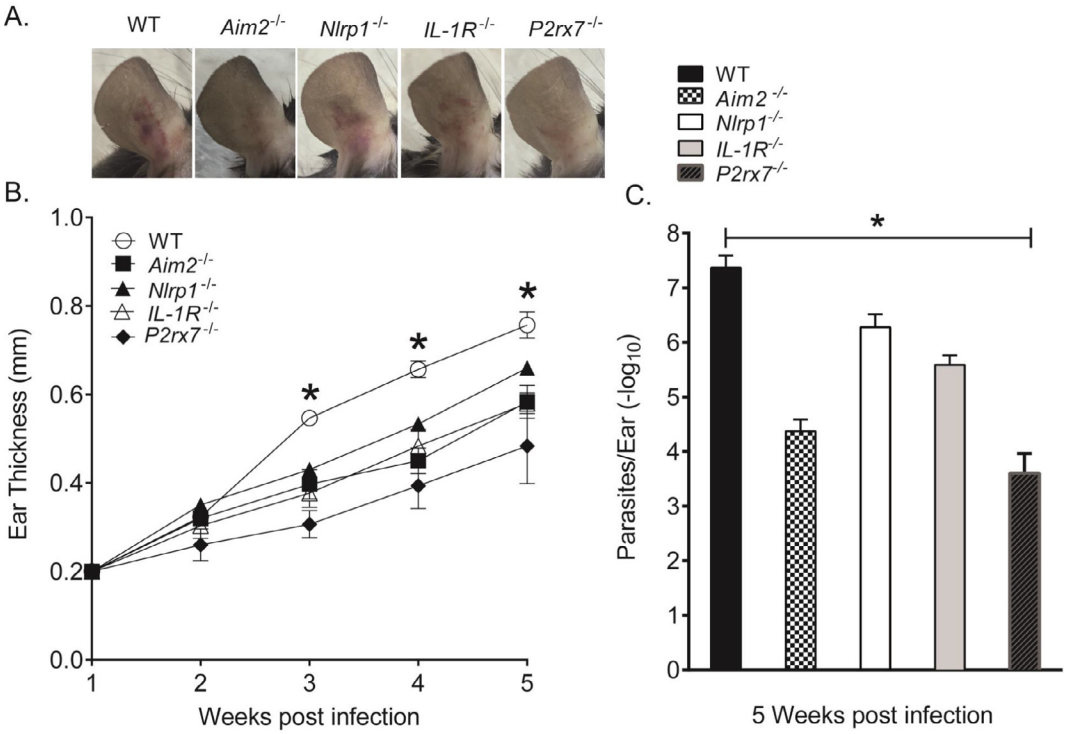


Fig. 5. AIM2, NLRP1, P2RX7 AND IL-1R confers susceptibility in experimental *L. braziliensis* infection. WT, *Aim2*$^{-/-}$, *P2rx7*$^{-/-}$, *Nlrp1*$^{-/-}$ and *IL-R*$^{-/-}$ mice were infected with 10^6 stationary phase promastigotes in left ear dermis. The course of lesion development was monitored for 5 weeks (A, B) and the parasite load was determined by a limiting dilution assay in the ears after 5 week post infection and was expressed as the mean values ± SDs (C). The results are representative of 3 independent experiments. ($^*p < 0.05$) compared to WT controls.

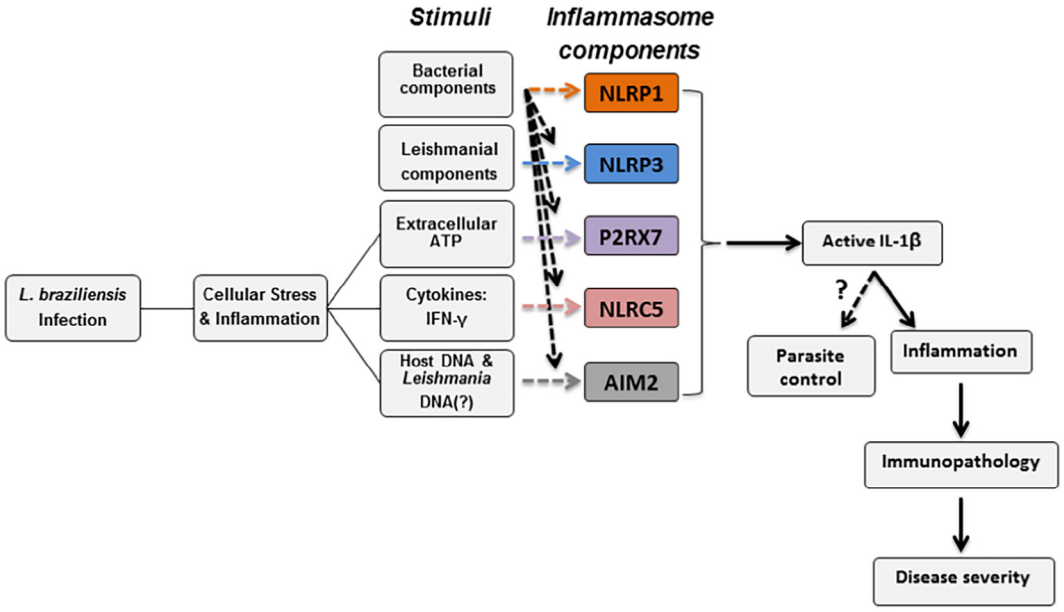


Fig. 6. A Inflammasome mediated regulation of immunopathology during CL. During *L. braziliensis* infection there are multiple parasite and host derived factors such as cytokines (IFN-γ), extracellular ATP, host and parasite components (DNA, cell membrane components and others) that are able to activate multiple inflammasome platforms such as NLRP1, NLRP3, NLRC5 and AIM2, resulting in the release of active IL-1β. As showed before, IL-1 mediates inflammation [18,25] and favors the parasite growth [25], ultimately resulting in immunopathology and disease severity.

Supplementary Fig. 1). However, these THP-1 macrophages might be predisposed to a specific inflammasome pathway due to the use of PMA for their differentiation. Recently, it was shown that AIM2 expression is related to disease severity [30] and is highly expressed in mucocutaneous lesions and among LCL patients who failed to respond to pentavalent antimonials. Our findings with human LCL patients also suggested that the elevated levels of AIM2 and inflammatory cytokines, such as IFN-γ, IL-6 and IL-1β, might play a pivotal role in regulating disease pathogenesis. AIM2 has a host protective role in the control of intracellular pathogens such as *Listeria, Aspergillus* and *Fransicella* [31–34]. In contrast, we observed that AIM2 mediates susceptibility in *L. braziliensis* infection, as AIM2-deficient mice showed reduced lesion size and parasite load compared to WT mice at 5 wpi. P2RX7, a plasma membrane receptor for extracellular ATP [35], also participates in ATP-

induced loss of intracellular K(+), which drives NLRP3 inflammasome activation and IL-1β secretion [36]. In this regard, a previous finding showed that *P2rx7*$^{-/-}$ mice were more susceptible to *L. amazonensis* infection [37]. Our results showed that *P2rx7*$^{-/-}$ mice had smaller lesion sizes and a reduction in parasite load at the site of infection. We also showed that *Nlrp1*$^{-/-}$ mice were resistant to *L. braziliensis* infection at 5 wpi compared to WT mice. The NLRP1 inflammasome contributes to the host-protective innate immune response to the parasite [38]. The absence of NLRP1 in pyroptosis-sensitive macrophages resulted in the enhanced replication of the parasite *Toxoplasma gondii* and protected them from cell death [38]. The expression of NLRP1 and AIM2 in human biopsies could play a role in controlling the immune response to avoid the progression of the lesions. Our findings suggested that *IL-1R*-deficient mice exhibits increased resistance to *L. braziliensis* infection and decreased edema at the inoculation site. In fact, the activation of inflammasomes can lead to severe pathology due to IL-1β production [18], which in turn promotes neutrophil recruitment, resulting in extensive tissue damage [25,39]. Inflammasome deficient mice such as Nlrp3, ASC, and caspase-1/11, or those lacking IL-1β or IL-1 receptor signaling components, displayed a reduction in the early influx of neutrophils, developed healing lesions and cleared the parasites from the lesion site [25]. Moreover, increased resistance of *IL-1R*$^{-/-}$ mice was also associated with an increased Th1 response along with down-regulation of IL-4, suggesting an indirect role of IL-1 signaling in the lesions development and parasite control [25]. According to our data and others previous published results, we proposed a working model to explain the involvement of inflammasomes in the disease process over time (Fig. 6). The involvement of nitric oxide (NO) in this mechanism is unclear, although the inducible nitric oxide synthase expression in lesions of LCL patients was previously reported, suggesting that it could be involved in both disease pathology, as well as parasite clearance [40,41]. Another inflammasome platform, MEFV, a pyrin encoding gene, was shown to negatively regulate NLRP3-ASC-dependent IL-1β release in autoimmune diseases [42]. The increased expression of MEFV in LCL biopsies appears to be a negative feedback loop for the down-regulation of inflammatory cytokines [43,44]. Another Pyrin-associated molecule, PSTPIP1 (proline serine threonine phosphatase-interacting protein 1), which is critical for recruiting pyrin to ASC, was also found to be upregulated in LCL lesions, as observed in other inflammatory disorders [45]. In the future, in-depth analysis of MEFV and PSPTPIP1 genes in LCL patients could identify disease-specific mutations that might affect the gene function, as reported in patients with familial Mediterranean fever [44,46–48]. Of particular interest to this study is the clinical and histopathological evolution of human cutaneous leishmaniasis in relation to time. The disease in humans initiates with an erythematous reaction at the site of a vector bite after a variable incubation period. The lesion develops into a papule and then ulcerates in a period of 2 weeks to 6 months from the first sign of infection [6]. As the disease progresses, the amastigote forms tend to be scarce, generally unapparent in chronic lesions [6]. Our studies, for the first time, showed a significant increase in the expression of almost all inflammasome genes, such as NLRP1, AIM2, Caspase5, Caspase1, ASC, NLRC5, P2RX7, MEFV, and PSPTPIP1, except IL-1β and NLRP3 in late lesions compared to early lesions. IL-1β, the main effector of the inflammasome pathway, was expressedinboth early and late lesions, again in line with the assumption that IL-1β may influence the maintenance of the cellular/Th1 response in leishmaniasis. Based on the above observations, we hypothesize that NLRP3-inflammasome activation during early LCL infection is eventually overtaken by AIM2 and NLRP1 inflammasomes during the late chronic phase of infection that continue to drive the production of IL-1β, leading to tissue destruction. These findings on inflammasome-specific genes could also be exploited for diagnostic and disease prediction tools, an immediate and imperative requirement for human ATL. Furthermore, IL-1β or NLRP3 blockade using small molecule pharmacological compounds, specific receptor antagonists or monoclonal antibodies, has been shown to have therapeutic success in experimental models of cutaneous leishmaniasis [18] and in inflammatory disorders such as heart failure, diabetes, rheumatoid arthritis [49–51]. Similar therapeutic approaches could be used for rapid and sustained reduction in disease severity associated with human ATL. Thus, this study highlights the importance of inflammasomes in disease pathology and host immunity in LCL patients, which could be exploited for therapeutics against LCL.

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Conflicts of interest

None.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.cellimm.2019.04.008.

References

[1] J. Alvar, et al., Leishmaniasis worldwide and global estimates of its incidence, PLoS ONE 7 (2012) e35671, , https://doi.org/10.1371/journal.pone.0035671.
[2] J. Alvar, Leishmaniasis and AIDS co-infection: the Spanish example, Parasitol. Today 10 (1994) 160–163.
[3] A. Barral, et al., Lymphadenopathy as the first sign of human cutaneous infection by Leishmania braziliensis, Am. J. Tropical Med. Hyg. 53 (1995) 256–259.
[4] J.M. Costa, et al., Disseminated cutaneous leishmaniasis in a field clinic in Bahia, Brazil: a report of eight cases, J. Tropical Med. Hyg. 89 (1986) 319–323.
[5] J.E. Martinez, Alba, L. Arias, M.A. Escobar, N.G. Saravia, Haemoculture of Leishmania (Viannia) braziliensis from two cases of mucosal leishmaniasis: re-examination of haematogenous dissemination, Trans. R. Soc. Tropical Med. Hyg. 86 (1992) 392–394.
[6] C.M. Gomes, et al., Complementary exams in the diagnosis of American tegumentary leishmaniasis, Anais Brasileiros de Dermatologia 89 (2014) 701–709.
[7] P.D. Marsden, M.S. Tada, A.C. Barreto, C.C. Cuba, Spontaneous healing of Leishmania braziliensis braziliensis skin ulcers, Trans. R. Soc. Trop. Med. Hyg. 78 (1984) 561–562.
[8] D.L. Costa, et al., Tr-1-like CD4+CD25-CD127-/lowFOXP3- cells are the main source of interleukin 10 in patients with cutaneous leishmaniasis due to Leishmania braziliensis, J. Infect. Dis. 211 (2015) 708–718, https://doi.org/10.1093/infdis/jiu406.
[9] H. Galdino Jr.et al., Interleukin 32gamma (IL-32gamma) is highly expressed in cutaneous and mucosal lesions of American Tegumentary Leishmaniasis patients: association with tumor necrosis factor (TNF) and IL-10, BMC Infect. Dis. 14 (2014) 249, https://doi.org/10.1186/1471-2334-14-249.
[10] A.P. Campanelli, et al., Chemokines and chemokine receptors coordinate the inflammatory immune response in human cutaneous leishmaniasis, Hum. Immunol. 71 (2010) 1220–1227, https://doi.org/10.1016/j.humimm.2010.09.002.
[11] F.O. Novais, et al., Genomic profiling of human Leishmania braziliensis lesions identifies transcriptional modules associated with cutaneous immunopathology, J. Invest. Dermatol. 135 (2015) 94–101, https://doi.org/10.1038/jid.2014.305.
[12] S. Benko, D.J. Philpott, S.E. Girardin, The microbial and danger signals that activate Nod-like receptors, Cytokine 43 (2008) 368–373, https://doi.org/10.1016/j.cyto.2008.07.013.
[13] D.S. Lima-Junior, et al., Inflammasome-derived IL-1beta production induces nitric oxide-mediated resistance to Leishmania, Nat. Med. 19 (2013) 909–915, https://doi.org/10.1038/nm.3221.
[14] V. Petrilli, C. Dostert, D.A. Muruve, J. Tschopp, The inflammasome: a danger sensing complex triggering innate immunity, Curr. Opin. Immunol. 19 (2007) 615–622, https://doi.org/10.1016/j.coi.2007.09.002.
[15] D.S. Zamboni, D.S. Lima-Junior, Inflammasomes in host response to protozoan parasites, Immunol. Rev. 265 (2015) 156–171, https://doi.org/10.1111/imr.12291.
[16] C. Pirmez, et al., Cytokine patterns in the pathogenesis of human leishmaniasis, J. Clin. Investig. 91 (1993) 1390–1395, https://doi.org/10.1172/JCI116341.
[17] E.A. Fernandez-Figueroa, et al., Disease severity in patients infected with Leishmania mexicana relates to IL-1beta, PLoS Negl. Trop. Dis. 6 (2012) e1533, , https://doi.org/10.1371/journal.pntd.0001533.

[18] F.O. Novais, et al., CD8+ T cell cytotoxicity mediates pathology in the skin by inflammasome activation and IL-1beta production, PLoS Pathog. 13 (2017) e1006196, , https://doi.org/10.1371/journal.ppat.1006196.
[19] J.L. Weirather, et al., Serial quantitative PCR assay for detection, species discrimination, and quantification of Leishmania spp. in human samples, J. Clin. Microbiol. 49 (2011) 3892–3904, https://doi.org/10.1128/JCM.r00764-11.
[20] C.M. Gomes, et al., Field validation of SYBR green- and TaqMan-based real-time PCR using biopsy and swab samples to diagnose american tegumentary Leishmaniasis in an area where Leishmania (Viannia) braziliensis is endemic, J. Clin. Microbiol. 55 (2017) 526–534, https://doi.org/10.1128/JCM.01954-16.
[21] L.C. Afonso, P. Scott, Immune responses associated with susceptibility of C57BL/10 mice to Leishmania amazonensis, Infect. Immun. 61 (1993) 2952–2959.
[22] L.M. Sousa, et al., IL-18 contributes to susceptibility to Leishmania amazonensis infection by macrophage-independent mechanisms, Cytokine 74 (2015) 327–330, https://doi.org/10.1016/j.cyto.2015.01.021.
[23] K.J. Bryson, X.Q. Wei, J. Alexander, Interleukin-18 enhances a Th2 biased response and susceptibility to Leishmania mexicana in BALB/c mice, Microbes Infect. 10 (2008) 834–839, https://doi.org/10.1016/j.micinf.2008.03.009.
[24] X.Q. Wei, et al., Host genetic background determines whether IL-18 deficiency results in increased susceptibility or resistance to murine Leishmania major infection, Immunol. Lett. 94 (2004) 35–37, https://doi.org/10.1016/j.imlet.2004.04.001.
[25] M. Charmoy, et al., The Nlrp3 inflammasome, IL-1beta, and neutrophil recruitment are required for susceptibility to a nonhealing strain of Leishmania major in C57BL/6 mice, Eur. J. Immunol. 46 (2016) 897–911, https://doi.org/10.1002/eji.201546015.
[26] P. Gurung, et al., An NLRP3 inflammasome-triggered Th2-biased adaptive immune response promotes leishmaniasis, J. Clin. Investig. 125 (2015) 1329–1338, https://doi.org/10.1172/JCI79526.
[27] S.M. Man, R. Karki, T.D. Kanneganti, DNA-sensing inflammasomes: regulation of bacterial host defense and the gut microbiota, Pathogens and Disease 74 (2016) ftw028, https://doi.org/10.1093/femspd/ftw028.
[28] L. Ponomareva, et al., AIM2, an IFN-inducible cytosolic DNA sensor, in the development of benign prostate hyperplasia and prostate cancer, Mol. Cancer Res.: MCR 11 (2013) 1193–1202, https://doi.org/10.1158/1541-7786.MCR-13-0145.
[29] V. Hornung, et al., AIM2 recognizes cytosolic dsDNA and forms a caspase-1-activating inflammasome with ASC, Nature 458 (2009) 514–518, https://doi.org/10.1038/nature07725.
[30] R.B. Moreira, et al., AIM2 inflammasome is associated with disease severity in tegumentary leishmaniasis caused by Leishmania (V.) braziliensis, Parasite Immunol. 39 (2017), https://doi.org/10.1111/pim.12435.
[31] T. Fernandes-Alnemri, et al., The AIM2 inflammasome is critical for innate immunity to Francisella tularensis, Nat. Immunol. 11 (2010) 385–393, https://doi.org/10.1038/ni.1859.
[32] R. Karki, et al., Concerted activation of the AIM2 and NLRP3 inflammasomes orchestrates host protection against Aspergillus infection, Cell Host Microbe 17 (2015) 357–368, https://doi.org/10.1016/j.chom.2015.01.006.
[33] V.A. Rathinam, et al., The AIM2 inflammasome is essential for host defense against cytosolic bacteria and DNA viruses, Nat. Immunol. 11 (2010) 395–402, https://doi.org/10.1038/ni.1864.
[34] J. Wu, T. Fernandes-Alnemri, E.S. Alnemri, Involvement of the AIM2, NLRC4, and NLRP3 inflammasomes in caspase-1 activation by Listeria monocytogenes, J. Clin. Immunol. 30 (2010) 693–702, https://doi.org/10.1007/s10875-010-9425-2.
[35] F. Di Virgilio, P2RX7: a receptor with a split personality in inflammation and cancer, Mol. Cell. Oncol. 3 (2016) e1010937, , https://doi.org/10.1080/23723556.2015.1010937.
[36] M. Karmakar, M.A. Katsnelson, G.R. Dubyak, E. Pearlman, Neutrophil P2X7 receptors mediate NLRP3 inflammasome-dependent IL-1beta secretion in response to ATP, Nat. Commun. 7 (2016) 10555, https://doi.org/10.1038/ncomms10555.
[37] S.P. Chaves, et al., Modulation of P2X(7) purinergic receptor in macrophages by Leishmania amazonensis and its role in parasite elimination, Microbes Infect. 11 (2009) 842–849, https://doi.org/10.1016/j.micinf.2009.05.001.
[38] K.M. Cirelli, et al., Inflammasome sensor NLRP1 controls rat macrophage susceptibility to Toxoplasma gondii, PLoS Pathog. 10 (2014) e1003927, , https://doi.org/10.1371/journal.ppat.1003927.
[39] R. Dey, et al., Gut microbes egested during bites of infected sand flies augment severity of leishmaniasis via inflammasome-derived IL-1beta, Cell host & microbe 23 (2018), https://doi.org/10.1016/j.chom.2017.12.002.
[40] M. Qadoumi, et al., Expression of inducible nitric oxide synthase in skin lesions of patients with american cutaneous leishmaniasis, Infect. Immun. 70 (2002) 44638–44642, https://doi.org/10.1128/iai.70.8.4638–4642.
[41] G. Serarslan, E. Atik, Expression of inducible nitric oxide synthase in human cutaneous leishmaniasis, Mol. Cell Biochem. 280 (2005) 147–149, https://doi.org/10.1007/s11010-005-8542-3.
[42] H. Van Gorp, et al., Familial Mediterranean fever mutations lift the obligatory requirement for microtubules in Pyrin inflammasome activation, PNAS 113 (2016) 14384–14389, https://doi.org/10.1073/pnas.1613156113.
[43] J.J. Chae, et al., Gain-of-function Pyrin mutations induce NLRP3 protein-independent interleukin-1beta activation and severe autoinflammation in mice, Immunity 34 (2011) 755–768, https://doi.org/10.1016/j.immuni.2011.02.020.
[44] P.R. Hesker, M. Nguyen, M. Kovarova, J.P. Ting, B.H. Koller, Genetic loss of murine pyrin, the Familial Mediterranean Fever protein, increases interleukin-1beta levels, PLoS ONE 7 (2012) e51105, , https://doi.org/10.1371/journal.pone.0051105.
[45] D. Holzinger, et al., Single amino acid charge switch defines clinically distinct proline-serine-threonine phosphatase-interacting protein 1 (PSTPIP1)-associated inflammatory diseases, J. Allergy Clin. Immunol. 136 (2015) 1337–1345, https://doi.org/10.1016/j.jaci.2015.04.016.
[46] P.J. Ferguson, et al., A missense mutation in pstpip2 is associated with the murine autoinflammatory disorder chronic multifocal osteomyelitis, Bone 38 (2006) 41–47, https://doi.org/10.1016/j.bone.2005.07.009.
[47] A. Golla, et al., Chronic recurrent multifocal osteomyelitis (CRMO): evidence for a susceptibility gene located on chromosome 18q21.3-18q22, Eur. J. Human Genetics: EJHG 10 (2002) 217–221, https://doi.org/10.1038/sj.ejhg.5200789.
[48] N.G. Shoham, et al., Pyrin binds the PSTPIP1/CD2BP1 protein, defining familial Mediterranean fever and PAPA syndrome as disorders in the same pathway, PNAS 100 (2003) 13501–13506, https://doi.org/10.1073/pnas.2135380100.
[49] R.C. Coll, et al., A small-molecule inhibitor of the NLRP3 inflammasome for the treatment of inflammatory diseases, Nat. Med. 21 (2015) 248–255, https://doi.org/10.1038/nm.3806.
[50] C.A. Dinarello, J.W. van der Meer, Treating inflammation by blocking interleukin-1 in humans, Semin. Immunol. 25 (2013) 469–484, https://doi.org/10.1016/j.smim.2013.10.008.
[51] E. Latz, T.S. Xiao, A. Stutz, Activation and regulation of the inflammasomes, Nat. Rev. Immunol. 13 (2013) 397–411, https://doi.org/10.1038/nri3452.