PpSP32, Phlebotomus papatasi immunodominant salivary protein, exerts immunomodulatory effects on human monocytes, macrophages and lymphocytes

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Research Article

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Abstract

Background: The saliva of sand flies, vectors of *Leishmania* parasites, contains several components that exert pharmacological activities facilitating the acquisition of blood by the insect and contribute to the establishment of the infection. Previously, we demonstrated that PpSP32 is the immunodominant salivary antigen in humans exposed to *Phlebotomus papatasii* bites and validated its usefulness as a predictive biomarker of the disease. PpSP32, whose functions are little known to date, is an intriguing protein due to its involvement in the etiopathogenesis of pemphigus, an auto-immune disease. Herein, we aimed to better decipher its role through the screening of several immunomodulatory activities either on lymphocytes or on monocytes/macrophages.

Methods: Peripheral mononuclear cells from healthy volunteers were stimulated with anti-CD3 / anti-CD28 antibodies, phytohemagglutinin, phorbol 12-myristate13-acetate / Ionomycin or lipopolysaccharide in the presence of increasing doses of PpSP32. Cell proliferation was measured after the addition of tritiated thymidine. Monocyte activation was tested by analyzing the expression of CD86 and HLA-DR molecules by flow cytometry. Cytokine production was analyzed in culture supernatants by ELISA. THP-1 derived macrophages were stimulated with LPS in the presence of increasing doses of PpSP32 and cytokine production was analyzed in culture supernatants by ELISA and multiplex technique. The effect of PpSP32 on NF-κB signaling was tested by Western blot. The anti-inflammatory activity of PpSP32 was assessed *in vivo* in an experimental inflammatory model, the carrageenan-induced paw edema in rats.

Results: Our data showed that PpSP32 down-modulated the expression of activation markers in LPS-stimulated monocytes and THP1-derived macrophages. This protein negatively modulated the secretion of Th1 and Th2 cytokines by human lymphocytes as well as pro-inflammatory cytokines by monocytes, and THP1-derived macrophages. PpSP32 treatment led to a dose-dependent reduction of the NF-κB phosphorylation. When PpSP32 was injected into the paw of carrageenan-injected rats, edema was significantly reduced.

Conclusions: Our data indicate that PpSP32 induces a potent immunomodulatory effect on monocytes and THP-1 derived macrophages. This inhibition could be mediated, among others, by the modulation of the NF-κB signaling pathway. The anti-inflammatory activity of PpSP32 was confirmed *in vivo* in the carrageenan-induced paw edema model in rats.

Background

The constraints imposed on haematophagous insects by the haemostatic and inflammatory response of vertebrates have led insects to put in place during their evolution several strategies to evade the host response. These are mainly through the production of a diversity of salivary proteins with various functions aiming to improve the blood meal while helping to thwart the inflammatory responses of the host [1,2]. Consistently, the saliva of sand flies, the vectors of leishmaniasis, contains a repertoire of components that exert different pharmacological activities facilitating the acquisition of the blood meal,
such as potent anti-hemostatic and vasodilator compounds and immunomodulatory molecules that contribute to the establishment of the infection [2–7]. For instance, maxadilan and adenosine found in the saliva of Lutzomyia (Lu.) longipalpis and Phelobotmus (P.) papatasi, respectively act as potent vasodilators [8,9]. A number of immunomodulatory molecules have also been reported in the saliva of sand flies. Such molecules can act on different effector cells and mediators of the immune system [2,10]. They can alter the function of antigen presenting cells such as macrophages and dendritic cells [11,12] and/or affect the expression of co-stimulatory molecules on their surface [13]. They can also alter the complement system by inhibiting both the classical and alternative complement pathways [14]. Furthermore, they can modulate the adaptive immune response by disturbing the Th1/Th2 balance. Accordingly, the whole salivary gland lysate of Lu.longipalpis downregulates the production of Th1 cytokines and upregulates the production of Th2 cytokines by human peripheral blood mononuclear cells (PBMC) [15]. Hence, the study of the structure and functions of salivary proteins of different vectors has aroused the interest of several researchers over the past two decades [7,10,16,17].

Phlebotomus (P.) papatasi the vector of zoonotic cutaneous leishmaniasis, a parasitic disease caused by Leishmania major widespread in the Old World mainly in North Africa and Middle East [18]. The salivary gland transcriptome of a colonized Tunisian strain of P.papatasi showed the presence of 10 families of salivary proteins namely the family of “odorant binding proteins” (OBP/D7) proteins, the family of “yellow proteins”, the family of “antigen 5 proteins”, the apyrase, the proteins “Salivary proteins 32-like” (SP32-like) or “Silk-related proteins”, the family of “SP16 proteins”, the 2.5 kDa proteins, the family of “38.8/Aegyptin-like proteins” and the family of “Lufaxin-like” or SP34 [19]. The family of Sp32-like proteins, initially characterized in the salivary glands of P. papatasi, is similar to the silk protein of Nephila clavipes[20]. This protein family is specific to sand flies [21]. A member of this family, the salivary protein PpSP32 of P. papatasi has been shown to be the immunodominant target of the antibody response in individuals living in endemic area for cutaneous leishmaniasis [22]. PpSP32 has homologies with collagen adhesion proteins and is predicted to be a mucin-based on the pattern of its O- and N-glycosylation [21]. Taking into account such features, we hypothesized and demonstrated that PpSP32 binds to specific proteins from the skin, the desmogleins leading to the loss of tolerance and the production of related autoimmune antibodies. The latter event may be the first step of the development of an autoimmune disease, the pemphigus [23]. PpSP32 is hence an intriguing protein whose functions remain unknown to date. Herein, we aimed to better decipher its functions through the screening of several immunomodulatory activities either on lymphocytes or on monocytes/macrophages.

Methods

Samples and cell lines

Peripheral blood samples were from 15 healthy volunteers with no history of leishmaniasis or travel to sand fly endemic areas. They were 10 females and 5 males with an age range from 20 to 38 years
(median of 28 years).

THP-1 cells, a human monocytic leukemia cell line, was obtained from American type culture collection (ATCC) and was maintained in RPMI 1640/Glutamax-1 medium (Invitrogen Life Technologies) supplemented with 10% heat inactivated fetal bovine serum (Gibco), 1% Sodium pyruvate (Gibco), 2% non-essential amino acids (Sigma), 100U/ml penicillin and 100µg/ml streptomycin (Gibco) and incubated at 37°C in a humidified atmosphere consisting of 5% CO₂.

To induce monocyte-macrophage differentiation, THP-1 cells were seeded in 6-well tissue culture plates at 5x10⁵ viable cells per well and treated with 20ng/ml ofphorbol 12-myristate13-acetate (PMA) (Sigma) for 72h at 37°C, 5% CO₂. Differentiated cells were then washed three times with RPMI 1640 and placed in complete media for 24h at 37°C, 5% CO₂.

**Culture media and reagents**

PBMC were cultured in RPMI 1640 medium (Capricorn) supplemented with 10% AB human serum (Sigma), 1% sodium pyruvate 100mM (Gibco), 1% nonessential amino acids (Gibco), 1% HEPES buffer (Gibco), 0.5% of β-mercaptoethanol10⁻²M (Gibco) and 0.2% of 10 mg/ml gentamicin (Gibco).

Recombinant form of PpSP32 was produced as previously described [22]. The following monoclonal antibodies were used for flow cytometry analysis: Fluorescein isothiocyanate (FITC), Allophycocyanin (APC), and Phycoerythrin (PE) conjugated with anti- cluster of differentiation (CD)14, anti-CD86 and human leukocyte antigen receptor (HLA-DR) antibodies, respectively (BD Biosciences).

**Cell proliferation assay**

The PBMCs were isolated on a Ficoll-Hypaque gradient then cultured in 96-well plates (10⁵ cells/well) for 72 hours in a 5% CO₂ humidified atmosphere at 37°C with anti-CD3 at 2 µg/ml and anti-CD28 at 2 µg/ml/phytotemagglutinin (PHA) at 10 µg/ml or PMA / Ionomycin at 50 ng/ml and 1 µg/ml, respectively in the presence or absence of different concentrations of the recombinant protein PpSP32 (0.5 µg/ml, 2 µg/ml, 5 µg/ml). All experiments were performed in triplicates. For proliferation studies, the uptake of (3H) thymidine (Amersham) was measured 6 hours after adding 0.4 mCi/well. Cells were harvested and the radioactivity was counted in a scintillation counter (Rack Beta; LKB Wallace). Results were expressed as a ratio of mean count per minute (cpm) of antigen-stimulated cultures /mean of cpm of unstimulated cultures.

**Co-stimulatory molecule analysis**

PBMCs were plated in 24-well plates (5x10⁵ cells/ml) then stimulated or not with lipopolysaccharide (LPS) (100 ng/ml) in the presence or absence of the various concentrations of PpSP32 (0.5 µg/ml, 2 µg/ml, 5 µg/ml) for 48 h. After incubation, the cells were washed then labeled with a mixture of specific antibodies each coupled to different fluorochromes: anti-CD14 (FITC), anti-CD86
(APC), anti-HLA-DR (PE) for 20 min at 4°C. The cells were then washed and resuspended in a fixing buffer (Cell Fix, Becton Dickinson) until read by the flow cytometer (FACS Canto II, Becton Dickinson).

**Cytokine detection assays**

The PBMCs were plated in 24-well plates (5x10^5 cells/ml) then stimulated or not by LPS at 100 ng/ml or PMA/Ionomycin at 50 ng/ml and 1μg/ml, respectively (Sigma). For each condition, the recombinant form of PpSP32 was added with increasing concentrations (0.5μg/ml, 2μg/ml and 5μg/ml). The plates were placed in an incubator at 37°C and in the presence of CO₂, for 48 hours. The culture supernatants were then collected and stored at -20°C until use.

THP-1 derived monocytes were treated or not with different concentrations of PpSP32 (0.5 μg /ml, 2 μg /ml or 5 μg /ml) for 48h. Cells were then stimulated with LPS (100ng/ml) for 18h. The supernatants were then collected and stored at -20°C until use.

Enzymelinked immunosorbent assay (ELISA) was performed on supernatants of PBMCs or THP-1 derived monocytes using human interleukin(IL)-6, Interferon-gamma(IFN-γ) or Tumor necrosis factor-alpha (TNF-α) ELISA sets (BD Biosciences) and IL-1β assay (Human IL-1β Duoset ELISA, R and D systems) according to manufacturer's instructions. For each cytokine determination, the results were interpolated from a standard curve using recombinant cytokines and expressed in pg/ml.

For some experiments, cytokine multiplex analysis was performed using the human inflammation 11 Plex assay kit (TNF-α,interferon-γ–induced protein-10 (IP-10), IL-1β, IL-27, IFN-γ, IL-8, IL-12p70,monocyte chemoattractant protein-1 (MCP-1), IL-1α, IL-6, IL-10) (Aimplex, Biosciences) according to the manufacturer’s instructions. Briefly, capture antibody-conjugated beads were first incubated with supernatants or standard controls for 60 minutes, then with biotinylated detection antibodies for 30 minutes and finally with streptavidin-PE for 20 minutes. Fluorescence signals of the beads were acquired by a flow cytometer (FACS Canto II, Becton Dickinson).

**Cell viability assay**

The effect of PpSP32 on the viability of THP1-derived macrophages cells was assessed using the 1,3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2Htetrazolium bromide) (MTT) method. Differentiated THP-1 cells were seeded at a density of 5x10³ cells per well in 96 well tissue culture plates and allowed to grow overnight at 37°C under 5% CO₂. Cells were cultured for 24h or 72h in the presence or not of different concentrations of PpSP32 (0.5μg/ml, 2μg/ml and 5μg/ml). MTT (0.5mg/ml) solution was then added and cells were incubated for a further 3 h. Dimethylsulfoxide (Sigma) was then added to solubilize formazan crystals and the optical density was measured at 560 nm to quantify the percentage of living cells. All experiments were performed at least twice in triplicate.

**Western blotting**
THP-1 derived macrophages were treated or not with different concentrations of PpSP32 (0.5μg/ml, 2μg/ml and 5μg/ml) for 48h at 37°C, 5% CO₂, then stimulated with 100ng/ml of LPS for 3h at 37°C, 5% CO₂. Total cell lysates were extracted at room temperature with 100µl of Laemmli Buffer (1x) per 5x10^5 cells. Protein concentration was determined by using the Bicinchoninic Acid Protein Assay Kit, (BCA, Sigma) with bovine serum albumin (BSA) as standard. Whole cell lysates (30µg/lane) were then separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinyl difluoride membrane (PVDFAmersham). After washing, the membrane was incubated with anti-phosphol kappa B alpha (anti-pIkB-α) antibody (Cell signaling technology) at 1:2000 overnight or anti-β actin at 1:1000 (Cell Signaling Technology) for 2 hours at room temperature. After washing and incubation with Horseradish peroxidase (HRP)-conjugated secondary antibodies (anti-rabbit IgG HRP at 1:2000), immunoblots were determined by enhanced chemiluminescence.

**Carrageenan induced paw edema**

The anti-inflammatory activity on carrageenan-induced paw edema was determined according to the method described by Winter et al. [24]. Young adult male rats of 125 to 165 g body weight were maintained in air-conditioned quarters with water and food. Naïve rats were randomly allocated to four groups: Control group received 2.5 ml/kg of physiological solution 0.9% NaCl used to resuspend the different drugs; Standard group received 1 mg/kg of dexamethasone; Positive control group received 15 mg/kg of carrageenan in 100 µl of 0.9% NaCl and Test group received 15 mg/kg of carrageenan and 5 µg de PpSP32. The drugs were administered into the left hind paw. Edema was followed by measuring changes in paw volumes using a sliding caliper at various times (0, 1, 2, 3 and 4 hours). The increase in paw volume was considered as an index of inflammation intensity.

**Statistical analysis**

Statistical analyses were performed using the one-way ANOVA with multiple comparisons when comparing paired groups or Mann-Whitney test when comparing independent groups. Statistical significance was assigned to a value of P< 0.05. All Statistical analyses and graphs were performed using GraphPad Prism v5.0 or v8.0 software.

**Results**

**Effects of PpSP32 on lymphocyte effector functions**

The effect of PpSP32 on lymphocyte effector functions was first assessed by studying their proliferative response to different stimuli (PMA/Ionomycin, PHA and CD3/CD28 antibodies). As shown in figure 1A, PpSP32 does not exert any effect on lymphocyte proliferation regardless of the dose of PpSP32 and the type of stimulus used.

When assessing the cytokine production upon activation with PMA/Ionomycin, we noted a significant downmodulation of IFN-γ at a concentration of 0.5μg/ml and 2 μg/ml as well as IL-4 at a concentration of
0.5µg/ml and 2 µg/ml and IL-6 levels with all concentrations used (0.5, 2 and 5µg/ml) (Figure 1B).

**Effects of PpSP32 on monocyte effector function**

To test the effect of PpSP32 on monocyte activation, we first assessed the expression of CD86 and HLA-DR surface molecules. PBMCs were thus stimulated with LPS in the presence of increasing doses of PpSP32 (0.5 µg/ml, 2 µg/ml or 5µg/ml) and surface molecule expression was analyzed by flow cytometry on CD14+ cells. As shown in Figure 2A, PpSP32 down-modulated the expression of both surface markers in LPS stimulated monocytes. Notably, such an effect was dose-dependent for CD86 expression.

In the next step, we analyzed the effect of PpSP32 on the production of pro-inflammatory cytokine upon LPS activation. LPS is a potent stimulus of monocytes and thus represents a more stringent test of the capacity of PpSP32 to modulate monocyte activation. As shown in Figure 2B, a significant and dose-dependent inhibition of LPS-induced secretion of IL-1β by PpSP32 was observed with all concentrations used (0.5, 2, and 5µg/ml). The production of TNF-α was slightly but not significantly decreased after the exposure to PpSP32. PpSP32 had no effect on the secretion of IL-6.

**Effects of PpSP32 on THP-1 derived macrophages**

The modulatory effects of PpSP32 on monocyte effector function led us to test its effects on THP-1 derived macrophages. The use of cell lines would also overcome the heterogeneity of responses noticed between the different donors.

We first established the optimal timing for production of IL-1β, IL-6 and TNF-α after LPS stimulation was 18h in such cell line. The cells were either pre-treated or not with different concentrations PpSp32 during 48h then stimulated with the LPS for additional 18 hours. PpSP32 exhibited an inhibitory effect of all tested cytokines (Figure 3A). A significant and dose-dependent inhibition by PpSP32 was observed for IL-1β and TNF-α while an inhibition was noticed for IL-6 for the high dose of PpSP32. The inhibitory effect of PpSP32 on pro-inflammatory cytokine production was confirmed by a multiplex assay (Figure 3B). This effect was noticed also for MCP-1, IP-10, IL-1α, IL-12, IL-8, IL-27 and IL-10, yet it was significant only for MCP-1, IL-27 and IL-1α in addition to IL-1-b, TNF-α and IL-6. Altogether, these data rather suggest a global inhibitory effect on macrophage cytokine production (Figure 3B).

To test whether the inhibitory effect of PpSP32 on the pro-inflammatory cytokine production was not related to a reducing cell viability of THP-1 derived macrophages, we used a MTT assay. Our data did not show any significant inhibitory effect on cell growth with different concentrations of PpSP32 after 24h or 72h incubation of THP-1 derived cells (Figure 4A).

To further confirm the anti-inflammatory effect of PpSP32 on THP-1 derived macrophages, we tested its effect on nuclear factor kappa B (NF-kB) signaling, the main pathway that regulates the expression of many inflammatory cytokines. THP-1 cells were pre-treated by increasing doses of PpSP32 then
stimulated or not by LPS during 3 hours. As shown in Figure 4B and 4C, PpSP32 treatment led to a dose-dependent reduction of the IkBphosphorylation.

**Effects of PpSP32 on carrageenan induced paw edema in rats**

The carrageenan-induced paw edema model in rats, one of the well-established acute inflammatory models *in vivo*, was used to test the anti-inflammatory activity of PpSP32. The injection of carrageenan into the rats hind paw induced an increase in paw edema which indicates the development of an inflammatory response. As shown in Figure 5, the edema was present as early as one hour after carrageenan injection, progressed rapidly and persisted for at least 4 hours after treatment. Dexamethasone, used as positive control, inhibited the inflammatory response due to carrageenan in rats. Treatment of rats with PpSP32 significantly reduced paw swelling from the second hour. This reduction was about 23% after second hour and reached a maximum of 30% at the third hour.

**Discussion**

This is the first evidence of immunomodulatory functions of PpSP32, the immunodominant salivary protein of *P. papatasi*. It is well known that sand fly saliva contains several molecules which impair the capacity of the hemostatic system by preventing vasoconstriction [9] and inhibiting platelet aggregation as well as blood coagulation cascade [25,26]. Some of these salivary proteins may also interfere with the host’s immune response by inhibiting the complement system or modulating the T cell response or antigen-presenting cell functions [2,7,10,27]. Despite the growing knowledge on the biology of saliva [21,28,29], the function of several components, including the PpSP32, the immunodominant salivary protein of *P. papatasi* [22], remains unknown to date. PpSP32 harbors structural homologies with other proteins such as a flagelliform silk-protein of *Nephila clavipes* as well as with collagen adhesion proteins [21]. PpSP32 was also predicted to be a mucin-based protein according to the pattern of its O- and N-glycosylation. Interestingly, it was demonstrated that PpSP32 binds to specific proteins from the skin, the desmogleins, leading to the loss of tolerance and the production of related auto-antibodies which causes pemphigus [23]. Herein, we aimed to screen one of the putative effects of this protein namely the immunomodulatory one.

To evaluate the potential effect of PpSP32 on the cellular immune response, we first assessed its effects on lymphocyte effector functions through testing the proliferative response as well as the cytokine production of such cells. Although PpSP32 has no effect on the proliferation of T lymphocytes, it exerted an inhibitory effect on Th1 response. Modulation of IFN-γ production following exposure to sand fly saliva has been already described in different species including *P. papatas*[15]. Since IFN-γ is a key factor that promotes leishmanicidal mechanisms of macrophages, its inhibition by the sand fly saliva could sustain the multiplication of amastigotes and promote the *Leishmania* infection. Our data, however, showed a parallel modulatory effect on Th2 cytokine production. Such results are consistent with those of Rohousova et al. which demonstrated that the salivary gland lysate of three different sand flies, *P. papatasi*, *P. sergenti* and *Lu. Longipalpis* inhibited the secretion of IL-4 by murine splenocytes [30].
However, another study from Mbow et al. reported a direct enhancing effect of *P. papatasi* saliva on IL-4 expression in the absence of *Leishmania* infection [31]. The latter results were obtained *in vivo*, perhaps explaining the difference with *in vitro* experiments.

In a second step, the immunomodulatory effect on antigen-presenting cell functions has been evaluated through the assessment of the expression of MHC class II and B7 (CD80 and CD86) molecules. In fact, modulation of these surface molecules could alter the response of T lymphocytes and promote tolerance towards *Leishmania* antigens. A significant down-modulation of both molecules was demonstrated even in the presence of low doses of PpSP32, a result consistent with this previously reported by Costa et al. using the salivary gland homogenate of *Lu. Longipalpis*[13]. In the latter report, the effect was mainly ascribed to maxadilan[12]. Contrastingly, in some other species such as *Lu.intermedia*, pretreatment with the salivary gland homogenate was able to significantly increase the expression of CD80, CD86 and HLA-DR on human monocytes [11]. Such discrepancy may be explained by the difference in the composition of such distinct sand fly species particularly by the difference in the amounts of maxadilan[32].

In a next step, we investigated whether the immune modulation exerted by PpSP32 would affect the secretion of pro-inflammatory cytokines by human monocytes. Our data showed that PpSP32 inhibited IL-1β production by LPS-stimulated PBMC but great heterogeneous responses found in the different donors for TNF-α and IL-6 production hampered drawing strong conclusions. To overcome such issues, we used derived macrophages from THP-1 cell line [33–35] and confirmed the inhibition of IL-1β, TNF-α and IL-6 in the latter cells. IL-1β and TNF-α play a crucial role in the clearance of *Leishmania* infection [36,37]. Downregulation of TNF-α has also been reported for other components of saliva, such adenosine, a pharmacologically active component of *P. papatasi* saliva [38,39]maxadilan, a salivary component of the sand fly *Lu. Longipalpis*[15] or RsP03 of *P. perniciosus*[40]. Interestingly, downmodulation of IL-6 production exerted by PpSP32 contrasts with several reports in the literature regarding other salivary sand fly components. Accordingly, either maxadilan, *P. papatasi, P. alexandri* or *P. duboscqi* salivary extracts have been reported to enhance the production of IL-6 by monocytes or PBMC [15,39,40]. To our knowledge this is the first report revealing a down regulation of IL-6 by a salivary protein of *P. papatasi*. This down-modulation has been shown in our work not only in LPS-stimulated monocytes/macrophages but also in PMA-ionomycin- stimulated lymphocytes.

In addition to TNF-α and IL-1β, number of other inflammatory cytokines is involved in macrophage functions [41]. Hence, a multiplex technique was used to measure several inflammatory cytokines after stimulation of THP-1 derived macrophages in the presence of different concentrations of PpSP32. Our results showed that PpSP32 decreases the secretion of IL-6, TNF-α and IL-1β and also IL-1α, IL-12, IL-27, IL-8, IP10, MCP1 and IL-10 yet a significant effect was reported only with for MCP-1, IL-27 and IL-1α in addition to TNF-α, IL-1β and IL-6. It is well known that IL-12, together with IFN-γ, participates in the polarization of the immune response by inducing the commitment of naive T cells into Th1 cells while inhibiting the development of Th2 lymphocytes [42]. Our data are consistent with those of Mbow et al. who reported that salivary gland homogenate of *P. papatasin* inhibits the expression of IL-12 in mice [31]. Since Link et al. demonstrated that adenosine from *P. papatasi* saliva inhibited IL-12 secretion by human
monocytes [43], we can suggest that the inhibition of IL-12 by P. papatasi sand fly saliva could be ascribed to PpSP32 along with adenosine.

Primarily expressed by macrophages and dendritic cells during the early phase of Leishmania infection, IL-27 contributes to the protection against L. major infection. This protection is exerted primarily through inhibition of IL-4-mediated Th2 cell responses and induction of IFN-γ production from CD4+ T and NK cells, thereby enhancing Th1 cell responses [44]. Furthermore, IL-27 enhances the differentiation of monocytes into macrophages and activates macrophages [45]. To our knowledge this is the first report which studies the effect of a salivary protein on IL-27 secretion by monocyte derived macrophages and which demonstrated a decrease in the secretion of this cytokine after PpSP32 stimulation.

IL-8, IP-10 and MCP-1 are potent chemotactic agents responsible for migration of several immune cells [46, 47]. Our results showed that PpSP32 decreased the secretion of all of them, particularly MCP-1. A previous report showed that pre-exposure of murine cells to Lu.intermedia salivary sonicates resulted in decreased expression of IP-10 during subsequent secondary exposures [48]. Another report showed that the saliva of Lu. longipalpis seems to increase levels of IL-8 secreted by LPS-stimulated human monocytes [13]. One work studied the effect of sand fly salivary components on MCP-1 expression but this report tested this expression by neutrophils and not macrophages. It revealed that Lu. longipalpis saliva in presence of L. chagasi induces MCP-1 expression by neutrophils which contrasts with our data showing an inhibitory effect of PpSP32 on these cytokines in monocyte/macrophages [49].

Contrasting with a global anti-inflammatory effect of PpSP32, our data showed that this molecule exerted an inhibitory effect also on IL-10, an anti-inflammatory cytokine. Divergent results have been reported regarding the effect of salivary components of sand fly saliva on IL-10 production. While Lu. longipalpis saliva decreases IL-10 secretion by human monocytes stimulated with LPS [13], adenosine and its precursor 5′-AMP, isolated from P. papatasi salivary glands have been reported to enhance IL-10 [43]. Maxadilan also stimulates the secretion of IL-10 by macrophages [50]. Regarding our data about the effects of PpSP32 on the cytokine production by monocytes/macrophages, the parallel inhibitory effect on either proinflammatory or anti-inflammatory mediators is intriguing. This could rather point to an overall inhibitory effect on monocyte/macrophage functions since IL-10 is also produced by these cells after activation. These findings also argue against the fact that the inhibitory effect exerted by PpSP32 on monocyte/macrophage function is mediated by the overproduction of IL-10.

Overall, our study provides new insights into the effects of PpSP32 on the cytokine production by human PBMC as well as by monocytes and human THP-1-derived macrophages. Herein, we showed for the first time that PpSP32 possesses rather anti-inflammatory properties. Leishmania parasites appear to exploit these immunomodulatory properties of PpSP32 to enhance its early survival in humans and exacerbate the infection. To better understand the mechanisms underlying our observations and make these findings more convincing, we investigated the mechanism by which PpSP32 modulates cytokine secretion. Hence, the effect of PpSP32 pre-treatment on the NFκB signaling pathway was assessed by testing the phosphorylation of IκB in LPS- stimulated THP-1 cells [51–53]. Our findings suggested for the first time
that PpSP32 inhibited LPS-induced inflammatory response in THP-1 cells through the inhibition of NFκB signaling pathway. However, we could not exclude that other cellular pathways may be involved in the biological activity of PpSP32.

Finally, the anti-inflammatory activity of PpSP32 was further assessed \textit{in vivo} in an experimental inflammatory model, the carrageenan-induced paw edema in rats. The injection of carrageenan produces a time-and dose-dependent edema in the paws of rats. Interestingly, when PpSP32 was injected into the paw, edema was reduced in the same manner that dexamethasone, a corticosteroid and anti-inflammatory molecule. The \textit{in vivo} inhibition of inflammation by salivary proteins has been already reported. Lufaxin, a novel factor Xa inhibitor from the salivary gland of \textit{Lu. longipalpis} has been demonstrated to inhibit inflammation \textit{in vivo}. In fact, lufaxin abrogates edema formation triggered by injection of factor Xa in the paw of mice [54]. \textit{In vivo} sub plantar injection of this factor induces an edema in the paws of mice that resembles the effects observed after administration of carrageenan [55].

Conclusion

This study indicates for the first time that PpSP32 induces a potent immunomodulatory effect on monocytes and THP-1 derived macrophages. This inhibition could be mediated, among others, through the modulation of the NF-kB signaling pathway. The underlying mechanisms are under study. Our study is a first step in understanding the functions of the immunodominant salivary protein of \textit{P. papatasi}, PpSP32. The investigation of other biological functions of PpSP32 such as anti-hemostatic effects is in progress in order to complete the overall picture.

Abbreviations


Declarations

Declarations

Ethics approval and consent to participate
The study was approved by the institutional ethics committee of Pasteur Institute of Tunis (2018/35/1/LR16IPT08). Informed consent of all participants was provided for the collection of blood samples and subsequent analyses. Rats used in this work were handled in strict compliance with animal ethics procedures and guidelines of the ethics committee of the Pasteur Institute of Tunis (No.2022/3/I).

**Consent for publication**

The authors agree for the publication of the article

**Competing interests**

The authors declare that they have no competing interests

**Author contributions**

CS, SM, IED and MBA designed and conceptualized the study. CS, SM and JJ carried out the experiments. CS, SM and MBA appraised the experimental results. OF, JGV, NS and SK provided reagents. MBA, SM, CS drafted the manuscript. FO revised the manuscript. All authors read and approved the final manuscript.

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Figures
Figure 1

**Effects of PpSP32 on lymphocyte effector functions:** (A) PBMC were stimulated or not with PMA/Ionomycin, PHA or anti-CD3/CD28 antibodies in the presence or absence of increasing doses of PpSP32 during 72h. Proliferative responses were assessed by (3H) thymidine uptake. Results are expressed as count per minute (cpm). Data are means of three independent experiments. (B) PBMC were stimulated with PMA (50ng/ml) and Ionomycin (1µg/ml) in the presence or absence of different concentrations of PpSP32 during 48h. Supernatants were collected and tested for cytokine production by ELISA. Data are presented as means ± SD. The P-value was determined by the ANOVA test with multiple
comparisons. * indicates $P<0.05$, ** $P<0.001$, *** $P<0.0001$. Abbreviations: PHA, Phytohemagglutinin; PMA, Phorbolmyristate acetate; Iono, Ionomycin; IFN-$\gamma$, Interferon gamma; Uns: unstimulated; IL-, interleukin; pg, picogram; ml, milliliter; µg, microgram; PpSP32, *Phlebotomus papatasi* salivary protein 32.

**Figure 2**

**Effects of PpSP32 on monocyte effector function.** Peripheral blood mononuclear cells (PBMC) were stimulated by LPS (100ng/ml) for 48 h in the presence or not of increasing doses of PpSP32. (A) The
mean fluorescence of CD86 and HLA-DR staining in CD14 positive cells is shown. * indicates $P<0.05$ when compared to condition without PpSP32. ** Cytokine production was tested in the supernatants by ELISA. Data are presented as means ± SD. The $P$-value was determined by the ANOVA test with multiple comparisons.* indicates $P<0.05$, ** $P<0.001$, *** $P<0.0001$ when compared to the condition without PpSP32. Abbreviations: HLA-DR, Human leukocyte antigen receptor; CD86, Cluster of differentiation 86; IL-, interleukin; TNF-α, Tumor necrosis factor alpha; PpSP32, *Phlebotomus papatasi* salivary protein 32; pg, picogram; ml, milliliter; µg, microgram.
Figure 3

**Effects of PpSP32 on cytokine production by THP1-derived macrophages:** THP-1-derived macrophages were pretreated with different concentrations of PpSP32, 0.5 µg/ml (0.5), 2µg/ml (2) and 5µg/ml (5) for 48h then stimulated with LPS (100 ng/ml) for 18h. Supernatants were collected and tested for cytokine production by ELISA (A) or by multiplex flow cytometer analysis (B). Data are presented as means ± SD. The *P*-value was determined by the ANOVA test with multiple comparisons. * indicates *P*<0.05, ** *P*<0.001, *** *P*<0.0001 when compared to the condition without PpSP32. Abbreviations: IL-, interleukin; TNF-α, Tumor necrosis factor alpha; IFN-γ, Interferon gamma; MCP-1, Monocyte chemoattractant protein 1; IP-10, interferon-γ–induced protein-10; Uns: unstimulated; pg, picogram; ml, milliliter; µg, microgram; PpSP32, *Phlebotomus papatasii* salivary protein 32.
Figure 4

Effects of PpSP32 on human THP-1-derived macrophages. (A) THP-1-derived macrophage viability assessed using the MTT was evaluated by incubating cells in the presence or absence of different concentrations of PpSP32 during 24h and 72h. Results of three independent experiments are expressed as mean of percentages ± SD of viability according to the control condition. (B) THP-1-derived macrophages were pretreated with PpSP32 (0.5, 2 or 5 μg/ml) for 48 h, then incubated with 100ng LPS for 3 h. Whole cell lysates (30μg/lane) were then separated, transferred and incubated with anti-p-κB-α.
antibody or anti-β actin. Immunoblots were determined by enhanced chemiluminescence after adding the HRP-conjugated secondary antibodies. Quantification of phospho-IκB level was performed by Image J (version 1.8.0). Data are presented as means ± SD. The P-value was determined by the ANOVA test with multiple comparisons. *indicates P<0.05. Abbreviations: LPS, Lipopolysaccharid; p-IκB-α, phospho I kappa B alpha; Uns: unstimulated; µg, microgram; ml, milliliter; h, hour; PpSP32, Phlebotomus papatasi salivary protein 32.

Figure 5
**Effects of PpSP32 on carrageenan-induced paw edema in rats.** Naïve rats were randomly allocated to four groups of three mice each: Control group received 2.5ml/kg of physiological solution 0.9% NaCl used to re suspend the different drugs; Standard group received 1mg/kg of dexamethasone; Positive control group received 15mg/kg of carrageenan in 100µl of 0.9% NaCl and Test group received 15mg/kg of carrageenan and 5µg de PpSP32. Edema was followed by measuring changes in paw volumes using a sliding caliper at various times (0, 1, 2, 3 and 4 hours). *(A)* Data are presented as means ± SD. The *P*-value was determined by the Mann-Whitney test.* indicates *P*<0.05, ** *P*<0.001, *** *P*<0.0001 when compared to the Test group. *(B)* Pictures from a representative experiment at 3 hours of injection are shown. Abbreviations: PpSP32, *Phlebotomus papatasi* salivary protein 32; h, hour; mm, millimeter.