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RESEARCH ARTICLE

# Atorvastatin inhibits the inflammatory response caused by anti-$M_3$ peptide IgG in patients with primary Sjögren's syndrome

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**Abstract** Experimental and clinical investigations have revealed that statins can down-regulate acute and chronic inflammatory processes. Whether statins express anti-inflammatory activities in the salivary glands in patients with primary Sjögren's syndrome (pSS) is not known. The in vitro and in vivo effect of atorvastatin on rat submandibular gland treated with anti-$M_3$ peptide IgG purified from SS patients was studied. The anti-inflammatory effects of atorvastatin were assessed by measuring the levels of IL-1$\beta$, $PGE_2$ and MMP-3 by ELISA. Atorvastatin inhibited the increase in the production of IL-1$\beta$, $PGE_2$ and MMP-3 in submandibular glands treated with anti-$M_3$ peptide IgG. A positive correlation between IL-1$\beta$ production with accumulation of $PGE_2$ and MMP-3 was observed. Rats pre-treated orally with atorvastatin (30 mg $kg^{-1}$) or vehicle (phosphate-buffered solution) once a day for three consecutive days impaired the increment in the production of IL-1$\beta$, $PGE_2$ and MMP-3 in the submandibular gland in the presence of anti-$M_3$ peptide IgG. In conclusion, the anti-inflammatory effects of atorvastatin are dependent upon inhibition of production of a pro-inflammatory cytokine (IL-1$\beta$) and pro-inflammatory mediators such as $PGE_2$ and MMP-3. These data suggest that atorvastatin may constitute an anti-inflammatory effect in SS.

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**Keywords** Atorvastatin · Submandibular gland · Anti-$M_3$ peptide IgG · $PGE_2$ · IL-1$\beta$ · MMP-3

## Introduction

Sjögren's syndrome (SS) is a complex chronic autoimmune disease of unknown aetiology which primarily targets the exocrine glands, resulting in eventual loss of secretory function. The pathophysiology of SS implies the presence of activated epithelial cells in the salivary glands. These cells express major histocompatibility complex (MHC) class-II molecules. Identification of inherited susceptibility markers suggests that environmental or endogenous antigens trigger a self-perpetuating inflammatory response in susceptible individuals. In addition, the continuing presence of active interferon pathways in SS suggests ongoing activation of the innate immune system (Ogawa et al. 2002; Gottenberg et al. 2006). Together, these findings suggest an ongoing interaction between the innate and acquired immune systems in SS.

Damage and/or cell death due to viral infection or other causes may provide triggering antigens to toll-like receptors in (or on) dendritic cells or epithelial cells which, by recognizing pathogen-associated patterns, are activated and begin producing cytokines, chemokines and adhesion molecules. T lymphocytes and B lymphocytes migrate into the gland, so they themselves become activated by dendritic cells and epithelial cells, thereafter acting as antigen-presenting cells (Fox 2005). Expressed antigens include SS-A/Ro, SS-B/La, alpha-fodrin and beta-fodrin or cholinergic muscarinic receptors (Gottenberg et al. 2003). Dendritic cells triggered by immune complexes formed from SS-A and anti-SS-A may propagate the ongoing innate and acquired immune activation.

The pathology of a typically involved salivary gland or lachrymal gland in SS reveals aggregations of lymphocytes that are initially periductal, then panlobular. These cells are primarily CD4 T cells (75 %) and memory cells, with 10 % B cells and immunoglobulin (Ig)-secreting plasma cells. Although individual lobules can be destroyed, salivary gland biopsy samples from patients with SS typically retain 40–50 % of their viable glandular structure. Therefore, inflammatory destruction of salivary glands and lachrymal glands may not fully account for the symptoms of SS (Bacman et al. 1998; Steinfeld et al. 2001; Waterman et al. 2000; Bolstad et al. 2003).

Recent studies focusing on the role of apoptotic mechanisms in the pathogenesis of primary Sjögren's syndrome (pSS) have suggested a neuroendocrine component. Pro-inflammatory cytokines released by epithelial cells and lymphocytes may impair neural release of acetylcholine. In addition, antibodies to acetylcholine muscarinic receptors may interfere with the neural stimulation of local glandular secretion (Bacman et al. 1998), perhaps by interfering with aquaporin (Steinfeld et al. 2001; Reina et al. 2004). Moreover, a recent study reports that $M_3$ muscarinic acetylcholine receptor (mAChR) antibodies may cause autonomic dysfunction in patients with pSS (Reina et al. 2004; 2007; Waterman et al. 2000; Bolstad et al. 2003; Pflugfelder 2004; Ng and Isenberg 2008).

Studies have also focused on the role of apoptotic mechanisms in the pathogenesis of pSS. A defect in Fas-mediated apoptosis (which is necessary for down-regulation of the immune response) can result in chronic inflammatory destruction of the salivary gland, resembling SS (Bolstad et al. 2003).

Owing to these structural and functional changes in lachrymal glands and salivary glands, their aqueous output is diminished. In the eye, tear hyperosmolarity results and is itself a pro-inflammatory stimulus, resulting in an inflammatory cascade on the ocular surface (Pflugfelder 2004) with evidence of immune activation of the conjunctival epithelium and local production of cytokines and metalloproteinases (MMPs). Damage to the corneal epithelium (already vulnerable due to inadequate protection of tear films) ensues, with resultant epithelial erosion and surface irregularity.

Statins are a well-known class of cholesterol-lowering drugs that inhibit the enzyme 3-hydroxyl-3-methylglutaryl coenzyme A reductase (Grundy 1988). They are the class of drugs most widely used for the prevention of primary or secondary coronary heart disease (Corsini et al. 1995; Heerey et al. 2000). The benefits of statin therapy may be ascribed (at least in part) to their actions on non-lipid factors (Rosenson 1999; Koh 2000). For instance, statins inhibit the inflammatory process in the vessel wall, an important feature of atherosclerosis (Solheim et al. 2001). Statins also slow the progression of atherosclerosis by inhibiting monocyte activation, metalloprotease synthesis in the vessel wall and the production of pro-inflammatory cytokines such as interleukin (IL)-1$\beta$ (Solheim et al. 2001) and prostaglandin $E_2$ ($PGE_2$) (Santodomingo-Garzón et al. 2006). Moreover, statins inhibit inflammatory responses in different models of autoimmune disease, such as collagen-induced arthritis, complete Freund's adjuvant-induced arthritis and experimental encephalomyelitis (Leung et al. 2003; Barsante et al. 2005).

Taking into account the anti-inflammatory and immunomodulatory properties of statins, we investigated the anti-inflammatory effect of atorvastatin on the action of anti-$M_3$ peptide IgG from pSS patients on the rat submandibular gland as well as the mechanisms underlying such an effect.

## Materials and methods

### Ethical approval of the study protocol

The study protocol complied with the tenets of the Declaration of Helsinki and accomplished with the rules established by the Ethics Committee of the University of Buenos Aires (Buenos Aires, Argentina). All subjects provided written informed consent.

### Study population

Females (age 35–55 years) were selected from the metropolitan area of Buenos Aires. Subjects were divided into two groups: 20 patients with pSS and 20 healthy volunteers. The diagnosis of pSS followed the $\geq$4 criteria stated by Vitali et al. (1993). Twenty out of 20 patients (100 %) gave a positive biopsy with a score focus of $3.8 \pm 0.07$. Serologic tests were carried out for anti-Ro/SS-A and anti-La/SS-B antibodies, rheumatoid factor (RF) and antinuclear antibodies (ANA) as previously reported (Reina et al. 2010).

### $M_3$ mAChR synthetic peptide

A 25-mer peptide K-R-T-V-P-D-N-Q-C-F-I-Q-F-L-S-N-P-A-V-T-F-G-T-A-I corresponding to the amino-acid sequence of the second extracellular loop of the human $M_3$ mAChR was synthesized by F-moc-amino acids activated using a 1-hydroxybenzotriazole/dicyclohexylcarbodiimide (HOBt/DCC) strategy with an automatic peptide synthesizer (Model 431A; Applied Biosystems, Menlo Park, CA, USA). The peptide was desalted and purified by high-performance liquid chromatography (HPLC). It was then subjected to amino-terminal sequence analysis by

automatic Edman degradation (470 A Sequence; Applied Biosystems).

### Purification of human immunoglobulin G (IgG)

The serum IgG fraction from 20 patients with pSS and 20 normal subjects was isolated by protein G affinity chromatography for protein A and standardized for protein G. Briefly, sera were loaded onto the protein G (Sigma-Aldrich, St. Louis, MO, USA) affinity column equilibrated with 1 M Tris–HCl (pH 8.0). Columns were then washed with 10 volumes of the same buffer. The IgG fraction was eluted with 100 mM glycine–HCl (pH 3.0) and immediately neutralized. IgG concentrations were determined by a radial immunodiffusion assay (9–10 mg/ml).

### Purification of anti-$M_3$ peptide IgG by affinity chromatography

The IgG fraction of 20 patients with pSS and 20 normal subjects was independently subjected to affinity chromatography on the synthesized peptide covalently linked to AffiGel 15 gel (Bio-Rad, Richmond, CA, USA) as described (Reina et al. 2004). Briefly, the IgG fraction was loaded onto the affinity column equilibrated with phosphate-buffered saline (PBS). The non-peptide fraction was first eluted with the same buffer. Specific anti-peptide antibodies were then eluted with 3 M KSCN and 1 M NaCl, followed by immediate extensive dialysis against PBS. The IgG concentration of non-anti-peptide antibodies and specific anti-muscarinic receptor peptide antibodies was determined by a radial immunodiffusion assay. Their immunological reactivity against muscarinic receptor peptides was evaluated by enzyme-linked immunosorbent assay (ELISA). The concentration of the affinity-purified anti-$M_3$ peptide IgG ($1 \times 10^{-7}$ M) that maximally increased the optical density (OD 2.4 ± 0.2) corresponded to a total IgG concentration of $1 \times 10^{-6}$ M (OD 2.2 ± 0.2). The non-anti-$M_3$ peptide IgG fraction eluted from the column showed OD values (0.27 ± 0.06) similar to those of normal IgG (OD 0.25 ± 0.04). The normal IgG fraction purified by affinity column chromatography gave a negative result (OD 0.24 ± 0.03).

### Animals

Male Wistar rats (250–300 g) from the Pharmacologic Bioterium (School of Dentistry, University of Buenos Aires) were used throughout. The animals were housed in standard environmental conditions and fed with a commercial pellet diet and water ad libitum. For surgical removal of submandibular glands, the animals were killed with an overdose of an i.p. ketamine/xylazine mixture (100 and 16 mg/kg, respectively). The experimental protocol followed the Guide to The Care and Use of Experimental Animals (DHEW Publication, NIH 80-23).

### $PGE_2$, MMP-3 and IL-1$\beta$ assays

Slices of submandibular glands (10 mg) from 12 rats/group were incubated for 60 min in 0.50 ml Krebs–Ringer bicarbonate (KRB) gassed with 5 % $CO_2$ in oxygen at 37 °C. IgG was added 30 min before the end of the incubation period. Blockers were added 30 min before the addition of different concentrations of IgG. Glands were then homogenized into a 1.5-ml polypropylene microcentrifuge tube. Thereafter, all procedures employed were those indicated in the protocol of the Prostaglandin $E_2$ Biotrak Enzyme Immunoassay (ELISA) System (GE Healthcare, Piscataway, NJ, USA). All the procedures employed to determine IL-1$\beta$ followed the instructions provided by the ELISA Kit from Cayman (Ann Arbor, MI, USA). For extraction of components of the extracellular matrix, the methods described by Rapraeger et al. (1986) were used with minor modifications. Briefly, samples were mixed with extraction buffer (0.5 M Tris HCl (pH 7.5), 1 % Triton X-100, 10 mM $CaCl_2$, 200 mM NaCl) at a ratio of 1:5 (weight/volume) at 4 °C and homogenized in a glass/glass conical homogenizer. The homogenate was then subject to three freeze–thaw cycles of 5-min each and centrifuged at 13,000 × *g* for 30 min at 4 °C. The detergent-soluble supernatant was recovered and stored at −70 °C for further analyses, and the insoluble pellet fractions discarded. Protein determination was according to the method of Lowry and Thiessen (1950). Detergent extracts of glands were analysed by ELISA to determine the enzymatic activity of MMP-3 according to manufacturer's instructions (Amersham Matrix Metalloproteinase-3 Biotrak Activity Assay System; GE Healthcare, NJ, USA). For cytokine (IL-1$\beta$) (Baturone et al. 2009), prostanoid ($PGE_2$) and metalloproteinase-3 (MMP-3) (Reina et al. 2011a, b) determination in sera of pSS patients, a blood sample was taken from each patient and healthy individuals (controls) and sera were stored at −80 °C until they were tested. Thereafter, all procedures employed for its determinations were those indicated in the protocols of the corresponding Kits (as described above) using 1:5 dilution of serum from each pSS patients or healthy individuals. The results of measurement of IL-1$\beta$, $PGE_2$ and MMP-3 were expressed as picogram per millilitre (pg/ml), nanogram per millilitre (ng/ml) and microgram per millilitre (μg/ml), respectively.

### In vivo atorvastatin treatment in rats

Rats were pre-treated with atorvastatin (30 mg $kg^{-1}$, p.o.) or PBS once a day for three consecutive days. Two hours

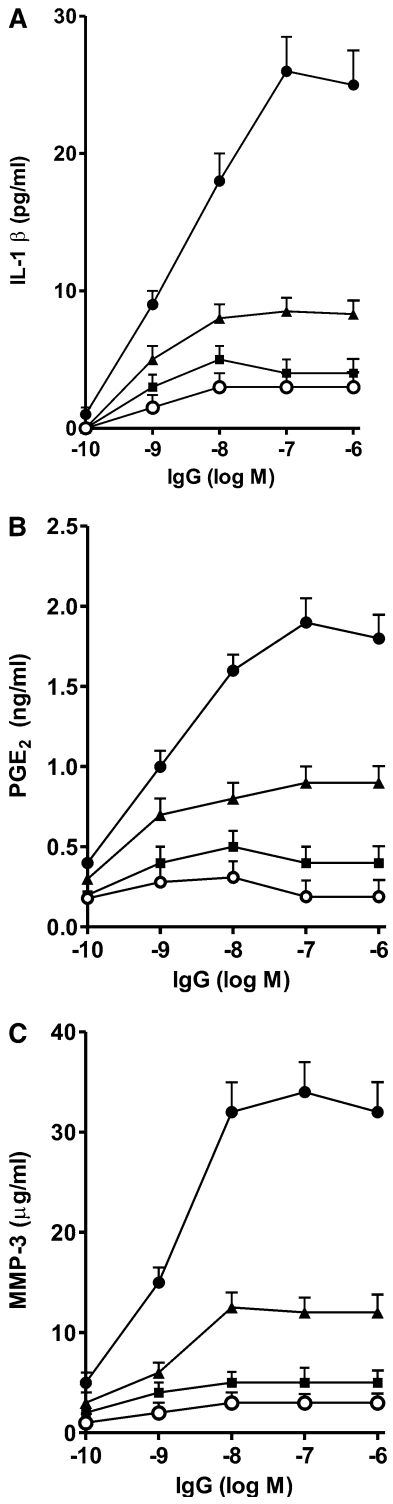



**Fig. 1** Stimulation of production of IL-1$\beta$ (**a**), $PGE_2$ (**b**) and MMP-3 (**c**). Concentration–response curve of anti-$M_3$ mAChR peptide IgG (IgG) from pSS patients alone (*filled circles*) or in the presence of $1 \times 10^{-5}$ M atorvastatin (*filled triangles*) or $1 \times 10^{-5}$ M $M_3$ synthetic peptide (*filled squares*) or normal IgG (*open circles*). Rats submandibular glands were incubated with each concentration of the anti-$M_3$ peptide IgG for 30 min and the concentration of IL-1$\beta$, $PGE_2$ and MMP-3 was assayed as described in "Materials and methods". Values are mean ± SEM of six pools from 20 pSS patients done in duplicate in each group. *$P < 0.001$ versus IgG. **$P < 0.0001$ versus anti-$M_3$ peptide IgG

after the last dose of atorvastatin (Santodomingo-Garzón et al. 2006), rats were killed as described above. The submandibular gland was extracted from the rat (10 rats/group) and was cut it into slices (10 mg) and then was incubated with anti-$M_3$ mAChR peptide IgG in the same manner as for in vitro studies. Then, we measured the production of IL-1$\beta$, $PGE_2$ and MMP-3 using ELISA Kits.

### Drugs

Atorvastatin was purchased from Pfizer (Nueva York, NY, USA) and cyclopentyl-α-hydroxyl-*N*-[1-(4-methyl-3-pentenyl)-4-piperidinyl] benzeneacetamide fumarate (J104129) was purchased from Tocris Cookson Incorporated (Baldwin, MO, USA) were freshly prepared in distilled water; 1-[4,5-bis(4-methoxyphenyl)-2-thiazolyl] carbonyl-4-methylpiperazine hydrochloride (FR-122047) and (5-bromo-2-(4-fluorophenyl)-3-[4-(methylsulfonyl)phenyl]-thiophene) (DuP 697) were diluted in dimethyl-sulfoxide (DMSO). The final concentration of DMSO was not more than 1:1,000 and they lacked pharmacological action. The drugs were diluted in the bath to achieve the final concentration stated in the text. Lyophilized sterile PBS solution containing 0.5 mg/ml of anti-human IL-1 RI neutralizing antibody was from R&D Systems (Minneapolis, MN, USA). Stock solutions were freshly prepared in the corresponding buffers. The drugs were diluted to achieve the final concentrations stated in the text.

### Statistical analyses

The Student's *t* test for unpaired values was used to determine the level of significance. If multiple comparisons were necessary, after analysis of variance, the Student–Newman–Keuls test was applied. Differences between means were considered significant if $P < 0.05$.

## Results

Knowing that the amino-acid sequence of the second extracellular loop of $M_3$ mAChR of rats and humans has strong homology (92 %), we studied the mAChR-mediated effect of autoantibodies from pSS patients on the rat submandibular gland. For this purpose, we used affinity-

purified anti-$M_3$ peptide IgG (IgG) from pSS patients and, as a control, anti-$M_3$ peptide IgG from healthy individuals.

Autoantibody stimulation resulted in the activation of several enzymatic pathways associated with salivary gland $M_3$ mAChR signalling. Figure 1a–c shows the ability of IgG to stimulate the production of IL-1$\beta$, $PGE_2$ and MMP-3, respectively, in a concentration-dependent manner, reaching the maximal capacity of stimulation at $1 \times 10^{-7}$ M. As a control, the anti-$M_3$ peptide IgG fraction from normal subjects and the influence of $M_3$ mAChR antagonist (J104291, $4.5 \times 10^{-9}$ M) were also taken. Normal anti-$M_3$ peptide IgG (Fig. 1a–c) gave negative results, and the $M_3$ mAChR antagonist blunted production of IL-1$\beta$, $PGE_2$ and MMP-3, indicating $M_3$ mAChR participation in those actions (Table 1). A reduction in anti-$M_3$ peptide IgG-stimulated production of IL-1$\beta$, $PGE_2$ and MMP-3 was observed in the presence of $1 \times 10^{-5}$ M atorvastatin or after pre-incubating the anti-$M_3$ peptide IgG with $1 \times 10^{-5}$ M synthetic $M_3$ peptide (Fig. 1a–c).

Figure 2 demonstrates that, under identical experimental conditions, there was a significant correlation between the serum concentration of $PGE_2$ and MMP-3 plotted as a function of IL-1$\beta$ levels in the sera of patients with pSS. These results indicated that activation of $M_3$ mAChR by anti-$M_3$ peptide IgG from pSS patients increased the production of $PGE_2$ and MMP-3, perhaps, as a result of stimulation of IL-1$\beta$.

Pre-treatment of rats with atorvastatin (30 mg $kg^{-1}$, p.o.) in vivo reduced the production of IL-1$\beta$, $PGE_2$ and MMP-3 induced by anti-$M_3$ peptide IgG to about the same level as that elicited by atorvastatin in an ex vivo system (Fig. 3). Again, normal anti-$M_3$ peptide IgG was ineffective in the study system. Rats injected with PBS gave identical results to those obtained with normal IgG (PBS: IL-1$\beta$: $3 \pm 1.8$ pg/ml; $PGE_2$: $0.4 \pm 0.1$ ng/ml; MMP-3: $3.6 \pm 1.1$ μg/ml).

Levels of IL-1$\beta$, $PGE_2$ and MMP-3 in serum from pSS patients were significantly higher ($P < 0.001$) than in normal individuals (control) (Fig. 4a). A positive correlation was observed between serum-level IL-1$\beta$ titres and serum levels of $PGE_2$ (Fig. 4b) and MMP-3 (Fig. 4c) from pSS patients. Also, a positive correlation was observed between serum level $PGE_2$ titres and serum levels of MMP-3 (Fig. 4d). Hence, if the serum level of IL-1$\beta$ was high, high levels of $PGE_2$ and MMP-3 were demonstrated.

To discern which arachidonic acid cascade enzymes are implicated in the inhibitory action of atorvastatin on the production of IL-1$\beta$, $PGE_2$ and MMP-3 by pSS anti-$M_3$ peptide IgG, we used cyclooxygenase (COX) inhibitors of this enzymatic cascade. The inhibition of COX-2 by DuP 697 ($5 \times 10^{-8}$ M) (Gans et al. 1990) and the COX-1 by FR 122047 ($5 \times 10^{-6}$ M) (Jouzeau et al. 1997) reduced the stimulatory effect of pSS anti-$M_3$ peptide IgG on the production of IL-1$\beta$, $PGE_2$ and MMP-3, having similar values as those seen for atorvastatin (Table 2). If J104091 ($M_3$ mAChR antagonist) was present before pSS anti-$M_3$ peptide IgG was added, the increment in the production of IL-1$\beta$, $PGE_2$ and MMP-3 was antagonized, suggesting the participation of the mAChR subtype $M_3$ in this phenomenon. On the other hand, the levels of IL-1$\beta$ were specific as they were blunted by the anti-human IL-1 RI neutralizing

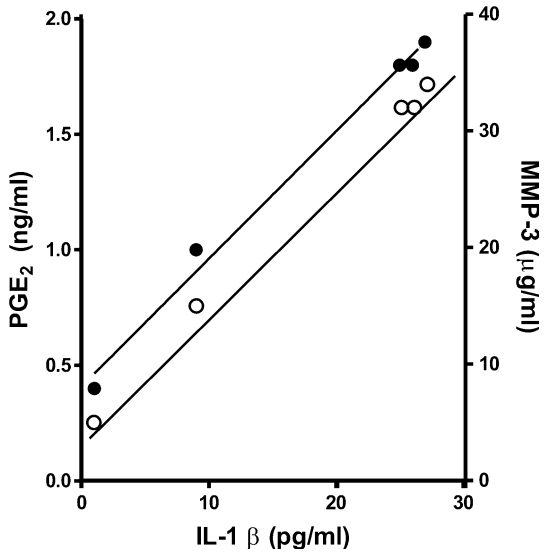


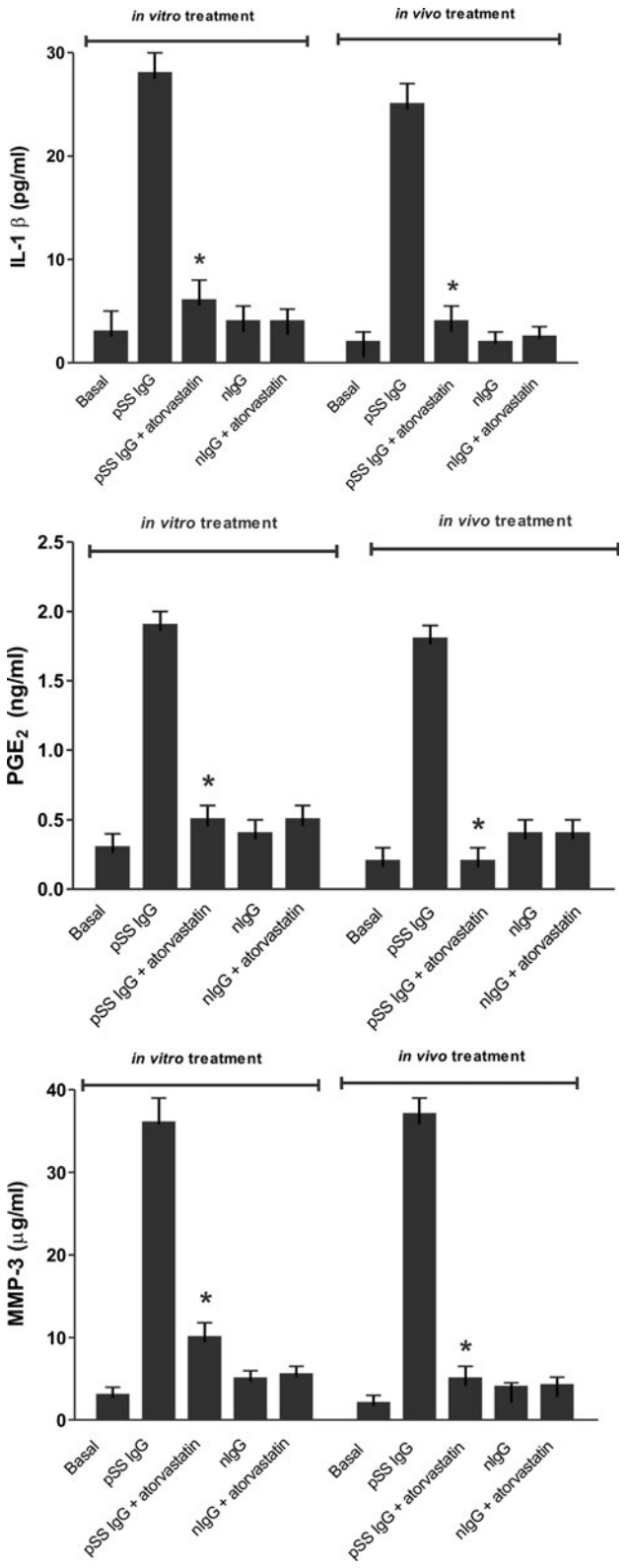

**Fig. 2** Correlation of the serum concentration of $PGE_2$ (*filled circles*) and MMP-3 (*open circles*), plotted as a function of IL-1$\beta$ levels in the sera of patients with pSS (Pearson $r$ 0.9988; $P$ value $P < 0.0001$)

**Table 1** Influence of $M_3$ mAChR antagonists on the production of IL-1$\beta$, $PGE_2$ and MMP-3 by anti-$M_3$ peptide IgG on the rat submandibular gland

| Addition | IL-1$\beta$ (pg/ml) | $PGE_2$ (ng/ml) | MMP-3 (μg/ml) |
|---|---|---|---|
| Basal | $3.4 \pm 0.28$ | $0.36 \pm 0.04$ | $3.8 \pm 0.31$ |
| anti-$M_3$ peptide IgG ($1 \times 10^{-7}$ M) | $27.2 \pm 2.81$* | $1.93 \pm 0.14$* | $36.4 \pm 3.12$* |
| Anti-$M_3$ peptide IgG + J104291 ($4.5 \times 10^{-9}$ M) | $11.2 \pm 1.52$** | $0.51 \pm 0.06$** | $10.3 \pm 0.89$** |
| Normal anti-$M_3$ peptide IgG ($1 \times 10^{-7}$ M) | $3.6 \pm 0.02$ | $0.31 \pm 0.03$ | $3.11 \pm 0.32$ |

Values are mean ± SEM of six experiments done in duplicate in each group

* $P < 0.001$ versus basal; ** $P < 0.0001$ versus anti-$M_3$ peptide IgG




antibody (0.5 ng/ml) and also, the anti-human IL-1 RI neutralizing antibody was able to diminish the production of $PGE_2$ and MMP-3 (Table 2).

**Fig. 3** Effect of atorvastatin in vivo and in vitro on the production of IL-1$\beta$, $PGE_2$ and MMP-3 induced by anti-$M_3$ peptide IgG. Animals were pre-treated for 3 days with atorvastatin (30 mg $kg^{-1}$, p.o.) once a day. The final dose was administered 2 h before anti-$M_3$ peptide IgG was added. Rats submandibular glands were incubated with each concentration of the anti-$M_3$ peptide IgG for 30 min and the concentration of IL-1$\beta$, $PGE_2$ and MMP-3 was assayed as described in "Materials and methods". Results are mean ± SEM of 10 animals per group. *$P < 0.001$ versus pSS IgG

## Discussion

The most common clinical manifestations described in pSS patients are alterations in salivary glands and lachrymal glands. This is characterized by lymphocytic infiltration into the glands with the presence of $M_3$ mAChR antibodies as salivary gland- and lachrymal gland-specific autoantibodies (Sumida et al. 2010; Mandl et al. 2010; Borda and Sterin-Borda 2001). Therefore, the nature of the cholinergic glandular dysfunction may be related to the inflammatory action of anti-$M_3$ peptide IgG (autoantibodies) on the salivary gland cholinergic system (Reina et al. 2007).

Binding of anti-$M_3$ peptide IgG on the $M_3$ mAChR of the submandibular gland involved the release of several inflammatory mediators which were impaired if the gland was in the presence of the $M_3$ mAChR antagonist J104291. This suggested the participation of the $M_3$ mAChR in the submandibular gland in this phenomenon. Immune-mediated xerostomia and xerophthalmia in pSS patients have been described (Nakamura et al. 2006). Here, we demonstrated the possible role of autoantibodies specific for the $M_3$ mAChR in inflammatory responses by the submandibular gland in pSS patients.

Autoantibodies could not only interact on a molecular level with human $M_3$ mAChR synthetic peptide, but also displayed agonistic activity by triggering the production of IL-1$\beta$, $PGE_2$ and MMP-3. Moreover, we observed a significant correlation between the increase in production of IL-1$\beta$ and $PGE_2$ and MMP-3 triggered by autoantibodies. Autoantibodies in the sera of pSS patients behaving as cholinergic agonists could also increase the endogenous amount of IL-1$\beta$, $PGE_2$ and MMP-3 in the sera of these patients. These specific pSS IgG activate glandular cholinergic receptors and maintained inflammation in the submandibular gland. Therefore, anti-$M_3$ mAChR autoantibodies present in the sera of pSS patients might play a part in the pathophysiological mechanisms underlying the inflammatory process described in SS. Not only did anti-$M_3$ peptide IgG behave as cholinergic agonists but they also diminished the reaction of submandibular gland to exogenous pilocarpine (cholinergic agonist), suggesting that although in an early step they were able to activate

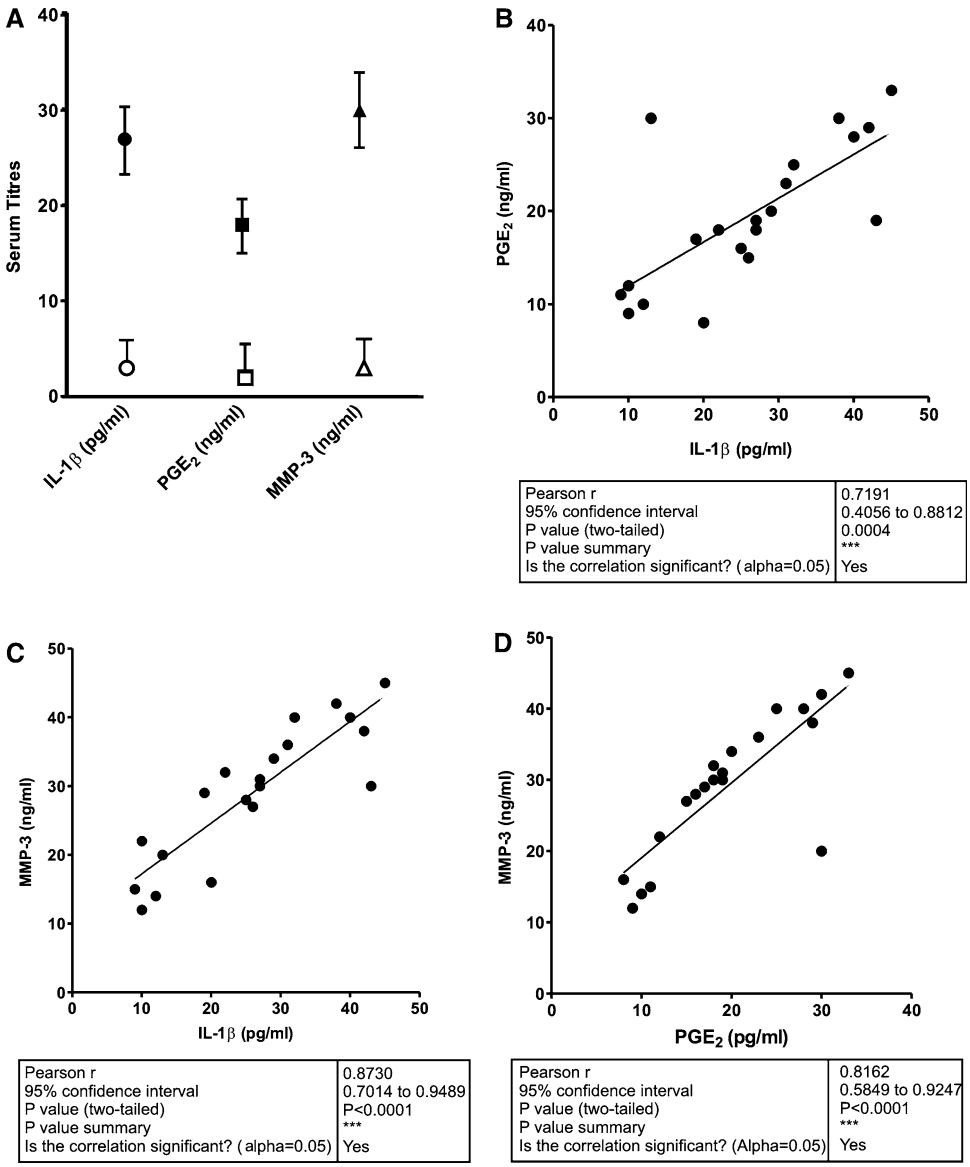



**Fig. 4** Serum titres of IL-1$\beta$, $PGE_2$ and MMP-3 (**a**) in patients with pSS and in healthy individuals. Correlation of $PGE_2$ (**b**), MMP-3 (**c**) serum titres plotted as a function of IL-1$\beta$ serum titres and $PGE_2$ serum titres plotted as a function of MMP-3 (**d**) serum titres are also shown. The results are mean ± SEM of seven pools from 20 pSS patients and 20 healthy individuals done in duplicate for each group. *$P < 0.001$ versus anti-$M_3$ peptide IgG

**Table 2** Influence of COX-1 and COX-2 inhibitors on the production of IL-1$\beta$, $PGE_2$ and MMP-3 by anti-$M_3$ peptide IgG in the rat submandibular gland

| Addition | IL-1$\beta$ (pg/ml) | $PGE_2$ (ng/ml) | MMP-3 (μg/ml) |
|---|---|---|---|
| Basal | 3.3 ± 0.21 | 0.37 ± 0.03 | 3.9 ± 0.35 |
| Anti-$M_3$ peptide IgG ($1 \times 10^{-7}$ M) | 28.4 ± 2.75* | 2.08 ± 0.21* | 35.6 ± 3.41* |
| Anti-$M_3$ peptide IgG + DuP 697 ($5 \times 10^{-8}$ M) | 15.2 ± 2.12** | 0.55 ± 0.91** | 12.4 ± 0.96** |
| Anti-$M_3$ peptide IgG + FR 122047 ($5 \times 10^{-6}$ M) | 18.4 ± 1.51** | 1.74 ± 0.06** | 21.3 ± 1.94** |
| Anti-$M_3$ peptide IgG + $M_3$ synthetic peptide ($1 \times 10^{-5}$ M) | 4.54 ± 0.32** | 0.75 ± 0.06** | 4.5 ± 0.38** |
| Normal anti-$M_3$ peptide IgG ($1 \times 10^{-7}$ M) | 3.6 ± 0.02 | 0.41 ± 0.05 | 3.61 ± 0.37 |
| Anti-$M_3$ peptide IgG + anti-human IL-1 RI neutralizing antibody (0.5 ng/ml) | 6.3 ± 0.07** | 0.79 ± 0.06** | 14.6 ± 1.87** |

Values are mean ± SEM of six independent experiments done in duplicate in each group

* $P < 0.001$ versus basal; ** $P < 0.0001$ versus anti-$M_3$ peptide IgG

glandular mAChR, they might ultimately bind irreversibly to those receptors as was observed previously in other tissues (Goin et al. 1997). All of these experimental data support the hypothesis that chronic interaction of anti-$M_3$ peptide antibody with glandular $M_3$ mAChR by impairing the action of acetylcholine (endogenous cholinergic

agonist), would promote a progressive blockade of glandular parasympathetic function in pSS patients.

Statins produce anti-inflammatory and immunomodulatory effects (Weitz-Schmidt 2002; Blanco-Colio et al. 2003). Hence, we investigated if atorvastatin exerted an anti-inflammatory action on rat submandibular glands in the presence of anti-$M_3$ peptide IgG from pSS patients. In vitro and in vivo treatment over 3 days with atorvastatin inhibited the increase in production of IL-1$\beta$, $PGE_2$ and MMP-3 provoked by anti-$M_3$ mAChR peptide IgG on the submandibular gland. Atorvastatin prevents inflammation by inhibiting the production of pro-inflammatory mediators (Santodomingo-Garzón et al. 2006). In general, IL-1$\beta$ is responsible for the release of prostanoids (Cunha et al. 2005). The anti-inflammatory effects of atorvastatin have been attributed to their capacity to inhibit the production of pro-inflammatory cytokines and $PGE_2$ (Santodomingo-Garzón et al. 2006). This statin has also been shown to inhibit $PGE_2$-induced hypernociception (Sachs et al. 2004; Tonussi and Ferreira 1994).

The mechanism by which atorvastatin inhibited the production of $PGE_2$/MMP-3 provoked by IgG from pSS patients in our model was mediated by COX-2 and COX-1 because it was prevented by specific blockade of these two enzymes. Therefore, activation of the $M_3$ mAChR in the submandibular gland of the rat by the antibody that triggers $PGE_2$/MMP-3 production was prevented by DuP 697. It is important to mention that the action of anti-$M_3$ peptide IgG increased cox-2 mRNA expression by an increase in intracellular calcium concentrations by interacting with glandular $M_3$ mAChR (Reina et al. 2007; Borda et al. 2011). If J104091 ($M_3$ mAChR antagonist) was present before pSS IgG was added, the increment in the production of IL-1$\beta$, $PGE_2$ and MMP-3 was antagonized (Table 1). This reflected the participation of the mAChR subtype $M_3$ in this phenomenon. Altered biosynthesis of $PGE_2$ is clearly involved in neuroinflammatory processes (Cao et al. 1996). Induction of MMP-3 has been detected in inflammatory processes, which is highly destructive and attacks several molecules in the extracellular matrix (Rosenberg 2002). The fact that anti-human IL-1 RI neutralizing antibody blunted the increment of IL-1$\beta$ observed in submandibular gland in the presence of anti-$M_3$ peptide IgG, and as a consequence, also inhibited the production of $PGE_2$ and MMP-3, argue in favour of our hypothesis that atorvastatin acts as an inhibitor of cytokine, prostanoid and metalloproteinase in our study model.

On the basis of the present study, we concluded that the production of IL-1$\beta$, $PGE_2$ and MMP-3 was inhibited by the anti-inflammatory action of atorvastatin. The cholinergic agonistic activity displayed by these autoantibodies may induce desensitization, internalization and/or intracellular degradation via glandular $M_3$ mAChRs stimulation that could lead to inflammation and/or gland destruction.

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**Conflict of interest** The authors deny any conflicts of interest related to this study.

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