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Autoantibodies against muscarinic acetylcholine receptor M_3 in Sjogren's syndrome and corresponding mouse models

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1. ABSTRACT

Muscarinic acetylcholine receptor M_3 (M3R) is a GPCR on exocrine gland cells involved in fluid secretion. In the last two decades, evidence has been accumulated arguing for a role of autoantibodies (aab) against M3R in the development of Sjögren's syndrome (SS). In this review, we provide an updated overview on this issue and critically discuss the relation between autoimmunity to M3R and SS pathogenesis. Clinical data as well as findings from experimental disease were summarized in categories addressing the presence of aab against M3R in SS patients, the function of anti-M3R aab, the association of aab against M3R with SS-related phenotypes, *in vivo* pathogenicity of transferred aab against M3R in mice, and mouse models induced via immunization with M3R. Based on these comprehensive data, we propose a hypothetic model for the role of aab against M3R in the pathogenesis of SS.

2. INTRODUCTION

Sjögren's syndrome (SS) is a chronic autoimmune disease mainly targeting the exocrine glands and leading to xerophthalmia and xerostomia (1), with a prevalence of0.4.-4% and a female-to-male ratio of 9:1 (2;3).This disease can develop as primary SS (pSS) exclusively, or in concert with other autoimmune diseases such as rheumatoid arthritis or systemic lupus erythematosus (secondary Sjogren's syndrome;sSS) (4). A essential role of B cells and autoantibodies in pathogenesis of SS has been suggested by both genetic (5;6) and clinical (5;6) studies. Furthermore, B cells and autoantibodies have also been demonstrated to be indispensable for the disease development in animal models of SS (7;8).SS is characterized by a panel of circulating autoantibodies (aab) including anti-SSA, anti-SSB, anti-M3R, and anti-α fodrin antibodies (9).Due to its functional relation to glandular secretion (10), M3R is of specific interest among the different autoantigens in SS.

M3R is one of the five members of the family of muscarinic receptors which mediate many physiological responses such as smooth muscle contraction, cardiac rate, and glandular secretion (11). M3R is expressed in multiple tissues including exocrine glands, indicating a potential role in the regulation of saliva and tear secretion (11). This view is supported by the observation that mice lacking M3R are unable to produce saliva under the stimulation of pilocarpine, an agonist of M3R (12). In the last two decades, an

accumulated body of evidence indicates the presence of aab against M3R with an antagonistic effect in SS (10;13). Based on these findings, a hypothesis on the pathogenic role of receptor-blocking M3R aab leading to impairment of secretory functions has been proposed (10).

It should be mentioned that a prominent role of M3R-specific auto-reactive T cells in SS pathogenesis has been claimed, as summarized and discussed previously (14). Here, we focus on the current state of knowledge regarding aab against M3R in SS and corresponding mouse models.

3.AUTOANTIBODIES AGAINST M3R IN SS PATIENTS

3.1. Detection of M3R-autoantibodies in patient sera

In 1996, Bacman and coworkers reported for the first time on the presence of aab in sera of SS patients, which were able to bind and activate muscarinic acetylcholine receptors of rat parotids (15). Furthermore, the blocking effect of these antibodies could be reversed by M3R-selective antagonists such as atropine and 4-Diphenylacetoxy-N-methylpiperidine methiodide (4-DAMP) (15). Since M3R is an important receptor regulating glandular secretion, aab against M3R were considered as a promising candidate for the pathogenic principle in SS. Thereafter, investigators applied various methods to detect aab against M3R in sera of SS patients (Table 1).

Given that the M3R is expressed on the cell surface, it appears to be ideal to determine disease associated M3R aab by using cell- or tissue-based methods, which provide conformational epitopes for aab binding. Using indirect immunofluorescence staining, Bacman *et al.* have shown that sera from SS patients, but not sera from healthy subjects, contain IgG with the capacity to bind to rat lacrimal gland acini (16). Furthermore, this binding could be partially inhibited by a synthetic peptide encoding the second extracellular loop (2ndEL) of M4R (16) (the authors considered it as derived from the M3R by mistake (see ref. 24)), suggesting the presence of autoantibodies against different subtypes of muscarinic acethylcholine receptors. Using a flow cytometric assay, Gao *et al.* demonstrated that 60% (3of 5) of pSS and 100% (n=6) of sSS patients revealed IgG with the capability to bind M3R-transfected CHO cells, but no control CHO cells. In contrast, none of the healthy controls (n=11) showed such aab (17). This study clearly demonstrates the presence of aab against M3R in SS patients. These findings were confirmed later by Zou *et al.* by a further cell-based method which is called as In-Cell Western assay using M3R-GFP expressing HEK293 cells (18). Here, sera from 75% (18 of 24) of the pSS patients were positive for anti-M3R IgG, which was significantly higher than the rate found in healthy controls (2 out of 23) (18).

Although cell-based methods are powerful in terms of both, sensitivity and specificity for detecting aab against M3R, they are not suitable as high throughput assays and, thus, not applicable for routine diagnostic purposes. Consequently, efforts have been made to adapt conventional methods like ELISA techniques to the detection of disease-relevant M3R-aabs.

To preserve the naive structure of the protein and its conformational epitopes, Bacman and coworkers developed an ELISA method using cell membrane material as immobilized antigen. Using this approach, they found 85% (17 of 20) of pSS patients and 82.4.% (12 of 17) of sSS patients positive foranti-M3R aab against the coated cell membranes of rat lacrimal gland acini while only 5.7.% (2 of 35) of the healthy controls scored positive (19). Antibody binding was sensitive to a blockade by a synthetic peptide corresponding to the 2ndEL of M4R. Based on these results, the authors claimed that anti-M3R aab represent a novel biomarker of SS, which can be detected using conventional ELISA tests. However, these promising results were not supported by a further study in which an ELISA based on M3R-transfected CHO cells as coated antigen was used. Here, Dawson *et al.* showed that neither sera from pSS patients nor from healthy controls contained IgG fractions, which were able to bind specifically to the immobilized M3R in the cell membranes, although the presence of anti-M3R IgG in pSS patients could be demonstrated by functional tests (20). Furthermore, other conventional methods employing denatured or linear target epitopes including western blot and immunoblotting with cell membrane as antigen also failed to determine aab against M3R (20). Therefore, it is still not clear whether conventional ELISA based on immobilized cell membrane extracts or whole cells as coated antigens are an appropriate strategy for detecting aab against M3R.

The most commonly used method for the recognition of anti-M3R IgG are peptide-based ELISAs. Since the 2ndEL of M3R is indispensable for ligand binding and receptor activation (21), most studies have focused on the use of peptides encoding the 2ndEL of the receptor for antibody detection. In 2005, Marczinovitis *et al.* reported that 77.5.% (31 of 40) of the pSS patients, but none (n=40) of the healthy controls were scored positive for aab against a linear peptide derived from the 2ndEL of M3R (22). Therefore, this approach could represent a promising tool for detecting the biomarker with high specificity and sensitivity. This notion was supported by three studies showing that IgG levels against peptides of

Table 1. Detection of autoantibodies against M3R in SS patients

Cell- or tissue-based assay					
Patients	**Detection methods**	**Frequency in SS patients**	**Frequency in controls**	**Significant**	**Reference**
pSS	Indirect IF staining with rat lacrimal gland acini	positive	negative	ND	(16)
pSS, sSS	Flow cytometric assay with M3R-transfected CHO cell	60% (3/5) in pSS, 100% (6/6) in sSS	0% (0/11)	Yes	(17)
pSS	In-Cell Western assay using M3R-GFP expressing HEK293 cell line	75% (18/24)	8.7.% (2/23)	Yes	(18)
Conventional ELISA					
Patients	**Coated antigen**	**Frequency in SS patients**	**Frequency in controls**	**Significant**	**Reference**
pSS and sSS	Cell membrane from rat lacrimal gland	85% (17/20) in pSS, 70.5.% (12/17) in sSS	5.7.% (2/35)	Yes	(16)
pSS	M3R-transfected CHO cells	0% (0/7)	0% (0/4)	no	(20)
pSS and sSS	Linear 2ndEL peptide (M4R_177-201)	90% (18/20) in pSS, 82.4.% (14/17) in sSS	5.7.% (2/35)	Yes	(16)
pSS and sSS	Linear 2ndEL peptide (M3R_213-237)	9.0.1% (11/122) in pSS, 13.7.% (14/102) in sSS	2.3.4% (3/128)	Yes	(24)
pSS	Linear 2ndEL peptide (M3R_208-230)	77.5.% (31/40)	0% (0/40)	Yes	(22)
pSS and sSS	Linear 2ndEL peptide (M3R_213-237)	14.7.% (11/75) in pSS, 16.3.% (5/32) in sSS	4.8.% (17/349)	no	(28)
pSS and sSS	Linear 2ndEL peptide (M3R_213-237)	54.8.% (23/42)	2.4.% (1/42)	Yes	(25)
pSS and sSS	Linear 2ndEL peptide (M3R_213-237)	7% (5/71)	5.4.% (2/37)	no	(27)
pSS	Linear 2ndEL peptide (M3R_205-220)	56.1.% (83 /148)	9.4.% (4/40)	Yes	(23)
pSS	Linear 2ndEL peptide (M3R_205-230)	1.9.2% (1/52)	3.5.7% (2/56)	no	(26)
pSS	Cyclic 2ndEL peptide (cM3R_205-220)	62.2.% (92 /148)	1.6.% (1/40)	Yes	(23)
pSS and sSS	Cyclic 2ndEL peptide (cM3R_213-237)	5.6.% (4/71)	2.7.% (1/37)	no	(29)
pSS	Cyclic 2ndEL peptide (cM3R_205-230)	1.9.2% (1/52)	3.5.7% (2/56)	no	(26)
pSS and sSS	GST-conjugated linear 2ndEL peptide (GST-M3R_213-228)	92.9.6% (66/71) in pSS, 28.5.7% (4/14) in sSS	0% (0/40)	Yes	(39)
pSS and sSS	GST-conjugated linear 2ndEL peptide (GST-M3R_213-228)	92.9.6% (66/71) in pSS, 28.5.7% (4/14) in sSS	0% (0/40)	Yes	(39)
pSS	GST-conjugated linear 2ndEL peptide (GST-M3R_213-228)	97% (39/40)	0% (0/40)	Yes	(22)
pSS	OVA_323-339 peptide-conjugated linear 2ndEL peptide (OVA_M3R_205-230)	3.84% 2/52	3.57% (2/56)	no	(26)
pSS	OVA_323-339 peptide-conjugatedcyclised 2ndEL peptide ((OVA_cM3R_205-230)	1.92% (1/52)	1.79% (1/56)	no	(26)
pSS and sSS	Streptavidin-coated plate withbiotin-M3R_213-237	9.1.% (3/33) in pSS,0% (0/13) in sSS	2.5% (1/40)	no	(29)
pSS	Streptavidin-coated plate with biotin-M3R_205-230	3.84% (2/52)	1.79% (1/56)	no	(26)
pSS	Streptavidin-coated plate with biotin-cM3R_205-230	5.77% (3/52)	3.57% (2/56)	no	(26)
pSS and sSS	Linear peptides of N-terminus	42.9.% (18/42)	4.8.% (2/42)	Yes	(25)
pSS and sSS	Linear 1stELpeptide (M3R_126-133)	47.6.% (20/42)	7.1.% (3/42)	Yes	(25)
pSS and sSS	Linear 1stELpeptide (M3R_125-134)	5.6.% (4/71)	2.7.% (1/37)	no	(27)
pSS and sSS	Linear 3rdELpeptide (M3R_511-530)	45.2.% (19/42)	2.4.% (1/42)	Yes	(25)

note. ND. not detected, EL. extracellular loop

the 2ndEL of M3R were significantly higher than those found in healthy controls (23-25). However, although using comparable strategies, results from three further studies are in sharp contrast to the above promising findings (26-28).

To improve the efficiency of the peptide-based ELISA, Marczinovitis *et al.* used linear peptides of the 2ndEL of M3R conjugated to GST as coating antigen. This modification significantly increased the sensitivity and specificity of their assay in which

97% (39 of 40) of pSS patients but none (n=31/40) of the controls scored positive for antigen binding (22). However, results of the latter study remain to be confirmed and reproduced. Recently, Chen *et al.* failed to demonstrate binding of IgG from pSS patients to a peptide of the 2ndEL of the M3R conjugated with OVA (26). As an alternative, the M3R-peptide from the 2ndEL was biotinylated and coupled to streptavidin-coated plates. Two studies applied this modified peptide-based ELISA to detect aab against M3R, but no significant difference was observed between SS patients and controls (26;29).

In summary the above results suggest that M3R aab playing a role in Sjögrens syndrome interact with a conformational epitope. Since the binding to conformational epitopes in M3R requires a correct three dimensional structure of the protein, approaches with linear peptides appear to be limited. As cyclic peptides are believed to mimic such conformational epitopes, cyclic peptide-based ELISAs have also been developed to detect aab against M3R. In 2011, He *et al.* reported that sera of 62.2.% (92 of 148) pSS patients but only 1.6.% (1 of 40) of healthy controls reacted positive for IgG directed against the cyclic peptide of the 2ndEL of M3R, demonstrating that the cyclic peptide is a better tool to detect the biomarker for pSS than the linear peptide in terms of both, sensitivity and specificity (23) . However, this finding could not be confirmed in two other independent studies (26;27).

Beside the peptide corresponding to the 2ndEL of M3R, peptides corresponding to other extracellular domains have also been used in peptide-based ELISA for the determination of aab in two studies. It has been reported that elevated levels of IgG binding to linear peptides derived from the N-terminus, the 1stEL, and 3ndEL were present in sera from pSS patients (25). However, these results were not confirmed in a second study (27).

Taken together, cell- or tissue-based methods are able to demonstrate aab against M3R in SS patients, approving their presence in SS. However, the applicability of conventional methods like ELISA for their detection is still under debate and such approaches need to be optimized.

3.2. Function of autoantibodies against M3R in SS

By definition, pathogenic aab have to be functionally related to the pathogenesis of the disease. Bacman *et al.* could demonstrate that IgG isolated from SS patient were able to mimic the effect of carbachol, an agonist of M3R, on isoproterenol-stimulated intracellular cAMP production in rat lacrimal glands. Furthermore, this effect could be inhibited by antagonists of M3R such as atropine or 4-DAMP (15) indicating for the first time that aab against M3R in SS patients are functional. The agonistic effect of SS IgG was confirmed by the same group in a later report by showing that SS IgG-triggered oxide synthase activation in rat lacrimal glands were abrogated by 4-DAMP (19) (Table 2)

Given that Ca^{2+} fluxes induced by M3R activation represents an essential intracellular signal for fluid secretion, many studies have focused on the effect of M3R aab on this process. Li and coworkers preincubated human submandibular gland cell line **(**HSG**)** with SS IgG or control IgG for 12 or 24 hours and determined the carbachol induced Ca^{2+} fluxes. They showed that preincubation with IgG from SS patients decreased the carbachol induced elevation of intracellular free Ca^{2+} as compared to control IgG, suggesting that aab against M3R act as an antagonist in this experimental system (30). This antagonistic effect of SS IgG on M3R agonist-evoked Ca^{2+}fluxes has been confirmed by many studies (31-33). Furthermore, monoclonal antibodies against M3R have been generated (33;34)exerting an antagonistic effect on M3R function. Interestingly, the antagonistic effect of SS-related IgG correlated positively with the time the cells were exposed to the IgG before the M3R-stimulus (i.e. carbachol) was added (20;31), which suggests an antibody-mediated internalization of M3R (31).

The antagonistic effect of anti-M3R IgG on M3R agonist-evoked Ca^{2+} fluxes in glandular cells support the hypothesis that the aab block the activation of the receptor and, as a consequence, impair the secretion of saliva and tears. This notion encouraged investigators to further define the relevant epitopes on the M3R. Based on the reactivity of SS-derived IgG to different synthetic peptides covering four extracellular domains of M3R, namely N-terminus, 1stEl, 2ndEL and 3rdEL, Tsuboi *et al.* categorized IgG from SS patients into different subgroups and tested corresponding IgG-effects on cevimeline hydrochloride-induced Ca^{2+}influx in HSG cells. Cevimeline is a M3R agonist. They could demonstrate that SS-derived IgG fractions directed against N-terminusor 1stEL enhanced agonist-induced Ca^{2+} fluxes while fractions reacting to the 2ndEL of M3R inhibited this function. However, SS-derived IgG directed against the 3rdEL did not modulate agonist-evoked Ca^{2+} fluxes (25),suggesting that aab against M3R with antagonistic effect recognizes the 2ndEL of M3R. The notion is supported by the fact that monoclonal antibodies against the 2ndEL of M3R have a similar antagonistic effect like the corresponding reactive fraction from patient sera (33;34). A different strategy to identify the relevant functional epitopes in M3R was applied by Koo *et al.* In this study, synthetic peptides encoding for four extracellular domains of the receptor were generated and exposed to SS-derived IgG. By analyzing the effect of that IgG on M3R

Table 2. Function of autoantibodies against M3R

Tested IgG	Functional assay	Effect of tested IgG	Reference
pSS IgG	Isoproterenol-stimulated intracellular cAMP production in parotid glands	Decreasing the cAMP production (agonistic effect)	(15)
pSS IgG	Oxide synthase (NOS) activity in rat lacrimal gland acini	Increasing the NOS activity (agonistic effect)	(16)
pSS IgG	Carbachol-evoked Ca^{2+} influx in mosue salivary gland acinar cells	no effect	(20)
pSS IgG and sSS IgG	Carbachol-evoked Ca^{2+} influx in HSG cell line	Inhibiting the Ca^{2+} influx (antagonistic effect)	(30)
pSS IgG and sSS IgG	Carbachol-evoked Ca^{2+} influx in HSG cell line	Inhibiting the Ca^{2+} influx (antagonistic effect)	(32)
pSS IgG	Carbachol-evoked Ca^{2+} influx in human salivary gland acinar cells	Inhibiting the Ca^{2+} influx (antagonistic effect)	(31)
pSS IgG	Carbachol-evoked Ca^{2+} influx in HSG cell line	Inhibiting the Ca^{2+} influx (antagonistic effect)	(33)
monoclonal antibodies against M3R_213-237	Cevimeline hydrochloride-induced Ca^{2+} influx in HSG cell line	Inhibiting the Ca^{2+} influx (antagonistic effect)	(34)
SS IgG positive for Nter of M3R	Cevimeline hydrochloride-inducedCa^{2+} influx in HSG cell line	Increasing the Ca^{2+} influx (agonist effect)	(25)
SS IgG positive for 1st EL of M3R	Cevimeline hydrochloride-inducedCa^{2+} influx in HSG cell line	Increasingthe Ca^{2+} influx (agonist effect)	(25)
SS IgG positive for2ndEL of M3R	Cevimeline hydrochloride-inducedCa^{2+} influx in HSG cell line	Decreasingthe Ca^{2+} influx (antagonistic effect)	(25)
SS IgG positive for3rd EL of M3R	Cevimeline hydrochloride-inducedCa^{2+} influx in HSG cell line	no effect	(25)
pSS IgG and sSS IgG	Pilocarpine-induced AQP-5 expression in rat parotid acinar cells	Inhibiting AQP5 trafficking (antagonistic effect)	(30)
pSS IgG	Carbachol-induced trafficking of AQP5 in AQP5-transfected HSG cell line	Inhibiting AQP5 traffiking (antagonistic effect)	(33)
pSS IgG and sSS IgG	Pilocarpine-induced AQP-5 expression in rat parotid acinar cells	Inhibiting AQP5 trafficking (antagonistic effect)	(30)
pSS IgG and sSS IgG	Carbachol-evoked mouse baldder contraction	Inhibiting the contraction (antagonistic effect)	(35)
pSS IgG and sSS IgG	mouse baldder contraction	acute agonist effect	(35)
pSS IgG and sSS IgG	Nerve-evoked mouse baldder contraction	Inhibiting the contraction (antagonistic effect)	(35)
rabbit anti-M3R_213-237 IgG	Carbachol-evoked colon longitudinal smooth muscle contraction	Inhibiting the contraction (antagonistic effect)	(37)
pSS IgG and sSS IgG	Nerve-evoked contraction of smooth muscle throughout gastrointestinal tract	Inhibiting the contraction (antagonistic effect)	(36)

agonist-evoked Ca^{2+} fluxes (32) these authors could show that only synthetic peptides corresponding to the 3rdEL of M3R were able to abrogate the antagonistic effect of SS-derived IgG (32), raising the possibility that the 3rdEL of M3R contains an epitope targeted by SS-pathogenic aab.

Beside changes in intracellular free calcium concentrations, activation of M3R also induces trafficking of aquaporin 5 (AQP-5) to the plasma membrane, which causes a rapid transport of water and thus contributes to fluid secretion. Li *et al.* have demonstrated that SS-derived IgG fractions are able to reduce the pilocarpine-induced AQP-5 trafficking in rat parotid acinar cells (30), suggesting an antagonistic effect on this M3R mediated glandular activation process. The above antagonistic action of SS-derived IgG on AQP-5 trafficking was confirmed by Lee and coworkers by using an AQP5-transfected HSG cell line (33). The effectsof SS IgG on AQP-5 trafficking thus demonstrated provide additional evidence for the hypothesis that anti-M3R aab attenuate secretion of saliva and tears via interference with physiological M3R activation.

Table 3. Association of autoantibodies against M3R with SS-related phenotypes

Study	Tsuboi *et al.* 2010 (25)	He *et al.* 2011 (23)	Kovacs *et al.* 2005 (39)	Jayakanthan *et al.* 2016 (38)	Zou *et al.* 2016 (18)
Tested IgG	IgG against all M3R extracellular peptides	IgG against cyclic M3R_213-237	IgG against GST-M3R_213-228	IgG against cyclic M3R_213-237	IgG against naive M3R
Number of SS patients	56 (pos. n=28, neg. n=28)	148 (pos. n=92, neg. n=56)	73 (pos. n=66, neg. n=7)	43 (pos. n=19, neg. n=24)	24 (pos. n=18, neg. n=6)
Disease duration	N.A.	n.s.	n.s.	N.A.	ND
Salivary flow rate	n.s.	ND	ND	ND	N.A.
Tears flow rate	n.s.	ND	ND	ND	ND
Extraglandular organ manifestation	n.s.	n.s.	P.A.	n.s.	ND
ESSDAI score	ND	P.A.	ND	n.s.	ND
Histopathology	n.s.	ND	ND	n.s.	P.A.
Anti-SSA	P.A.	n.s.	n.s.	n.s.	P.A.
Anti-SSB	n.s.	n.s.	n.s.	n.s.	n.s.
Rheumatoid factor	n.s.	P.A.	ND	n.s.	ND
ANA	ND	P.A.	n.s.	n.s.	n.s.
IgG concerntration	P.A.	n.s.	ND	P.A.	ND
Leucopenia	ND	P.A.	P.A.	ND	ND
Anaemia	ND	P.A.	n.s.	ND	ND
Thromocytopenia	ND	P.A.	ND	ND	ND

note. ND. not detected, P.A. positive association, N.A. negative association, n.s. not significant

Since the M3R is also expressed on smooth muscle cells and regulates muscle contraction, some functional tests have been performed with regard to this aspect. In 2000, Waterman *et al.* demonstrated that SS-derived IgG was able to inhibit both carbachol- and nerve-evoked mouse bladder contraction (35), suggesting that these aab can have an antagonistic effect on M3R mediated muscle contraction. Consistent with this, Park *et al.* demonstrated an antagonistic effect of SS IgG on nerve-evoked contraction of smooth muscle throughout the gastrointestinal tract (36). Furthermore, Cavill *et al.* reported that IgG raised in rabbits against a peptide of the 2nd EL of human M3R was able to inhibit directly carbachol-induced smooth muscle contraction of murine colon (37), confirming the antagonistic potency of anti-M3R antibodies. Thus, SS-derived IgG or anti-M3R IgG from immunized rodents have exhibited consistently antagonistic effects on smooth muscle contraction mediated by agonist-activation of M3R. However, when such experiments were performed in absence of M3R agonist, SS-derived IgG exhibited an acute agonistic effect on smooth muscle contraction (35).

In summary, results of a variety of functional assays demonstrate that aab against M3R in SS sera show an antagonistic effect on agonist-induced M3R activation. Moreover, in the absence of M3R agonists, aab against M3R display an acute agonist effect on M3R activation (Table 2). It must be doubted, however that the later effect plays a role in the disease, given that *in vivo* M3R receptors are constantly exposed to the natural ligand in a pulsatile fashion.

3.3. Association of autoantibodies against M3R with SS-related clinical and immunological phenotypes

Correlations between the presence of antagonistic M3R IgG and disease activity strongly suggests a pathogenic role of these aab in SS. Therefore, many studies have been performed to analyze the association between M3R aab and corresponding clinical and immunological phenotypes of SS.

In general, SS-related disease phenotypes encompassing disease duration, salivary flow rate, tears flow rate, extra-glandular involvements, histopathology, score of disease activity, histopathology, anti-SSA, anti-SSB, rheumatoid factor (RF), antinuclear antibodies (ANA),serum IgG concentration, leucopenia, anemia and thromocytopenia (18;23;25;38;39) were all correlated with the reactivity IgG from patient sera with M3R peptides (23;25;38;39) or native M3R (18) (Table 3).Among the different clinical parameters, disease duration (25;38)and salivary flow rate (18) have been

Table 4. Effect of transferred anti-M3R antibodies *in vivo*

Transferred IgG	Epitope	Recipient mice	Effect	Reference
pSS IgG with anti-M3R activity	unknown	NOD-*scid*, C57BL/6-*scid*	decrease the salivary production	(7)
F (ab)2 of pSS IgG with anti-M3R activity	unknown	NOD-*scid*	decrease the salivary production	(7)
mouse anti-hM3R IgM monoclonal antibodies	conformational epitope within the extracellular domain	NOD-*scid*, C57BL/6-*scid*	decrease the salivary production	(41)
mouse IgG with anti-M3R activity	linear or cyclic pepitde encoding the 2ndEL of M3R	Balb/c	no effect onsalivary or tears production	(26)

Table 5. Mouse models of SS induced by immunization with M3R

Study	Lizuka *et al.* 2010 (42)	Chen *et al.* 2016 (26)
Immunized antigen	Six peptides encoding extracellular domains of M3R	OVA peptide conjugated linear or cyclic peptide of the 2ndEL of M3R
Immunized mice	M3r-/- mice with C57BL/6 backgroud	Balb/c
Reciepient mice	Rag-/- mice with C57BL/6 backgroud	Not detected
Autoantibodies against immunized peptide of M3R	Detected	Detected
Autoantibodies against conformation epitopes of M3R	Not detected	Not detected
Lymphocytic infiltration in lacrimal glands	Not detected	Not detected
Lymphocytic infiltration in salivary glands	Detected	Not detected
Pilocarpine induced saliva production	Detected	not affected
Pilocarpine induced tears production	Not detected	not affected

shown to correlate negatively with the presence of aab against M3R, while the European league against rheumatism (EULAR) Sjögren's syndrome disease activity index (23) and extraglandular organ manifestation (39) have been shown to be positively associated. Notably, such associations were not consistently found throughout all studies. Reasons for these discrepant results may be heterogeneity of the detected aab with regard to their target epitopes among different studies or a low number of patients in some studies, which limits the power of statistics.

4. AUTOANTIBODIES AGAINST M3R IN MOUSE MODELS OF SS

Mouse models are powerful research tools for investigating the pathogenesis of human diseases as well as for searching for novel therapeutic targets (40). In the meanwhile, mouse models themselves are accepted as a proof for the pathogenicity of autoantibodies or autoantigens. For example, many of these models are based on an active immunization with homologous or heterologous autoantigens (40) demonstrating their pathogenic relevance. In addition, in some mouse models the pathogenicity of aab could be demonstrated by their passive transfer to healthy animals. To investigate the role of M3R in SS, both, passive transfer of aab against M3R and immunization with M3R, have been performed in mice (Tables 4 and 5).

4.1. Passive antibody transfer models

In 1998, Robinson and coworkers investigated for the first time the pathogenicity of pSS IgG by transferring SS IgG to NOD-scid and C57BL/6-scid mice (7). They demonstrated that both pSS IgG and F $(ab)_2$ of pSS IgG were able to decrease the salivary production induced by pilocarpine together with isoproterenol which activate M3R and β-adrenergic receptor, respectively (7). Since the transferred pSS IgG were able to inhibit the binding of M3R agonist which was demonstrated by radioligand binding assay (15), these results indicated a pathogenic role of anti-M3R aab in the impairment of a production of saliva. Two years later, the same group verified their hypothesis by transferring monoclonal antibodies against conformational epitopes of M3R into mice (41). They described two anti-M3R IgM monoclonal antibodies capable of binding to the extracellular surface domain of M3R-expressing COS-7 cells which decreased the pilocarpine-induced production of saliva and inhibit the membrane-associated expression of AQP-5 (41). In addition, most recently Chen *et al.* investigated whether aab against M3R recognizing linear epitopes are pathogenic *in vivo* (26).In these

studies, transfer of aab derived from mice immunized with linear or cyclic peptides encoding for the 2ndEL of M3Rto Balb/c mice revealed no inhibitory effect on pilocarpine-induced saliva or tears production in the recipient animals (26). These results suggest that aab against linear epitope within the 2ndEL of M3R may not be pathogenic with regard to impairment of secretion function of exocrine glands.

Taken together, experimental evidence from passive transfer-induced mouse models conforms to the above notion that SS-pathogenic aab target conformational epitopes associated with M3R activation/inactivation, while linear epitopes seem to be apathogenic with respect to the SS disease phenotypes (Table 4).

4.2. Mouse models of pSS induced by immunization with M3R

In 2010, Lizuka and coworkers established a novel mouse model for pSS via immunization with peptides covering extracellular domains of M3R (42). To overcome the immunotolerance to the self-antigen, M3R-/- mice were immunized with murine M3R peptides and splenocytes of the immunized mice were subsequently transferred into $Rag1^{-/-}$ mice. 45days after inoculation of splenocytes, $Rag1^{-/-}$ mice developed major pSS-like features, including lymphocyte infiltration into exocrine glands and impaired production of saliva (42).As expected, the $Rag^{-/-}$ mice generated antibodies against the immunized M3R peptide after splenocyte transfer, but whether the aab thus induced were causal in the SS-related disease phenotype of the acceptor animals or even directed against conformational epitopes of the receptor remained unclear. Moreover, induction of a pSS-like disease in $Rag1^{-/-}$ mice by transfer of purified CD3 T cells derived from immunized $M3R^{-/-}$ mice, strongly argues for an essential pathogenic role of M3R-reactive T cells in this novel pSS mouse model.

A novel approach to induce aab against M3R in mice and to investigate their role *in vivo* was applied by Chen *et al*. (26). The authors immunized Balb/c mice with a synthetic peptide comprising a linear or cyclic peptide encoding for the 2ndEL of M3R anda peptide sequence derived from ovalbumin ($OVA^{323-339}$),which provides a strong T cell epitope recognized by the H2-d haplotype. As expected, these mice produced high titer of aabdirected against the linear or cyclic peptides encoding the 2ndEL of M3R. The antibodies displayed a weak capacityof binding to freshly isolated salivary gland cells. However, as compared to controls, the pilocarpine-induced secretion of saliva and tears was not impaired in the immunized mice, suggesting that the antibodies thus induced were not pathogenic (26).

Taken together, at least one study indicated that the induction of an autoimmune response against M3R can result in the development of a SS-like disease. However, other studies did not provide evidence for a pathogenic role of aab against M3R in the disorder.

5. CONCLUSION AND PERSPECTIVES

In summary, studies from the last two decades regarding the role of aab against M3R in SS provide the following insights:

1. Aab against M3R are present in SS patients, but their detection is technically demanding.
2. Aab against M3R in SS are functional. They reveal antagonistic properties on agonist-mediated M3R activation and, possibly, in addition agonistic effects on the M3R in the absence of a bona fideM3R agonist.
3. The presence of aab against M3R in SS is associated with some clinical or immunological features of the disease.
4. Passive transfer of aab recognizing conformational epitopes, but not linear epitopes, into mice can impair the pilocarpine-induced secretion of saliva (i. e. elicit one symptom of SS).
5. An active immune response to M3R can lead to a SS-like disease, but these animal model fail to provide any evidence for a pathogenic role of aab against M3R in the disorder.

Results of the different studies discussed here support in part the hypothesis of a pathogenic role of aab against M3R in SS, which antagonize the secretion of saliva and tears. Although this hypothesis is promising and straightforward, some abovementioned findings need to be strengthened in future studies. First, although cell-based assays have shown that aab against M3R are present in SS patients, the number of patient samples analyzed in these assays was relative small and needs to be increased to provide more convincing evidence. Second, associations between M3R-IgG in patients and SS-related clinical and immunological phenotypes are not consistent among different studies. Future approaches on this issue will require optimized experimental settings, e.g. by evaluating in large cohorts the association of aab against specific conformational epitopes of M3Rwith SS-related phenotypes. Third, in the models where disease was induced by active immunization, no evidence for a pathogenic role of aab against M3Rhas been shown so far. One reason for this might be that immunized mice did not generate aab against conformational epitopes of M3R. Therefore, this issue needs to be addressed by new mouse models in which it is ascertained that M3R immunization entails production aab capable of antigonising M3R activation. Finally, previous reports suggest that M3R

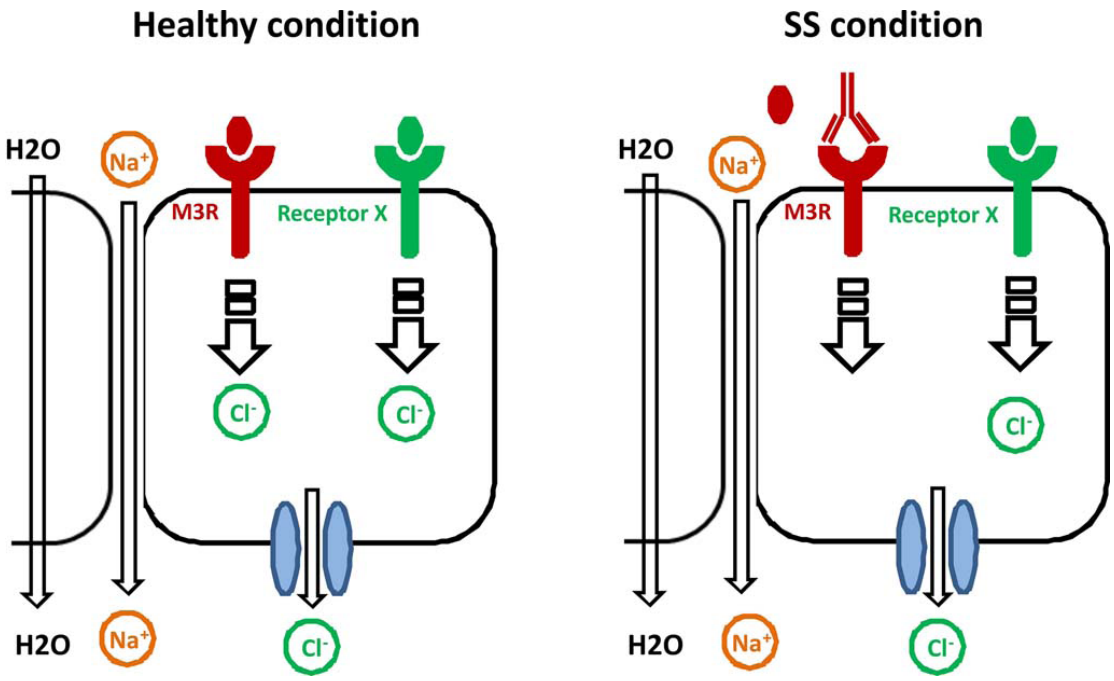

Figure 1. A simplified model of the role of anti-M3R autoantibodies in control of fluid secretion in acinar cells of exocrine glands. Under normal condition (left panel), fluid secretion is regulated by M3R and other receptors (depicted as receptor X). Activation of a specific receptor alone or several different receptors initiate their corresponding signaling pathways and consequently activate the apical membrane Cl^-channel.The efflux of Cl^-ions draws Na^+ions across the cells, resulting in fluid secretion. Undercondition of SS disease, autoantbodies against M3R with antagonistic activity block M3R activation-mediated fluid secretion.When this effect cannot be compensated via other secretion-regulating mechanisms, a pathologicalimpairment of the secretory function is observed.

aab could serve as promising diagnostic biomarker for SS. However, in terms of sensitivity and specificity, the outcomes from different studies were inconsistent. A major reason for the discrepancy of results is the diversity of methods used for antibody determination. As a consequence, standardized high throughput methods, which allow the detection of the potential biomarker with good sensitivity and specificity, need to be developed.

Notably, $M3R^{-/-}$ mice are unable to produce saliva when challenged pilocarpine while the response to isoproterenol (β-adrenergic agonist) is not impaired (12). This observation suggests that exocrine secretion is regulated beside M3R by multiple further mechanisms. Furthermore, $M3R^{-/-}$ mice are normal in terms of basal level of saliva secretion and are free of dry mouth-related complications, suggesting that M3R activation indispensable for controlling the basal exocrine secretion. These important findings from $M3R^{-/-}$ mice need to be taken into account when a pathogenic role of aab against M3R in SS is suggested. Here, we propose a novel model explaining the role of aab against M3R in the pathogenesis of SS (Figure 1). According to our new hypothesis, fluid secretion of acinar cells of exocrine glands is regulated by multiple receptors, including M3R and others. Activation of a specific receptor alone or of several different receptors initiates corresponding signaling pathways and consequently activates the apical membrane Cl^- channel. To maintain electro-neutrality, the efflux of Cl^- ions into the lumen draws Na^+ ions across the cells, resulting in fluid secretion. Under the SS condition, aab against M3R with antagonistic activity block M3R activation-mediated fluid secretion. However, whether aab against M3R lead to pathological impairment of exocrine secretion, such as dry eyes and dry mouth, is largely dependent on whether the anti-M3R autoantibody-mediated decrease in secretion can be compensated by other mechanisms regulating secretion. When the latter mechanism (s) are also somehow impaired ,e.g. by other aab or factors other than antibodies, then aab against anti-M3R mediated decrease in secretion will cause pathological symptoms.

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