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Monocytes from Sjögren's syndrome patients display increased vasoactive intestinal peptide receptor 2 expression and impaired apoptotic cell phagocytosis

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Summary

Sjögren's syndrome (SS) is a chronic autoimmune disease characterized by salivary and lacrimal gland dysfunction. Clinical observations and results from animal models of SS support the role of aberrant epithelial cell apoptosis and immune homeostasis loss in the glands as triggering factors for the autoimmune response. Vasoactive intestinal peptide (VIP) promotes potent anti-inflammatory effects in several inflammatory and autoimmune disease models, including the non-obese diabetic (NOD) mouse model of SS. With the knowledge that VIP modulates monocyte function through vasoactive intestinal peptide receptors (VPAC) and that immune homeostasis maintenance depends strongly upon a rapid and immunosuppressant apoptotic cell clearance by monocytes/macrophages, in this study we explored VPAC expression on monocytes from primary SS (pSS) patients and the ability of VIP to modulate apoptotic cell phagocytic function and cytokine profile. Monocytes isolated from individual pSS patients showed an increased expression of VPAC2 subtype of VIP receptors, absent in monocytes from control subjects, with no changes in VPAC1 expression. VPAC2 receptor expression could be induced further with lipopolysaccharide (LPS) in pSS monocytes and VIP inhibited the effect. Moreover, monocytes from pSS patients showed an impaired phagocytosis of apoptotic epithelial cells, as evidenced by reduced engulfment ability and the failure to promote an immunosuppressant cytokine profile. However, VIP neither modulated monocyte/macrophage phagocytic function nor did it reverse their inflammatory profile. We conclude that monocytes from pSS patients express high levels of VPAC2 and display a deficient clearance of apoptotic cells that is not modulated by VIP.

Keywords: apoptotic cell clearance, monocytes, Sjögren's syndrome patients, VPAC receptors

Introduction

Sjögren's syndrome (SS) is a chronic autoimmune disorder that affects 0·5–1% of the adult population, with a high economic impact in health care. The disease hallmark is a salivary and lacrimal gland dysfunction, although co-morbidities are found depending upon genetic background, hormones and environmental triggers [1–5]. Clinical observations and results from patient cell approaches and animal models of SS point to the loss of salivary gland homeostasis as a triggering factor for the autoimmune response which would, in turn, promote further damage to the gland [6–11]. In line with this, evidence of aberrant expression of inflammatory and apoptosis mediators in salivary gland epithelial cells from pSS patients and murine models was reported [12–17]. Immune homeostasis depends strongly upon a rapid and immunosuppressant apoptotic cell clearance by monocytes/macrophages to prevent an inflammatory response and self-tolerance breakdown [18–21]. Consistently, evidence on an impaired or delayed clearance of apoptotic cells by macrophages was reported in systemic lupus erythematosus (SLE) patients,

Table 1. Characteristics of primary Sjögren's syndrome (pSS) patients and healthy volunteers (control).

Participants	Age (mean ± s.d.)	Extraglandular manifestations (number of patients)	Disease duration (mean ± s.d.)	Systemic treatment (number of patients)
pSS Patients $n = 38$	29–79 years (55·9 ± 11·9)	Autoimmune thyroid disease (7); synovitis (2); cutaneous vasculitis (4); Raynaud phenomenon (2)	2–33 years (12·2 ± 9·8)	Hydroxicloroquine (19), levothyroxine (6), prednisone (3), pilocarpine (1)
Control $n = 16$	24–61 years (42·2 ± 13·2)	n.a.	n.a.	n.a.

Patients fulfilled the American–European Consensus Group Criteria for pSS and all of them were positive for anti-Ro (SSA) serum antibodies as described in Material and methods. Drug prescription to pSS patients is indicated, except for 12 patients who only received local symptomatic management. Control subjects received no drug treatment. All the participants were women. s.d. = standard deviation; n.a. = not applicable.

although its direct aetiopathogenic role is still unclear [22–24]. Regarding SS, a deficient phagocytosis of apoptotic cells was described in the non-obese diabetic (NOD) mouse model of the disease [25–27]. In particular, macrophages isolated from NOD mice at the SS-like stage expressed a predominant M1 inflammatory activation profile and presented a defective engulfment of apoptotic acinar cells [26].

Vasoactive intestinal peptide is a prosecretory and vasodilating neuropeptide with potent immunomodulatory effects through the activation of vasoactive intestinal peptide receptor VPAC1 and VPAC2 receptors on monocytes, macrophages and T cells [28–32]. VIP promotes anti-inflammatory and tolerogenic effects in several inflammatory and autoimmune disease models [30,33–37]. Particularly in the NOD mouse model of SS, a local gene therapy with an adenoviral construct encoding VIP restored salivary secretion and reduced autoimmune markers [38]. Also, a predominant M2 alternative activation profile was promoted by VIP in NOD mice macrophages at the SS-like stage [26,27,39].

In this study we analysed Sjögren syndrome individual patients' monocytes to explore their VPAC receptor expression profile and function, particularly the effect of VIP on the phagocytosis of epithelial apoptotic cells. We observed that monocytes from pSS patients showed increased expression of VPAC2 that was absent in normal subjects' monocytes, and that this effect paralleled an impaired phagocytosis of apoptotic cells, with reduced engulfment and failure to express an immunosuppressant cytokine profile that was not restored by VIP.

Materials and methods

Patients

Blood samples were collected from patients that fulfilled the American–European Consensus Group Criteria for pSS [40] ($n = 38$), followed-up at the Rheumatology Unit, Department of Medicine of the CEMIC, Buenos Aires, Argentina, and from healthy volunteers as the control group ($n = 16$). All the participants were women who signed an informed consent to participate in this study, approved by the Argentine Society of Clinical Investigation Review Board and Ethical Committee. The age range, disease duration, extraglandular manifestations and systemic treatments are indicated in Table 1. All patients were positive for anti-Ro (SSA) serum antibodies, and 12 patients received only local symptomatic management.

Peripheral blood mononuclear cells (PBMC)

PBMC from patients and controls were isolated from heparinized peripheral blood by density gradient centrifugation on Ficoll-Hypaque (Amersham Pharmacia Biotech, Uppsala, Sweden). Monocytes were purified by attachment to a culture plate for 2 h and washed three times with warm phosphate-buffered saline (PBS) to remove non-adherent cells, and adherent monocytes were recovered with a cell scraper. Cell population purity was checked by fluorescence activated cell sorter (FACS) analysis using anti-CD14 monoclonal antibody (mAb) and was found to be > 85% for each set of experiments. In some experiments $CD14^+$ cells were separated by performing positive selection with $CD14^+$ micro-magnetic beads, according to the manufacturer's instructions (Miltenyi Biotec, Bergisch Gladbach, Germany), with a purity of 95%. $CD14^+$ cells from each patient or control sample were used separately for VPAC determination, plated for phagocytosis experiments or incubated with 1 μg/ml lipopolysaccharide (LPS) in the presence or absence of 100 nM VIP for 24 h before being homogenized for reverse transcription–quantitative polymerase chain reaction (RT–qPCR) determinations.

Epithelial cell apoptosis induction

The human salivary gland (HSG) epithelial cell line was kindly provided by Dr Bruce Baum (NICDH-NIH, Bethesda, MD, USA) and cultured in 24-well flat-bottomed polystyrene plates (Becton Dickinson, Franklin Lakes, NJ, USA) in complete Dulbecco's modified Eagle's medium (DMEM) 10% fetal calf serum (FCS) (Gibco, Invitrogen, Buenos Aires, Argentina). HSG cells displayed secretory

Table 2. Primers used in this study. Oligonucleotide primers were designed using the online tool Primer3® (Whitehead Institute for Biomedical Research).

Gene	Forward	Reverse
Vpac1	CCCCTGGGTCAGTCTGGTG	GAGACCTAGCATTCGCTGGTG
Vpac2	CCAGATGTCGGCGGCAACG	GCTGATGGGAAACACGGCAAAC
Vip	CAGTAACAGCCAACCCTTAGCC	TGAGAAGAGTCAGGAGCACAAGG
Gapdh	TGATGACATCAAGAAGGTGGTGAAG	TCCTTGGAGGCCATGTAGGCCAT
Cd14	CAAGTGTGAAGCCTGGAAGCCG	AGCAGCAACAAGCAGGACGC

properties and markers according to their salivary gland epithelial phenotype [41]. Apoptotic HSG cell suspension was obtained by incubating HSG cells at 70% confluence during 24 h with 50 nM staurosporine (Sigma Chemical Co, St Louis, MO, USA). The frequency of apoptotic HSG cells was assessed by propidium iodide (PI) and fluorescein isothiocyanate (FITC)-annexin V staining following the manufacturer's recommendations (BD Biosciences, San José, CA, USA) and analysed by flow cytometry (FACS) using WinMDI software®.

Phagocytosis of apoptotic HSG cells by patients' monocytes

Phagocytosis of apoptotic HSG cells by monocytes was determined by FACS. HSG cells were induced to apoptosis during 24 h with 50 nM staurosporine and stained with carboxyfluorescein succinimidyl ester (CFSE; eBioscience, San Diego, CA, USA). Apoptotic HSG cell suspension was added to each well of the 24-well plate containing adherent monocyte monolayers from individual patients or control subjects for 60, 90 or 120 min at 37°C at a 1:1 or 3:1 relationship (apoptotic HSG : monocyte cells) in the presence or absence of 100 nM VIP (Polypeptide Group, Strasbourg, France). Non-ingested cells were washed out and monocytes detached by addition of Tryple® (Gibco, Grand Island, NY, USA). The monocyte population was stained with phycoerythrin (PE)-conjugated anti-CD14 mAb (BD Biosciences) and the percentage of phagocytosis was determined as CD14/CFSE double-stained cells by FACS. Ten thousand events were acquired in a FACSAria II® cytometer and results were analysed using the WinMDI software®. Samples were incubated in parallel with a non-relevant, isotype-matched CD14 antibody as a background control.

Cytokine production

To assess tumour necrosis factor (TNF)-α, interleukin (IL)-10 and IL-6 production by monocytes after phagocytosis experiments, cells were incubated further for 24 h and supernatants collected for cytokine determination by enzyme-linked immunosorbent assay (ELISA) (e-Bioscience).

RT–PCR and RT–qPCR

Total RNA isolation was performed using TRIzol (Invitrogen, Carlsbad, CA, USA) and cDNA were generated from 1 μg of RNA using a Moloney murine leukaemia virus (MMLV) reverse transcriptase, RNAsin RNAse inhibitor and oligo dT kit (Promega-Biodynamics, Buenos Aires, Argentina). Each cDNA was then amplified using specific primers for VPAC1, VPAC2, VIP and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) or CD14 antigen were used as housekeeping genes (Table 2). PCR products and DNA size markers were fractionated on 2% agarose gels, visualized with ethidium bromide staining, and band density was expressed in arbitrary units (AU) normalized to GAPDH. Bands were semi-quantified with ImageJ® and intensity expressed in AU relative to GAPDH. qPCR was performed using Mezcla Real (Biodynamics, Buenos Aires, Argentina), according to the manufacturer's instructions.

Statistical analysis

The significance of the results was analysed by Student's *t*-test and Mann–Whitney *U*-test for non-parametric samples. When multiple comparisons were necessary, the Student–Newman–Keuls test was used after analysis of variance. Differences between groups were considered significant at $P < 0{\cdot}05$ using the GraphPad Prism4 software (GraphPad, San Diego, CA, USA).

Results

VPAC2 is highly expressed in pSS monocytes

We first analysed the expression of both subtypes of VIP receptors, VPAC1 and VPAC2, on monocytes isolated from pSS patients and control subjects. Figure 1a shows that pSS monocytes express the VPAC2 subtype of VIP receptors, which were absent in monocytes from normal subjects. The expression of VPAC2 in pSS monocytes was similarly high, regardless of whether the housekeeping gene used was GAPDH or CD14 as a monocyte specific marker (Fig. 1b). In contrast, VPAC1 subtype was expressed in both patient and control $CD14^+$ cells at similar levels (Fig. 1c). We could

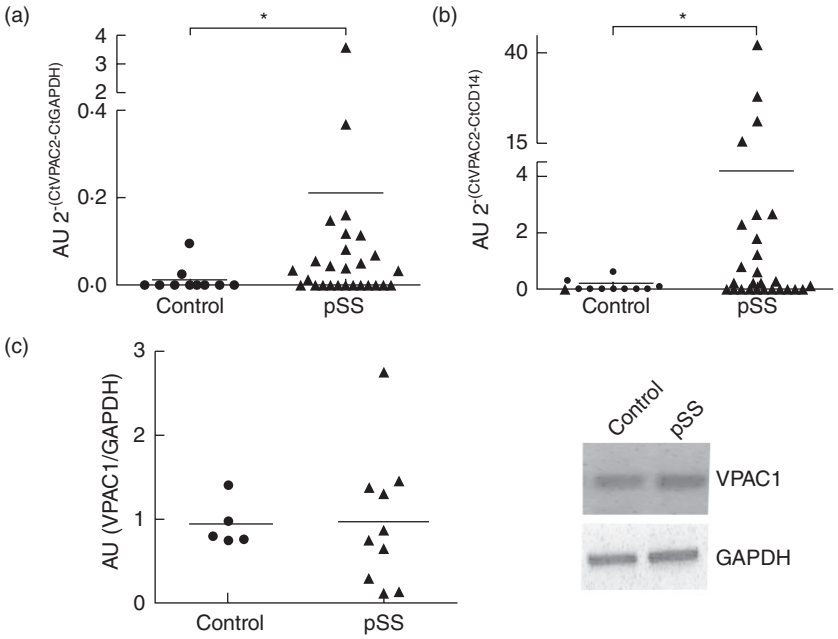

Fig. 1. Vasoactive intestinal peptide receptor (VPAC) expression in patients' CD14$^+$ cells. (a,b) Monocytes were isolated from primary Sjögren's syndrome (pSS) or control subjects and analysed for VPAC2 expression by quantitative reverse transcription–polymerase chain reaction (qRT–PCR) as described in Material and methods using glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and CD14 to calculate relative expression. Values represent VPAC2 relative expression to GAPDH ($2^{-(CtVPAC2\text{-}CtGAPDH)\times 10\ exp3}$) or to CD14 ($2^{-(CtVPAC2\text{-}CtGAPDH)\times 10\ exp3}$) calculated from each pSS or control subject monocyte sample. Media calculated is also shown. *$P < 0{\cdot}05$, Mann–Whitney *U*-test. (c) VPAC1 receptor expression was assessed in the same samples of (a) by RT–PCR, as described in Material and methods. Band intensity expressed in arbitrary units (AU) relative to GAPDH was semi-quantified with ImageJ®. Values represent VPAC1 expression in AU obtained with each different pSS or control subject monocyte sample and the media value is also depicted.

not detect VIP expression in the monocyte population of any of the patients' or control subjects' blood samples tested (not shown).

VPAC2 expression in pSS monocytes is increased by LPS and inhibited by VIP

Because the VPAC2 receptor subtype was up-regulated in mouse peritoneal macrophages as well as in a murine macrophage cell line after stimulation through Toll-like receptors (TLRs) [28,42], we explored whether this receptor could be induced through TLR-4 in monocytes from pSS patients. Thus, we incubated pSS monocytes with 1 μg/ml LPS for 24 h *in vitro*. A more than threefold increase in VPAC2 expression over basal levels (incubated in the absence of LPS) was observed only in pSS monocytes (Fig. 2, right panel). In contrast, VPAC2 receptors, absent in normal monocytes, could not be induced further by LPS treatment in our experimental conditions (Fig. 2, left panel). Interestingly, VIP inhibited the effect of LPS on VPAC2 expression on pSS monocytes (Fig. 2, right panel).

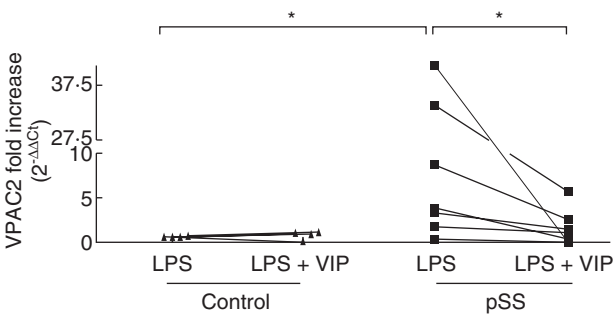

Fig. 2. Lipopolysaccharide (LPS) induction of vasoactive intestinal peptide receptor (VPAC)2 expression in patients' CD14$^+$ cells and vasoactive intestinal peptide (VIP) modulation. Monocytes from different primary Sjögren's syndrome (pSS) patients or control subjects (control) were isolated and incubated for 24 h with 1 μg/ml LPS in the presence or absence of 100 nM VIP as described in Material and methods before they were analysed for VPAC2 expression by quantitative reverse transcription–polymerase chain reaction (qRT–PCR). Values represent the fold increase of VPAC2 expression in each patient or control monocyte sample incubated with LPS for 24 h with or without VIP, over VPAC2 expression in the same incubation period in the absence of stimuli. *$P < 0{\cdot}05$, Mann–Whitney *U*-test.

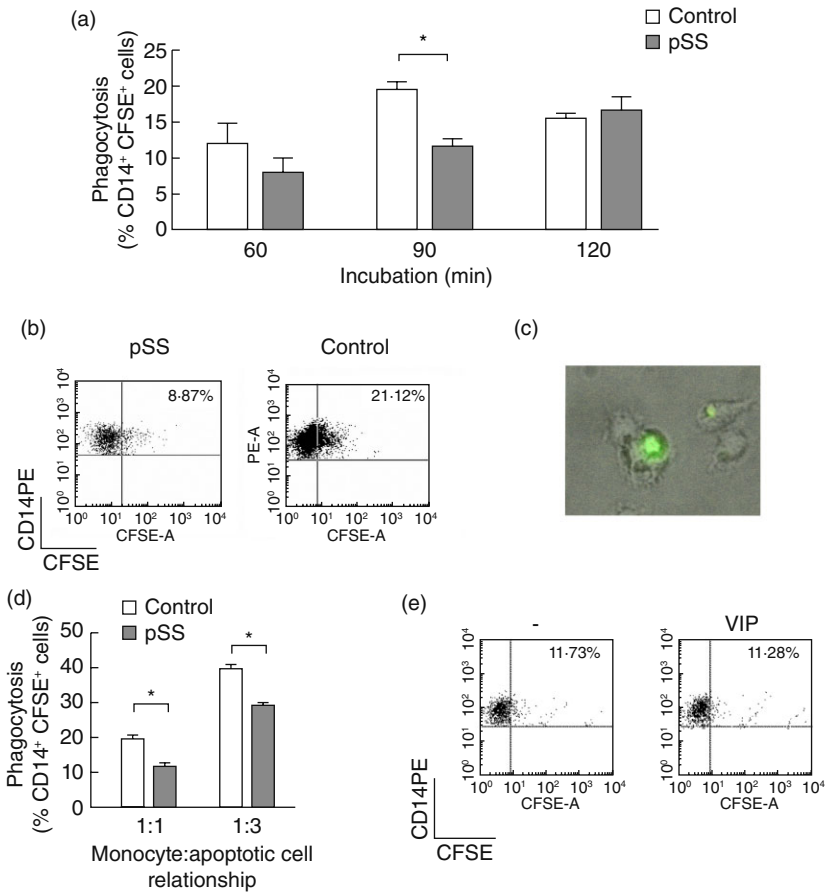

Fig. 3. Impaired phagocytosis of apoptotic human salivary gland (HSG) cells by primary Sjögren's syndrome (pSS) monocytes. Monocytes were isolated from pSS or control subjects' peripheral blood mononuclear cells (PBMCs) and plated for 24 h for phagocytosis experiments, as indicated in Material and methods. (a) HSG cells stained with carboxyfluorescein succinimidyl ester (CFSE) and induced to apoptosis for 24 h with 50 nM staurosporine were added to monocytes from individual pSS patients or control subjects plated on 24-well plates for 60, 90 or 120 min at a 1:1 apoptotic HSG : monocyte relationship. Values are mean ± standard error of the mean (s.e.m.) frequency of $CD14^+CFSE^+$ cells of at least six pSS or control monocyte samples determined by fluorescence activated cell sorter (FACS). $^*P < 0{\cdot}05$, Mann–Whitney *U*-test. (b) Representative dot-plot of 1:1 apoptotic cell : pSS or control monocyte incubated for 90 min. (c) Microphotography (×400) representative of at least eight experiments with pSS monocytes. (d) Monocytes from pSS patients or control subjects plated for 90 min at a 1:1 or 1:3 monocyte : apoptotic cell. Values are mean ± s.e.m. frequency of $CD14^+CFSE^+$ cells of six pSS and three control monocyte samples for a 1:1 relationship or four pSS and three control samples for a 1:3 relationship determined by FACS. $^*P < 0{\cdot}05$. (e) Monocytes from pSS patients incubated for 90 min at a 1:1 apoptotic cell:monocyte relationship in the presence or absence of 100 nM VIP. Representative dot-plot of three similar experiments.

Impaired phagocytosis of apoptotic cells by pSS monocytes is not restored by VIP

Sjögren's syndrome is associated with an increased apoptosis of salivary gland epithelial cells and loss of gland immune homeostasis. Considering that apoptotic cell clearance by phagocytic cells in a suppressant manner is essential for tissue homeostasis maintenance, we next explored the phagocytosis of apoptotic epithelial HSG cells by pSS patients' and control subjects' monocytes and the effect of VIP. Figure 3a shows an impaired phagocytic function of pSS monocytes at 90-min incubation time, as determined by FACS compared with monocytes from normal subjects. Representative cytometry plots in Fig. 3b show the percentage of phagocytosis of HSG apoptotic cells by control and pSS monocytes at 90 min. A microphotograph of CFSE-stained HSG apoptotic cell body ingested by pSS monocytes is shown in Fig. 3c. The impairment in apoptotic cell phagocytosis observed in $CD14^+$ cells from pSS patients was assessed at two different monocyte : apoptotic cell relationships (Fig. 3d). As shown in Fig. 3e, 100 nM VIP had no effect on phagocytosis of apoptotic cells by pSS monocytes at a 1:1 relationship and 90-min incubation setting. As expected for normal monocytes/macrophages, phagocytosis of apoptotic cells induced a suppressant cytokine profile with lower levels of the proinflammatory cytokine TNF-α and increased levels of IL-10 only in normal subjects' monocytes (Fig. 4a,b). In contrast, TNF-α production increased in pSS $CD14^+$ cells, whereas IL-10 did not, supporting an impaired apoptotic cell clearance (Fig. 4a,b).

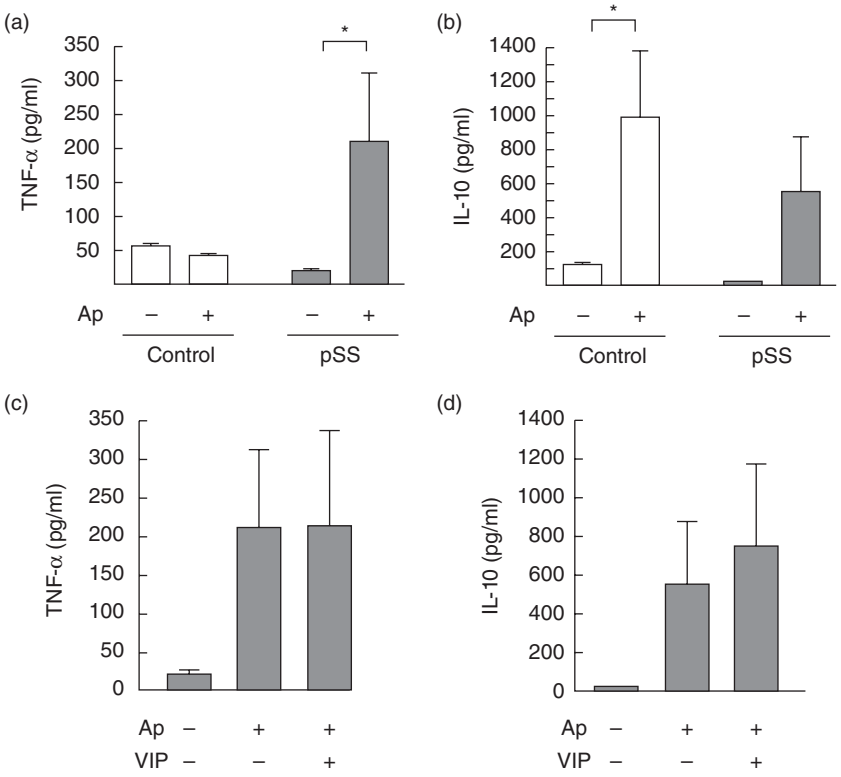

Fig. 4. Inflammatory profile upon phagocytosis of apoptotic cells by primary Sjögren's syndrome (pSS) monocytes not modulated by vasoactive intestinal peptide (VIP). Monocytes from pSS patients or control subjects were incubated for an additional 24 h after phagocytosis of human salivary gland (HSG) apoptotic cells (Ap) or without apoptotic cells added (–) and the levels of tumour necrosis factor (TNF)-α (a) and interleukin (IL)-10 (b) were determined by enzyme-linked immunosorbent assay (ELISA). Values are mean ± s.e.m. of at least six different pSS or control monocytes. *$P < 0{\cdot}05$. (c) Monocytes from pSS patients were incubated for an additional 24 h in the presence or absence of 100 nM VIP after phagocytosis of HSG Ap or without Ap added (–) and the levels of cytokines were determined by ELISA. Values are mean ± s.e.m. of six different pSS monocyte samples.

IL-6 levels also increased when pSS patients' $CD14^+$ cells engulfed apoptotic HSG cells (data not shown). We next investigated if VIP could reverse the proinflammatory profile of monocytes after apoptotic cell phagocytosis. Figure 4c,d shows that 100 nM VIP, the same concentration that limited LPS-induced VPAC2 expression (Fig. 2), could not reduce TNF-α or increase IL-10 production by pSS monocytes.

Discussion

In this study we present data to indicate that pSS patients' monocytes display a high expression of VPAC2 which can be modulated by LPS and VIP. Conversely, we showed that pSS patients' monocytes exhibit an impaired phagocytosis of apoptotic epithelial cells that could not be restored by VIP treatment *in vitro*. To our knowledge, there are no previous reports on apoptotic epithelial cell clearance defects by pSS patients' monocytes. Accordingly, our results indicate that TNF-α and IL-6 were released only by pSS monocytes when they phagocytized apoptotic HSG cells, consistent with a proinflammatory non-silent defective process. However, VIP did not improve phagocytosis of HSG apoptotic cells by pSS monocytes, nor did it contribute to a suppressant microenvironment by down-regulating TNF-α, IL-6 or enhancing IL-10.

A defect of *in-vitro* apoptotic cell clearance was described in systemic lupus erythematosus (SLE). Cultured macrophages differentiated from monocytes or stem cells of SLE patients displayed morphological abnormalities and showed an impaired phagocytosis of apoptotic cells [22,23]. Macrophages from SLE and rheumatoid arthritis (RA) patients were smaller, with less ability to differentiate and to adhere to apoptotic Jurkat T cells than control cells [43]. However, only SLE macrophages showed reduced engulfment of apoptotic cells, indicating that not only adherence and differentiation but multiple signals were impaired in the phagocytic process, similar to the results shown here for pSS monocytes, where phagocytosis of apoptotic HSG cells appears hampered at different levels. The observation that engulfment ability was reduced but not completely abolished in the short incubation time assayed here suggests that a basal impairment can be compensated *in vitro* through rapidly conveying monocyte signalling. Finally, our results on defective apoptotic epithelial cell clearance by macrophages is also consistent with previous observations in patients' glands and murine models of SS, where increased apoptosis of epithelial cells and aberrant expression of apoptosis markers and inflammatory mediators were proposed to have a role in the initiation and perpetuation of SS [14].

Previous observations in the NOD mouse model of SS indicated that VIP injected locally into NOD females at the SS-like stage reduced proinflammatory cytokines and normalized salivary secretory function [38]. Consistently, VIP promoted an alternative activation profile in NOD mice macrophages and favoured a suppressant apoptotic cell clearance by NOD macrophages [26,27]. However, which

biological circuits will be mediated preferentially by VIP *in vivo* is not easily predictable, as was shown clearly by Abad and coworkers in VIP knock-out mice that are resistant to experimental autoimmune encephalitis and LPS-induced endotoxaemia [44,45].

Monocytes from pSS patients but not those from control subjects expressed VPAC2 receptors as revealed by qRT–PCR, and the expression of this subtype receptor was increased when the cells were stimulated *in vitro* with LPS. VPAC1 is the unique subtype expressed in normal human resting monocytes [46,47]. However, various stimuli are known to modulate VPAC expression in immune cells. In particular, the VPAC2 receptor subtype was up-regulated in mouse peritoneal macrophages and macrophage cell lines when they were primed *in vitro* with inflammatory stimuli through TLR-4 [42]. Here we showed that the effect of LPS on VPAC2 expression occurred only in pSS but not in normal monocytes, and that it averaged a threefold increase, in line with previous observations in murine macrophages. Interestingly, a higher expression of VPAC2 was observed on fibroblast-like synovial cells from rheumatoid arthritis patients compared with osteoarthritis patients [48], suggesting that receptor differential expression might regulate VIP local effects *in vivo*. The increased expression of VPAC2 receptors in resting pSS monocytes shown here might reflect a compensating mechanism operating *in vivo*.

Of note, the higher expression of VPAC2 receptors in pSS monocytes did not suffice to modulate their functional profile in the presence of VIP *ex vivo*. In fact, this enhanced VPAC2 expression, and even the expression of VPAC1 comparable to normal subjects' monocytes, could not favour a suppressant phagocytosis when the cells were treated with VIP *in vitro*. This observation strongly supports a prominent role for a deficient phagocytosis of apoptotic cells in the pathogenesis of pSS that is refractory to the anti-inflammatory effect of VIP. The observation also confirms that the anti-inflammatory efficacy of VIP is more evident when immune cells are primed *in vitro* with potent proinflammatory stimuli. Certainly, in rheumatoid arthritis and ostheoarthritis patients' synovial cells, VIP potently inhibited proinflammatory signals when cells were stimulated *in vitro* with poli I : C [49]. Similarly, in the present results, the inhibitory effect of VIP was stated only on VPAC2 induction by LPS.

Our results indicate collectively that pSS patients' monocytes highly express VPAC2 receptors and display an impaired phagocytic function of apoptotic epithelial cells that could not be modulated by VIP. The functional relevance of the higher expression of VPAC2 receptors in pSS monocytes and whether it is a compensatory mechanism or just an epiphenomenon due to damage-associated molecular pattern (DAMP) ligand stimulation of pSS monocytes *in vivo* is a matter of future studies. However, the consistency of this observation in pSS patients, regardless of the disease outcome and duration, and the absolute absence of VPAC2 expression in the control group monocytes with normal apoptotic cell clearance, suggest its potential as a functional biomarker in pSS.

Acknowledgements

V. H., L. F. and E. G. carried out all the experiments and statistical analysis. A. E. and O. H. followed-up pSS patients and provided blood samples. R. R., O. H. and C. P. L. designed the study, discussed the results and prepared the manuscript. All authors read and approved the final manuscript. This work was funded by the National Research Council CONICET (PIP 2012-2015); the University of Buenos Aires (UBACyT 20020100100505) and the National Agency of Sciences and Technology ANPCyT (PICT 2011-0144).

Disclosure

No conflicts of interest to declare.

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