# The Efficacy and Safety of Bone Marrow-Derived Cells Extract in the Management of Sjögren's-like Disease in NOD Mouse Model

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## Abstract

**The Efficacy and Safety of Bone Marrow-Derived Cells Extract in the Management of Sjögren's-like Disease in NOD Mouse Model**

Sjögren's syndrome (SS) is a common, chronic, and debilitating autoimmune diseases, affecting nearly 4 million North Americans. It affects females approximately 14 times higher than males, most of which are postmenopausal women. It is characterized by exocrine glands lymphocytic infiltration and glandular destruction. SS is a slowly progressing disease that starts decades before the patients experience any symptoms. Patients will experience dry eyes and mouth, and a wide range of extra-glandular manifestations, including fatigue, arthritis, arthralgia, pulmonary, neurological, cutaneous, and muscular manifestations, and lymphoma. The generalized manifestations lead to a significantly compromised quality of life. The pathogenesis is complex and still not fully understood due to the difficulty of studying the disease in humans and the ambiguity of its etiology. Unfortunately, no cure has been found yet. The current management is directed toward dryness symptoms palliation, prevention of further complications, and when serious systemic conditions emerge, immunosuppressive agents are indicated.

The aim of this PhD thesis is to test a new treatment modality that combines the characteristics of cell and biologic therapies. The risks associated with both treatments complicate the clinical condition of significantly compromised patients. Therefore, we employed the success of BM-derived cells in managing SS in the form of a biologic therapy, by extracting their proteins and using it as a potential SS therapy. We tested the ability of bone marrow BM-derived cells extract in decreasing inflammation and promoting trophic changes in the glandular tissue in the NOD mouse model, when injected before SS immune dysregulation intensifies.

First, we tested the effectiveness of BM-derived mesenchymal stromal cells extract (MSCsE) versus BM-derived mesenchymal stromal cells in preventing or alleviating the symptoms of SS. The treated groups (MSCs and MSCsE) showed comparable positive results regarding these treatments efficacy in preserving the salivary and lacrimal flow rates. This preservation was the

outcome of maintained cell proliferation and specialized cell subpopulations and genes associated with regeneration. Additionally, SS immune dysregulation was, somewhat, managed via the elevation of IL-10 and suppression of B cells.

Second, we assessed the effectiveness of BM cell extract (BMCE) in hindering SS immune dysregulation in NOD mice and we evaluated its systemic safety. The treated mice showed comparable results to those of MSCsE and MSCs; suggesting a common and/or possibly different, proteins composition in both therapies, that might have led to the recorded regenerative outcomes. Additionally, we tested the systemic safety of BMCE as an example of the cell extract concept. Surprisingly, the BMCE-treated NOD mice showed no difference in comparison to the control (saline-treated) NOD mice and the non-SS and non-treated control mouse model, ICR

In conclusion, BM-derived cells extract treatment has shown promising results in managing SS, and we may recommend the use of BMCE and MSCsE as potential treatment modality for patients. They exerted immune regulatory and trophic effects to injured exocrine tissues. Additionally, the various risks associated with biologic and cellular therapies are minimal when using the extract. Biologic therapies are associated with opportunistic infections due to a complete interference with cytokines or blocking B cells as strategies to manage SS; however, this does not apply to cell extracts. Additionally, cellular therapies require myeloablative conditioning and their use is complicated with Graft versus Host Disease (GvHD) and malignancy. However, using the cell extract did not lead to complications in our mice after a follow up period of 16 weeks and no prior preparative procedures were required. Yet, we may not exclude possible changes which might appear with longer in vivo follow up periods.

**Résumé**

**Vérification de l'efficacité et l'innocuité d'extrait de cellules dérivées de la moelle osseuse dans le modèle de souris NOD pour la gestion de maladie semblable au Syndrome de Sjogren.**

Le syndrome de Sjögren (SS) est l'une des maladies auto-immunes les plus courantes, touchant près de 4 millions de Nord-Américains. Le syndrome cible 14 fois plus les femmes que les hommes et en majorité affecte les femmes ménopausées. Le syndrome se caractérise par une infiltration et attaque des lymphocytes dans les glandes exocrines. Le SS progresse lentement et débute des décennies avant la présentation des symptômes. Lors du diagnostic les patients présentent une sécheresse oculaire et buccale, ainsi que des manifestations non glandulaires. Ces manifestations peuvent inclure, la fatigue, l'arthrite, l'arthralgie, des maux musculaires, pulmonaires, neurologiques, cutanées, ainsi qu'un lymphome. Ces manifestations diminuent la qualité de vie du patient. La pathogénie est encore mal comprise en raison de la difficulté d'étudier la maladie et de l'ambiguïté de son étiologie. À ce jour, il n'existe aucun traitement. La prise en charge actuelle vise à pallier les symptômes de la sécheresse, à prévenir leurs complications et, en cas systémiques graves, utiliser des immunosuppresseurs.

Cette thèse vise à tester un traitement qui combine des thérapies cellulaires et biologiques. Les risques associés à ces dernières pour les patients cliniquement compromis sont élevés. Par conséquent, nous tirons du succès des cellules dérivées de la moelle osseuse dans la gestion du SS. Sous forme de thérapie biologique, des protéines extraites des cellules de la moelle ont été testées. Nous avons évalué la capacité de l'extrait de cellules souches de la moelle osseuse à réduire l'inflammation dans les glandes de la souris NOD. Ce modèle animal développant au cours de sa vie des symptômes du SS, nous avons effectué des injections précédant le dérèglement immunitaire du SS.

En premier lieu, nous avons testé l'efficacité de l'extrait des cellules de souche mésenchymateuse de la moelle osseuse (MSCsE) et l'efficacité des cellules complètes de souche mésenchymateuse de la moelle osseuse (MSC) pour pallier les symptômes du SS. Les groupes traités (MSC et

MSCsE) aident à préserver les débits salivaires et lacrymaux et il y a des taux comparables entre ces deux groupes. La prolifération cellulaire, une certaine sous-population de cellule, des gènes associés à la régénération, une élévation de l'IL-10 et la suppression des lymphocytes B semblent tous contribuer à l'amélioration.

Ensuite, nous avons évalué l'efficacité de l'extrait de cellules BM (BMCE) pour empêcher le dérèglement immunitaire du SS chez les souris NOD. Les souris traitées montrent des résultats comparables à ceux du MSCsE et des MSC; suggérant une composition de protéines commune ou différente entre les deux thérapies, qui pourrait avoir mené à la régénération. De plus, nous avons testé la sécurité systémique de BMCE. Étonnamment, les souris NOD + BMCE ne démontrent aucune différence par rapport aux souris NOD de contrôle (traitées à la saline) et au modèle de souris de contrôle non-SS et non traitées, ICR.

Nous recommandons l'utilisation du BMCE et MSCsE en tant que modalités de traitement potentielles pour les patients atteints de SS. Elles offrent des effets immunitaires régulateurs et trophiques aux tissus exocrines lésés. De plus, l'extrait minimise les risques liés aux thérapies biologiques et cellulaires. Celles-ci sont associées aux infections opportunistes en raison de l'immunosuppression par l'interférence complète des cytokines ou du blocage des cellules B. Avec l'extrait biologique non cellulaire, il y a un moins grand risque d'infection. D'autre part, les thérapies cellulaires nécessitent un conditionnement myéloablatif, avec des risques de rejet du tissu et de formation de tumeurs. Comparativement, l'extrait ne cause pas de complications chez nos souris après une période de 16 semaines et aucune préparation préalable n'a été requise. Cependant, nous ne pouvons pas exclure les changements qui pourraient apparaître avec un suivi in vivo à plus long terme.

The following hypothesis were investigated in this thesis:

**Hypothesis 1**

Mesenchymal stromal cells extract (MSCsE) and mesenchymal stromal cells (MSCs) have comparable efficacy in the management of Sjögren's-like disease in NOD female mice.

**Rationale**

SS is a complex autoimmune disease that affects 0.1-0.72% of the populations worldwide with a female preponderance [1-6]. It is a slowly progressive disease that starts many years before patients experience any symptoms and seek medical assistance. Dry eyes and mouth are cardinal signs and their severity depends on various factors. Extraglandular manifestation are common in patients and might involve every organ and system. [7]. Until now, no drug has cured SS or induced remission [8]. Presently, SS management is limited to symptoms alleviations and prevention, and systemic immunosuppression is administered whenever extraglandular symptoms arise [9]. Unfortunately, most of the tested drugs, particularly biologic therapies, offered limited therapeutic efficacy with very limited improvement in the exocrine glands function, rather, the reported improvement was for systemic symptoms like fatigue [10].

Our group and others have successfully tested the efficacy of MSCs in alleviating SS in NOD mice [11, 12]. However, tumorigenic concerns and other complications exist [13, 14]. On the other hand, several studies have reported that MSCs paracrine signaling in inflammation is the main mode of action which means, in our opinion, their secretome/proteome can accomplish the mission when used [15-17]. In a recent study, proteome extracted from BM-derived MSCs contained around 2220 proteins of different functions, such as cell signaling and growth [17]. Therefore, the therapeutic potential of MSCs which is exerted by secreting proteins can be obtained by extracting the cells proteome and applying it as a therapy.

**Hypothesis 2**

Bone marrow cell extract is effective in managing SS-like disease in NOD mice in both salivary and lacrimal glands.

**Rationale**

Bone marrow (BM) is an important reservoir for mesenchymal and hematopoietic stem cells [18]. This unique composition of the cells in addition to the secretome in their niche are a valuable source of therapeutic proteins for various diseases [17]. We have previously reported that BM (preceded by myeloablative conditioning with CFA) and BMCE successfully alleviated SS-like symptoms in NOD mice in different studies [19-21]. NOD female mice exhibit inflammatory infiltration in all salivary glands, particularly the submandibular; however, only 52% exhibit similar pattern in the lacrimal glands [22]. NOD mice seem to display heterogenous clinical and histological pictures similar to that of humans. Therefore, in the third chapter, we tested weather BMCE is effective in restoring the lacrimal glands function and preserving the structural integrity of the cornea. Additionally, we have made two modifications: 1) BM collection methodology was performed with less manipulation and we preserved the secretomes in the BM niche, and 2) BMCE injection timing was scheduled to extend its effect.

**Hypothesis 3**

Cell extract is a safe treatment modality up to 16 weeks posttreatment.

**Rationale**

The extract is composed of proteins mainly, whereas cell membrane fragments/nuclei have been excluded by the centrifugation force. Therefore, cell extract is considered less immunogenic due to the lack of membranous MHC I and II molecules. Additionally, primary tumor formation from cells (BM or MSCs) is minimized due to the breakdown of these cells. However, we cannot exclude that the extract might promote the growth of an existing tumor; therefore, longer follow up period should be carried out with general tissue assessment. Thus, we believe BMCE and MSCsE are less immunogenic and less tumorigenic. To test this hypothesis, we have evaluated the

histopathology of four organs: the liver, the kidneys, the spleen, and the pancreas. Blood biochemistry for liver kidney damage were also assessed.

Generally, the **aim** of this thesis is to test the effectiveness of different BM-based cells extract in alleviating SS-like in NOD mice. In our opinion, a successful treatment is capable of restoring/preserving the glandular function, managing the immune dysregulation, and be relatively safe when injected systemically.

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Author contributions and statements of originality

**1- Studying Sjögren's Syndrome in Mice: what is the best available model?**

**Authors: Ghada Abughanam,** Ola Maria, Simon D. Tran.

**Contributions: G.A.** designed the review and drafted the manuscript; S.D.T. and O.M. reviewed and edited the manuscript**.**

**Originality:** This is the most recent and comprehensive review of murine models used in studying Sjögren's Syndrome. The aim of this manuscript is to provide researchers working in the field of Sjögren's Syndrome with the most updated and comprehensive review paper regarding the murine models used. Firstly, the paper discusses the characteristics of an ideal murine model regarding the clinical and laboratory aspects. Secondly, we have searched all the models that have been already been considered suitable and the new ones that has not been listed in any other review. Additionally, we have also included unpublished data from our lab work regarding a specific model and discussed its suitability. Lastly, we have expressed our opinion about the most suitable model for the pathogenesis and drug testing purposes based on our own experience in the field.

**2- Compact Bone-Derived Multipotent Mesenchymal Stromal Cells (MSCs) for the Treatment of Sjögren's-like Disease in NOD Mice.**

**Authors: Ghada Abughanam**, Younan Liu, Saeed Khalili, Dongdong Fang, and Simon D. Tran

**Contributions: G.A.** designed and conducted experiments; drafted the manuscript; **S.D.T**. designed experiments; reviewed the manuscript, **Y.L**., **D.F**., **S.K.** designed and conducted experiments.

**Originality:** This book chapter is a continuation of an already published paper authored by S.K. However, in this book chapter we repeated all the materials and methods in the previous paper and added a new section (the outgrowth of compact bone MSCs) to the culturing and expansion of compact bone section that has enhanced and sped up the expansion of the cells.

**3- Mesenchymal Stem Cells Extract (MSCsE)-Based Therapy Alleviates Xerostomia and Keratoconjunctivitis Sicca in Sjögren's Syndrome-Like Disease**

**Authors: Ghada Abughanam,** Osama A. Elkashty, Younan Liu, Mohammed O. Bakkar and Simon D. Tran.

**Contributions: G.A.**, designed and conducted the experiments, analyzed the data, performed the statistical analysis; drafted the manuscript, **S.D.T**. designed the experiments; reviewed the data; edited the manuscript; **O.A.E**. conducted the experiments; reviewed the manuscript; **M.O.B**. designed experiments; **Y.L.** designed experiments.

**Originality:** This is the first study that evaluated MSCs/MSCsE therapeutic effect on salivary and lacrimal glands function, and the immune dysregulation in NOD mouse model. The results of this study show that the MSCsE has a therapeutic potential that is very close to that of the MSCs; however, MSCsE is easier to transfer, store, and manage for clinical use in comparison to MSCs. Moreover, with the increasing reports linking MSCs to tumor formation, MSCsE is only the intracellular contents cannot initiate the formation of tumors.

**4- Allogenic Bone Marrow Cells Extract (BMCE) Alleviated Sjögren's-Like Disease in NOD Mice.**

**Authors: Ghada Abughanam**, Younan Liu, and Simon D. Tran.

**Contributions: G.A.** designed and conducted the experiments; analyzed the data; performed the statistical analysis; drafted the manuscript, **S.D.T.** designed the experiments; reviewed the data; edited the manuscript, **Y.L**. designed and conducted the experiments.

**Originality:** This is the first study that evaluated BMCE therapeutic effect on salivary and lacrimal glands function, and the immune dysregulation in NOD mouse model. Additionally, this is the first study that evaluate the toxicity effect of BMCE systemically, which could also be applied to ant cell extract therapy. The results show an improvement in the salivary and lacrimal glands function and a resolution in the inflammation systemically and locally in the salivary and lacrimal glands. it is also a safe to use as no organs damage was evidenced and the blood markers for liver and kidney toxicity were all within normal for the BMCE group.

**Abbreviations**

| | |
|---|---|
| AE | Annular erythema |
| AEGD | American European consensus group criteria |
| ANA | Antinuclear antibodies |
| ACR | American Rheumatology Group |
| AIDS | Acquired Immune Deficiency Syndrome. |
| APC | Antigen Presenting Cells |
| ATDs | Autoimmune thyroid diseases |
| AQP5 | Aquaporin 5 |
| ArKO | Aromatase-Deficient Mice |
| BAFF | B-cell activating factor |
| bFGF | basic fibroblast growth factor |
| bHLH | basic Helix-loop-helix |
| CA | Carbonic anhydrase |
| CD3 | Cluster of differentiation 3 |
| CD4 | Cluster of differentiation 4 |
| CD8 | Cluster of differentiation 8 |
| Ck5 | Cytokeratine 5 |
| CMV | Cytomegalo virus |
| mCMV | Murine Cytomegalo virus |
| DHEA | Dehydroepiandrosterone |
| DLCO | Carbon monoxide diffusing capacity |
| EBV | Epstein–Barr virus |
| EGF | Epidermal growth factor |
| ELG | Extraorbital lacrimal gland |
| EULAR | European League Against Rheumatism |
| FoxP3 | Forkhead box P3 |
| FS | Focus score |
| GM-CSF | Granulocyte macrophage-colony stimulating factor |

| | |
|---|---|
| GvHD | Graft versus host disease |
| HIV | Human Immunodeficiency Virus |
| HRT | Hormonal replacement therapy |
| HTLV | Human T-cell leukemia virus 1 |
| idd | Insulin-dependent diabetes |
| IFN-y | Interferon gamma |
| IgA | Immunoglobulin A |
| IgG | Immunoglobulin G |
| IgM | Immunoglobulin M |
| IL-10 | Interleukin-10 |
| IL-12 | Interleukin-12 |
| IL-14α | Interleukin-14 alpha |
| IL-17 | Interleukin-17 |
| IL-6 | Interleukin-6 |
| MALT | Mucosa-associated lymphoid tissue |
| MHC | Major histocompatibility complex |
| MPGN | Membranoproliferative glomerulonephritis |
| M3R | Muscarinic 3 receptor |
| NK | Natural killer |
| NKCC1 | Na-K-Cl cotransporter |
| NOD | Non-obese diabetic |
| NSAIDs | Non-steroidal anti-inflammatory drugs |
| PCR | Polymerase chain reaction |
| PG | Parotid gland |
| PI3K | Phosphoinositide 3-kinase |
| pSS | Primary Sjögren's Syndrome |
| RA | Rheumatoid arthritis |
| RCTs | Randomised controlled trials |
| RF | Rheumatoid factor |
| SLE | Systemic lupus erythematosus |
| SLG | Sublingual gland |

| | |
|---|---|
| SMG | Submandibular gland |
| SjS | Sjögren's Syndrome |
| SS-A | Sjögren's Syndrome Antigen A |
| SS-B | Sjögren's Syndrome Antigen b |
| sSjS | Secondary Sjögren's Syndrome |
| Tg. | Transgenic |
| TGF-β1 | Tumor growth factor beta 1 |
| TLRs | Toll-like receptors |
| TNF-α | Tumor necrosis factor-alpha |
| TSP-1 | Thrombospondin-1 |

## Chapter one

# Background and literature review

## Preface

In this chapter we discuss the literature review that is related to Sjögren's Syndrome in humans and mice. We have covered the structure and function of salivary and lacrimal glands. Sjögren's Syndrome classification, etiology, pathogenesis, signs and symptoms, and current clinical management were also discussed. Finally, we have highlighted the clinical trials that have tested biological therapies (most related to our therapies) and the associated complications.

## Background and literature review

### 1.1. Human Salivary glands

In humans, saliva is produced by major salivary glands (>95%) and minor salivary glands (<5%). The major glands are the left and right submandibular, parotid and the sublingual (Fig.1). Minor salivary glands are distributed throughout the oral cavity mucosa and submucosa. Major salivary glands share the same anatomical architecture which is composed of secretory endpieces and a duct system (Fig 2) [23]. The secretory endpieces are either spherical or tubular, depending on the secretion nature. They are formed of secretory epithelium surrounded by extracellular matrix and multiple cell types: myoepithelial, fibroblasts, immune, endothelial, and stromal cells, and nerve endings (Fig 3) [23, 24]. Histologically, the glands are branched tubuloalveolar enveloped with a capsule that sends septa which divide the gland into lobes and smaller lobules. The gland connective tissue element includes thin connective tissue sheath around the acini. The secretory portion is composed of three cell types; serous, mucous and myoepithelial cells that surrounds the acini. The duct portions are highly branched structures that begin from the acini and ends by opening in the oral cavity as the main duct. The intercalated ducts are composed of simple layer of low cuboidal cells that starts the duct system by collecting saliva from the acini and merge together to form the striated ducts; a simple layer of cuboidal to low columnar cells. The intralobular ducts are the merge of several striated ducts together and they are composed of more than one layer of epithelial cells. The interlobar ducts are the main ducts that collect the saliva from each lobe, then converge together to form the stratified main duct that delivers the saliva to the oral cavity in a specific anatomical location [23].

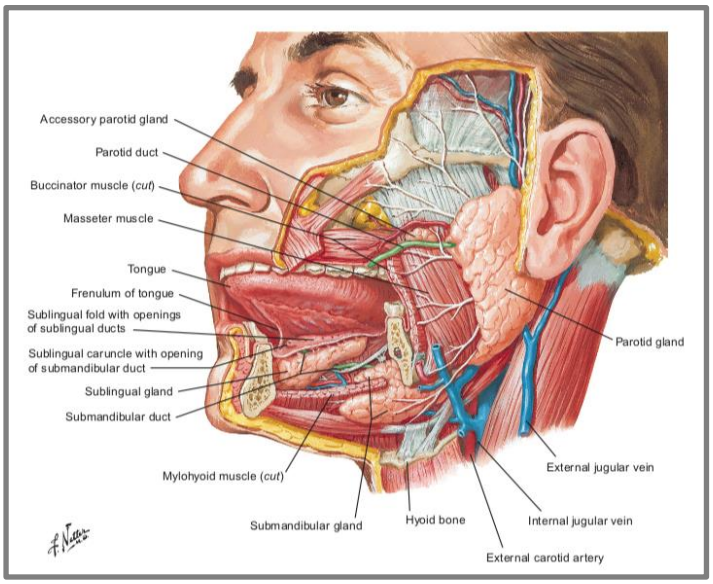


**Figure 1**. **Human major salivary glands**. Copyright © 2018 Elsevier Inc.

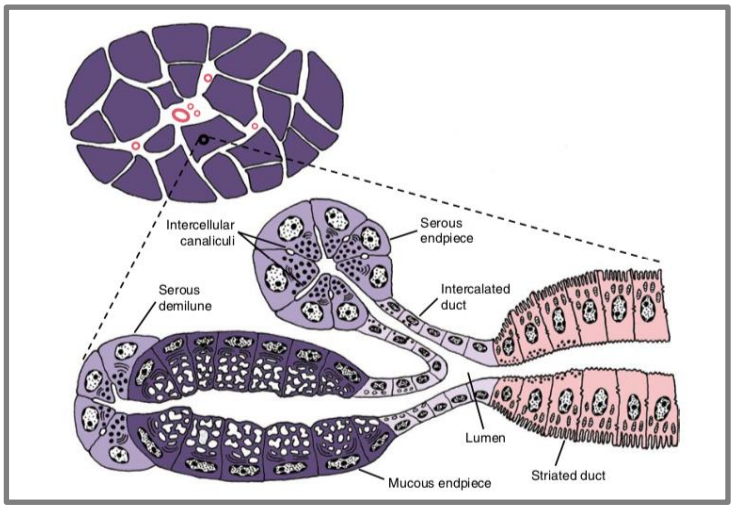


**Figure 2. Secretory endpieces**. A diagram showing the serous/mucous endpieces and intercalated/striated ducts. Copyright (2014) John Wiley & Sons [24].

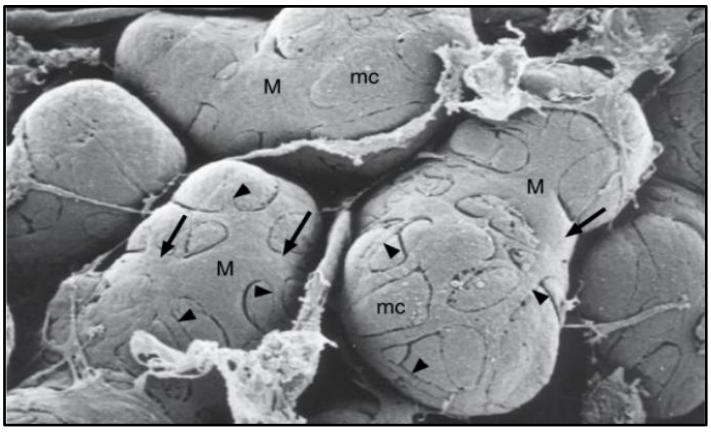


**Figure 3**: **Myoepithelial cells**. Scanning electron micrograph showing the myoepithelial cells (M) surrounding the tubular mucous acini (mc). The arrows are pointing to the myoepithelial broad processes that taper into small ones (arrowheads) [24]. Copyright (2014) John Wiley & Sons.

The human **submandibular** gland weighs 7-16 gm and is located in the digastric triangle in the posterior area of the floor of the mouth, medial to the mandible and wraps around the posterior border of the mylohyoid muscle (Fig 2) [25]. The submandibular glands delivers the saliva into the oral cavity via the Warton's duct that opens the sublingual caruncula under the tongue [23]. The submandibular gland is a mixed gland, it is composed of serous endpieces (mainly) and some mucous endpieces (Fig 4B). The serous cells produce proteins similar to what the parotid gland secrets except for a number of mucins that are only produced by the submandibular gland [24]. Although it is not the largest, the submandibular gland secrets around 65% of saliva [26].

The paired human **parotid** gland is the largest of the major salivary glands where it weighs 20–30 gm [27]. The gland is located in the preauricular region along the posterior surface of the mandible. The passage of the facial nerve through the gland divides it into superficial and deep lobes. The superficial lobe overlies the lateral surface of the masseter muscle and is located lateral to the facial nerve. The deep lobe is medial to the facial nerve and is located between the mastoid process of the temporal bone and the ramus of the mandible [28]. The parotid is a pure serous gland, despite being the largest, it only produces 20% of human saliva [26]. The striated ducts are prominent in the parotid. Scattered adipocytes are present, and they increase with age [24]. The parotid delivers

saliva via its main duct, Stenson's duct, which opens opposite to the upper second molar. Histologically, the parotid is composed mainly of serous secreting acinar cells (Fig 4A).

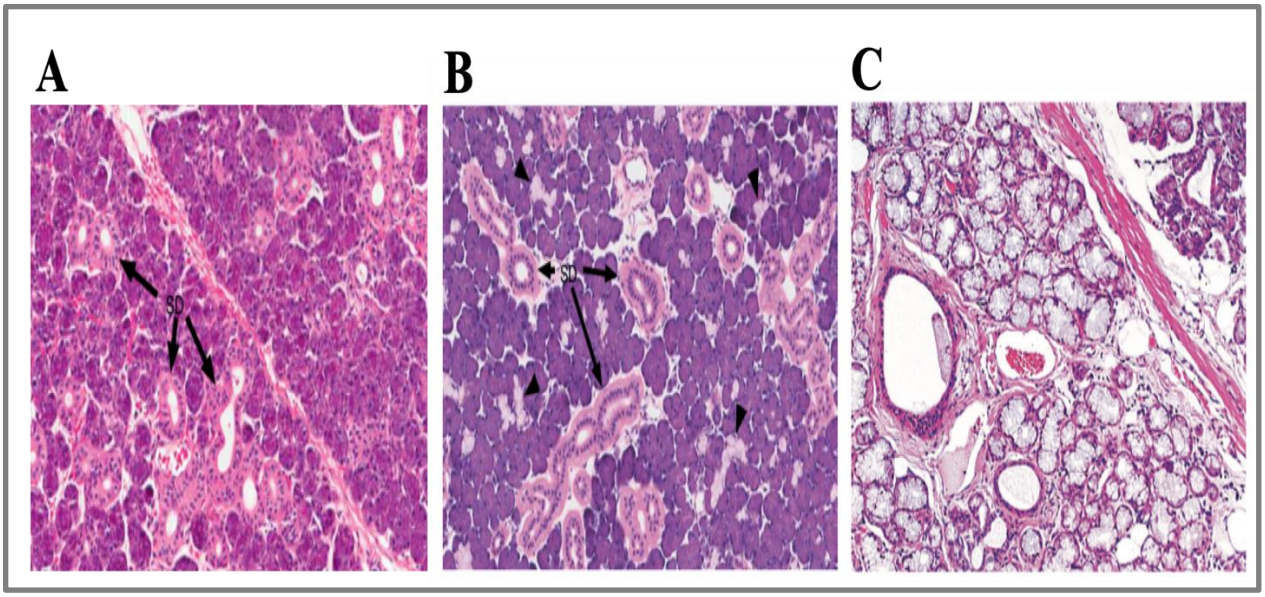


**Figure 4. Human parotid and submandibular glands histology**. **(A)** Parotid gland is composed of serous endpieces and many striated ducts (SD). **(B)** Submandibular gland is a mixed gland composed of serous endpieces (mainly) and some mucous endpieces (arrowheads) [24]. **(C)** Sublingual gland is a pure mucous gland. Copyright (2014) John Wiley & Sons.

The human **sublingual glands** are located in the ventral cervical region superio-lateral to the submandibular glands (Fig. 1). A sublingual gland is composed of a single lobe drained by a major duct that opens caudal to the lower incisors. The secretory unit is formed of compound tubuloalveolar structure which secretes pure mucous saliva. The sublingual gland is a mixed gland, but contains mainly mucous endpieces [24], and it produces around 7-8% of the secreted human saliva [26].

### 1.2. Mouse salivary glands

Like humans, mice have 3 pairs of major salivary glands: submandibular, parotid, and sublingual, and several minor salivary glands. The anatomy and the histology of these glands are somewhat different from human; therefore, the nature and consistency of the secreted saliva is different [27].

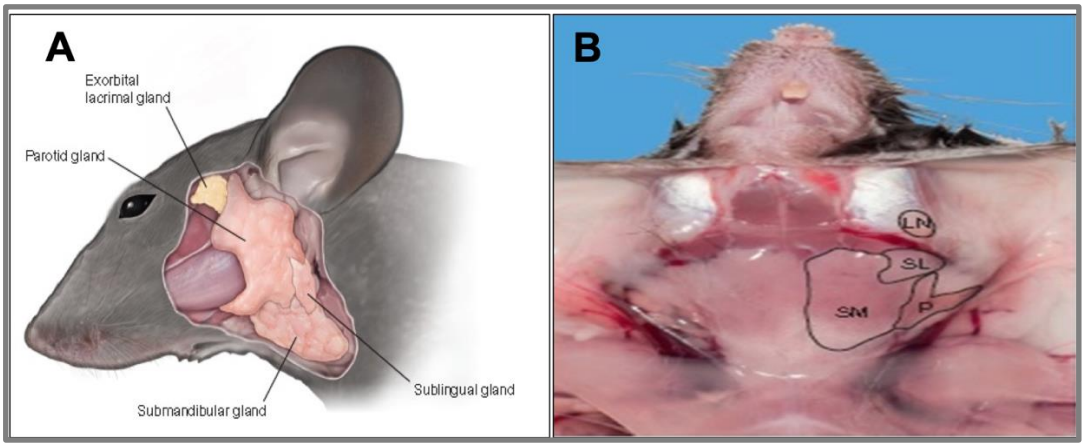


**Figure 5. Mouse salivary glands. (A)** Latero-inferior view of mouse cervical region showing the three major salivary glands: submandibular gland (SMG), parotid gland (PG) and sublingual gland (SLG) and the associated lymph nodes. **(B)** A photograph of a dissected mouse ventral cervical region showing the paired major salivary glands outlined in black: Submandibular gland (SM), Sublingual gland (SL), the Parotid gland (P) and lymph nodes [27]. Copyright (2012) Elsevier Inc

The mouse **submandibular gland** is the largest of the major glands. It is located in the central ventral cervical region and the two glands meet in the midline (Fig 5). The general architecture of the gland is formed of lobes that further divide into lobules (Fig 6). The lobules are composed of secretory units: compound tubule alveolar acini that produce mainly serous and some mucous secretion (Fig 7). The duct system starts by draining the secretory portion via low cuboidal intercalated ducts which merge into larger striated ducts composed mainly of cuboidal epithelium. A unique part of the duct system that connects the intercalated to the striated ducts is called the convoluted duct. It is present in males only and has a granular content and appearance.

The **parotid gland** is in the ventral cervical subcutaneous region extending from the lateral and upper border of submandibular glands to the periauricular area and up to the lacrimal glands (Fig

5). The gland is not encapsulated and therefore it is a diffuse multilobed (Fig 6). The lobes are formed of smaller lobules which form the secretory portions. The secretory units are compound tubuloalveolar structure producing a pure serous secretion (Fig 7) [27].

The **sublingual glands** are in the ventral cervical region superio-lateral to the submandibular glands (Fig 5). It is composed of a single lobe drained by a major duct that opens caudal to the lower incisors. The secretory unit is formed of compound tubuloalveolar structure which secretes pure mucous saliva (Fig 6,7) [27].

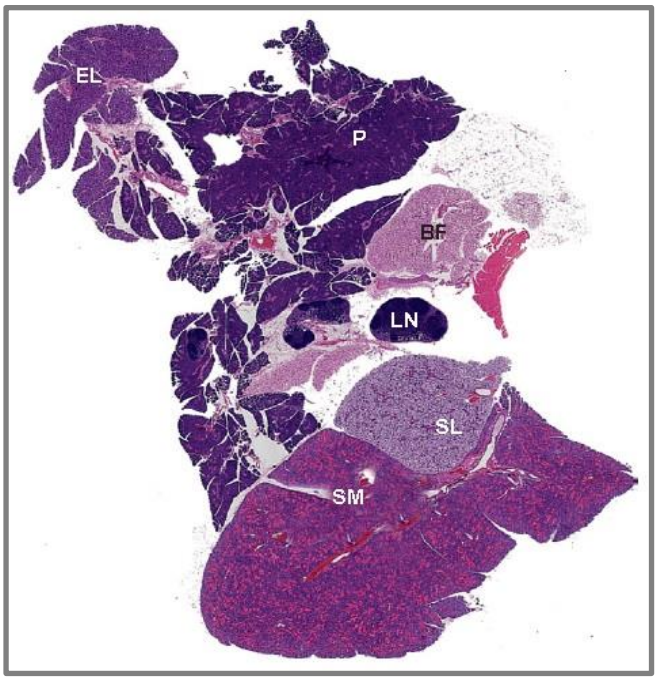


**Figure 6. Histology of mouse salivary glands.** H&E stained image of the cervical soft tissues from a male mouse: submandibular gland (SM), parotid gland (P) and sublingual gland (SL), exorbital lacrimal gland (EL), associated lymph nodes and brown fat (BF) [27]. Copyright (2012) Elsevier Inc.

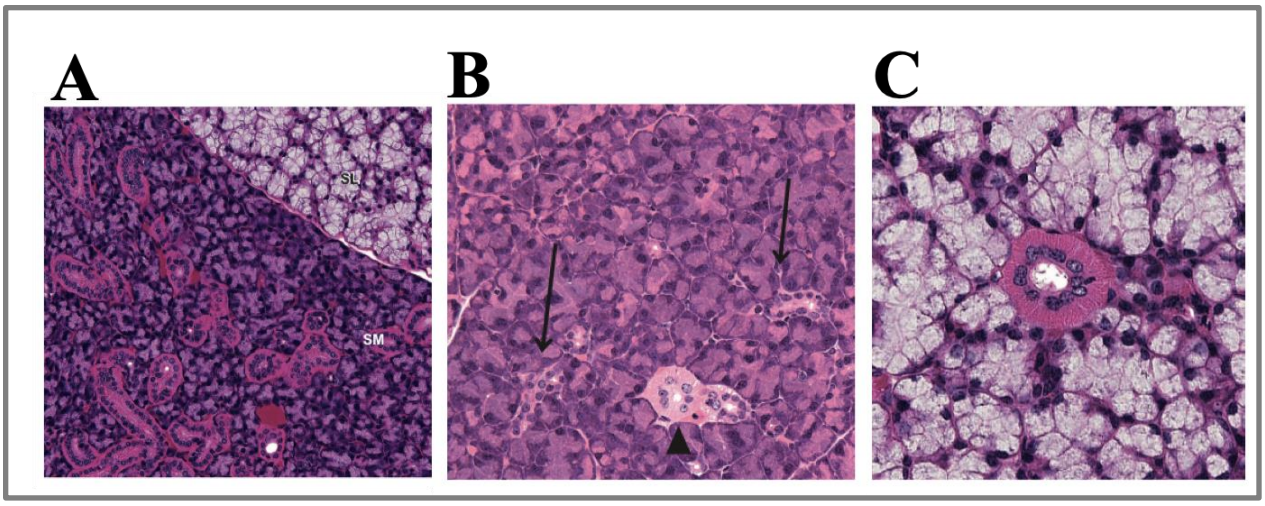


**Figure 7. Histology of mouse major salivary glands (H&E). (A**) Submandibular gland is a mixed gland containing both serous (mainly) and mucous endpieces **(B)** Parotid gland is composed of serous endpieces. Striated ducts (arrowhead) and Intercalated (arrows). **(C)** Sublingual gland is a mucous secreting gland [27]. Copyright (2012) Elsevier Inc.

### 1.3. Saliva composition, formation and function

Saliva is a dilute hypotonic fluid composed of water, mainly (>99 %), in addition to electrolytes (sodium, potassium, calcium, magnesium, bicarnodbonate and phosphates), hormones, enzymes, immunoglobulins, urea and ammonia. It is slightly acidic (pH 6-7); however, it is more acidic in low flow, and more basic in peak flow [26, 29]. Major salivary glands secrete most of the saliva with a small contribution from the scattered minor salivary glands. Parotid glands secrete 20% of saliva, submandibular glands secrete 60%, and 7–8 % is secreted by sublingual glands, and the remaining 12-13 % comes from the minor salivary glands [30]. Secretion by each major gland differs from others in composition and consistency. The submandibular and parotid secretions are more aqueous while the sublingual secretion is more viscous. Saliva synthesis starts in acinar cells, modified by the ductal cells then carried to the oral cavity via the major ducts. Salivary flow rate is variable, but a rate above 0.1 mL/min for unstimulated saliva and 0.2 mL/min for stimulated is considered normal. When salivary flow decreases below 0.1 mL/min hypofunction concerns are raised [31]. Saliva is actively secreted and controlled by the sympathetic nervous system leading

to protein rich saliva, whereas the parasympathetic system activation favours a water rich secretion [32, 33].

### 1.3.1. Fluid secretion

Formation of fluids in saliva is a work orchestrated by water and ion transporters, and channel proteins. Primary saliva is initiated at the secretory endpieces which are called acinar cells. Acinar cells secrete an isotonic proteins-rich fluid which passes through the duct system and gets modified to become hypotonic [33]. Binding of a neurotransmitter to acinar cells elicits a cascade of events [30, 33]. Acetylcholine (Ach) binding is responsible for water and electrolytes secretion. It is secreted by the parasympathetic nerve endings and binds to its receptors leading to several cellular events that result in the release of $Ca^{2+}$ from intracellular stores. Norepinephrine (NE) binding to α-adrenergic receptors also participate in the fluid secretion [24]. Elevated $Ca^{2+}$ concentration opens the luminal (apical) $Cl^-$ channels and the basolateral $K^+$ channels. The movement of $Cl^-$ towards the lumen creates an electrochemical gradient favouring the movement of $Na^+$ into the lumen from the tight junctions followed by water molecules which pass via tight junctions and the AQP5 channels on the apical surface (Fig 8) [24]. The movement of $Cl^-$ and $K^+$ creates a transepithelial electrical potential difference that is neutralized by paracellular $Na^+$ movement across the tight junctions [34, 35]. The process is started by $Cl^-$ transcellular movement through basolateral NKCC1cotransporters, mainly, and $Cl^-/HCO_3^-$ exchanger concentrating $Cl^-$ intracellularly against its intracellular gradient [36]. NKCC1cotransporters facilitate the movement of two $Cl^-$ ions intracellularly seven folds above its electrochemical gradient concentration with 1 $Na^+$ and 1 $K^+$ ions [34]. Cells keep the concentration of $Na^+$ low by using $Na^+/K^+$ ATPase; utilizing ATP for the export of 1 $Na^+$ molecule and import of 1 $K^+$ molecule. $Cl^-/HCO_3^-$ exchanger is considered an alternative mechanism to aid in the $Cl^-$ concertation rather than the main mechanism in concentrating $Cl^-$ intracellularly. $Cl^-/HCO_3^-$ exchanger depends on the activity of $Na^+/H^+$ exchanger. As primary saliva passes the ductal cells, neural impulses arrive turning $Na^+/K^+$ ATPase on [30]. The resulting fluid is isotonic rich in $Cl^-$ and $Na^+$ which travels to the duct system.

Ductal cells are polarized, and they share similarities and differences with acinar cells. Once saliva passes through striated ducts, NaCl is reabsorbed and $KHCO_3$ is secreted but not water. Ductal tight junctions are watertight which leads to a hypotonic saliva [36, 37]. Ductal cells reabsorb $Na^+$

through their apical channels. Sodium ions are removed from the cytoplasm through $Na^+/K^+$ ATPase located basolaterally, this enzyme maintains low $Na^+$ in the cytoplasm and ensures the passive flow of $Na^+$ ions from saliva, intracellularly. Furthermore, $Na^+/H^+$ exchangers participate in the intracellular uptake of $Na^+$ [36]. Membrane chloride channel proteins ensure the moving of $Cl^-$ ions from the lumen intracellularly which is favoured by low intracellular $Cl^-$ concentration and the influx of $Na^+$, leading to depolarization of the apical membrane. $Cl^-$ ions then enter the interstitium via the same channels, this is facilitated by the depolarization of the basolateral membrane which is caused by the opening of $K^+$ channels [34]. Furthermore, the presence of $Cl^-/HCO_3^-$ exchanger, which is coupled to $Na^+/H^+$ exchangers apically, participates in the reuptake of chloride ions and enriching saliva with $HCO_3^-$. Finally, Potassium ions get imported into the cytosol via the $Na^+/K^+$ ATPase and secreted into saliva via the $K^+/ H^+$ antiport (exchanger) [38].

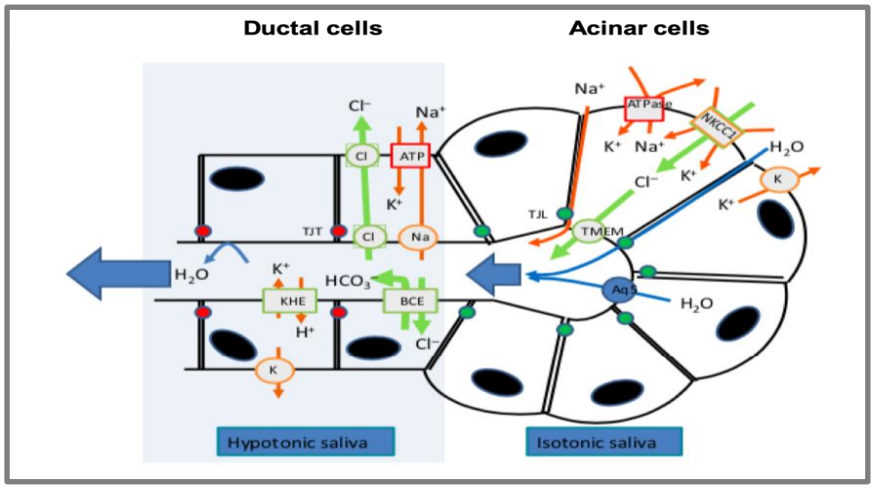


**Figure 8. Saliva formation.** A diagram showing the mechanism of saliva secretion in salivary glands. It shows the transporters and channels involved in the process of saliva bulk formation. Saliva is first formed in the acinar cells (isotonic) then the ductal cells modify its composition to produce the final form (hypotonic) that will enter the oral cavity **[**37**].** Copyright (2015) John Wiley & Sons.

### 1.3.2. Protein secretion

Protein secretion is controlled mainly by the sympathetic system. Binding of norepinephrine to its receptors on acinar cells initiates an intracellular cascade that results in the exocytosis of protein [39]. The release of $Ca^{+2}$ from its intracellular stores initiates phosphorylation of several proteins that leads to fusion of the secretory vesicles to the luminal membrane of acinar cells and the release of their contents into saliva. However, parasympathetic signaling also promotes the secretion of mucins by mucous cells and low exocytosis by acinar cells [24]. Not only neural signals promote the release of proteins in saliva, but gastrointestinal hormones in the blood, like cholecystokinin, influence the process also [24, 37, 39].

### 2.3.3. Saliva protein composition

Saliva is a complex and unique fluid. It is composed of water (95–99.4%), minerals, electrolytes, metabolites, nucleotides, polynucleotides, hormones, immunoglobulins, proteins, enzymes, cytokines, and other components (Fig 9) [29, 40]. Whole saliva is a heterogenous fluid containing secretions from major and minor salivary glands, oral epithelium, and the crevicular fluid secreted by the gingival cells [29]. Salivary proteins serve numerous functions, including simple coating of the oral structures for protection, defensive mechanism against pathogens, and starting the digestion process [26].

Saliva contains a number of proteins that are designated for a defense function, including immunoglobulin, lysozyme, mucins and AMPs (antimicrobial peptides). Immunoglobulin A (IgA) is found as a dimer in saliva and attached to hinge protein called J chain produced by plasma cells [29]. It acts as a first line of defense in the innate arm of the immune system by immobilizing antigens. Lysozyme is another antimicrobial enzyme which is strongly cationic, and it hydrolyzes the cell wall of bacteria. Mucins are large insoluble glycoproteins; they coat and protect the oral mucosa from the dehydration and mechanical insults, such as abrasion. Salivary peroxidase (also called sialoperoxidase or lactoperoxidas) oxidizes bacterial by-products that contain thiocyanate ion into hypothiocyanite which is very toxic [29, 41]. AMPs or alarmins or HDPs (host defense peptides) is a family of a large number of ribosomal synthesized peptides which are a major component of the innate immune system [29]. Cystatins help in preventing periodontal diseases by inhibiting cysteine-proteinase; in addition, it helps regulating salivary calcium [26]. A group of

salivary proteins aggregates bacteria and prevents it from adhering to oral surfaces, including glycoproteins, statherins, agglutinins, histadine-rich proteins, and proline-rich proteins [26]. Amylase is another major protein constituent in the saliva. It helps starting the digestion in the oral cavity by breaking down polysaccahrides, many electrolytes and proteins in its composition. Its composition differs depending on the rate of saliva secretion, the age of the patients, and the presence of any clinical conditions. The three main electrolytes in saliva are Na, K, and Cl, in addition to others at a much less concentration. Saliva contains a wide array of proteins, ranging from small polypeptides to larger functional-specific proteins, like amylase.

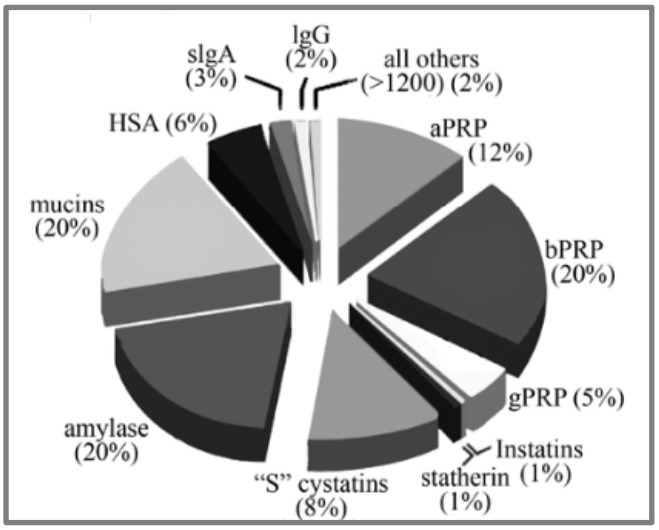


**Figure 9. Major proteins in the human saliva.** A diagram showing the major salivary proteins and their percentages [42]. Copyright (2010) Società Italiana di Otorinolaringologia e Cshirurgia Cervico-Facciale

### 1.3.4. Functions of saliva

Saliva has many important functions and its loss compromises the patient's quality of life [32, 43]. It moistens and lubricates the oral cavity which helps protecting the oral mucosa from abrasion and washes off exfoliated cells, microorganism and food debris by swallowing. For taste to occur, a substance must dissolve in a solution; therefore, saliva acts as a solvent for the ingested food to

be recognized by taste buds [44]. Saliva aids in the digestion via its content of α-amylase enzyme [32]. Alpha amylase is secreted by all major salivary glands, but mainly the parotid gland [45]. It breaks starch down into simpler sugars like maltose, maltotriose, maltotetrose, and some higher oligosaccharides. Saliva incorporates into the bolus of food and forms a cohesive mass covered with mucin which facilitate the ingestion [46]. This latter process is greatly appreciated by patients suffering from dry mouth due to either SS or head and neck irradiation. As a compensation mechanism, they keep drinking water and other fluids during eating and after swallowing to help smoothen the food bolus and prevent its lodging in their throats.

### 1.4. Human lacrimal glands

The human lacrimal gland is composed of two portions: the main (intraorbital) which is located in the lacrimal fossa (frontal bone), and the accessory (extraobital) which resides in the temporal segment of the superior conjunctival fornix. The two parts are divided by the lateral horn of the levator palpebrae muscle [47, 48]. The lacrimal gland is an exocrine gland; formed of acini and a duct system. The acini are the secretory part of the gland which are composed of a basally located myoeoithelial cells supporting a pyramidal layer of epithelial cells with scattered lymphocytes, mast, and fibroblasts cells [49, 50], acini account for 80% of the whole gland [51]. The ductal part forms 20% of the gland, it aids in the modification and the transportation of the tear. The gland is divided into several lobules by loose connective tissue which allows the neurovascular bundle and the interlobular ducts to run through. The lobules comprise many acini and intralobular ducts. The interlobular ducts then converge and form around 12 excretory ducts which open on the conjunctival fornix. The large pyramid-shaped acinar cells form a lumen in the center, where the initial tear secretion is first collected [47]. The acinar cells, resembling exocrine acinar cells, contain many secretory vesicles and a basally located nucleus.

The **accessory lacrimal gland** is divided into two parts**:** Wolfring and Krause glands. They are both small serous and share similar structure but have different locations. They both empty in the palpebral conjunctival surface [47, 52]. The accessory lacrimal gland has a similar histology to that of the main lacrimal gland. It is unknown how significant its secretion is to the whole secreted tears. However, a study reported that the removal of the main lacrimal glands in monkeys did not

affect the tear layer that was maintained by the secretions of their accessory lacrimal glands [47, 53].

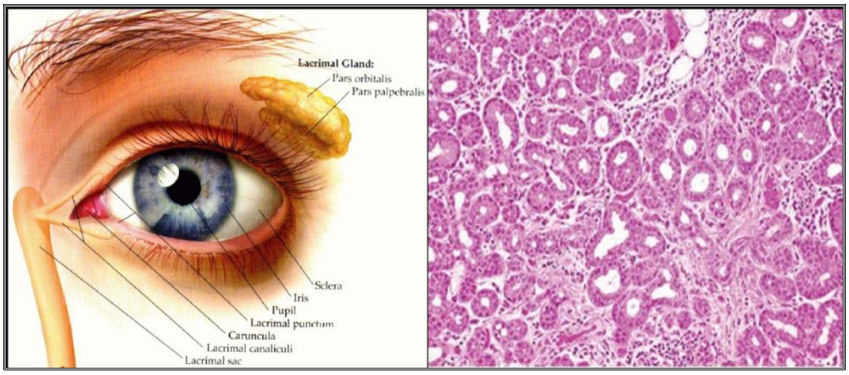


**Figure 9. Human Lacrimal glands**. (**Lef**t) Frontal view of the right orbit showing the excretory ducts of the lacrimal gland opening into the fornix of the conjunctiva. (**Right**) H&E stained histological section of the human main lacrimal gland [48]. Copyright Pearson Education Inc.

### 1.5. Mouse Lacrimal glands

Mice have a lacrimal apparatus that is composed of lacrimal glands and a connective pathway. There are three lacrimal glands: the extraorbital, the orbital (intraorbital) and the Harderian gland [54]. The **extraorbital** is larger than the intraorbital in size and located subcutaneously ventral and anterior to the eye. The **intraorbital** is located under the upper eyelid laterally; however, the histology is similar in both glands [51]. Both glands are classified as serous tubulo-alveolar glands; however, they resemble the histomorphology of the parotid salivary gland with some differences. The lacrimal gland acini are larger, paler in color and less packed with secretory granules when compared to the parotid, in addition; the nuclei size of the lacrimal cells are irregular (anisokaryosis) and they have variable sizes [27]. The **Harderian gland**, also called the gland of the third eyelid, is only present in animals that have a nictitating membrane. This gland occupies most of the orbit. It is a tubular gland that secrets an oily secretion with unclear function [54, 55].

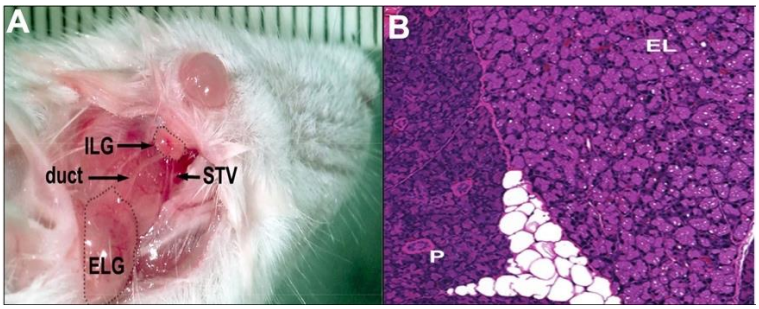


**Figure 10. Mouse Lacrimal gland. (A)** A photograph of a dissected temporal region of the mouse showing extraorbital lacrimal glands (ELG) and the intraorbital lacrimal gland [56, 57]. **(B)** An H&E stained histological section of the extraorbital gland (EL) and for comparison reasons, the parotid is left to it (P) [27].

### 1.6. Tears composition, formation and function

Tears are an exceptionally complex stable fluid that is composed of a wide array of proteins, lipids, electrolytes, and water [58]. They serve various functions to maintain the health and integrity of the ocular surface. Tears are continuously produced to cover and protect the exposed surfaces of the ocular system, the cornea and conjunctiva [58]. Tears are produced by several glands surrounding the eyes in addition to the epithelium covering the ocular surfaces. The tear film is trilaminar; composed of three layers, from the outermost to the innermost: a lipid layer, an aqueous layer, and a mucous layer [59]. The layers are variable in thickness; the lipid layer is the thinnest (around 0.1 mm), the middle aqueous layer is the thickest of all (around 7-10 mm); however, the inner mucous layer thickness is somewhat controversial [60]. Each layer of the three is secreted by a specific gland. The lipid layer is secreted by the Meibomian glands. The aqueous layer is secreted largely by the main lacrimal gland with a small contribution from the accessory gland and the corneal epithelium; however, leakage of water and electrolyte from the conjunctival junctions contributes to the formation of this layer [58, 61, 62]. The last layer, the mucous layer, is secreted by the goblet cells in corporation with the conjunctival and the corneal epithelia with an unknown contribution.

Tears are composed of multiple components: water, proteins, ions, and electrolytes [63, 64]. Tears contain many proteins secreted by the ocular glands [58, 64], which serve as antimicrobial and growth enhancers [65]. One of the most important functions of tear proteins is the prevention of bacterial and viral infections. This role is carried out by lysozyme, secretory immunoglobulin (IgA), lactoferrin, lipocalin, and peroxidase as antibacterial; however, glycoproteins (mucins) act as antibacterial and antiviral [66]. Mucins cover the outmost layer of epithelium and protect it by binding via their heterogeneous carbohydrate side chains, and trapping pathogens; preventing them from penetrating any further [66]. During wound repair, tear proteins promote cellular proliferation and migration [67]. Other important functions of tears are; promoting growth and aiding in the healing process via their content of growth factors and cytokines.

### 1.6.1 Water and electrolytes secretion.

The secretion process of tears is somewhat similar to that of saliva. Acinar cells are responsible for the secretion of the primary tears and its protein, then the ductal cells modify the electrolyte composition [68]. The secretion of tears is initiated by binding of acetylcholine (Ach) to M3R muscarinic receptors leading to an intracellular increase of $Ca^{+2}$ level and an activation of protein kinase C (PKC). PKC (directly/indirectly) and $Ca^{+2}$ (directly) phosphorylate ion channels [59]. $Na^{+}$, $K^{+}$ and $Cl^{-}$ ions and water and their movement across the lacrimal gland epithelial membrane are responsible for the formation of tears. The production of tears goes through two phases. The first phase is accomplished by the acinar cells which secrete NaCl rich tears. The second phase is carried out by the ductal cells; they modify the passing tears by reabsorbing $Na^{+}$ ions and secreting $K^{+}$ ions. The first step of tears formation starts at the basolateral membrane of the acinar cells by $Na^{+}/K^{+}$ ATPase pumps [58]. $Na^{+}/K^{+}$ ATPase pumps $Na^{+}$ actively from the cells into basolateral space against its electrochemical gradient utilizing ATP. The latter process decreases the concentration of $Na^{+}$ and elevates $K^{+}$ intracellularly. $Na^{+}$ then enters the lumen passively through a paracellular route via its channels. $Cl^{-}$ ions enters the cells from the interstitium via $Cl^{-}/HCO_3^{-}$ exchanger, then reach the lumen apically via passive flow. $K^{+}$ ions diffuse passively toward the interstitium space and the lumen, when its concentration becomes continuously elevated due to the activity of $Na^{+}/K^{+}$ ATPase [59]. $H_2O$ flows passively toward the lumen, apically, as a result of hydrostatic and osmotic gradients following $Na^{+}$ electrolyte. Generally, tears are hypertonic at the

normal rate and isotonic at the high rates [58]. The latter suggests a role of the ductal cells in reabsorbing the water from tears when they reside long enough in the lacrimal glands.

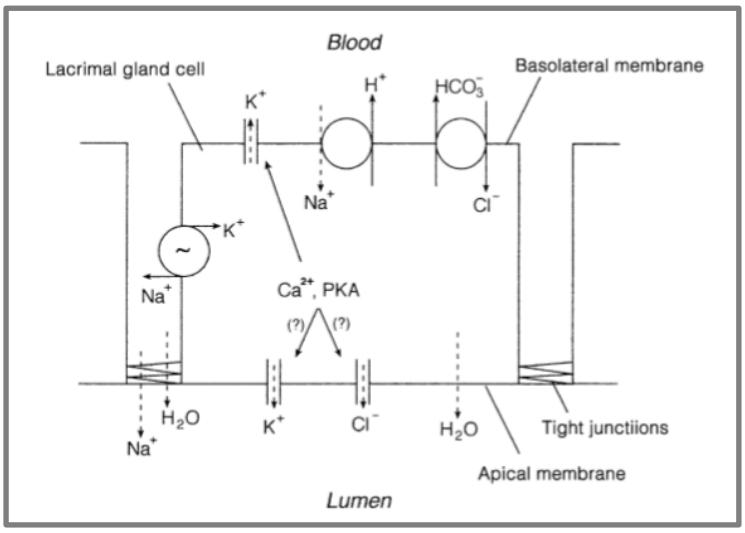


**Figure 11. Tear fluid secretion** A schematic diagram showing the electrolyte and water movement in tears formation in lacrimal glands [58]. Copyright (2006) Elsevier B.V.

### 1.6.2. Protein secretion

Neural and hormonal signals are integral regulators of lacrimal glands secretion which generate a second messenger, such as $Ca^{+2}$, cAMP, cGMP, or DAG [59]. The arrival of the proper stimulus determines the type of protein secretion that is either regulated or constitutive, such as tear IgA [59, 69]. Hormones are responsible for stimulating constitutive protein (short-lived) secretion which is synthesis-controlled secretion and the neural signals are responsible for the release-controlled secretion. The granules which contain proteins controlled by consecutive secretion fuse easily with the membrane to release their secretion; they are not stored [59]. On the contrary, the granules that are controlled by the regulated mode of secretion are stored in the cytoplasm and have a relatively long half-life. They are prevented from fusion until the intracellular level of secondary messenger rises [59].

### 1.6.3 Tear protein composition

Proteins in the tears come from different sources: main and accessory lacrimal glands, the epithelium covering the ocular surface, and the conjunctival blood vessels. The table below (Table 1), adapted from Dartt et al, shows the documented tear proteins secreted by the main lacrimal gland; derived mainly from animal studies.

**Table1. Main lacrimal gland secreted proteins**

| | |
|---|---|
| Apolipoprotein D | Secretory Immunoglobulin A |
| Convertase decay-accelerating factor | Transforming growth factor-β1 |
| Endothelin-1 | Basic fibroblast growth factor |
| Lacritin | Cystatin-related protein |
| Group II phospholipase $A_2$ | Granulocyte-monocyte colony-stimulating factor |
| Immunoglobulin M | Immunoglobulin G |
| Lactoferrin | Interlukin-1β |
| Peroxidase | Monomeric Immunoglobulin A |
| Prolactin | Polymeric Immunoglobulin A |
| Secretory component | Transforming growth factor-α |
| Tumor necrosis factor-α | β-Amyloid protein precursor |
| Cystatin | Epidermal growth factor |
| Hepatocyte growth factor Interlukin-1α | Lysozyme |
| Plasminogen activator | Retinoic acid |
| Tear lipocalins | Transforming growth factor- $\beta_2$ |

### 1.7. The Cornea

The cornea is a dome-shaped, clear structure in front of the eye. It is a highly specialized and multilayered structure. In humans, the layers from most externally inward are: epithelium,

Bowman's membrane, stroma, Descemet's membrane, and endothelium. It is also divided into central and peripheral cornea depending on the location [70]. In humans, the cornea is 0.52 mm thick centrally and increases peripherally [70]. In mice, the reported thickness is variable probably due to the differences in strains and the difference in the technique used; however, in BALB/c it is 170 um measured by confocal microscope and decreases peripherally [71]. It was also reported that the epithelial layer is 51 um and 119 for the stroma when cryostat sections were used [71]. The cornea occupies 15% and 50% percent of the total eye surface area in humans and mice, respectively. The two major layers in thickness are epithelium (1/3 of the total thickness) and stroma (2/3 of the total thickness). The epithelium is stratified, squamous, and non-keratinized. The stroma is rich in collagen fibers and the transparency of the cornea is dependent on the three characteristics of these fibers. First, the fibers have constant uniform diameter throughout the cornea; second, the fibers are uniformly spaced from each other and the same applies to their bundles; third, the cornea is relatively dehydrated [72].

### 1.8. Bone Marrow and Bone Marrow-Derived Mesenchymal Stromal Cells (MSCs)

BM is the largest hematopoietic organ which comprise 3-5% of the total body weight [73]. It houses various stem and progenitor cells that are essential for hematopoiesis and regeneration [74]. BM transplant (BMT) has been widely used for the management of various conditions including autoimmune diseases [75-77]. In SS, we have successfully tested BMT in NOD mice; however, it has not been tested in patients yet [19, 20]. BM-derived MSCs were also tested in patients and NOD mice and promising results were achieved [12, 78]. BM executed a dual therapeutic action; it reduced the inflammation and preserved the glandular secretory functions [19, 20].

Mesenchymal stem cells (MSCs) are multipotent cells which are characterized by plastic adherence and self-renewal [79-82]. MSCs have been utilized therapeutically due to their immunomodularity potentials, tissue repair/regeneration, and their anti-fibrotic properties [83, 84]. MSCs are considered immune privileged because they lack MHC II and express low levels of MHC I; hence, their immune rejection is low, allowing their wide use in cell-based therapies [85]. MSCs, and their derivatives, have been utilized for the management of a wide array of diseases, such as autoimmune diseases, neural injuries, and graft versus host disease (GvHD) [11, 12, 86-

102]. Autogenous MSCs are easily accessible from different sources in the body, such as bone marrow, peripheral blood, dental pulp, periodontal ligaments, and adipose tissue [80, 103-105]. The patient's own cells are extracted, expanded in vitro, and injected back for the intended use. MSCs are well documented for their immunomodularity and anti-inflammatory properties [106-108].

### 1.9. Xerstomia and Keratoconjunctivitis Sicca (Dry Mouth and Dry Eyes)

Xerostomia and keratoconjunctivitis sicca are clinical conditions resulting from decreased or diminished saliva and tears production, respectively. Saliva/tears decrease when the secreting cells, acini, are damaged either by an autoimmune attack as in SS, or due to irradiation to the head and neck, medications, or aging [109]. For some known and unknown reasons, acinar cells are more prone to be damaged due to external insults than the ductal cells; this is evident in both SS and irradiated patients. Xerostomia is accountable for multiple oral complications which lead to a severely compromised quality of life, such as difficulty in chewing and swallowing, recurrent oral and pharyngeal infections, ulcerations, and dental caries [8, 110-114]. Keratoconjunctivitis sicca leads to a constant irritation of the ocular surface, recurrent infection, and corneal erosions; scaring; and a possible perforation [3, 110, 115]. Management of SS starts with educating the patient and applying prophylaxis measures. Patients are advised to keep their oral cavity and ocular surface hydrated by drinking water more often and applying artificial tears. When a residual tissue is present, Salagen (secretagogue) is prescribed to increase the secretion of saliva and tears. In serious systemic manifestations, immunosuppression and immunomodulatory drugs are prescribed [116, 117].

### 1.10. Sjögren's Syndrome (SS)

SS was first described by the Swedish ophalmologist, Henrik Sjögren, where he presented his thesis entitled; "Zur Kenntnis der Keratoconjunctivitis sicca" that was published in the Acta Ophthalmol journal in 1933 [111]. SS is one of most common autoimmune diseases which manifests primarily in the exocrine glands, the salivary and lacrimal [118, 119]. It is associated with lymphocytic infiltration in these glands, and other epithelial tissues, leading to destruction and later, loss of glandular secretory function. Subsequently, patients experience dry mouth/eyes and a wide array of extraglandular symptoms [110]. It affects women and men unequally; women,

mainly postmenopausal, are prone 15 times more than men [120]. The prevalence of SS varies among countries ranging from 0.1-0.72% depending on the classification criteria used; several studies carried out in different countries yielded different results which might be due to ethnic differences [4-6, 121-124].

### 1.10.1. Primary and Secondary Sjögren's Syndrome

The disease is classified into primary (pSS, sicca syndrome) when it manifests alone with extraglandular involvement , or secondary (sSS) if it coexists with another connective tissue autoimmune disease, such as RA, SLE, or Hashimoto's thyroiditis [125-127]. Although the two SS syndromes have much in common, but there are numerous differences at the clinical, immunological and genetic levels [110, 128]. Upon diagnosis, the classification criteria for both are the same except that the positivity for autoantibodies is not mandatory for the secondary SS, according to American-European Consensus Group (AECG) [129, 130]. A study was performed to evaluate the similarities between pSS and sSS regarding dry eye test and antibody level and found no difference. However, the positivity for anti-SSA was more related to the severity in pSS and to Rose Bengal test score [131]. Additionally, higher autoantibodies levels is strongly correcelated with parotid glands enlargement and higher infiltrating B cells in pSS [130].

### 1.10.2. Immune Dysregulation in SS

Innate and adaptive immunity alterations were evident in SS patients and SS animal models [132]. Although we achieved a significant progress in understating the SS immune dysregulation but, we are still missing some key events, especially the very early ones. The initial trigger that causes exocrine glands injury remains unidentified; however, a combination of genetic predisposition and environmental factors are believed to be critical. The genetic background alone does not lead to disease emergence, this is supported by the fact that identical twins lack concurrence of SS, and the same applies to the environmental factors [133, 134]. However, viral infection is strongly proposed as one of the initiating factors when the supposed genes are present; however, it is not definitive [132, 133]. The viral infection provides antigens to toll-like receptors (TLRs) located on dendritic and epithelial cells of the exocrine glands which activates them to present the major histocompatibility complex class II (MHC-II) and secret inflammatory cytokines.

### 1.10.3. Aetiology and Risk Factors

The aetiology of SS is controversial and multifactorial, it is also intrinsic and extrinsic. The late onset of the disease, especially in pSS, makes characterization of the triggering factors and early cellular and molecular events very difficult. However, until now, series of four distinct cellular events have been identified in SS human patients: 1) initiation by an exogenous factor, 2) disruption of salivary gland epithelial cells, 3) T-lymphocyte migration and infiltration of exogenous glands, and (4) B lymphocyte hyper-reactivity accompanied with production of rheumatoid factor and autoantibodies to Ro (SS-A)/La (SS-B) [112, 135]. SS was first presented by Dr. Henrik Sjögren in 1933 [114, 136]. Thereafter, a substantial amount of research was directed toward identifying the mechanisms that leads to SS. Several hypotheses have been proposed to explain the complex nature of the disease, using data from patients and studying several animal models. However, there are four mechanisms that are believed to be very crucial in the development of SS. These include: the effect of epithelial cells apoptosis and the role of autoreactive T and B lymphocytes [137].

#### 1.10.3.1. Genetic Predisposition

Most autoimmune disorders are prevalent as low as 0.1-1.0% in the general population. The chances that any patient develops an autoimmune disease jump five times higher if a first degree relative is affected and this possibility is doubled when your monozygotic twin has developed it [138]. Siblings of SS patients exhibited the highest risk among relatives [139]. Numerous genes have been linked to SS etiopathogenesis, but these genes are not rare variants, they are rather polymorphic [140]. Additionally, SS patients showed a significantly higher gene expression for: MHC (HLA), antigen processing, type 1 interferon regulated genes, and lymphocytes development [141]. MHC genes encode components of the human leukocyte antigens (HLA) class I and II. MHC II genes associated with HLA-DR and HLA-DQ have been considered the most important genes linked to SS etiology [139]. These molecules, when expressed on salivary gland epithelial cells, can present autoantigen and exogenous antigens to T-cells which will invite more lymphocytes and a massive infiltration occurs [142, 143].

### 1.10.3.2. Viral Infection

As a multifactorial and complex disease, SS has been linked to several viruses. Glandular infection with one of the accused viruses leads to dendritic cells activation and a subsequent activation of HLA-independent innate immune system [144]. Many viruses have been linked to SS, including cytomegalovirus, Epstein–Barr virus (EBV), human herpes virus type 6, human T lymphotropic virus 1, retroviruses, hepatitis C virus, and enteroviruses [145-147]. One of the premier studies investigating the presence EBV in patients' salivary glands was conducted by FOX et al. [148]. They found that 67% of the patients' salivary glands were positive for EBV, whereas neither the control nor the other SS patients' organs showed any positivity toward the EBV antibody or DNA probe.

### 1.10.3.3. Hormones

The predominance of women affected with SS has led to more investigations to unveil the connection between the female sex hormones and higher disease susceptibility. At first, an assumption was made that the decrease of blood level of sex hormones after menopause has participated in the pathogenesis of SS. On the contrary, sex hormones have an aggravating factor on the disease severity. A study was carried out to evaluate the effect of hormonal replacement therapy (HRT) on women has found that an increase in duration of the HRT has caused an increase in dry eye syndrome and they recommended that patients should be protected from this side effect [149]. Another hormone along with the ovarian estrogen, is the adrenal dehydroepiandrosterone (DHEA). DHEA which decreases after menopause has been linked to the emergence of SS [150] and its replacement has decreased oral dryness [151]. Estrogens receptors (ER α/β) are expressed on most immune cells. These receptors influence both the innate and the adaptive immunity [152]. Therefore, androgens themselves are not directly immunogenic, but do regulate the immune system indirectly [153]. Another study have found that the treatment of adult female MRL/Ipr mice with testosterone, estradiol or cyclophosphamide had an immunosuppressant effect measured by the lymphocytic infiltration in the lacrimal gland; lower focus score [154]. In NOD.B10.$H^{2b}$ mice, ovariectomy has led to lymphocytes infiltration in the lacrimal gland and subsequently, death of the epithelial cells, but the treatment with dihydrotestosterone or 17b estradiol at the time of the ovariectomy had arrested these changes [155, 156]. Low levels of vitamin D has been found lately to be associated with autoimmune diseases including SS [156]. SS patient who had low levels of

vitamin D could be at a higher risk of neuropathy and lymphoma [115]. These findings suggest that vitamin D might be involved in the etiology and its low level affects the prognosis; therefore, it was advised to be included in the treatment protocol [115].

### 1.10.4. Pathophysiology

The pathogenesis of SS is very complicated and not fully understood despite the vast advancement of our understanding in the last decades achieved by the sophisticated technology and the use of several mouse models. However, due to the gap between the onset of the disease and the time of the diagnosis, that is somewhat big, we are missing the critical early pathophysiological events. Unfortunately, we cannot determine the chronological order of SS progress since several pathological events are happening at the same time [157]. However, there is an agreement among researchers that the initiation, is most likely due to a viral infection; however, not confirmed, the pathology might have started at the epithelial level, provided that the patient is genetically susceptible.

#### 1.10.4.1. Glandular Epithelium Dysfunction

SS symptoms are believed to arise because of glandular epithelial dysfunction that is caused by the progressive inflammation and its by-products: function-modulating cytokines, autoantibodies and apoptotic signals [158]. However, the degree of the gland dysfunction is not necessarily correlated with the severity of the inflammation, which supports the fact that epithelial cells are involved [159]. The secretion of these cells is somewhat altered due to intrinsic and extrinsic factors. It is believed that the cytokines associated with inflammation interfere with neurotransmission signals coming to the glands. Additionally, autoantibodies against muscarinic receptor 3 (M3R) have been associated with reduced salivary function in patients [158]. Parasympathetic signaling via M3R, directly affect the intracellular trafficking of AQP5, affecting the water content in saliva [158] [160, 161]. This disruption of AQP5 expression will take part in the epithelial dysfunction; furthermore, several studies reported that the localization of AQP5 is shifted basally instead of the normal apical location but, some reports have found that TNF-α, as an inflammatory cytokine, is involved in this translocation of the water channel, whereas blocking TNF-α has restored AQP5 apical location [162, 163]. Mucins are part of the saliva constituents,

but irregular over-expression and accumulation have been linked to inflammatory cytokines and reported in SS patients [164].

Researchers have always emphasized on the pivotal role of epithelial cells apoptosis in the pathogenesis of SS and several studies have reported an increased rate of apoptosis in patients [165, 166]. Three major autoantigens which are often targeted by immune system are SSB/La, Ro52/TRIM21 and Ro60/TROVE2. Anti-Ro/SSA and anti-La/SSB are found in the serum of 60-70% of primary SS patients [112]. During the initial phases of apoptosis, they are translocated to the cytoplasm; later, they are expressed on apoptotic blebs [158]. Eventually, antigen presenting cells (APC) present these proteins to T-cells , this step is considered the major event in SS initiation [161]. However, the epithelial cellular events that have led to their apoptosis are still not understood. Several mechanisms are thought to participate in the apoptosis mechanism in exocrine glands of SS patients [158]. Epithelial cells, taken from patients, are involved in initiation of apoptosis because they express Toll-like receptor (TLR) and some costimulatory molecules which take part in the inflammation by recruiting lymphocytes [135]. However, it could also be a results of an imbalance between the apoptosis inhibitor Bcl-2 and the apoptosis inducer Bax factors or the activation of FAS/FASL axis [167]. Cytotoxic T and B cells have been reported to be associated with apoptosis by secreting cytokines and enzymes that critically influence the process [158, 161]. Additionally, circulating anti-SSB/La and anti-SSA/Ro have been linked to increased levels of caspase-3 [115]. Lastly, Estrogen deficiency, which is present in the majority of SS female patients, has been linked to the epithelial cell apoptosis. Mice that are deficient in estrogen have shown symptoms similar to SS in human patients. These mice were found to overexpress RbAP48 which leads to dry eyes and mouth via promoting apoptosis and expression of autoantigens [168, 169].

#### 1.10.4.2. Role of T Cells

Fox et al has reported that $CD4^+$ T cells (polarized toward Th1) comprised the majority of infiltrating lymphocytes in the minor salivary glands biopsies from SS patients [170, 171]. Although both cell subsets are present in the lymphocytic foci; however, $CD4^+$ seems to lead the attack then $CD8^+$ follows as the disease progresses [172]. $CD4^+$ was found to comprise around 70% and $CD8^+$ forms the rest of the lymphocytes detected in minor salivary glands biopsies [173].

$CD4^+$ cells were found located in the periductal area, whereas $CD8^+$ were in close proximity to the acinar epithelial cells. [174]. $CD4^+$ glycoprotein defines a major T cell subset called the T helper (Th) [175]. T helper is further divided into two major families; the Th1 and Th2 which secrete different sets of cytokines [173]. Th1 cytokines are considered more pathogenic whereas Th2 exerts a more protective function; however, Th2 was also found to be play a pathogenic role under specific conditions [173]. Th1 secrets IFN-γ and IL-2, which lead to the activation of macrophages. Th2 secrets IL-4, IL-5, and IL-13; important cytokines in humoral response and other immune responses. On the other hand, a significant percentage of acinar cells stained positive for an apoptosis marker were in close contact with $CD8^+$ [174]. However, SS etiopathogenesis is far more complex to be assigned to a specific pathogenic pathway. Recently, various T cells were found to be involved in the initiation and the progression of the disease, including Th17, T reg, and others [171]. In the next section, a more detailed role of each cytokine is discussed.

#### 1.10.4.3. Cytokines in Sjögren's Syndrome

Cytokines are powerful mediators that regulate the two arms of the immune system: the innate and the adaptive[176]. They are small pleotropic regulators mediating several cellular events, such as immune reactions, hematopoiesis, wound healing and chemotaxis proteins secreted. Cytokines function and secretion are tightly controlled, and any deviation might lead to allergy, immune deficiency, or autoimmunity. They often commit as pro- or anti-inflammatory; however, this is not always the case and it is governed by the surrounding conditions and environment [176]. Cytokines over-secretion or imbalance is a hallmark in SS pathogenesis. Analysis of the cytokine profile in the salivary glands and peripheral blood of SS patients revealed that type I IFN pathway is the most important and most influential in SS pathogenesis [177].

**IFNs**

IFN (interferons) pathway is activated in several autoimmune diseases, including SS; at least 50% of SS patients were positive for IFNs when their blood and salivary glands were tested [173, 177]**.** Type I INFs are secreted by infected cells, viral infections in particular, to limit spreading of the pathogen by inducing intrinsic antimicrobial state, activating APCs/NK cells, and activating the adaptive immune system which shifts the immune reaction toward an antigen-specific T- and B-cell response [177, 178]. They are secreted by plasmacytoid dendritic cells (pDC). Type II-IFN-γ

is a major cytokine in Th1 reaction. It is secreted by T cells, macrophages and natural killers (NKs), and is directed to clear intracellular infections has been involved in the onset of the disease [179]. It was found at high concentrations in SS-prone NOD mice; furthermore, when these mice were double knocked out for IFN (NOD.IFNγ$^{-/-}$) or IFNγ-R (NOD.IFNR$^{-/-}$), no sialadenitis was evidenced [180]. However, in SS patients; analysis of glandular infiltrating cells showed high concentration but normal blood level , in the same patient [176, 181]. Moreover, it was found at higher levels in glands with sicca symptoms but no infiltration, suggesting a role in the dysfunction of the acinar cells. When HSG cells were incubated with IFN-γ they were found with slower growth and depleted $Ca^{+2}$ stores [176, 182, 183]. These previous findings explain the impact of IFN-γ high concentration on the exocrine glandular cells.

**TNF-α**

Tumor necrosis factor alpha (TNF-α) is a pleiotropic cytokine that can activate several pathways involved in several biological processes [177]. It is produced by several cells, including monocytes, CD4+ T cells, and epithelial cells. TNF-α upregulates the apoptotic receptor Fas in targeted cells [176]. However, , perhaps it is the cellular mediators iNOS/NO that are influenced by high TNF-α in the serum that are actually leading to its toxic effect in inflammation [137, 184]. A study published in 2012, has reported a negative effect of TNF-α on the expression of AQP5 in a cell line [163]. In SS patients, high levels of TNF-α are detected in the serum and in minor salivary gland biopsies [177]. However, anti-TNF-α therapies failed to achieve satisfactory results [163].

**BAFF**

B-cell activating factor (BAFF) critically promotes survival and maturation of splenic B cells [185]. It is a member of the TNF family expressed as membrane-bound that can be cleaved and released in a soluble form [186]. It is expressed by immune and non-immune cells, such as monocytes, dendritic cells, to a lesser extent T cells, and salivary/airway epithelial cells. After production, BAFF is activated by several factors, including IFN-α, IFN-γ, GCS-F, and CD40. BAFF transgenic mice exhibit a severe form of autoimmunity that expresses as SS and SLE which later leads to renal failure [187]. High levels of BAFF in saliva and minor salivary glands biopsy were detected in SS patients. Excessive production of BAFF is connected to resistance to apoptosis

signals and lengthened B cells survival, hyperglobulinemia, higher autoantibodies, and ectopic germinal centers in salivary glands [186, 188]. BAFF-targeted treatment has achieved satisfactory results in a clinical trial; however, no improvement in the salivary or lacrimal function was detected [189].

**TGF-β**

Transforming growth factor beta (TGF-β) family is composed of a group of cytokines that are involved in many immune and non-immune processes [190, 191]. TGF-βs receptors are expressed by almost all cell types which explains its pleiotropic action [192]. It is critically involved in immune tolerance and homeostasis; perturbations in its signaling pathway was connected to cancer development and initiation of inflammatory diseases [193]. Certain populations of $T_{reg}$ and Th3 secret TGF-β1 to induce their immunoregulatory function [192]. TGF-β was detected in the minor salivary glands of SS patients with variable level in the adjacent infiltrating cells [194]. TGF-β is associated with overstimulation of the immune system and its overexpression is connected to fibrosis [176].

**IL-1**

Interleukin-1 (IL-1) is a group of cytokines and receptors that affect a broad spectrum of immunological responses, and is associated with innate immunity [195]. IL-1 family is associated with more destructive inflammation than any other cytokine family; however, some of the members are essential in the non-specific infections resistance (innate immunity) [195]. High levels of IL-1β were found in minor salivary glands biopsies, saliva, and blood of SS patients [196, 197]. Additionally, both IL-1α and IL-1β were found at higher levels in tears of SS patients [198], whereas another study reported an imbalance of IL-1/IL1Ra in the oral mucosa [199]. Other studies found a higher IL-1β serum level in anti-SSA/Ro positive patients [200]. The previous reports, strongly associated IL-1 with the pathogenesis of SS in humans.

**IL-4**

Interleukin-4 (IL-4) is an anti-inflammatory cytokine and the dominant player in Th2 response. However, SS is one of the autoimmune diseases with an imbalance between Th1 and Th2 which is reflected on the pattern of cytokines detected in the patients [201]. IL-4 was not detected or was

low in SS patients' biopsies, and in other reports, much higher ratios of IFN-γ/IL-4 were also detected in the salivary glands when compared to the peripheral blood in SS patients [176, 181].

**IL-6**

Interleukin-6 (IL-6) is a pleiotropic cytokine which is secreted by lymphoid and non-lymphoid cells and display a hormone-like mode of action and mediate homeostatic processes [202]. It is released from macrophages and monocytes in response to pathogen invasion and damaged tissue, then transmitted via blood to elicit a cascade of immune responses, including acute phase responses [203, 204]. However, when the insult is removed, its level goes back to normal, but excessive or persistent secretion leads to various inflammatory conditions and cancers formation [204]. It is also involved in the generation and proliferation of Th 17 cells [205]. However, its deficiency can significantly affect the innate and adaptive immunity functions toward viral, parasitic, and bacterial infections [202]. High level of IL-6 was connected to fatigue in SS patients along with IL-1β and TNF-α [206]. SS patients displayed high levels of IL-6 in the saliva of which was correlated with other features of the disease, such as SFR, focus score, or autoantibodies [207]. However, no significant difference of serum IL-6 level was detected between SS patients and control subjects [208].

**IL-10**

Interleukin-10 (IL-10) family is divided into three groups where IL-10 comprises the first group [209]. IL-10 induces immunosuppression of innate and adaptive immune responses to limit the tissue damage in exaggerated immune responses [209]. It is secreted from various cell types, including $T_{reg}$ , macrophages, T and B cells, NK, and dendritic cells [209]. It potently inhibits the secretion of proinflammatory cytokines, including IFN-γ, TNF-α, IL-1β, and IL-6 in several cell types [210]. On the contrary, IL-10 has immunostimulatory functions; it can induce $CD8^+$ T cells to secret IFN-γ, and support the growth and differentiation of B cells, mast cells, and thymocytes [210]. IL-10 serum analysis revealed higher level of IL-10 in SS patients [211]. Moreover, IL-10 level was found to correlate with higher SS susceptibility, and autoantibodies level [127, 212].

**IL-12**

IL-12 family is composed of inflammatory cytokines; IL-12 and IL-23, and anti-inflammatory cytokines; IL-27 and IL-35 [213]. IL-12 is a heterodimeric cytokine secreted by macrophages, monocytes, B cells, and a number of immune cells [214]. IL 12 induces the secretion of IFN-γ which in its turn amplifies the intensity of the immune reaction. IL-12 was detected in the cytoplasm of most acinar and ductal cells in salivary glands of SS patients, and in the scattered lymphocytes [214]. In a recent study, they found that SS patients have higher serum levels of IL-12p70 which positively correlated with the activity of the disease [215].

**IL-18**

IL-18 is a member of IL-1 family and its structure is homologous to that of IL-1β, but its function is different [216, 217]. It is expressed by a wide range of lymphoid and non-lymphoid cells, such as T and B cells, macrophages, dendritic cells, osteocytes, and keratinocytes [216]. IL-18, in synergy with IL-12, is a key cytokine in Th1 induction and the major inducer of IFN-γ synthesis and release; however, it also instruments Th2 activation via IL-4-independent process [216, 218]. In addition, IL-18 is involved in the secretion of TNF-α and IL-1β from Th1, macrophages, and NK cells [218, 219]. Elevated IL-$18^{+}$ cells count has been linked to parotid involvement and C4-hypocomplementemia which are indicators of poor prognosis and lymphoma development [220]. SS patients exhibited high serum levels of IL-18, and even higher levels were found in patients with myalgia symptoms [212].

**IL-17**

Th17 is derived from T memory cell, it secretes IL-17, IL-23, and IFN-γ when activated by IL-12 [176]. IL-17 family is composed of six members which areIL-17 A, B, C, D, E, and F [221]. IL-17 association to autoimmunity is well established [221, 222]. Strong positive staining in lip biopsies for IL-17A was detected in infiltrating lymphocytes and salivary ductal cells, and to a lesser extent the acinar cells of SS patients [223]. No difference was detected for the level of IL-17 in saliva and serum between SS patients and healthy controls [223]. In SS patients, no clear correlation between increased Th17 activity and SS symptoms has been established; however, its activity supports autoreactive B cells which leads to disease progression [224].

### 1.10.5. Signs and Symptoms

The patients' chief complaints are dryness in the mouth, difficulty swallowing, and a grainy sensation in their dry eyes. However, a significant percentage (30-40%) of patients will also present with multiple extraglandular symptoms, particularly, fatigue. As time passes, the patient's quality of life will deteriorate with more systemic complications to arise.

#### 1.10.5.1. Oral Manifestations

Oral signs and symptoms arise when more than 50% of salivary secretion is lost [225]. Swelling of the salivary glands is a common finding; 25-66% of SS patients have an enlarged parotid [226]. Many patients report symptoms of dry mouth or an associated complaint that is due to dryness like the presence of an unpleasant taste, difficulty swallowing of dry food, and difficulty retaining their dentures. As the disease progresses, patients lose the pooling of the saliva in the floor of the mouth and the oral mucosa appear glazed; in some cases, fine wrinkles might appear. On the other hand, the tongue appears lobulated, might stick to the palate with partial or complete depapillation in severe cases [227]. Saliva maintains certain composition of oral flora due the presence of several compounds, such as IgA, defensins, proteases, histatins, and lysozyme, lactoperoxidase; however, in patients with diminished salivary flow, this composition is altered [227, 228]. It was reported that the number of oral bacteria from SS patients and control was similar but there was a shift toward certain types of bacteria, such as Lactobacillus acidophilus, Streptococcus mutans, and Candida albicans fungi, which explains the increased risk of dental caries and oral candidiasis in SS patients [228]. The loss of the flushing and buffering capacity of saliva have manifested as higher dental caries, especially the types that are usually rare in healthy subjects, such as root and incisal caries [117].

#### 1.10.5.2. Ocular Manifestations

The symptoms of dry eyes vary from one patient to another, depending on the severity of the tear flow loss, the ability of the remaining tears to maintain the function, and the patient's tolerance for ocular dryness [229]. The ocular symptoms are expressed by the patients as: grittiness, having a foreign body in the eyes, irritation, photosensitivity, rope-like secretion at the inner canthus of the eye, erythema, eye fatigue and decreased visual acuity [230, 231]. The cornea will show erosion and thinning due to desiccation, and in severe cases filamentary keratitis is seen in the damaged

areas [232]. Conjunctivitis may occur due to Staphylococcus aureus infection. In severe cases, ulceration, opacification, and perforation may occur in few cases [227].

#### 1.10.5.3. Extraglandular (Systemic) Manifestations

Extraglandular manifestations occur in 30-40% of SS patients [233]. SS has a wide clinical spectrum, ranging from a mild exocrinopathy to life threatening condition [234]. Thus, the immune dysregulation in SS can simply affect any organ due to the lymphocytic infiltration around epithelial cells and B cells hyperactivity. The latter causes the formation of autoimmune complexes that leads to more serious complications, like palpable purpura, cryoglobulinemia-associated glomerulonephritis [233, 234]. Peripheral lymphocytic infiltrations were found in the liver, kidney, and lungs around bronchi/bronchioles. The cryoglobenemia and autoantibodies produced by B cells hyperactivity are responsible for the extraglandular manifestations, such as vasculitis, purpura, glomerulonephritis, peripheral nephropathy, and most seriously lymphoma [234]. However, extraglandular manifestations may present in some patients alone without glandular involvement [235]. Systemic involvements include fatigue, skin, neural, pulmonary, hepatic, musculoskeletal, renal, hematological, hematological, thyroid, and vaginal manifestations [236-241].

**Fatigue**

It is one of the most common extraglandular complications with a prevalence of 68-85% of patients [241, 242]. Some have attributed it to a relation between SS and fibromyalgia [242] while others found no correlation [243]. However, the autonomic nervous system dysfunction, both the sympathetic and parasympathetic, was found to cause fatigue, depression and anxiety [244].

**Cutaneous Manifestations**

Patients suffer numerous cutaneous complications, such as dry skin (xeroderma), burning sensation, skin rash, vasculitis, Raynaud's phenomena, eyelid dermatitis, angular cheilitis, and erythema annulare [227, 245]. The skin manifestations are caused by the disruption to the biochemical biology of the epidermis, expressed as increased epidermal proliferation and perturbation of epidermal differentiation [246]. Patients, estimated at 23-67%, experience pruritis and dryness in some areas, which lead to scratching and subsequently pigmentation due to trauma

to the melanocytes [246]. Cutaneous vasculitis is the most serious skin manifestation which affects small vessels [245]. It is often clinically presented as purpura on the lower extremities [238]. Patients who develop vasculitis frequently have poor prognosis because they are at a higher risk of developing glomerulonephritis and non-Hodgkin lymphoma [245].

**Pulmonary Manifestations**

Lung involvement is common among SS patients, but it is usually subclinical and mainly affects the bronchioles in the form of peribronchial lymphocytic infiltration [247]. Pulmonary symptoms are reported as dry upper respiratory tract, dry crusty nose and bronchial hypersensitivity [248]. However, dry cough or xerotrachea is the main symptom which was reported in nearly 50% of patients [247, 249]. Pulmonary symptoms are not serious and are basically a results of epithelial tissue damage caused by the immune system [247].

**Gastrointestinal and Hepatic Manifestations**

Dysphagia has been reported in 30-81% of patients [250]. It is caused by the lack of saliva, and the longer duration that it takes the tongue to contact the posterior wall of the pharynx. Dyspepsia is another symptom that is found in 15.6-23% of screened patients. $CD3^+$ lymphocytes, mainly $CD4^+$, were found in the wall of the stomach of SS patients, similar to those found in their salivary glands and are responsible for the atrophy of the gastric glands [250]. Liver involvement is a common extra-glandular manifestation in SS patients; thus, patient's liver should be examined, especially when they have positive autoantibodies, such as anti-Ro, anti-Ro/La, and/or anti-RNP [251]. Primary biliary cirrhosis (PBC), autoimmune hepatitis (AH), non-alcoholic fatty liver disease, and HCV are the most common causes of liver disease in SS patients [250]. Primary Biliary Cirrhosis (PBC) is a serious liver specific disorder characterized by chronic loss of interlobular bile ducts [252]. It was found that 4-9% patients had PBC. Hepatomegaly is reported in 11-21% of patients [250]. B cell clonality is another hepatic feature that is found in 77.8% of patients; however, it was not associated with an increased risk of lymphoma [250].

**Musculoskeletal Manifestations**

Musculoskeletal complications are relatively common in SS patients, specifically, arthralgia and arthritis [253]. An estimated 40% of patients have been found to have nonerosive arthritis, and 60-70% of patients were RF positive [8]. In addition, an estimated 20% of patients suffered from a diffuse musculoskeletal ache that might indicate un underlying fibromyalgia syndrome.

**Neural Manifestations**

Neural complications were found to occur in 2-70% of SS patients [241, 253]. Peripheral involvement is a common complaint among patients. [241]. The peripheral clinical spectrum of neuropathy is broad and includes pure sensory neuropathy, sensorimotor polyneuropathy, and autoimmune demyelinating neuropathy. Central nervous system is less involved; reported in 20% of cases only [253], and most importantly misdiagnosed [241]. It manifests frequently as diffuse, non-focal neurological signs and includes numerous features, such as cognitive deficits, psychiatric abnormalities, and migraine [241].

**Renal Manifestations**

Renal complications are relatively rare in SS patients and may precede the sicca symptoms [241]. It manifests as interstitial nephritis; the presence of white blood cells in the interstitium combined with tubular atrophy and eventually fibrosis. In rare cases, some patients might develop glomerulonephritis due to the deposition of immune complexes; however, it usually manifests late in the disease course due to small vessels involvements [241]. Glomerulonephritis was found to be associated with formation of lymphoma [254]. Additionally, SS patients have shown higher tendencies toward developing urinary tract infection more than healthy individuals [235].

**Hematological Manifestations**

Considering the nature of SS as an autoimmune dysregulation disease, clinical cytopenia were not a complication to expect; however, it was found that 40% of SS patients had cytopenia, such as autoimmune haemolytic anaemia, immune thrombocytopenia, myelodysplastic syndrome, neutropenia, aplastic anaemia and red cell aplasia [255]. Idiopathic CD4+ T-lymphocytopenia which is a risk factor for non-Hodgkin malignant lymphoma was found to occur frequently in SS patients, particularly in anti-SSA positive patients [241, 256].

**Lymphoma**

Lymphoma was found to occur in around 5% of SS patients [1]. It is one of the most serious complications associated with pSS; it was found that pSS patients are at 44 times higher risk than the normal population [112, 257]. Patients who have cryoglobulinemia are 9 times more likely to develop lymphoma than other pSS patients [112]. Non-Hodgkin (low grade B cells) lymphomas, also known as mucosa-associated lymphoid tissue (MALT) [258], are the main type that pSS patients develop [259]. These lymphomas are usually found in the salivary glands. Predictive factors for possible underlying lymphomas are many, including parotid swelling, palpable purpura, and splenomegaly [260]. MALT is a slow-growing tumor with a good prognosis.

**Thyroid Manifestations**

A strong association was found between SS and autoimmune thyroid diseases (ATDs) [261]; in fact, SS and ATDs are the most frequent coexisting disorders [262]. SS was found to be 10 times higher in ATDs patients and ATDs was nine times higher in SS patients**.** The previous findings suggest a solid correlation between the genetics and the etiopathogenesis of both diseases. The strong association between both diseases was due to shared antigens between tissues [261].

**Vaginal Manifestations**

Exocrine glands in the wall of the vagina are a common site for lymphocytic infiltration, leading to vaginal dysfunction and dryness. Dyspareunia (vaginal dryness) and vulvar pruritus are common complaints among female SS patients [263, 264]. Females report painful intercourse and pruritis; upon examination**,** an active fungal or bacterial infection are present in those female patients, similar to the oral complications [246].

### 1.10.6. Sjögren's Syndrome Classification Criteria

Since 1965, establishing classification criteria for SS has been attempted in clinics by physicians and multidisciplinary specialists [265-270]. The classification criteria were given more attention to be widely used by clinicians as a diagnostic tool, providing that high sensitivity and specificity are met [129]. However, as the complete pathogenic picture has not been unveiled yet, the classification was updated several times to accommodate the new findings. Since 2002, classification of SS has been based primarily on the American European consensus group criteria

(AEGD) [129, 271]. In 2016, these criteria included subjective and objective findings, summarised in Table 2. The AEGD suggests that the validation of SS diagnosis depends on the presence of any 4 of the 6 signs, listed in table 2, as long as sign IV (Histopathology) or IV (Serology) is positive [272]. However, recently, the American Rheumatology Group (ACR) has proposed a new classification criterion for diagnosis of SS giving more weight to the objective findings and excluding subjective findings due to the arising use of expensive biologic treatments (Table 3). According to this classification, patients presenting with subjective signs and symptoms of SS can have a confirmed diagnosis if two of the three objective tests are positive [272].

**Table 2**. **American-European Consensus Group (AECG)** [272]

1. **Ocular symptoms**: a positive response to at least one of the following questions:

- Have you had daily, persistent dry eyes for more than 3 months?
- Do you have a recurrent sensation of sand or gravel in the eyes?
- Do you use tear substitutes more than 3 times a day?

2. **Oral symptoms**: a positive response to at least one of the following questions:

- Have you had a daily feeling of dry mouth for more than 3 months?
- Have you had recurrently or persistently swollen salivary glands as an adult?
- Do you frequently drink liquids to aid in swallowing dry food?
-

3. **Objective ocular signs** - a positive result for at least one of the following two tests:

- Schirmer's I test, performed without anesthesia (≤5 mm in 5 minutes).
- Rose Bengal score or other ocular dye score (≥4 according to van Bijsterveld's scoring system).

4. **Histopathology:** Biopsy from minor salivary glands and examined by an expert histopathologist with a focus score ≥ 1, defined as number of lymphocytic foci (which are adjacent

to normal-appearing mucous acini and contain more than 50 lymphocytes) per 4 $mm^2$ of glandular tissue.

5. **Salivary gland involvement**: objective evidence of salivary gland involvement when one of the following tests is positive:

- Unstimulated whole salivary flow (≤1.5 ml in 15 min).
- Parotid sialography showing the presence of diffuse sialectasias (punctate, cavitary or destructive pattern), without evidence of obstruction in major ducts.
- Salivary scintigraphy showing delayed uptake, reduced concentration and/or delayed excretion of tracer.

6. **Autoantibodies**: presence in the serum of the following autoantibodies: Antibodies to Ro (SSA) and/or La (SSB) antigens.

**Table 3**. **American College of Rheumatology criteria (ACR)** [270]

1. Keratoconjunctivitis sicca with ocular staining score ≥3
2. Labial salivary gland biopsy exhibiting focal lymphocytic sialadenitis with a focus score ≥1 focus/4 $mm^2$.
3. Autoantibodies: presence in the serum of the following autoantibodies:

- Antibodies to Ro (SSA) and/or La (SSB) antigens.
- Positive rheumatoid factor.
- ANA titer ≥1:320.

In 2016, the two main rheumatology communities: the American College of Rheumatology and the European League Against Rheumatism, have finally released together the "The 2016 ACR/EULAR classification criteria" [273]. This new classification did not introduce any new measures, but in fact, has changed the weight of each of the previous criteria. The importance of each criterion in the diagnosis is given certain points depending on how sensitive this criterion is,

in the diagnosis, with a total of 9 points. The presence of autoantibodies and a confirmatory positive histopathological assessment, each were given three of the total nine points. However, the salivary and ocular flow rates, as well as, the ocular staining, each received a point, if the determined reading is met (Table 4) [273, 274]. In addition, for a diagnosis to be initiated, the patient must meet certain inclusion criteria which includes subjective oral and ocular dryness, experienced by the patient for at least three months. Exclusion criteria were also applied to confirm that the patient has no other coexisting diseases or has received radiation in the head and neck area (Table 4) [273, 274].

**Table 4. The 2016 ACR/EULAR classification criteria for SS** [275]

**1. Consensus criteria items for the classification of SS**:

- Abnormal unstimulated salivary flow rate* (⩽0.1 ml/min). **(1 point)**
- Abnormal Schirmer's test (<5 mm in 5 min). **(1 point)**
- Abnormal findings with lissamine green or fluorescein staining ⩾5 in Ocular Staining Score or ⩾4 in Van Bijsterveld Score. **(1 point)**
- Presence of anti-Ro/SSA antibodies. **(3 points)**
- Histological evidence of focal lymphocytic sialadenitis, with a focus score ⩾1 focus/4 $mm^2$, (1 focus = 50 lymphocytes/4 $mm^2$). **(3 points)**

**2. Inclusion criteria**:

- Dryness of eyes or mouth for at least 3 months, not explained otherwise (e.g. medications, infection).

**3. Exclusion criteria**:

- Status post head/neck radiation.
- HIV/AIDS.
- Sarcoidosis.
- Active infection with hepatitis C virus (PCR replication rate).

- Amyloidosis, graft versus host disease, IgG4-related disease.

A diagnosis is established if a score ⩾4 points is achieved after application of inclusion and exclusion criteria.

### 1.10.7. Sjögren's Syndrome Treatment

Treating SS is very difficult, complicated, and costly, due to the complex nature of the disease and the involvement of various organs and tissues [116]. Management of SS patients should be team-based, which should include at least a rheumatologist, a dentist, and an ophthalmologist [258, 276]. The current universal protocol for SS is basically based on educating the patients about the nature of the disease and the aggravating factors, alleviating the dryness symptoms, and prescribing immunosuppressant and/or immunomodulatory drugs for serious complications and unresponsive patients [116, 258].

#### 1.10.7.1. Current Clinical Treatment for dry mouth and eyes

This section will cover the current treatment protocols that are recommended by physicians and dentists to SS patients for managing the symptoms.

**Dry Mouth and Oral Lesions**

Saliva is essential for maintaining oral structures and oral environment [277]. The treatment protocol starts with patients' education about the importance of saliva. They should be advised to compensate for the lost saliva by drinking water and fluids more frequently, to reduce the dryness complications, such as dental caries, periodontal diseases, and oropharyngeal infections [248]. Managing SS depends on the remaining salivary tissue and the severity of hyposalivation; mild, moderate or severe [278, 279]. In mild hyposalivation, where tissue is still preserved, artificial saliva and lubricants along with mechanical saliva stimulators, like sugar-free gum is the first protocol to adopt. Topical 5% fluoride varnish at least twice a year is recommended, for its important role in preventing dental caries, combined with a daily use of fluoride toothpaste and a weekly use of fluoride mouth rinses. Patient's education about better dietary choices is

recommended, including the decrease of sugar and sticky food [280]. When saliva decreases substantially, moderate to severe hyposalivation, with decreased remaining gland function, a more profound treatment is recommended. Oral sympathomimetic drugs, like pilocarpine or cevimeline are the best choice, unless any contraindications are present, such as hypertension [116, 281, 282].

**Dry eyes**

The damage that occurs to the corneal and conjunctival epithelia is a result of tear volume decrease, changed tear composition, and tear-film break-up [258]. Dry eyes or xeropthalmia management depends on symptoms severity and the damage to the ocular apparatus. Mild episodic dry eye symptoms are usually managed with preventive measures, like avoidance of any systemic drugs that might cause dryness of the eyes, like antihypertensive drugs, avoidance of activities that require a prolonged opening of the eyes, like reading and video games, as well as maintaining a good eyelid-margin hygiene to reduce Meibomian gland dysfunction [258, 275]. When the previous regimens are no longer relieving the symptoms, the use of artificial tears should be considered, at least twice daily [258]. Their ingredients do not mimic the composition of human tears, they contain lubricant with a polymer base and a viscous agent. It was found that the use of more muco-adhesive artificial tears, the greater the reduction of dry eye symptoms [283]. Lubricating ointments are usually prescribed for bed time as they have a prolonged effect, due to strong adhesive property compared to artificial tears, while the patient is asleep; however, they might hinder sight if used during the day [258, 275]. Topical anti-inflammatory eye drops can also be prescribed in moderate to severe cases but for no longer than 6 months, in cases where optimal aqueous therapy has been achieved but the patient is experiencing discomfort [258, 275]. As in dry mouth, pilocarpine and cevimeline have been found effective in alleviating dry eye symptoms. In severe cases, where all the above interventions have failed to relief the patient's symptoms, blockage of the tear drainage system by punctual occlusion is advised. It will increase the residence of the artificial tears in the eyes and hence alleviate the dryness symptoms [258], and it can be performed temporarily or permanently [284, 285].

### 1.10.7.2. Current Clinical Treatment for Extraglandular Manifestations

Symptoms like fatigue, pulmonary and renal involvements…etc., are usually treated systemically, unlike sicca symptoms which are managed topically [286].

#### Fatigue

Most pSS patients suffer from fatigue, depression and anxiety, in addition to the dryness-related discomfort; leaving patients with impaired quality of life [287]. SS patients show different psychological profiles and express high levels of fatigue [286]. Evaluation of fatigue is somewhat difficult due to the presence of other psychological disturbances, like depression and anxiety, in addition to the high prevalence of anxiety in the general population [288]. Several therapies have been tested in randomized clinical trials (RCTs), like omega-6 fatty acid gamma-linolenic acid, dehydroepi-androsterone, and hydroxychloroquine but no improvement was reported by SS patients [289-293]. Furthermore, some biological therapies have shown no promise in managing fatigue in SS patients, including Etanercept and Infliximab (TNF inhibitors), and Belimumab (monoclonal antibody targeting BAFF) [294, 295]. However, Abatacept, (TNF inhibitor) was effective in alleviating fatigue in a small study SS group [296]. On the other hand, Rituximab (anti-CD20) use showed conflicting results where it was effective in some trials but not in others [297, 298].

#### Lymphoglandular Involvement

Enlargement of salivary glands is recurrent, often affects the parotid gland; however, it is rare [258, 299]. It usually begins as unilateral and later becomes bilateral [300]. The current treatment protocol of enlargement is based on clinical experience. In acute enlargement, NSAIDs and short-term glucocorticoids are the first choice by clinicians when the cause of enlargement is pSS [258]. In chronic cases, surgery is the treatment of choice [258]. Lymphoma is a possible cause for the lymphoglandular enlargement in acute and chronic cases; therefore, it should be investigated before commencing the aforementioned management [258].

#### Articular Involvement: Arthralgia and Arthritis

Arthralgia, usually mild, is present is almost half of pSS patients [301, 302]. The current drugs used are usually NSAIDs, hydroxychloroquine, methotrexate, leflunomide and glucocorticoids

[258]. Methotrexate is the first choice to treat arthritis in pSS, as well as RA and SLE. However, low doses of glucocorticoids are considered effective but should not be used for a long period [302].

**Cutaneous Manifestations**

Cutaneous manifestations are common in SS patients, such as xeroderma (23-68%), eyelid dermatitis, annular erythema (AE), and cutaneous vasculitis [241, 303]. Annular erythema which is an elevated wide red periphery with a pale center, is reported in 10% of SS patients [304]. A high percentage of SS patients with an AE test that is positive for anti-Ro/SSA or anti-La/SSB, or most frequently both [305]. These lesions are mainly seen on the face or disseminated, less likely on the neck and upper limb, and tend to leave no scarring when treated [304, 305]. AE is managed with topical glucocorticoids unless the condition is extensive; thereafter, the systemic route is taken into consideration [258]. Xeroderma can be treated with emollient-based moisturizers, taking frequent showers and avoiding skin irritants [306].

**Pulmonary Involvement**

Pulmonary complications were reported in 10-20% of patients [240, 307, 308]. The lung interstitium is affected mainly, but bronchiolitis and bronchiectasis are common also [308, 309]. Several diagnostic tools are utilized to diagnose the complications, including chest-x-ray, CT, lung function test, carbon monoxide diffusing capacity (DLCO) and 6-min walk test. The management of pulmonary complications includes antitussive drugs and β2 adrenergic agonist for the treatment of cough. In general, other interstitial complications are not treated but are subjected to re-evaluation every 6 months as they are often not severe; however, in severe cases, glucocorticoids are usually prescribed [258].

**Renal Involvement**

SS affects renal system causing tubulointerstitial nephritis or immune complex-mediated glomerulopathy [310]. The prevalence of renal diseases in pSS is relatively low, <10% of patients, and it is mostly tubulointerstitial nephritis [241, 311]. Renal involvement could manifest as hypertension, proteinuria, and renal failure. pSS patients must be screened regularly, every 1-2 years, for low molecular weight proteinuria, bicarbonaturia, uricosuria, phosphaturia, glycosuria,

hypokalaemia, nephrocalcinosis, and acidosis [258]. Bicarbonate and/or electrolyte supplementation for a long term is a standard intervention to prevent serious complications. Tubulointerstitial nephritis is usually treated with glucocorticoids; however, life-threatening conditions, like membranoproliferative glomerulonephritis (MPGN) are usually controlled with immunosuppressant [258, 310].

**Muscular Involvement**

Patients experience two distinct types of muscular pain; a mild form in the absence of weakness and no elevated creatine kinase, and a more severe, chronic, and widespread form. The first one is usually treated with analgesics and is considered as a neuropathic pain. However, the second form, which seems to be much more frequent, affecting 30-50% of patients, is more serious and associated with myositis [258]. Myositis is indicative of moderate to severe disease activity and requires management with glucocorticoids [258]. Methotrexate is usually prescribed to SS patients with moderate/severe disease activity to reduce the side effects of glucocorticoids [258].

**Neurological Involvement**

Peripheral neuropathies have been reported in 2.0 - 64% of pSS patients; however, the central neuropathies are only in 5% of patients [300, 312, 313]. Patients with a severe and active disease form, are more likely to develop neurological manifestations [313]. The spectrum of pSS-related neurological lesions is wide [314]. They can be manifested as pure sensory, sensorimotor which is associated with pro-lymphomatous manifestation, or as autonomic neuropathies which is rare [312]. Pure sensory lesions present as distal symmetric sensory loss due to demyelination of nerve axons [312]. Peripheral neuropathies are usually treated with glucocorticoids. Patients with refractory disease and elevated autoantibodies are managed with plasma exchange [315].

**Cryoglobulinemia**

Cryoglobulins are immunoglobulins that precipitate when subjected to a temperature below 37 degrees [316, 317]. They are associated with multiple diseases, such as infections, autoimmune diseases, and malignancies [316]. Cryoglobulinemia is associated with the presence of activated polyclonal B-cells secreting different immunoglobulins [258, 318]. There are three types of cryoglobulins; I, II, and III, pSS is associated with the latter two, a condition called mixed

cryoglobulinemia. Type II consists of polyclonal IgG and polyclonal IgM, whereas type III is composed of polyclonal IgG, monoclonal IgM and RF. Cryoglobulins mediate medical complications via two routes: vascular sludging as in type I, or immune-mediated mechanisms as in Type II and III which are associated with pSS [316]. Cryoglobulinemia leads to various clinical conditions ranging from mild to life-threatening. Mild clinical conditions include fatigue, purpura, and arthralgia. Severe complications involve the nervous system, both central and peripheral, and cutaneous involvements, whereas life-threatening conditions include the MPGN and widespread vasculitis [258, 318]. Treatment of cryoglobulinemia complications depends on the severity and the extent of the condition; it includes glucocorticoid, immunosuppressant drugs, plasma exchange, rituximab, azathioprine or mycophenolate mofetil. Rituximab is considered the first line of treatment in case of systemic manifestations; however, in case of systemic vasculitis, plasma exchange is added to the treatment protocol [258, 319].

**Lymphomas**

Surgical removal (of the gland) along with chemotherapy and radiotherapy are possible options that clinicians often consider; however, close monitoring is also an option with asymptomatic and localized MALT associated with low SS activity [320].

### 1.10.7.3. Clinical Trials

SS is considered the most difficult rheumatic disease to control [116]. The rheumatologic community have not been able to establish evidence-based clear clinical guidelines for SS treatment; consequently, clinicians will rely on the experts' opinions and the treatment of other related autoimmune diseases [321]. However, the advanced understanding of the disease pathophysiology has enabled researchers to design and test biologic therapies. The selection of these treatments was based on the expanded knowledge of certain pathways that are profoundly involved in the disease initiation and/or progression. Factors that affect the function, differentiation and proliferation of T and B cells have received great attention. They are both involved in the pathogenesis; infiltrate the exocrine glands and tends to go through clonal expansion inside the glands [322]. However, other molecules that are critically involved in the immune dysregulation, such as TNF-α have been tested as well [323].

#### 1.10.7.3.1. Biological Treatment

SS is considered the most difficult rheumatic disease to control due to its complex pathogenesis [116, 324]. As previously mentioned, the current available treatments are for symptomatic-based relief unless patient develops extra-glandular manifestations; afterwards, immunosuppressants/immunomodularity agents are prescribed; however, some patients appear to be resistant; thus, the development and testing of biological therapies is mandated [325]. Biological therapies received great attention in the field of autoimmune diseases including SS. They are large synthesized agents that target specific molecules, such as cytokines, cell surface marker, cluster of differentiation, and receptors [298, 323].

The biological therapies tested for SS were often tested in other autoimmune diseases. Improvement was somewhat evident, but with limited efficacy for several reasons [326]. The patients and disease heterogeneity were an obstacle to the success of these therapies in clinics and clinical trials. More sophisticated patients' selection criteria must be established before testing any drug. Patients are at different disease stages, severity, and inflammatory markers making the outcomes unpredictable; however, it might have been different has the appropriate patients' group been selected [326].

Generally, TNF inhibitors are not beneficial for most SS patients, because TNF has an immunoregulatory function rather than an inflammatory one [10, 326]. Rituximab-treated patients showed some improvement in fatigue, glandular function, and disease activity in small studies; however, in large randomized-controlled trials, it failed to achieve the desired results [10]. **Belimumab** and **Abatacept** have been tested in uncontrolled trials and showed a promise [10]; however, a confirmation of the results must be verified by larger randomized controlled trails. TNF-alpha inhibitor (e.g. Infleximab and Entanercept) have been approved for use in clinics to treat various autoimmune diseases, such as rheumatic arthritis and psoriatic arthritis, ankylosing spondylitis, inflammatory bowel disease, and plaque psoriasis [8]. **Infleximab** was tested in a multicenter randomized double-blind placebo-controlled trial on 103 patients but failed to improve joints pain, fatigue, tender joint count, swollen joint count, subjective and objective measures of salivary/lacrimal gland functions, and serum inflammatory markers [10, 295]. **Entanercept** was also tested in a randomized double-blind placebo-controlled trial on 28 patients but was found

ineffective [8]. Further studies were carried out to understand why TNF inhibitors were inefficient, inflammatory markers did not improve and some were elevated, including IFN-α, BAFF, and TNF-α [123, 327-329].

SS patients have higher than normal levels of B and plasma cells, and their associated serum cytokines, such as BAFF. Additionally, a higher activity of B cells is evident with hyperglobulinemia and a wide range of autoantibodies, including anti-SSA, anti-SSB, and RF [10]. Anti-B cells therapies are monoclonal antibodies directed against several B cell markers. The three major anti-B cell therapies used in clinical trials are: Rituximab (anti-CD20), Epratuzumab (anti-CD22), and Belimumab (anti-BAFF) [10, 120]. **Rituximab** treatment has led to a drastic decrease in circulating B cells; thus, it is approved for the treatment of non-Hodgkin's lymphoma, chronic lymphocytic leukemia, RA, and anti-neutrophil cytoplasmic antibodies (ANCA)-associated vasculitis [10]. Five clinical trials have been conducted on the efficacy of Rituximab in managing SS [330-334]. The design of the trials and the number of recruited patients were different among the studies. The two larger studies yielded a non-beneficial use of Rituximab in the treated SS patients except for little improvement in salivary function [330] and some improvement in fatigue [334]; however, the results are not cost-effective [330]. The other three studies show some improvement in the salivary/lacrimal glands functions, fatigue, inflammatory markers; yet, the evidence is not statistically significant or did not meet the study primary endpoint [10] In the foregoing, the treatment outcomes were heterogenous which are due to the heterogeneity of the recruited patients, which has led to an inconclusive answers [10]. Moreover, patients were allowed to take their medication while taking Rituximab, which might have affected the treatment results [335].

**Belimumab** is a fully human IgG1λ recombinant mono-clonal antibody against BAFF which has been approved for the use in SLE patients [336]. The treated SS patients showed normalized BAFF-R on the targeted B cells, and lowered the level of Ig, RF, and ANAs [327]. However, Belimumab was found to decrease the serum level of EGF in treated patients [336]. An open-label clinical trial reported decreased disease activity, and improvement in quality of life, pain, and fatigue; however, the salivary and lacrimal functions did not improve [10, 189, 328].

**Abatacept** is a CTLA-4 inhibitor that was approved for the treatment of RA, juvenile RA, and psoriatic arthritis [10]. This is a co-receptor on antigen presenting cells that is important for the activation of T cells [10]. Three clinical trials have been conducted to evaluate the efficacy of Abatacept in treating SS. Two studies reported a very small improvement in salivary function and the third reported no change. They also reported an improvement in the extraglandualr manifestation in treated SS patients such as fatigue, however, none of the trials was a placebo controlled [329, 337, 338].

**Anakinra** is an IL-1 receptor inhibitor approved for RA [10]. A placebo-controlled trial revealed a 50% reduction in fatigue by visual analog scale [339]. **Tocilizum** is an IL-6 inhibitor that has been approved for several diseases management including RA [10]. No trial has been conducted for testing its efficacy in SS treatment; yet, one patient had neuromyelitis optica spectrum disorder complicated with SS did not show any improvement with various interventions, had shown gradual improvement in her neurological signs when treated with Tocilizum [340]. Another patient had SS with pulmonary involvement was responsive and showed improvement when treated with Tocilizum [341].

Biologic drugs which have been tested in SS have already been approved for the management of other diseases; hence, their risks and complications have been investigated. Various complications have been reported with these biologic therapies, including opportunistic infections, depression, cardiovascular complications, viral infection, neurological complications, and tumors [231, 342-347]. The most reported treatment risks are atypical opportunistic infections, such as tuberculosis, herpes zoster, legionella pneumophila, and Listeria monocytogenes [348]. There was no clear association between the drug of choice and the possibility of infection; however, factors like age, gender, and comorbidity were better predictors [349].

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## Chapter Two

# Studying Sjögren's Syndrome in Mice: What Is the Best Available Model?

## Preface

Mice are an essential animal model in the study of human diseases including SS. The search and the engineering of a mouse model that can facilitate the study of SS is ongoing. However, an ideal mouse model has not been found; yet, the data generated from the available models have advanced our knowledge about the pathogenesis significantly.

In this chapter, we highlight the basic clinical, histopathological, and serological characteristics that an ideal model should display for the best recapitulation of the disease. We have updated the list of the available models with all the reported SS features they exhibit. Lastly, we have expressed our opinion about the best available model, based on our experience in SS treatment in preclinical studies.

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# Studying Sjögren's Syndrome in Mice: What Is the Best Available Model?

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## Abstract

Sjögren's syndrome (SS) is a common autoimmune disease characterized by lymphocytic infiltration and destruction of exocrine glands. SS manifests primarily in the salivary and lacrimal glands, but many organs are also involved, leading to dry mouth, dry eyes, and other extra-glandular manifestations. Studying the disease in humans is complicated with many limitations and restrictions; therefore, the need for a proper mouse model is mandatory. SS mouse models are categorized depending on the disease emergence either spontaneously or due to an experimental manipulation. The usefulness of each mouse model varies, depending on the SS features exhibited by that model; however, each model has advanced our understanding of the disease pathogenesis significantly. In this review article, we list all the available murine models with their SS characteristics to assist scientists in their selection of the appropriate model for their specific studies. Finally, we highlight the most relevant model, based on our experience that we acquired during previous and current investigations.

**Key words:** Sjögren's Syndrome, autoimmunity, salivary glands, lacrimal glands.

## Introduction

Sjögren's syndrome (SS) is a multisystem rheumatoid inflammatory disease that manifests primarily in exocrine glands and affects mainly middle-aged women [1-3]. Its prevalence is variable among different populations ranging from 0.1- 0.72% [4-9]. In addition to the glandular dysfunction, several extra-glandular manifestations associated with lymphocytic infiltration and B cells hyperactivity in other organs are also present [10, 11]. Similar to other autoimmune diseases, SS can be found solitary (Primary SS) or accompanying other autoimmune diseases (Secondary SS) such as Systemic Lupus Erythematosus (SLE) and Rheumatoid Arthritis (RA) [3, 12]. Despite major differences in the immune defence mechanisms, evolutionary distance, and living environment, murine models have been used extensively in biomedical research to avoid the ethical challenges in human research [13]. Murine models have taken a large share, much more than other animal models, due to their availability as natural and genetically engineering models.

SS usually develops as a result of triggering environmental factors in genetically susceptible individuals [14]. However, the ambiguity involving the etiology of the disease and the initial pathological processes are due to the time gap between the onset of the disease and the emergence of clinical symptoms. In other words, to advance our understanding of these missing pathological steps, we must identify at-risk individuals and perform longitudinal studies involving several minor salivary gland biopsies and blood samples. However, such studies are challenging for several reasons. Firstly, our understating of SS genetics is still underway, unlike other autoimmune diseases like SLE and RA [15]. SS does not follow a simple Mendelian-like pattern and is a complex autoimmune disease due to the polygenic inheritance [16]. Therefore, identifying the susceptible individual is difficult. Secondly, the morbidity associated with longitudinal human studies involving the harvest of tissues and blood has ethical and patient recruitment difficulties. Giving all the previous difficulties associated with human studies, an alternative animal option is strongly justified.

Mice have been an invaluable tool for studying SS etiopathogenesis and for drug testing due to their small size, easy breeding, and a relatively low maintenance cost in comparison to bigger animal models [17, 18]. Several spontaneous and engineered murine models have been used extensively and a substantial knowledge about SS etiopathogenesis has been generated. However,

each of these models is unique but is inherently incapable of providing all answers to our SS investigations due to genetic and phenotypic differences from humans [13]. Theoretically speaking, an ideal murine model must display a range of characteristics, such as etiology, etiopathology, clinical features, serology and immunobiology [19]; nonetheless, in reality, this ideal model, does not exist. However, some models display more of these characteristics than the others, which makes them better candidates for studying SS.

In this review, we list all the available murine models used as possible candidates for SS studies. We have also listed all the reported characteristics that these murine models exhibit to enable the researchers to decide which model will be more suitable for their intended use. Finally, we provide our opinion as to which model has recapitulated the key features of SS the best, based on our previous and current investigations.

**The Ideal Murine Model**

SS patients display a panel of clinical and laboratory features that distinguish the disease from other autoimmune diseases, more specifically, other dry eyes/mouth conditions caused by certain factors. The clinical features are fundamental and necessary for the selection of a murine model (Table1). Other laboratory characteristics, such as autoantibodies and certain blood cytokines, are also important where they represent key markers in diagnosing the disease and are utilized as key parameters for therapeutic success. Therefore, we could consider the key features for establishing a diagnosis in patients as our checklist for how close or far a model is for being ideal. For the most recent classification criteria established as with an agreement between the two main international accepted rheumatologic groups, the American Rheumatology Group (ACR) and the European League Against Rheumatism (EULAR) see (Table 2).

**Table 1** SS characteristics that must be present in an ideal animal model [20, 21].

- **Clinical features**

- Dry mouth.
- Dry eyes.

- **Histopathology features**
  - Lymphocytic infiltration in the lacrimal and/or salivary glands.
  - Destruction of the gland stroma and decreased acinar cell number.
  - Persistent lesion.

- **Serological features**
  - Hypergammaglobulinemia
  - Anti-SSA
  - Anti-SSB
  - Anti-M3R (anti-muscarinic 3 receptor)
  - RF (rheumatoid factor).
  - Anti-CPP (anti-citrullinated cyclic peptide).
  - ACA (anti-centromere antibody).
  - AMA (anti-mitochondrial antibody.

- **Extra-glandular features**
  - Multiple organs involvement including skin, respiratory system, liver, and blood vessels.

- **Genetic features**
  - Abnormal MHC I and II genes expression, especially HLA-DR and HLA-DQ.
  - Non-MHC-related genes: INF signaling pathway; B-cell activation and autoantibody production.

**Table 2. The 2016 ACR/EULAR classification criteria for SS** [22]

**4. Consensus criteria items for the classification of SS**:

- Abnormal unstimulated salivary flow rate* (⩽0.1 ml/min). **(1 point)**
- Abnormal Schirmer's test (<5 mm in 5 min). **(1 point)**
- Abnormal findings with lissamine green or fluorescein staining ⩾5 in Ocular Staining Score or ⩾4 in Van Bijsterveld Score. **(1 point)**
- Presence of anti-Ro/SSA antibodies. **(3 points)**
- Histological evidence of focal lymphocytic sialadenitis, with a focus score ⩾1 focus/4 $mm^2$, (1 focus = 50 lymphocytes/4 $mm^2$). **(3 points)**

**5. Inclusion criteria**:

- Dryness of eyes or mouth for at least 3 months, not explained otherwise (e.g. medications, infection).

**6. Exclusion criteria**:

- Status post head/neck radiation.
- HIV/AIDS.
- Sarcoidosis.
- Active infection with hepatitis C virus (PCR replication rate).
- Amyloidosis, graft versus host disease, IgG4-related disease.

A diagnosis is established if a score ⩾4 points is achieved after application of inclusion and exclusion criteria.

**1. Spontaneous Mouse Models**

Genetic predisposition is strongly related to SS etiopathogenesis [1, 18, 23, 24]. The development of SS in spontaneous models involves several genes which gives us an invaluable tool to study the effect of these genes on the disease onset and progression, whereas it is challenging to perform such studies in SS humans' patients. NOD mouse model and its derivatives are the best available models for studying SS pathogenesis and drug testing in addition to several other models.

### 1.1. NOD Mice

Non-obese diabetic (NOD) mouse is an inbred strain that was established by Makino *et al.,* 37 years ago, from a cataract-prone sub-line of outbred ICR (Institute of Cancer Research, USA) mouse [25]. NOD mice were originally used as an animal model for diabetes mellitus type1, due to lymphocytic infiltration leading to insulitis and β-islet destruction. Around 70% of the female NOD mice and 20% of the males develop spontaneous diabetes by 13 weeks of age. This strain shares many similarities with diabetic human patients, like weight loss, hyperglycemia, hypercholesterolemia, glycosuria, ketonuria, polyuria, polydipsia, and polyphagia. However, their use for Sjögren's-like disease did not start until the late '90s. It is one of the most popular strains for studying SS due to the several similarities it shares with human SS, such as decreased glandular secretions and lymphocytic infiltration [26]. Lymphocytic infiltration was evident in submandibular and lacrimal glands, starting at 12 weeks of age with a subsequent glandular dysfunction at 20 weeks of age [25]. Successive studies have also revealed that specific autoantibodies, like anti-SSA/Ro, anti-SSB/La and anti-muscarinic receptor III were elevated [27-32]. Genetic analysis of the involved insulin-dependent diabetes (*idd*) loci revealed that only *idd3* and *idd5* are linked to SS exocrinopathy in NOD mice, whereas the rest are associated with diabetes type I only. Although the link between MHC-associated genes and SS-like disease is weak, several studies support that MHC-II genes are linked to defected central autoimmunity [33]. Female NOD mice show submandibular glands sialoadenitis as early as 8 weeks of age. Lacrimal dysfunction and lymphocytic infiltration are evident in nearly 52% of the female NOD mice (unpublished data). Dryness of the eyes is further complicated into thinning of the corneal epithelium in the mice with lacrimal involvement (unpublished data). Despite the various SS features this model displays, diabetes remains a significant complication that affect the survival rate and overall wellbeing of this model.

### 1.2. NOD.B10.H2$^{b}$ (NOD.H2$^{b}$) Mice

NOD.B10.H2$^{b}$ is a congenic line of the NOD strain where the MHC I-A$^{g7}$ *Idd1* susceptibility locus is replaced by the MHC I-A$^{b}$ locus from C57BL/10 mice: they are negative for NOD MHC class I and II antigens [34]. These mice exhibited lymphocytic infiltration into the salivary and lacrimal glands the way SS-like disease does in the NOD mice [32], but infiltration is less severe (unpublished data). Almost all the female mice showed submandibular infiltration by the age of

11 months in a varying degree of severity. Male mice showed less severe inflammation and only 9% were infiltration free [34]. They suffer from salivary gland dysfunctions without the accompanying pancreas infiltration (insulitis) and the resulting severe diabetes [32]; however, our investigation did not reveal glandular dysfunction when the mice were followed up for 56 weeks (unpublished data). Serum analysis revealed the presence of anti-nuclear antibodies against: double-stranded DNA (dsDNA), single-stranded DNA (ssDNA), and U1-snRNP68 in female mice [35].

### 1.3. C57BL/6.NOD*Aec1Aec2*

C57BL/6.NOD*Aec1Aec2* mouse strain was established by Cha *et al.* in 2006 [36] and was further described by Nguyen *et al* [37]. Successive studies have revealed that two genetic regions on chromosomes 1 and 3 termed *Aec2* and *Aec1*, respectively, are sufficient to recapitulate SS-like disease in disease-free C57BL/6 mice [37]. When this mouse strain was first established, it was verified for the suitability of using it as SS mouse model. The lymphocyte infiltration in the salivary gland was detected at 10 and 19 weeks in males and females, respectively. However, female mice showed more severe infiltration, and were larger at 22 weeks of age. Surprisingly, males exhibited a more severe form of dacryoadenitis while females showed none. Caspase-3 levels in the submandibular gland were elevated between 4-14 weeks of age then decreased afterwards; however, the lacrimal glands showed an opposite trend. Serine protease level was detected at 10 weeks of age, and ANAs (Antinuclear antibodies) were evident as early as 5 weeks of age in males but 10 weeks in females. In additions, all mice examined were found to have positive IgM against mM3R-transfected Flp-In CHO cells in their sera. Upon examining salivary function, females and males both lost 35–40% in salivary flow rates between 5-19 and 5-22 weeks of age, respectively [37].

### 1.4. Other NOD Derivative Mice

A number of engineered NOD mice were established to verify the role/involvement of specific genes or proteins in the pathogenesis of SS [18]. This list includes: NOD.*IFN-γ*$^{-/-}$, NOD.*IFN-γ R*$^{-/-}$, NOD.*IL4*$^{-/-}$, NOD *Igμ*$^{-/-}$.

Interestingly, NOD.*IFN-γ*$^{-/-}$ and NOD.*IFN-γ R*$^{-/-}$ mice showed no salivary function loss, lymphocytic infiltration, increase in acinar cells apoptosis, or abnormal salivary protein expression by 20 weeks of age. The previous findings emphasize on the pivotal role of IFN-γ in the pathogenesis of SS in NOD mice. Beyond 30 weeks of age, sparse leukocytes were found in the submandibular glands similar to the healthy control [38]. Surprisingly, lymphocytic infiltration was apparent in lacrimal glands, particularly males, similar to that of NOD mice. Apoptosis signals in the submandibular and lacrimal glands using Caspase 3 and TUNEL assays showed comparable level to the healthy control mice.

NOD.*IL4*$^{-/-}$ mouse model was created to study the role of IL-4 in the pathogenesis of SS. Upon examining the targeted tissue, several proinflammatory cytokines were found but not IL-4. Although the cytokine was not found at the time of examination, it was still important in the pathogenesis of SS. Lymphocytic inflammation was evident as early as 8 weeks, similar to NOD mice, but at 20 weeks the infiltration was more severe than that in NOD mice. Although the lymphocytic infiltration was severe, it did not lead to dry mouth. Assessment of apoptosis, measured by the level of positive acinar cells for TUNEL, revealed a comparable level to NOD mice and higher than that of healthy mice. Serum analysis revealed the absence of M3R autoantibodies which might, partially, explain the absence of xerostomia despite the presence of lymphocytic inflammation. This mouse model provides a strong evidence that IL4 does participate in the pathogenesis of SS regardless of their undetected level in the tissue [39].

To study the role of B lymphocytes, Robinson established the NOD Igμ$^{-/-}$ mouse model that lacked functional B lymphocytes in their immune system [40]. Although these mice exhibited salivary and lacrimal gland T cell infiltration at 8 weeks, they had normal salivary function. Older mice (> 20 weeks) showed higher cysteine protease activity (apoptosis indicator for acinar and ductal cells) in comparison to their younger 8-week-old counterparts. This suggested the strong role of autoantibodies produced by B lymphocytes on the function of the exocrine glands [40].

### 1.5. NZB/W F1 (NZB/NZW F1) Mice

The first spontaneous mouse model utilized in the study of SS was NZB/W F1 [41]. It was generated by crossing the first filial generation New Zealand black (NZB) with the New Zealand

white (NZW). This model spontaneously develops SS and SLE disease characteristics. Both NZB and NZB/NZW F1 share similar disease characteristics while NZW does not. Both salivary and lacrimal glands showed lymphocytic infiltration by 16 weeks of age, but it was more intense in the lacrimal gland. Histological sections from both glands showed periductal and perivascular infiltration. Acinar cells showed a washed-out appearance while the connective tissue looked more edematous. However, cross sections of the gland showed well-preserved gland architecture. The severity of the mononuclear infiltration was influenced by the age, sex and type of the gland; therefore, females, lacrimal glands, and older mice showed much more lesions than their counterparts. Although not all parotid glands showed lesions, they were found to be the most severely affected gland measured by the degenerative lesions, whereas sublingual glands were the least affected. When the mononuclear infiltration was evident, not always, it did not exceed 1-2 small lesions [41]. However, autoantibodies, anti-SS-A and anti-SS-B, were not detected in their serum [42].

### 1.6. MRL/lpr (MRL/Mp-lpr) Mice

MRL/lpr is a congenic strain that has obtained the *lpr* (lymphoproliferation) mutation from MRL/MpJ strain [43]. Mice with *lpr* mutation lack Fas which is a cell surface protein that transduces apoptosis. These mice develop immune complex disease with features of SLE, SS, and RA-like disease [42, 43]. Submandibular gland lesions were found at the age of two months [44]. Over expression of IL-1β and TNF-α was evident before salivary gland lesions, whereas IL-6 elevation was in accordance with the lesions [45]. Hoffman *et al*., have conducted one of the earliest studies on the exocrine involvement of this strain. They have found that more mice showed infiltration in the submandibular gland than in the parotid or sublingual. Lacrimal glands involvement was present in nearly all the mice studied. No anti-SSA/Ro or anti-SSB/La was detected in the sera of these mice, but later on, studies have found that some mice do develop these autoantibodies. The presence of autoantibodies was shown in MRL/lpr as a congenic mouse from the MRL strain. However, autoantibodies, anti-SSA/Ro and anti-SSB/La, were not detected in their serum [42].

### 1.7. NFS/sld Mice

NFS/s1d mouse model has an autosomal recessive mutation that arrests sublingual gland differentiation. Several cytokines, IL-1ß, TNF-α, IL-2, IFN-γ, IL-6, IL-10, IL-12p40, and adhesion molecules, ICAM-1, LFA-1, CD44, Mel-14, were upregulated in this model [46]. NFS/sld mice develop a spontaneous inflammatory change in the salivary and lacrimal glands in thymectomised mice 3 days after birth. No significant inflammation was found in other organs or in other non-thymectomised mice. Females were affected significantly higher and showed more lesions in their glands than males. The lymphocytic infiltration was more prominent in females and composed mainly of $CD3^+$ and $CD4^+$ T cells with some $CD8^+$ T cells and B220 B cells. Sera analysis revealed anti-salivary duct autoantibodies in mice with autoimmune lesions [47]. A 120-kilodalton autoantigen α-fodrin was purified from the salivary glands of these mice, this autoantigen induces proliferation of T cells, in vitro. Neonatal immunization with this autoantigen prevented disease development [48].

### 1.8. Aly/aly Mice

Mice homozygous for an autosomal recessive mutation aly (alymphoplasia) lack both lymph nodes and Peyer's patches, and show defects in both humoral and cellular immunity. Histopathological analysis revealed chronic inflammatory changes in exocrine organs, such as salivary glands, lacrimal glands, and pancreas of the homozygotes (aly/aly), but not the heterozygotes (aly/+). In these exocrine organs, mononuclear cells consisting mainly of CD4+ T cells, infiltrate periductal areas, and, in some cases, the cell infiltration extended to glandular lobules. The inflammatory changes in exocrine organs were transferred by a T cell-enriched fraction of spleen cells from homozygous animals. These results suggest that autoimmune mechanisms mediated by self-reactive T cells may be involved in the inflammatory lesions of various exocrine organs in the homozygous mice, although these mice show immunodeficiency. Inflammatory changes were also observed in the lungs of the homozygotes. Since SS is characterized by diffuse lymphocyte infiltration in the periductal areas of the lacrimal and salivary glands and is occasionally associated with pulmonary disease, aly/aly mice may serve as a unique spontaneous model of SS [49].

### 1.9. IQI/Jic Mice

IQI/Jic strain is an inbred strain established from ICR mice [50]. These mice produce antinucleolar autoantibodies in response to mercuric chloride exposure [51]. Lymphocytic focal infiltration is evident in the salivary and lacrimal glands of these mice. Females showed more involvement of the salivary glands than their male counterparts; up to 80% of all females at all ages showed sialadenitis that worsened after 6 months of age. However, males showed slight lesions, not independent of their age, but the incidence increased with age [50]. Infiltrating immune cells were detected as young as 4 weeks of age, involving MHC $II^+$, $CD11c^+$ and $B7\text{-}2^+$ dendritic cells (DCs). At 8 weeks of age, the infiltrating lymphocytes were seeded in the submandibular glands of females and the lacrimal glands of males. These lymphocytes were B cells and $CD4^+$ T cells in similar ratios [52]. This strain was also found to have multiple organs involvement. The lungs, pancreas and kidneys were infiltrated with $CD4^+$ T-cells and B-cells at advanced ages, like SS patients [53]. When these mice were subjected to neonatal thymectomy, severe lesions were found in the lacrimal glands suggesting a crucial rule of $CD4^+$ $CD25^+$ $T_{reg}$ cells [54].

## 2. Transgenic Mouse Models

### 2.1. HTLV-1 Tax Transgenic Mice

Human T-cell leukemia virus 1 (HTLV-1) is a retrovirus involved in adult T-cell leukemia and in the pathogenesis of autoimmune diseases, such as SS and RA [19, 55]. This model contains the HTLV-1 *tax* gene under the control of the viral long terminal repeat (LTR) which leads to a phenotype that involves exocrine glands [19, 56]. Diffuse and multifocal ductal epithelial cells proliferation is evident in these mice at an early age which later intensifies leading to distortion of glandular architecture, particularly the submandibular and parotid [56]. As the proliferation advances, lymphocytic infiltration starts surrounding the enlarged epithelial masses, and later plasmas cells will appear [56]. Lacrimal glands will also undergo epithelial cells proliferation; however, it is not as severe as in salivary glands and appears late. The severity of the glandular pathology corresponds to *tax* gene expression. Tax protein production increases with age and it is produced equally in males and females [56].

### 2.2. IL-6 Transgenic Mice

Interleukin-6 (IL-6) is a cytokine that is originally known to be necessary for the maturation of B cells. Later, its multifunctionality was revealed to involve a critical role in immune responses, hematopoiesis and the acute phase immune response. Dysregulation of IL-6 leads to several autoimmune diseases, such as rheumatoid, osteoporosis and psoriasis [57, 58]. The effect of IL-6 on the development of SS and other autoimmune diseases was studied by using transgenic hybrid mice for graft versus host disease (GvHD) model with MHC class II disparity. These mice showed elevated IL-6. Systematic investigation of these mice showed a larger spleen index and autoimmune-like lesions that left the animal weakened. In addition, these mice showed elevated antimitochondrial antibodies. The previous findings strongly correlated with the elevated level of IL-6 and the progression of the autoimmune diseases [19, 59].

### 2.3. IL-10 Transgenic Mice

IL-10 (interleukin-10) is known for the maturation and regulation of T and B cells, as well as enhancing MHC II antigen expression; therefore, an abnormal level of IL-10 might play a role in the pathogenesis of autoimmune diseases. Furthermore, IL-10 controls cytokine production by natural killer cells and immunoglobulins by B cells [60]. IL-10 is generally considered as the most important anti-inflammatory interleukin that prevents inflammation-mediated tissue damage [61]. This mouse model was created by microinjection of IL-10 mouse cDNA in C57BL/6 fertilized eggs under the amylase promoter [62]. This model showed epithelial apoptosis accompanied with glandular infiltration of Fas-ligand $(FasL)^+$ $CD4^+$ T cells, and less than 10% were $CD8^+$. The glandular infiltration was evident as early in 8-week-old mice and increased in intensity as they aged. The glandular tissue stained positive for MHC class II I-$A^K$. Clinically, the mice exhibited lower saliva and tear secretion than their control group at 8-week-old, and it continued to decline overtime. No differences were found between males and females.

### 2.4. IL-12 Transgenic Mice

IL-12 is a proinflammatory heterodimeric cytokine produced by APCs, B cells and phagocytic cells. It is responsible for the production of several cytokines, especially IFN-y. IL-12 acts as a growth factor for activated T and NK cells and is best known for induction of the differentiation of $CD4^+$ T lymphocytes from a Th0 to a Th1 [63, 64]. This mouse model was created by Kimura

*et al*. 2005 to investigate the effect of chronic exposure to IL-12 in murine thyroid glands [64]. The strain was engineered to express IL-12 p70 under the transcriptional control of the thyroglobulin promoter [64]. Stimulated salivary flow rate was measured in both females/males and was found statistically lower in IL-12 Tg mice when compared to their wild type counterparts. However, this decrease was age-dependant in males; salivary flow rate was decreased at 16 weeks of age when compared to control mice; however, females were not age dependant as they showed lower salivary flow rate at all time points (7-20 weeks of age). Histological analysis of the salivary and lacrimal glands revealed lymphocytic infiltration composed mainly of $B220^+$ B cells and $CD4^+$ T cells. Strong correlation was found between the glandular hypofunction and lymphocytic infiltration in females. ANAs were found statistically higher in IL-12 Tg mice when compared to control counterparts at 13, 32 and 36 weeks of age. However, when anti-SSA/Ro levels were investigated, higher values were found in the transgenic mice but not significant at all the assessed time points [65].

### 2.5. IL-14α Transgenic Mice

IL-14 α is a cytokine that induces activated B cell proliferation and inhibits immunoglobulin secretion and expands certain B cells subpopulations [66, 67]. Analysis of peripheral blood leukocytes IL-14 α transcripts in primary and secondary SS patients were found higher than the age-, ethnic- and sex-matched controls [68]. IL-14 transgenic mice were created to study the role of IL-14 in vivo [69]. Transgenic mice at different ages were examined and their sera were evaluated for immunoglobulins and autoantibodies production. By six months of age, the mice developed hypergammaglobulinemia, including IgM and IgG. A significant increase in IgA and IgG2a levels was found in the serum by 9 months of age. The analysis of autoantibodies associated with SS and SLE-like IgG ANA, anti-dsDNA, anti-chromatin, anti-Ro, anti-La, anti-Sm, and anti-nRNP, showed that some mice had one or two autoantibodies elevated but the majority did not express any [65]. Histological assessment of the parotid glands and kidneys revealed lymphocytic infiltration and further IgM deposition in the kidneys. Aged mice developed $CD5^+$ B cell lymphoma similar to what is found in some SS and SLE patients [19, 70].

## 2.5. BAFF Tg Mice

BAFF transgenic mice produce a very high level of B cell activating factor (BAFF). BAFF is involved in the B-cell survival; however, excessive levels can lead to inability to respond to censoring death signals and escaping critical tolerance checkpoint [71, 72]. BAFF Tg mice exhibit enlarged marginal zone (MZ) B-cell compartment and show MZ-like B cells circulating in the blood, lymph nodes, and salivary glands. They are also characterized by the presence of excessive levels of autoantibodies, leading to kidneys and salivary glands destruction, similar to SLE and SS, respectively [73]. The previous features highlight the possible link between the active autoimmune cells and BAFF [73]. BAFF Tg mouse tended to develop SS as they age. Older mice (> one year) exhibited larger submandibular glands, decreased saliva flow rates, as well as, submandibular gland destruction, due to severe lymphocytic infiltration [74]. The disease severity was variable among the mice and was not affected by their sex. No anti-SSA/Ro or anti/SSB/La autoantibodies were detected in their sera, regardless of the disease severity. Cells population was composed of a larger proportion of B cells but it was variable in this strain [74].

## 2.6. Id3 Knockout Mice

DNA binding inhibitors (1,2,3, and 4 (IDs)) are nuclear proteins, when present at high concentration, that bind to basic Helix-loop-helix transcription factors (bHLH). bHLH is a family of proteins that control cell fate, differentiation and proliferation, forming ID-bHLH dimers, which lack the basic binding site, therefore, no interaction with DNA takes place [75, 76]. Id3 is important in the development of T lymphocytes [77, 78] and B lymphocytes, as well [79]. Id3 expression is high in proliferating cells and down regulated in differentiating cells [79]. Id3 null mice showed selective defects in humoral immunity because Id3 is necessary in BCR-mediated B lymphocytes proliferation [79]. This phenotype was partially explained by the study of purified B cells, which showed that Id3 was required for BCR-mediated B-cell proliferation. It could also be explained by the study of Id3 mouse T cells, which showed that Id3 was required for optimal expression of IFN-γ [79]. Id3 null mice recapitulated many of the primary SS symptoms that human patients present [80]. They secreted significantly less saliva and tears in both males and female, as early as 2-4 months of age. Both males and females were unable to keep their eyelids opened at around six months of age due to severe dryness. Histological assessment of the salivary and lacrimal glands revealed lymphocytic infiltration at around two months of age which was more significant at six

months. T lymphocytes ($CD4^+$ and $CD8^+$) and B cells were identified in the lymphocytic infiltrations. Serum analysis revealed the presence of autoantibodies at a significantly high frequency after one year of age but not before. The pancreas, kidneys, lungs, thyroid and liver did not display any gross abnormalities. However, histological assessment of some old animals (> one-year-old) showed occasional infiltration in the lungs and kidneys [80].

### 2.7. TGF-β1 Knockout Mice

TGF-β1 (Transforming Growth Factor-β1**)** is a pleotropic cytokine secreted by T cells and is essential for immune homeostasis [81, 82]. It is responsible for innate and adaptive immune cells regulation in vitro. It has an important role in immune regulation and tolerance. In addition, it has a suppressive action on several immune cells, including T/B cells and macrophages. The immune system increases the release of TGF-β1 to protect and/or recover from autoimmune diseases [83]**.** Mice carrying a homozygous mutation for TGF-β1 die at around three weeks after birth, due to organ failure caused by Wasting Syndrome. Upon organ examination, animals exhibited a marked tissue infiltration and necrosis [84]. The infiltration involved many organs, including liver, heart, stomach, lung, pancreas, salivary glands and striated muscles. Salivary glands infiltration was slight to moderate multifocal in the periductal regions [84]. Inflammation was evident around one week after birth; however, it was variable among mice [85]. The composition of the infiltrating cells was mainly lymphocytes with some plasm cells. As the infiltration proceeded, the acinar cells were the most affected cells, where they were shrunk and eventually atrophied.

### 2.8. PI3K Knockout Mice

Phosphoinositide 3-kinase (PI3K) is an enzyme that is activated by receptors for antigen, cytokines, costimulatory molecules, immunoglobulins and chemoattractant. PI3K is responsible for immune cells proliferation and differentiation [86, 87]. In class 1A PI3K deficient mice, autoimmunity developed around two months of age but became more profound at 4 months in females and males equally [88]. Large infiltration in the lacrimal glands was found in the periductal area with acinar cells destruction. Infiltration foci were composed mainly of $CD4^+$ T cells, some $CD8^+$ T cells and $B220^+$ B cells. Other organs, including liver, lungs and intestines showed signs of infiltration but with less penetration. Serum analysis revealed the presence of ANAs in almost

two thirds of the studied mice. Anti-SSA titre was higher in the PI3K deficient mice than the control and it tended to increase with age [88].

### 2.9. TSP-1-Deficient Mice

Thrombospondin-1 (TSP-1) is largely responsible for the activation of the latent form of TGF-β1 extracellularly in vivo [89]. TGF-β1 is a bipolar cytokine which plays an important role in immunity development [90]. Its overexpression is associated with fibrosis and exaggerated immune response; however, TGF-β1 is responsible for limiting innate and adaptive immune responses to reinstate immune homeostasis [83, 90, 91]. Based on the previous information, the effect of TSP-1 on SS pathogenesis was investigated. When TSP-1-deficient mice were created, they appeared normal at birth, but later they developed SS-like disease in the lacrimal glands. The lacrimal glands were invaded with the lymphocytic which led to apoptosis, glandular deterioration and eventually, abnormal tear formation. These mice develop ocular surface abnormalities similar to SS in humans, including deterioration of the cornea and crusty eyes. Serum analysis of TSP-1 null mice revealed the presence of anti-SSA and anti-SSB autoantibodies. $CD4^+$ T lymphocytes were found to secret IL-17 which is strongly linked to chronic inflammation. Isolated antigen presenting cells were capable of activating T-lymphocytes in vitro which, in turn, were able to secrete IL-17 [92]. Further studies are needed to investigate the changes in the salivary glands in TSP-1 null mice in the future.

### 2.10. RbAp48 knock in Tg Mice

Retinoblastoma Associated Protein 48 (RbAp48) also known as RBBP4 is a protein that interacts with multiple cellular proteins that mediate its action in cell growth and apoptosis [18, 93]. RbAp48 Tg mice were created by microinjection of gene fragments containing RbAp48 cDNA, regulated by the salivary gland-specific promoter, into fertilized eggs from C57BL/6 [94]. Ovariectomized C57BL/6 mice showed enhanced salivary and lacrimal glands apoptosis via p53-mediated overexpression of RbAp48 [94]. Therefore, RbAp48 Tg mice are a useful strain to study the role of estrogen deficiency in autoimmunity. Mice examined at 24 weeks of age exhibited autoimmune exocrinopathy similar to SS; however, lymphocytic infiltration in salivary and lacrimal glands was more frequent at 30-50 weeks. Females displayed a more severe form of the disease at all ages. The majority of infiltrating cells were $CD4^+$ with some $B220^+$, $CD8^+$, and $CD11^+$. A significant

decrease in saliva and tears volumes was evident, starting at 30 weeks of age. High levels of anti-SSA/Ro, anti-SSB/La, and anti-α-fodrin (120-kD) autoantibodies were detected in their sera [95]. MHC II expression was evident on exocrine epithelial cells which enable them to act antigen presenting cells. Therefore, these cells might express exocrine antigens to $CD4^+$ cells which leads to the initiation of the autoimmune reaction [95].

### 2.11. Aromatase knockout (ArKO) Mice

Aromatase is a cytochrome P450 that is responsible for estrogen biosynthesis [96]. Estrogen receptors α- and β-knockout mouse models showed other abnormalities, like autoimmune nephritis but not SS; therefore, an animal model that lacks estrogen itself not the receptors was the model of choice [97]. ArKO mice lack the aromatase gene and consequently lack estrogen. The examined mice (12-17)-month-old, developed mild splenomegaly, bone marrow hypercellularity, and impaired renal function due to chronic estrogen deficiency. Additionally, they spontaneously develop SS-like symptoms in both females and males. The gross examination of the salivary glands showed enlargement and massive lymphocytic infiltration, mainly $B220^+$ B cells, which led to the destruction of the acinar cells. Alpha-fodrin fragments were detected in the salivary gland, and anti-α-fodrin antibodies were also found in the sera of these mice due to the destruction of the protein filaments. The later findings are the important hallmarks of SS [98].

### 2.12. *Opn* Tg mice.

Osteopontin (OPN) is a multifunctional protein that is involved in various physiological processes. T cells activation has been linked to high upregulation of OPN gene; therefore, various autoimmune diseases exhibited OPN overexpression including SS [99-101]. In *Opn* Tg mice, OPN overexpression was achieved by the immunoglobulin enhancer/SV40 promoter which led to OPN overexpression of in the bone. These mice exhibited a significant saliva loss by 16 weeks of age. Histological assessment revealed that 62% of female *Opn* Tg mice showed lymphocytic infiltration in the submandibular gland, 50% for the lacrimal gland, and 12.5% were positive for both [100]. Immunohistochemical analysis of the submandibular glands showed higher staining for OPN in the ductal cells and colocalization of OPN with lymphocytic infiltration. Serum analysis showed elevated IL-4, IL-6, IL-2 and TNF-α levels.

### 2.13. CD25 knockout mice (IL-2Rα$^{-/-}$)

CD25 is an interleukin 2 receptor alpha subunit (IL-2Rα). IL-2 receptor is composed of IL-2Rα (CD122) and γ chains complexed with α subunit (CD25) [102]. The binding of IL-2 to its receptor leads to the interruption of Th17 differentiation; therefore, in the absence of IL-2 receptor α subunit, this favors a more differentiated Th17 production which leads to autoimmunity [102, 103]. This mouse model, as the name depicts, lacks the expression of IL-2Rα and exhibit a multi-organ inflammatory condition evident in the exocrine glands and the gastrointestinal tract [104]. Rahimy *et al.* ran an analytical study of this model investigating the lacrimal gland [102]. Upon excision, the lacrimal glands were enlarged, red, and inflamed as early as 8 weeks of age. Further histological analysis revealed acinar atrophy and fibrosis, and periductal fibrosis accompanied with severe lymphocytic infiltration. However, at 16 weeks, the glands were small and atrophied. Histological analysis revealed generalized disarrangement, acinar cells loss, and glandular atrophy [102]. Cytometry analysis indicated the abundance of $CD4^+$ and more $CD8^+$ cells at all ages. Unlike other mouse models, CD25 KO displayed similar pathology in males and females. Peroxidase and EGF were both evaluated as two markers for lacrimal gland activity. Peroxidase was never detected at any age, and EGF was very low. Young CD25 KO mice showed higher Th17 (TGF-β1, IL-17A, IL-23R, IL-21, CCL20, and Th1 (IFN-α, IL-2, IL-12, IL-12RB1, IL-18R, T-bet) cytokines [102, 105]. The cornea showed higher irregularities and $CD4^+$ and $CD8^+$ infiltration in the conjunctiva similar to that of the lacrimal glands [105].

## 3. Immunization mouse models

### 3.1. CA II Immunization

CA (Carbonic Anhydrase) is a basic zinc metalloenzyme with a wide distribution in the tissues where it regulates acid base status in vivo [106, 107]. The autoantibodies against CA were found in the sera of SS and SLE patients, and the titers correlated to the disease activity [107]. Experimental sialadenitis was induced by immunizing PL/J ($H\text{-}2^u$) mice with human CAII intradermally. Immunized mice showed a significant infiltration in the salivary glands compared to untreated mice. The infiltration was observed around intercalated ducts and intralobular, causing atrophy to the acinar cells. Some mice showed lymphocytic infiltration in the pancreas and the kidneys. Mice bearing $H\text{-}2^s$ and $H\text{-}2^u$ were found susceptible to CAII immunization [108].

### 3.2. M3R peptide immunization

M3R (M3 muscarinic acetylcholine Receptor) is expressed in exocrine glands, including salivary and lacrimal glands [109]. It delivers the neural command from the parasympathetic system to initiate secretion from these glands. Several studies reported that 40% of SS patients has M3R reactive T cells in their blood, and 9-100% of these patients tested positive for M3R autoantibodies [109]. Iizuka *et al.* have established M3R mouse model to study the effect of this receptor on the development of sialadenitis in the presence of autoantibodies against it. They have injected fragments of murine M3R into $M3R^{-/-}$, then, the splenocytes were inoculated and injected into $Rag1^{-/-}$ mice ($M3R^{-/-}/Rag1^{-/-}$) Upon examination of $M3R^{-/-}/Rag1^{-/-}$, very high levels of M3R autoantibodies were found in the serum accompanied with low saliva secretion [110]. Histological assessment revealed a significant lymphocytic infiltration in the salivary glands that were later identified as $CD4^+$ (mainly) with few B cells and IFN-γ and IL-17- secreting cells. A few apoptotic cells were also found in the salivary glands. Thus, the previous data strongly suggest that blocking M3R by autoantibodies is an important event in the development of SS.

### 3.3. Ro Immunization

Autoantibodies against ribonucleoproteins SSA/Ro (Anti-SSA/Ro) and SSB/La (anti-SSB/La) existed in > 75% of SS patients when measured via a sensitive technique [111]. Their levels serve as a diagnostic marker for SS and other autoimmune diseases [112, 113]. Scofield *et al.* have tested the ability of short peptides from the 60-kDa Ro (or SSA) Ag immunization to induce SS-like disease in BALB/c mice. Immunized mice developed an immune reaction and produced antibodies against both Ro/La antigens, similar to SS human patients. Upon histological examination, lymphocytic infiltration was found in the salivary glands, which were composed of $CD4^+$ (45%), $CD8^+$ (18%) T lymphocytes and $CD19^+$ (38%) B lymphocytes. Salivary flow was lower in the immunized but not in the control mice [114].

## 4. Infection mouse models

### 4.1. Murine Cytomegalovirus

Environmental factors have been documented as aggravating factors for autoimmune diseases in are genetically predisposed patients [14, 19]. Several viruses were found to be involved in the pathogenesis of SS, including Epstein-Bar virus (EBV), hepatitis C virus and cytomegalovirus (CMV) [115]. Despite the usefulness of murine models for studying the role of CMV in the etiopathogenesis of SS, the virus targets different cell types in humans and mice. Human CMV (HCMV) usually targets the ductal cells while the murine CMV (mCMV) prefers the acinar cells, and it seems to induce an inflammatory response leading to cell death followed by regeneration [116-119]. Four mouse strains: C57B1/6 [B6]-$^{+/+}$, Fas-deficient B6-lprllpr, TNFRI-deficient B6-tnfrl$^{-/-}$, and B6-tnfrlo$^{-/-}$-lpr/lpr mice were found useful as they recapitulated specific phenotypes of SS-like disease when transfected with mCMV. At 28 days post-infection, extensive inflammatory cell infiltration was detected in the salivary glands of C57BL/6 [B6]-$^{+/+}$, B6-*tnfr*1$^{-/-}$ and B6-*lpr/lpr*. However, at 10 days post infection, no inflammation was observed in C57BL/6 [B6]-$^{+/+}$ and B6-*tnfr*10/0 which was the time at which the infectious mCMV was no longer detectable. On the other hand, in B6-*lpr/lpr*, only salivary glands showed inflammation despite the absence of mCMV in the gland after 100 days. B6-*lpr/lpr* infected mice showed a high level of anti-SSB/La, anti-SSA/Ro, and rheumatoid factor (RF) 100 days post-infection while C57BL/6 [B6]-$^{+/+}$ did not [120].

**Conclusion**

After reviewing all the available murine models for SS and based on our experience in this field, we came to a conclusion that NOD strain is the model that recapitulates the disease characteristics the best and it is the most suitable for drug testing. NOD mice exhibit a heterogenous clinical features and laboratory features, comparable to that of SS in humans, better than any other available mouse model. However, this should not lead us to abandon the other mouse models or neglect the important data that was generated. On the contrary, these data, often, supported and complemented to results obtained from NOD mice.

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# Studying Sjögren's Syndrome in Mice: what is the best available model?

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| Abstract: | Sjögren's syndrome (SS) is a common autoimmune disease characterized by lymphocytic infiltration and destruction of exocrine glands. The disease manifests primarily in the salivary and lacrimal glands, but other organs are also involved, leading to dry mouth, dry eyes, and other extra-glandular manifestations. Studying the disease in humans is entailed with many limitations and restrictions; therefore, the need for a proper mouse model is mandatory. SS mouse models are categorized depending on disease emergence either spontaneously or due to experimental manipulation. The usefulness of each mouse model varies depending on the SS features exhibited by that model; each SS mouse model has advanced our understanding of the disease pathogenesis. In this review article, we list all the available murine models which have been used to study SS and comment on the characteristics exhibited by each mouse model to assist scientists to select the appropriate model for their specific studies. Also, we recommend relevant models with the ideal SS features, based on our experience acquired during previous and current investigations. |
| Suggested Reviewers: | |

## Chapter Three

# Compact Bone-Derived Multipotent Mesenchymal Stromal Cells (MSCs) for the Treatment of Sjögren's-like Disease in NOD Mice

## Preface

This chapter was prepared to facilitate the access to a comprehensive and detailed protocol that was followed to prepare compact bone derived MSCs for the management of SS-like in NOD mice published in a previous study. The chapter lists all the materials used and the methodologies followed starting from the excision of bones until treatment administration. Additionally, this book chapter includes experiments and steps that were not mentioned in the previous paper. We aimed at optimising MSCs isolation and characterization which we have conducted later in chapter four.

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# Compact Bone-Derived Multipotent Mesenchymal Stromal Cells (MSCs) for the Treatment of Sjögren's-like Disease in NOD Mice

**Ghada Abu Elghanam, Younan Liu, Saeed Khalili, Dongdong Fang, Simon D. Tran**

## Abstract

Compact bone (cortical or dense bone) is among the organs that contain multipotent mesenchymal stromal cells (MSCs). Unlike bone marrow plugs where MSCs were initially isolated, compact bone has minimal hematopoietic cells and thus facilitates the MSCs isolation process. In vitro, MSCs from compact bone show multipotency and differentiation into mesenchymal tissues such as bone, adipose, and cartilage, under certain conditions. MSCs therapy has been promising in pre-clinical and clinical studies against autoimmune diseases. Not only can MSCs replace the lost tissue through their regenerative properties, but they can also control the autoimmune attacks by immunoregulatory cytokines. This protocol describes the use of compact bone-derived MSCs to preserve salivary function (saliva flow/output) in the NOD (non-obese diabetic) mouse model affected with Sjögren's-like disease.

## Keywords

Compact bone, Mesenchymal Stromal Cells, MSCs, Autoimmune diseases, Sjögren's Syndrome, Sjögren's-like disease, Saliva, Hyposalivation, Xerostomia, Non-obese diabetic (NOD) mouse.

## 1. Introduction

Saliva is a secretory fluid that protects the oral tissues and aids in swallowing; thus, its loss is problematic. Sjögren's Syndrome (SS), radiotherapy to the head and neck area for cancer treatment, and the intake of some drugs (e.g. antihypertensive agents) can all cause saliva loss (salivary hypofunction, xerostomia). SS is a common chronic autoimmune disease characterised by focal lymphocytes infiltration into the salivary and lacrimal glands leading to xerostomia and

keratoconjunctivitis sicca [1]. Women are affected nine times more than men and are mostly postmenopausal. SS can be restricted to the secretory glands only, primary SS, or coexists with other autoimmune diseases like rheumatoid arthritis (RA) or systemic lupus erythematosus (SLE), secondary SS. The etiology of SS is believed to be multifactorial; certain environmental factors seem to provoke an existing genetic predisposition [2].

Current treatments of SS are mostly palliative; no curative treatment exists until now. Therapeutic options include artificial saliva and tears, parasympathomimetic agents like pilocarpine (Salagen) to overstimulate the residual functioning salivary tissue. In more severe cases with serious systemic involvement, immunosuppressant drugs like methotrexate might be used [3]. Unfortunately, all previously mentioned treatments cannot restore the damaged cells nor enhance the formation of new ones. This has encouraged several research attempts to find a suitable treatment for SS via stem cells therapy or tissue engineering [4-6].

MSCs have been used to treat autoimmune diseases [7-10] due to their immunomodulatory and anti-inflammatory properties that can alleviate and attenuate the immune attack against the organs [11, 12]. In addition, these cells have a powerful regenerative capacity that will support the existing tissue and probably trigger the formation of new cells. Differentiation of MSCs into local tissue cells type is possible if these cells are from a mesenchymal origin whilst the formation of new cells in the non-mesenchymal tissue is mainly the responsibility of the local progenitor cells. In this chapter, we describe a protocol that uses mesenchymal stromal cells from compact bone to preserve the saliva flow rate in non-obese diabetic mice (NOD).

NOD mouse is a commonly used animal model to study SS [10, 13-16]. These mice show salivary and lacrimal lymphocytic infiltration and a subsequent secretion loss similar to SS human patients. Recent studies have reported that MSCs from NOD bone marrow (BM) have a very low expression of the surface marker CXCR4 which is responsible for homing BM stem cells to the inflammation site [17]. Transplantation of normal MSCs from healthy donors to NOD mice will eliminate this obstacle. The newly introduced cells can be trafficked to the inflammation site and arrest the attack against the glandular cells with anti-inflammatory and immunomodulatory properties. This chapter will demonstrate the steps for isolating compact bone MSCs and their enrichment, to the final injection into the NOD mice.

## 2. Materials

### 2.1. Preparing an Aseptic Condition

It is important to maintain the sterility throughout this procedure. Once the animal (donor) is euthanized, all procedures are carried out in the laminar flow hood. All surgical equipment and tools should be sterilized prior to their use. All surfaces, gloves, and any items introduced into the laminar flow hood should be sprayed generously with 70% ethanol.

### 2.2. Animals

1. 6–8-weeks-old CByB6F1-eGFP male mice were used for transplantation experiments (male GFP transgenic mouse [C57BL/6TgH (ACTbEGFP)10sb/J (stock # 003291) were bred with female BALB/c (stock # 00651)]
3. Five animals were kept in the same cage and provided with standard animal care in the animal facility.

### 2.3. Animal Surgery

1. $CO_2$ chamber.
2. 70% ethanol.
3. Sterilized dissection straight scissors.
4. Sterilized straight tweezers.
5. Sterilized scalpel (Feather).
6. Falcon 50 mL centrifuge tubes (Falcon™, Fisher Scientific).
7. Antibiotic-Antimycotic 100%, (Gibco, Life Technologies).
8. Fetal bovine serum (FBS), (Gibco, Life Technologies).
9. PBS.
10. Washing buffer: 1.0 mL antibiotic-antimycotic + 2.0 mL fetal bovine serum (FBS) + 97 mL PBS.

### 2.4. Isolation of Compact Bone $CD45^-/TER\text{-}119^-$ Multipotent Mesenchymal Stromal Cells

1. 70% ethanol.
2. Sterilized straight tweezers.

3. Sterilized scalpel (Feather).
4. 70 mm ceramic mortar and pestle.
5. Antibiotic-Antimycotic 100% (Gibco, Life Technologies).
6. PBS.
7. 70 µm nylon cell strainer (BD Biosciences).
8. Falcon 50 mL centrifuge tubes (Falcon $^{TM}$, Fisher Scientific).
9. MesenCult$^{TM}$ MSC Basal Medium (Mouse), (STEMCELL).
10. MesenCult$^{TM}$ Mesenchymal Stem Cell Stimulatory Supplements (Mouse), (STEMCELL).
11. MSCs growth medium: 1 bottle of MesenCult$^{TM}$ MSc Basal Medium + 1 bottle MesenCult$^{TM}$ Mesenchymal Stem Cell Stimulatory Supplements + 1.0 mL antibiotic-antimycotic.
12. Fetal bovine serum (FBS) (Gibco, Life Technologies).
13. Collagenase type I (Worthington).
14. Collagenase I solution: 0.25 gm collagenase I + 80 mL PBS + 20 mL fetal bovine serum (FBS) then filter sterilize the solution.
15. 2, 5, 10 mL sterile pipette (Falcon$^{TM}$, Corning).
16. Parafilm
17. 60 and 100 mm cell culture dish (SARSTEDT).
18. 5.0 mL round-bottom polystyrene tube (BD Biosciences).
19. 3% acetic acid in methylene blue.
20. Glass cover slip 22×22 mm (Fissure Scientific)
21. Hemocytometer.

### 2.5. Selection and Culture of Compact bone CD45$^{-}$ /TER-119$^{-}$ Multipotent Mesenchymal Stromal Cells

1. EasySep$^{TM}$, Mouse Mesenchymal Progenitor Enrichment Kit (STEMCELL).
2. EasySep$^{TM}$ Magnet (STEMCELL).
3. MSCs growth medium. (*see* **2.4.11**).
4. 100 mm cell culture dish (SARSTEDT).
5. 15 mL conical centrifuge tubes (Falcon$^{TM}$, Fisher Scientific).
6. 2, 5, 10 mL sterile pipette (Falcon$^{TM}$, Corning).
7. 0.25% Trypsin-EDTA (Gibco, Life Technologies).

8. Sterilized pipette tips (1-5, 20-200 µL and 1.0 mL)
9. Trypan blue stain 0.4% (Gibco, Life Technologies).
10. Glass cover slip 22×22 mm (Fissure Scientific).
11. Hemocytometer.

**2.6. Osteogenic Differentiation**

1. Osteoblast differentiation medium: α-MEM, 1% antibiotic-antimycotic (100 U/mL penicillin-G, 100 µg/mL streptomycin and 0.25µg/mL Amphotericin B), supplemented with 10% FBS, 0.1mM ascorbic acid, and $10^{-8}$ M dexamethasone, 2 mM β-glycerophosphate.
2. 6 well cell culture plate (SARSTEDT).
3. 2, 5, 10 mL sterile pipette (Falcon™, Corning).
4. 5% silver nitrate solution in $dH_2O$.
5. 95%, 80%, 50% and 20% ethanol.
6. PBS.
7. Distilled water ($dH_2O$).

**2.7. Adipogenic Differentiation**

1. Adipogenic differentiation medium: α-MEM, 1% antibiotic-antimycotic (100 U/mL penicillin, 100 µg/mL streptomycin and 0.25 µg/mL Amphotericin B), $10^{-8}$ M dexamethasone, 10 µg/mL insulin, 0.5 mM 1-Methyl- 3 – Isobutylxanthine (IBMX), 0.5 µM hydrocortisone, 60 µM Indomethacin.
2. 6 well cell culture plate (SARSTEDT).
3. 2, 5, 10 mL sterile pipette (Falcon™, Corning).
4. Oil red O stain: 0.3% oil red O staining solution. 0.3 g oil red O (ICN Biomedicals) stain dissolved in 100 mL isopropanol.
5. PBS.
6. 10% Neutral Buffered Formalin.
7. 60% Isopropanol.
8. Distilled water ($dH_2O$).

### 2.8. Chondrogenic Differentiation

1. StemXVivo™ Chondrogenic Base Media (R&D Systems).
2. StemXVivo™ Human/Mouse Chondrogenic Supplement (R&D Systems).
3. Antibiotic-Antimycotic 100% (Gibco, Life Technologies).
4. Chondrogenic differentiation medium: 1.0 mL StemXVivo Chondrogenic Base Media + 10 µL StemXVivo Human/Mouse Chondrogenic Supplement with + 1.0 mL antibiotic-antimycotic.
5. 2, 5 mL sterile pipette (Falcon™, Corning).
6. Anti-Collagen II antibody (R&D Systems).
7. 15 mL conical centrifuge tubes (Falcon™, Fisher Scientific).

### 2.9. Flow Cytometry Analysis

1. Anti-Mouse TER-119 APC, clone: TER-119 (eBioscience).
2. Anti-Mouse CD11b, clone: M1/70 (eBioscience).
3. Anti-Mouse CD106 (VCAM-1) eFlour® 450, clone: 429 (eBioscience).
4. Anti-Mouse CD105 eFlour® 450, clone: MJ7/18, (eBioscience).
5. Anti-Mouse Ly-6A/E (Sca-1), clone: D7 (eBioscience).
6. Anti-Mouse CD73 PE-Cyanine7, clone: eBioTY/11.8 (TY/11.8), (eBioscience).
7. Anti-Mouse/Rat CD29 PE-Cyanine7 (Integrin beta 1), clone: eBioHMb1-1(HMb1-1) (eBioscience).
8. Anti-Mouse CD44 PE-Cyanine7, clone: IM7 (eBioscience).
9. Anti-Mouse CD45, clone: 30-F11 (BD Bioscience).
10. BD LSRFortessa cell analyzer (BD Bioscience).
11. Flowjo software (Tree Star).

### 2.10. Colony Forming Unit- Fibroblast (CFU-F) Assay

1. 6 well culture plate (Sarstedt).
2. MSCs growth medium. (*see* **2.4.11**).
3. 2, 5, 10 mL sterile pipette (Falcon™, Corning).
4. Giemsa stain (Sigma).
5. Methanol.

6. Distilled water ($dH_2O$).

### 2.11. Cell Transplantation

1. MSCs growth medium. (*see* **2.4.11**).
2. 0.25% Trypsin-EDTA (Gibco, Life Technologies).
3. Fetal bovine serum (FBS), (Life Technologies).
4. 2, 5, 10 mL sterile pipette (Falcon™, Corning).
5. PBS.
6. Distilled water ($dH_2O$).
7. Normal saline.
8. 0.5 mL Insulin syringes (BD Bioscience).
9. Sterilized 1.5 mL eppendorf tube.
10. Sterilized pipette tips (1.0 mL).
11. Mouse restrainer.
12. Alcohol swap.
13. Red heat lamp.

## 3. Method

### 3.1. Animal Surgery

This section describes the steps for the surgical dissection of the animal, removing muscle tissue from the limbs and preparing the compact bone for $CD45^-/TER\text{-}119^-$ cells isolation.

1. Euthanize the animal using the accepted method at your facility.
2. Place the body on the supine position and disinfect generously the whole body with 70% ethanol.
3. Use a pair of dissection straight scissors and straight tweezers to cut the fur and the skin from the base of the neck toward the pelvic region. Cut the skin toward the wrist region and the ankle region respectively. Remove completely the skin that covers the limbs.
4. Remove as much as possible of the muscles and the attached ligaments with the aid of dissection straight scissors and a scalpel. Avoid cutting the bone at this stage.

5. Cut the leg/forearm just below the ankle/wrist joint and the femur/humerus just above the hip/shoulder joint.

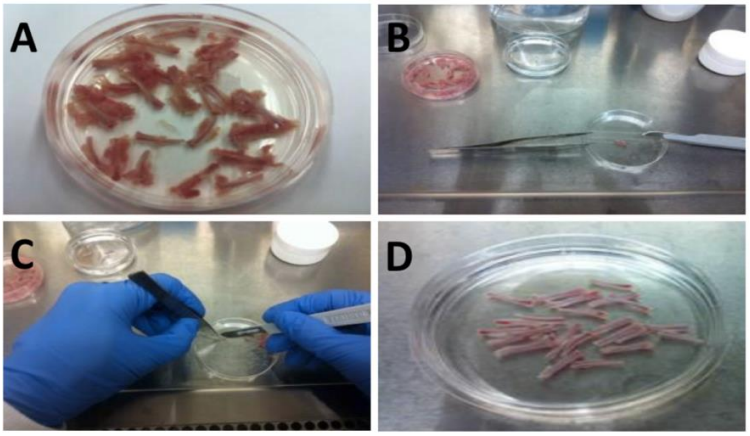


**Figure 1. Surgical resection of long bones and removal of any surrounding soft tissues**. (a) Long bones after resection from the body of the mouse. (b) Instrumentations setup for the complete removal of soft tissues still attached to the long bones. (c) Removal of the soft tissues with the aid of straight tweezers and a scalpel. (d) Long bones denuded of soft tissues were immersed in washing buffer. Copyright (2017) Springer Science+Business Media LLC.

6. Transfer the limbs to a new 100 mm cell culture dish containing the washing buffer.
7. Remove any remaining muscles or ligaments and then separate the bones.
8. Transfer the bones to a 50 mL conical centrifuge tube containing the washing buffer, and gently shake the tube. Change the buffer three times. (*see* **Figure 1**).

### 3.2. Isolation of Compact Bone CD45$^-$/TER-119$^-$ Multipotent Mesenchymal Stromal cells

This section describes the steps for the isolation of compact bones cells from 5 mice by collagenase I digestion and outgrowth from cultured bone fragments.

1. All solutions used are at room temperature.
2. Sterilize the mortar with 70% ethanol and allow it to dry in the laminar flow hood.
3. Place the bones in the sterilized mortar.
4. Gently crack the bones with the pestle and break them down into smaller pieces. (*see* **Note 1**).

5. Add 10 mL of the washing buffer to the bone fragments then pipette the solution up and down several times. (*see* **Note 2**) Discard the buffer and add another 10 mL, repeat pipetting up to six times or until the bone fragments turn white in color. At this step, the depletion of bone marrow cells is reached.

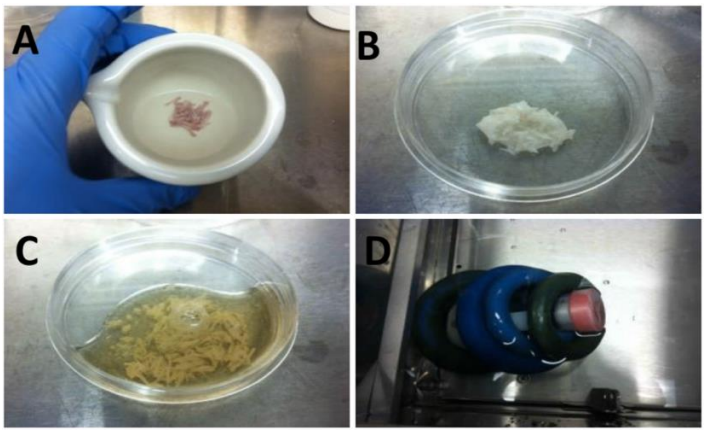


**Figure 2. Bone marrow separation and isolation of mesenchymal stromal cells from the compact bone**. (a) Long bones in a sterilized mortar. (b) The marrow-free bone fragments in a 100 mm cell culture dish. (c) Collagenase I solution with bone fragments in 100 mm cell culture dish. (d) Incubation of bone fragments with collagenase I in a 37 °C shaker water bath. Copyright (2017) Springer Science+Business Media LLC.

6. Place the bone fragments in a 100 mm cell culture.
7. Add enough collagenase I solution (5-7 mL) to cover all the bone fragments.
8. Leave the collagenase I solution for 3-5 minutes; cut the softened bone fragments with straight tweezers and a scalpel into 1-2 mm. Higher cell yield will be obtained with smaller fragments.
9. Add more collagenase I solution (8-10 mL) to the fragments to make the final volume 15 mL then transfer them to a new 50 mL conical centrifuge tube. (*see* **Note 3**).
10. Seal the tube with parafilm and place it in a 37 °C shaker bath for 45 minutes.
11. Stop the enzymatic digestion by adding 20 mL washing buffer, making the final volume 35 mL.

12. Transfer the supernatant to a new 50 mL conical centrifuge tube and wash the bone fragments with an additional 10 mL of washing buffer.
13. Aspirate and add the buffer to the supernatant from previous step and filter them through a 70 µm nylon cell strainer.
14. Count the cells with 3% acetic acid in methylene blue. (*see* **Note 4**).
15. Centrifuge at 300 x g for 10 minutes.
16. Aspirate the supernatant and resuspend the cells in MSCs growth medium. Seed cells at 3.0 - $5.0\times10^5$ cells/cm in 100 mm cell culture dish. Culture the cells in 37 °C humid incubator with 5% $CO_2$. Change the media every 2-3 days.
17. Transfer the bone fragments from the 50 mL conical centrifuge tube to a new 100 mm cell culture dish.
18. Add MSCs growth medium to cover bone fragments.
19. Culture the bone fragments following the same procedure (*see* **3.2.18, Note 4, Figure 3**).
20. When the cells reach 70-80% confluency (*see* **Note 5**), detach the cells with 0.25%Trypsin-EDTA for 2- 3 minutes in the 37°C humid incubator or until the cells become rounded and lose attachment to the culture vessel. (see **Note 6**).
21. Centrifuge the cells at 300 x g for 5 minutes.
22. Aspirate the supernatant and resuspend the cells in 5.0 mL of MSCs growth medium, next perform cell counting.
23. Centrifuge the cells at 300 x g for 5 minutes.

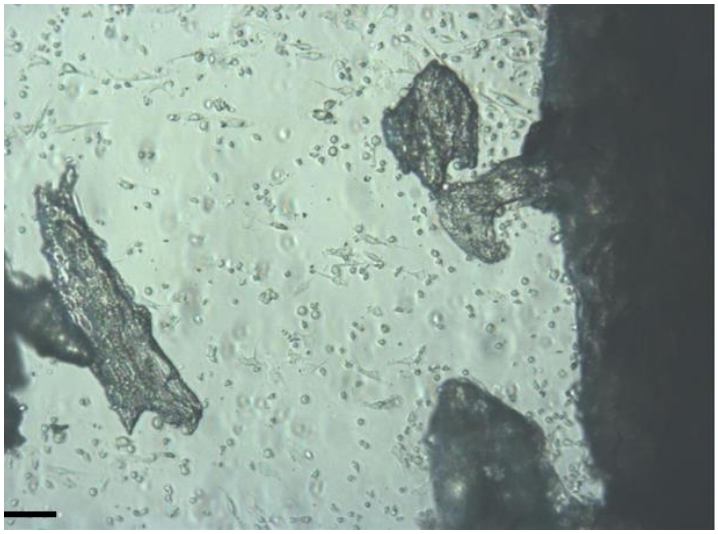

**Figure 3. Compact Bone cells by outgrowth method**. Phase contrast photomicrograph showing the cell outgrowth from the edges of the compact bone fragments (shown as black irregular objects; for example, on the right side of the photomicrograph). Scale bar = 38 μm. Copyright (2017) Springer Science+Business Media LLC.

24. Aspirate part of the supernatant and leave a volume to make the final concentration 2.0-5.0×$10^7$ cells/mL.
25. Transfer the cells to 5.0 mL polystyrene round-bottom tubes, which will fit later in the EasySep™ magnet.
26. Keep the cells on ice until the selection. (*see* **Figure 2**).

### 3.3. Selection and Culture of Compact Bone CD45$^-$/TER-119$^-$ Multipotent Mesenchymal Stromal Cells

This section describes the enrichment of CD45$^-$/TER-119$^-$ MSCs by using a cocktail of biotinylated antibodies designed against non-MSCs (CD45$^+$, TER-119+ cells) whilst the MSCs are not labelled.

1. Isolation of the CD45$^-$/TER-119$^-$ cells was performed using EasySep™ mouse mesenchymal progenitor enrichment kit (STEMCELL).
2. Use the cells from the previous step (*see* **3.2**).

3. Add the mouse mesenchymal progenitor enrichment cocktail to the cell suspension at a ratio of 50 µL/mL, mix well and incubate at 4°C refrigerator for 15 minutes.
4. Add 4.0 mL of MesenCult MSc Basal Medium and centrifuge the suspension.
5. Aspirate the supernatant and add fresh medium to suspend the cells at 2.0-5.0×$10^7$ cells/mL.
6. Add 250 µL of Biotin selection cocktail to every 1.0 mL of cells, mix well and incubate in 4°C refrigerator for 15 minutes.
7. Vortex the M Prog™ Magnetic Microparticles for 30 seconds or until no visible clumps inside the tube then add 150 µL to each 1.0 mL of the cells suspension. Mix well and incubate in 4°C refrigerator for 15 minutes.
8. Add more medium to the suspension to bring the final volume to 2.5 mL then pipette up and down gently 2-3 times.
9. Place the tube uncapped in the EasySep™ magnet for 5 minutes in laminar flow hood.
10. Invert the EasySep™ magnet with the tube inside and pour the solution containing the target cells into a new conical centrifuge tube in one motion. (*see* **Note 7**).
11. Add more MSCs growth medium to the cell suspension, then perform cell counting.
12. Plate cells at 2.0-5.0×$10^5$cells/$cm^2$ in 100 mm cell culture dish. (*see* **Note 8**)
13. Passage cells at 70-80% confluency with 0.25% Trypsin-EDTA.

### 3.4. Multilineage differentiation

This section describes the osteogenic, adipogenic and the chondrogenic differentiation procedure and the final verification by the appropriate staining method.

#### 3.4.1. Osteogenic Differentiation

1. Cells are seeded in a 6 well cell culture plate with growth medium and incubated in a 37°C humid incubator until they reach approximately 50-70% confluency.
2. Aspirate the growth medium and replace with 2.0 mL of the osteogenic inductive medium per well.
3. Incubate the cells in a 37°C humid incubator and change the medium every 2-3 days.

4. After 3 weeks of induction, osteogenic differentiation is visualised by Von Kossa staining method.

### 3.4.2. Adipogenic Differentiation

4. Cells are seeded in a 6-well plate with growth medium and incubated in a 37℃ humid incubator with 5% $CO_2$ until they reach approximately 90-100% confluency. (It takes approximately 1-4 days)
5. Aspirate the growth medium and replace it with 2.0 mL of the adipogenic inductive medium per well.
6. Incubate the cells in a 37℃ humid incubator with 5% $CO_2$ and change the medium every 2-3 days.

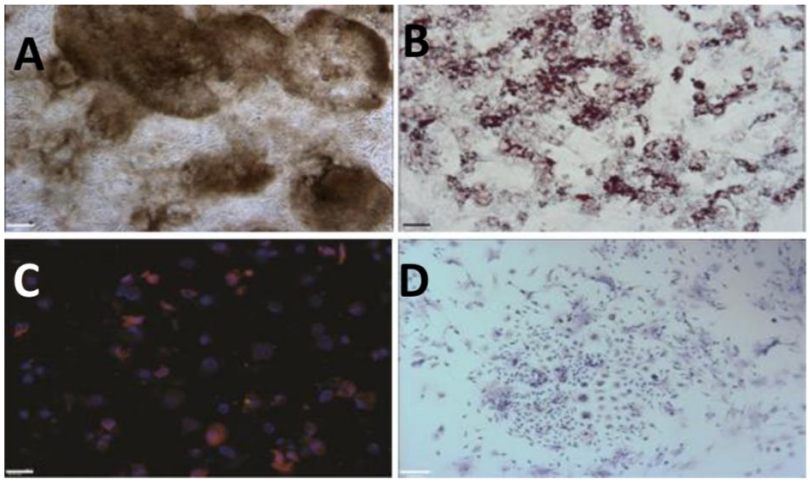


**Figure 4.** Multilineage differentiation of compact bone and CFU formation. (a) Von Kossa staining for osteogenic differentiation. (b) Oil red O staining for Adipogenic Differentiation. (c) Collagen type II immunofluorescent staining for chondrogenic differentiation (from Ref. 16). (d) CFU formation stained with Giemsa stain. Scale bar = 38 μm. Copyright (2017) Springer Science+Business Media LLC.

7. After 3 weeks of induction, adipogenic differentiation is visualised by Oil Red O staining (*see* **Figure 3**).

### 3.4.3. Chondrogenic Differentiation

1. Transfer 250,000- 500,000 cells to a 15 mL conical centrifuge tube and centrifuge at 300 × g for 5 minutes.
2. Aspirate the supernatant and resuspend the cell pellet with 0.5 mL of chondrogenic differentiation medium then gently pipette up and down 2-3 times.
3. Centrifuge the cells again at 300 × g for 5 minutes without discarding the medium. Allow the detachment of the cell pellet from the bottom of the tube without disturbing it. Loosen the cap of the tube and place it in a rack vertically inside the 37°C humid incubator with 5% $CO_2$.
4. Change the medium with freshly prepared chondrogenic differentiation medium every 2-3 days (*see* **Note 9**).
5. Culture the cells for up to 21 days. Cell pellet is retrieved, cryopreserved in OCT, and sectioned with a microtome at 7µm thickness.
6. Chondrogenic differentiation is further confirmed with the immunofluorescence staining for Collagen II. (*see* **Figure 3**).

### 3.5. Colony Forming Unit (CFU) Assay

1. Seed the cells (*see* **3.3.12**) at 10,000/$cm^2$ and culture them for 10-20 days.
2. Change medium every 2-3 days.
3. When the colonies are formed, the staining step can be started.
4. Remove the media and gently wash the cells twice with PBS.
5. Allow the cells to dry, and then add enough methanol to cover the cells, and incubate for 5 minutes at room temperature (around 25 °C) in a fume hood.
6. Remove the methanol and allow drying for 5 minutes.
7. Add a diluted Giemsa (1:20 in distilled water) and incubate for 5 minutes.
8. Remove the Giemsa stain and wash gently with $dH_2O$ until the water is clear (*see* Figure **3**).

### 3.6. Flow cytometry

1. Harvest the cells at 70-80% confluency at passage 3 with 0.25% Trypsin-EDTA.
2. Cells are stained with the provided antibodies (*see* **2.9**) and prepared for the sorting using BD LSRFortessa cell analyzer.
3. Data obtained from the analyzer was analyzed using Flowjo software.

### 3.7. Cell Transplantation

This section describes the steps for harvesting the cells and the final transplantation into the mice via the tail vein**.**

1. Cells used for the transplantation are harvested shortly prior to the procedure.
2. Add enough 0.25% Trypsin-EDTA to a 70-80% confluent cell culture dish at passages 3-5, incubate in a 37°C humid incubator with 5% $CO_2$ for 2-3 minutes or until the cells detach. (*see* **Note 10**).
3. Deactivate the 0.25% Trypsin-EDTA by adding an equal volume of the MSCs growth medium.
4. Collect the cells and centrifuge at 300 × g for 5 minutes.
5. Aspirate the supernatant and wash the cells with normal saline three times then centrifuge at 300 × g for 5 minutes.

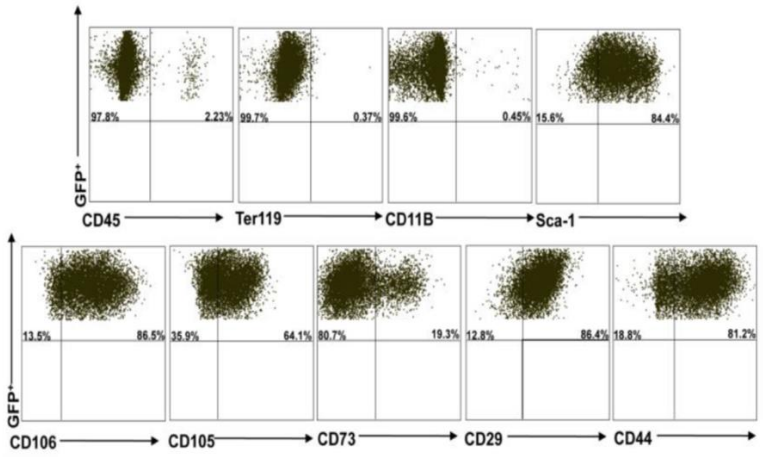

**Figure 5. FACS analysis** of MACS purified $CD45^{-}$ /$TER\text{-}119^{-}$ cells from compact bone shows 97.8% $CD45^{-}$, 99.7% $TER\text{-}119^{-}$, 99.6% $CD11b^{-}$ and 84.4% $Sca1^{+}$, 86.5% $CD106^{+}$, 64.1% $CD105^{+}$, 19.3% $CD73^{+}$, 86.4% $CD29^{+}$, 81.2% $CD44^{+}$ cells (16). Copyright (2017) Springer Science+Business Media LLC.

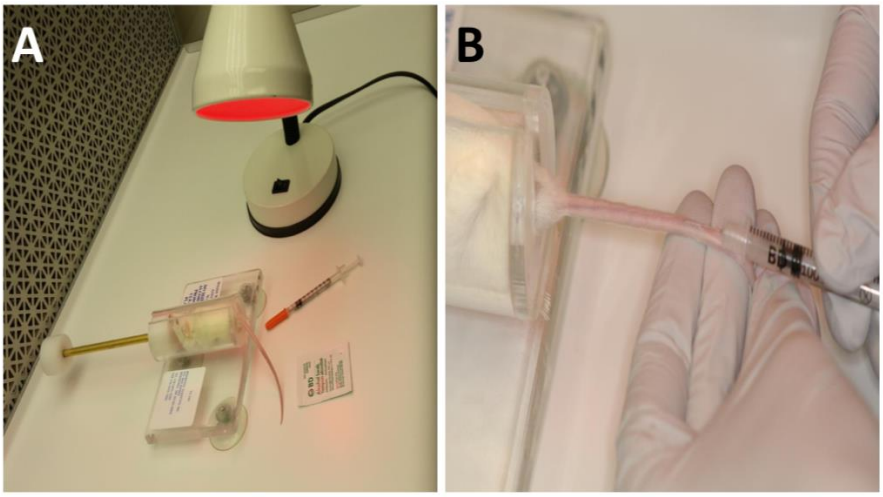


**Fig. 6 Cell Transplantation**. (a) The apparatus and the materials required for the cell transplantation. (b) The needle should be inserted parallel to the tail vein in the middle third. Copyright (2017) Springer Science+Business Media LLC.

6. Suspend the cells in normal saline at a ratio of $1.0\times10^{7}$/100 µL in a 1.5 mL Eppendorf tube and keep it on ice until the injection time.
7. Warm up the cell suspension to body temperature by holding the Eppendorf tube between your fingers and vortex it prior to the injection.
8. Using a 0.5 mL insulin syringe, aspirate 100 µL of the cell suspension for each mouse.
9. Place the mouse in the restrainer in sternal position (*see* **Note 11, Figure 6**).
10. Direct the red heat lamp at the lower two thirds of the mouse tail for 5-10 minutes or until the veins are visible and dilated.

11. Rub the tail with an alcohol swab and insert the syringe needle (the bevel facing upward) in the middle third section of the lateral tail vein, parallel to the tail. Inject 100 µL slowly at a constant speed (*see* **Note 12, Figure 6**).
12. Apply a gentle pressure at the injection site to stop the bleeding with an alcohol swab for 1-2 minutes.
13. Monitor the mouse after the injection for any adverse reactions.
14. Repeat the injection twice a week for two consecutive weeks.
15. For saliva collection, sedate the mouse with isoflurane then inject 0.05 mg/kg pilocarpine subcutaneously. Collect whole saliva by placing a micropipette (inside a 0.5 mL eppendorf tube) intraorally for 10 minutes from the start of pilocarpine injection. Saliva is collected every four weeks after the cell therapy.

## 4. Notes

1. Avoid trituration that may devitalize the cells.
2. This step is performed to remove the bone marrow from the bone fragments
3. Bone from one mouse requires 3.0 mL of collagenase I solution.
4. Acetic acid with methylene blue is used to remove any remaining RBCs that may cause inaccuracy of the cells number
5. When the bone fragments are cultured, it is advised not to move the plate for three days after the initial plating.
6. Cells from the bone fragments outgrowth are harvested after the removal of the bone fragments. The bigger fragments are suctioned individually using a 2.0 mL sterile pipette whilst the smaller ones are collected at one side of the cell culture dish by washing them with PBS then suctioned together all at once.
7. Avoid any tapping of the tube inside the magnet as you may get the unwanted cells
8. Selection is recommended after the first passage as the cell number is higher than the freshly isolated cells.
9. Avoid disturbing or losing the cell pellet during medium exchange.
10. Prepare cells instantly before the injection, as the cells form clumps with longer time.

11. Selecting the appropriate size of the mouse restrainer/holder is an important factor. Smaller-size restrainer can interfere with the mouse breathing, while larger-size restrainer allows mouse movements during injection.
12. Change to another injection site if the injection does not flow passively (e.g. high pressure on the piston of the syringe), as the injected cells most likely diffused into the surrounding tissue instead of inside the vein.

## 5. Acknowledgments

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# Chapter 3

## Compact Bone-Derived Multipotent Mesenchymal Stromal Cells (MSCs) for the Treatment of Sjogren's-like Disease in NOD Mice

**Ghada Abu Elghanam, Younan Liu, Saeed Khalili, Dongdong Fang, and Simon D. Tran**

### Abstract

Compact bone (cortical or dense bone) is among the organs that contain multipotent mesenchymal stromal cells (MSCs). Unlike bone marrow plugs where MSCs were initially isolated, compact bone has minimal (amount of) hematopoietic cells and thus facilitates the MSCs isolation process. In vitro, MSCs from compact bone show multipotency and differentiation into mesenchymal tissues such as bone, adipose, and cartilage, under certain conditions. MSCs therapy has been promising in preclinical and clinical studies against autoimmune diseases. Not only can MSCs replace the lost tissue through their regenerative properties, but they can also control the autoimmune attacks by immunoregulatory cytokines. This protocol describes the use of compact bone-derived MSCs to preserve salivary function (saliva flow/output) in the NOD (nonobese diabetic) mouse model affected with Sjogren's-like disease.

**Key words** Compact bone, Mesenchymal stromal cells, MSCs, Autoimmune diseases, Sjogren's syndrome, Sjogren's-like disease, Saliva, Hyposalivation, Xerostomia, Nonobese Diabetic (NOD) mouse

### 1 Introduction

Saliva is a secretory fluid that protects the oral tissues and aids in swallowing; thus its loss is problematic. Sjogren's syndrome (SS), radiotherapy to the head and neck area for cancer treatment, and the intake of some drugs (such as antihypertensive agents) can all cause saliva loss (salivary hypofunction, xerostomia). SS is a common chronic autoimmune disease characterized by focal lymphocytes infiltration into the salivary and lacrimal glands leading to xerostomia and exophthalmia [1]. Women are affected nine times more than men and are mostly postmenopausal. SS can be restricted to the secretory glands only, primary SS, or coexists with other autoimmune diseases like rheumatoid arthritis (RA) or systemic lupus erythematosus (SLE), secondary SS. The etiology of SS is

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## Chapter Four

# Mesenchymal Stem Cells Extract (MSCsE)-Based Therapy Alleviates Xerostomia and Keratoconjunctivitis Sicca in Sjögren's Syndrome-Like Disease

## Preface

SS is a complex autoimmune disease and no drug has been able to cure it or induce remission; therefore, the development of a treatment is ongoing. In clinical trials and preclinical studies, MSCs has successfully managed SS. However due to cell therapy risks and side effects, an alternative and a more convenient biological therapy is preferred.

This is the first preclinical study we have carried out to examine the efficacy of MSCsE (in comparison to parent, MSCs) in alleviating SS-like disease in female NOD mice. Our results show that MSCsE was efficient in hindering the immune dysregulation via re-establishing peripheral tolerance in a comparable efficacy to that of MSCs. The salivary and lacrimal glands both showed specialized cellular preservation and higher proliferation rates. This suggests that MSCsE might replace MSCs or could be combined with other biologic agents for better therapeutic outcomes.

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# Mesenchymal Stem Cells Extract (MSCsE)-Based Therapy Alleviates Xerostomia and Keratoconjunctivitis Sicca in Sjögren's Syndrome-Like Disease

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**Abstract:** Sjögren's Syndrome (SS) is an autoimmune disease that manifests primarily in salivary and lacrimal glands leading to dry mouth and eyes. Unfortunately, there is no cure for SS due to its complex etiopathogenesis. Mesenchymal stromal cells (MSCs) were successfully tested for SS, but some risks and limitations remained for their clinical use. This study combined cell- and biologic-based therapies by utilizing the MSCs extract (MSCsE) to treat SS-like disease in NOD mice. We found that MSCsE and MSCs therapies were successful and comparable in preserving salivary and lacrimal glands function in NOD mice when compared to control group. Cells positive for AQP5, AQP4, α-SMA, CK5, and c-Kit were preserved. Gene expression of AQP5, EGF, FGF2, BMP7, LYZ1 and IL-10 were upregulated, and downregulated for TNF-α, TGF-β1, MMP2, CASP3, and IL-1β. The proliferation rate of the glands and serum levels of EGF were also higher. Cornea integrity and epithelial thickness were maintained due to tear flow rate preservation. Peripheral tolerance was re-established, as indicated by lower lymphocytic infiltration and anti-SS-A antibodies, less BAFF secretion, higher serum IL-10 levels and $FoxP3^+$ $T_{reg}$ cells, and

selective inhibition of $B220^+$ B cells. These promising results opened new venues for a safer and more convenient combined biologic- and cell-based therapy.

**Keywords:** Sjögren's Syndrome (SS); autoimmune diseases; biologic therapy; bone marrow; cell extract; lacrimal gland; mesenchymal stromal cells (MSCs); non-obese diabetic mice (NOD); salivary glands; submandibular glands.

## Introduction

SS is a common progressive autoimmune disease that affects females predominantly [1–3]. The prevalence of SS is variable worldwide; ranging from 0.1% to 0.72% of the population [4–11]. SS progresses slowly and patients exhibit clinical symptoms years after the disease onset [12]. The immune system targets epithelial tissues, infiltrates it with lymphocytes, and later forms autoantibodies against specific glandular antigens [3,13–16]. The aberrant immune dysregulation leads to the destruction of epithelial tissues, especially salivary and lacrimal glands, and to several extra-glandular manifestations. The secretory function of the glands diminishes gradually resulting in dryness of the mouth (xerostomia), eyes (keratoconjunctivitis sicca), and organs containing exocrine glands, such as the nose and vagina [17–20]. The current SS management is symptomatic based to alleviate the dryness severity and complications [21,22]. However, patients with systemic involvement and serious complications are prescribed immunosuppressant and disease-modifying antirheumatic drugs [23–25]. Unfortunately, the current management is not adequate nor satisfactory, leading to a compromised quality of life [26–28].

MSCs are multipotent cells that can self-renew and give rise to specialized cell types, such as bone, cartilage, and muscles [29–32]. They were firstly isolated from the bone marrow, and later were extracted from various tissues, including peripheral blood, umbilical cord, adipose tissue, periodontal ligaments, and dental pulp [30,33–35]. Under normal homeostasis, MSCs are actively involved in the connective tissue maintenance. During tissue repair, they are responsible for secreting bioactive molecules that result in tissue regeneration and restoration [36]. MSCs are hypoimmunogenic because they lack the expression of MHC II and they express low levels of MHC I [37,38]. MSCs have demonstrated promising therapeutic potentials when used in different diseases and in tissue regeneration. They were successfully deployed in the management of neural

injuries, GvHD, cardiac regeneration, and most importantly autoimmune diseases [39–58]. MSCs display a unique combination of immunoregulatory/immunosuppression, tissue regeneration/repair, and anti-fibrotic properties [59,60] which make them a suitable therapeutic modality for autoimmune diseases.

MSCs are well documented for their immunomodularity and anti-inflammatory properties [61–64]. Several studies have reported that MSCs suppressed T and B cells proliferation when injected at the peak or at the onset of the disease [65–67]. However, Xu et al. have reported a defective MSCs immunoregulatory function in SS patients and NOD mice [50]. They incubated BM-derived MSCs from NOD mice and SS patients with PBMCs (Peripheral Blood Mononuclear Cells), a significantly higher proliferation rate of PBMCs was found in comparison to MSCs isolated from healthy donors. Thus, the previous findings rationalize the replacement of MSCs in NOD mice or SS patients with adequately functioning ones from healthy donors to compensate for the defective immunoregulation function. Nonetheless, the utilization of MSCs in treatments is not risk-free. These cells possess an attractive self-renewal and unlimited proliferation capacities, but these characteristics can be unpredictable and uncontrollable in vivo. MSCs might form tumors or enhance the progression of an existing one [68,69]. Therefore, transforming MSCs into extract/lysate can eliminate, theoretically, the tumorigenic risk. Our group and others have reported the therapeutic potentials of bone marrow cell extract (soup) in the management of irradiation-induced and SS damage of salivary glands and myocardial infraction, respectively, indicating the success of the concept [70–72]. Yet, to the best of our knowledge, the efficacy of MSCs extract (MSCsE) has not been tested in any field.

The main aim of this study is to evaluate the efficacy of MSCsE in preserving the exocrine function of the salivary and lacrimal glands in NOD mice in comparison to MSCs. We hypothesized that MSCsE treatment executes this task via two mechanisms. Firstly, through their trophic and regenerative capacities, and secondly, by re-establishing peripheral tolerance which protects the glands against the autoimmune attack and eventually preserving the tissues from the autoimmune destruction.

## Materials and Methods

### Animal Models

All experimental procedures were performed following the guidelines imposed by the Canadian Council on Animal Care. Our protocol (2007-5330) was approved by the University Animal Care Committee (UACC) at the McGill University.

### Recipient

Eight-week-old female NOD mice with Sjögren's-like disease purchased from Taconic Farms (Germantown, NY, USA) were randomized into three groups. Group 1: NOD mice treated with bone marrow-derived MSCs (n =8), Group 2: NOD mice treated with bone marrow-derived MSCsE (n=12) and Group 3, Control: NOD mice treated with normal saline (n=11).

### Donors

Eight-week-old male C57BL/6 were purchased from Jackson Laboratory (Bar Harbor, ME, USA). Mice were kept at the animal facility according to the animal care rules and regulation.

### Wild Type Control

Eight-week-old ICR (Institute of Cancer Research) mice were purchased from Taconic Farms (Germantown, NY). ICR is an outbred strain from which NOD subline was derived [50,122]. This strain was used as a wild type and SS-free control mouse model. ICR mice received no treatment. Their saliva and tear flow rates were measured at the same time the treated and control NOD groups were (n = 6).

### Blood Glucose Monitoring

Starting at 12 weeks of age, fasting blood glucose was monitored for all mice once weekly using Accu-Check® (Roche) system where mice were bled at the tail middle third. All mice with diabetes (> 250 mg/dL) were administered insulin subcutaneously and checked daily afterwards. All mice with borderline (150–250 mg/dL) level were checked twice weekly. Mice with normal glucose levels were monitored weekly until hyperglycemia was evidenced then the above protocol was followed.

**Mesenchymal stromal Cells (MSCs) Culture, Preparation of the Extract (MSCsE), and Their Transplantation**

Bone marrow (BM) cells were harvested from 8-week-old male C57BL/6 mice. Briefly, mice were euthanized according to the animal ethics protocol at McGill University. Mice were disinfected thoroughly with 70% ethanol then the femur and the tibia of the hind limbs were obtained [52]. The isolated bones were washed with cold 1× PBS to remove any remaining blood or tissues and kept on ice throughout the procedure. The bones were placed inside a pre-cut sterilized 1.0 mL pipette tip which was adapted inside a sterilized 1.5-mL Eppendorf tube. The Eppendorf tubes were then placed in a 4 °C centrifuge at a speed of 4040× g for 30 s then the pellets were collected and kept on ice. The BM pellet was reconstituted and mixed thoroughly with the MSCs media (MesenCult™ MSC Basal Medium+ MesenCult™ Mesenchymal Stem Cell Stimulatory Supplements (STEMCELL)). The cell suspension was filtered using a 40-µm nylon cell strainer. The cell number was determined then the cells were cultured in 75 $cm^2$ flasks (Corning Inc, Corning, NY, USA) and seeded at a ratio of $40 \times 10^5$ cells/$cm^2$. The flasks were closed tightly and incubated unmoved for five days at 37 °C in a 5% $CO_2$ incubator. Thereafter, floating cells were discarded, and a fresh media was added. When the cells (spindle in shape) reached 70–80% confluency, they were passaged at a ratio of 1:3. MSCs were enriched by passaging as the non-MSCs and hematopoietic cells tend to attach strongly to the culture vessels. When cells reached passage 8, they were detached and prepared for treatment. Each mouse received $2.0 \times 10^6$ cells/100 µL in normal saline once weekly for four consecutive weeks starting at 8 weeks of age via the tail vein.

For MSCsE preparation: cells from passage 8 were reconstituted in normal saline at a ratio of $2.0 \times 10^6$ cells/100 µL. The cells suspension was placed in liquid nitrogen to freeze then thawed at room temperature; the process was repeated three times to ensure complete cell rupture. At the end of the third cycle, the tubes were thawed and placed in the centrifuge at 4545× g speed for 30 min at 4 °C temperature. At the end of the centrifugation cycle, the supernatant (i.e., the cell extract) was collected for immediate use or stored at −80 °C freezer. Treated mice received 100 µL of MSCsE once weekly for four consecutive weeks starting at 8 weeks of age.

**Secretory Function of the Saliva and Lacrimal Glands (Saliva Flow Rate: SFR and Tear Flow Rate: TFR)**

Secretory function of the salivary glands (Saliva Flow Rate: SFR) was measured by inducing mild gas anesthesia in NOD mice using 1.5–3% isoflurane, 5% halothane and 1 L/min oxygen. When the mice were sedated, SFR was stimulated by injecting 1.0 mg pilocarpine/kg body weight subcutaneously in the dorsal side of the neck. Whole saliva was obtained from the oral cavity by placing a micropipette into a pre-weighed 0.5 mL microcentrifuge tubes at the corner of the mouth. Five minutes after the pilocarpine injection, saliva collection was done for 10 min; however, any saliva produced in the first 5 min was discarded. Saliva volume was determined gravimetrically and then stored in -20 °C freezer. SFR was measured pre-treatment at 8 weeks of age (week 0) then at 4, 8, 12, and 16 weeks post-treatment.

TFR was measured at the same appointment as SFR to reduce the animal discomfort. After 10 min of the injection of pilocarpine, phenol red threads (Zone Quick, FCI Ophthalmics, Japan) were placed gently in the medial canthus of both eyes with the aid of fine tip tweezers for 5 min; any tear secretion in the previous 10 min was removed before the final measurement was recorded. The thread was measured by a ruler to the approximate mm and then placed in a sterilized 1X PBS containing tube and stored in −20 °C freezer. The readings of both eyes were averaged, and then a mouse group average was calculated. Only mice that showed lymphocytic infiltration in the lacrimal gland were included in the TFR, lacrimal focus score, lacrimal focus area, and corneal thickness assessments in the results. The percentage of positive mice for lacrimal gland infiltration were 40–75% among the groups.

**Submandibular and Lacrimal Gland Tissue and Serum Analysis**

Al analysis were carried out 16 weeks post-treatment (24-week-old NOD mice).

**Serum Preparation and Analysis**

Shortly after the animals were euthanized, the blood was drawn via cardiac puncture. Blood was left to clot for 30 min at room temperature then centrifuged at 1212× g for 8 min. Serum was isolated, aliquoted and then stored in −80 °C freezer for further analysis later. ELISA was used for

the analysis of serum EGF (ab100679, abcam), Anti-SSA/Ro (5710, Alpha Diagnostics), anti-SSB/La (5810, Alpha Diagnostics), and IL-10 (ab46103, abcam).

**Focus Score and Focus Area**

Focus score is defined as the number of the lymphocytic infiltrates/4mm$^2$, where a focus is an aggregate of ≥ 50 lymphocytes). It is evaluated under the microscope using serial H&E stained histological sections cut at different levels. Focus area defined as the area occupied by the lymphocytic infiltrates in the glands measured in μm$^2$. It was performed by using 200 or 400× magnified images that were acquired using Volocity software. Thereafter, the size of each focus was assessed using ImageJ software. The average for each group was then calculated and represented in μm$^2$.

**Immunohistochemistry**

Formaldehyde-fixed paraffin-embedded (FFPE) submandibular and lacrimal glands sections were blocked for endogenous peroxidases by using fresh 3% $H_2O_2$ after the antigen retrieval step with acetic acid pH 6. The nonspecific binding of the primary antibodies was blocked with 1% BSA and 5% normal goat serum in PBS for one hour at room temperature. The primary antibodies, B220 (550286, BD Biosciences), FoxP3 (14–5773, eBioscience), BAFF (11021244, Enzo Life Sciences) were incubated overnight in 4°C refrigerator. Polyclonal rabbit anti-Rat secondary antibody was applied for 1 h at room temperature. Visualization was performed using the DAB+ system (k3468, Dako). Counter-staining with hematoxylin stain was run for one minute. Brightfield microscopy was used to obtain magnified images using Volocity software. The percentage of positive cells was performed using Image J software. For B220 and BAFF analysis, the positive signal was measured as the intensity and was divided by the focus area (surface area of the lymphocytic infiltrate) and an intensity percentage was generated. For FoxP3, the positive cells were counted then divided by the total area of the focus then the data was represented as cell/μm$^2$. Data were represented as mean ± S.D.

**Immunofluorescence**

Submandibular and lacrimal glands frozen sections were blocked with 1% BSA and 5% normal donkey serum in PBS for one hour at room temperature. The primary antibodies: AQP5 (ab78486, abcam), AQP4 (ab9512, abcam) CK5 (PRB-160P, Covance), α-SMA (ab7817, abcam), c-Kit

(ab5506, abcam), Ki-67 (9129S, Cell Signaling Technology) were incubated for 24 h in 4°C refrigerator. Polyclonal donkey anti-mouse or rabbit fluorophore-conjugated secondary antibodies in 1X PBS were applied for 1 h at room temperature. Finally, 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI) (d1306, Invitrogen) was applied for 3 min. Images were acquired using Volocity software and the intensity was analyzed using 4–6 200× magnified fields using ImageJ. Using ImageJ software, the positive signal occupying area was calculated then divided by the total area of the tissue and a percentage was generated. An average per mouse then per group were calculated and represented as mean ± S.D.

**Quantitative Real-Time PCR**

Total RNA extraction was performed with PureLink RNA Mini kit (Thermofisher:12183018A). High-Capacity cDNA Reverse Transcription kit (Thermofisher:4368814) was utilized to create the cDNA strands. Triplicate quantitative RT-PCR assays were performed by Step One Plus (Life Technologies) in TaqMan Universal Master Mix II (4440040, Applied Biosystem, Foster City, Canada). The probes used were: EGF (assay ID: Mm00438696), AQP5 (assay ID: Mm00437578), BMP7 (assay ID: Mm00432102), FGF2 (assay ID: Mm00433287), IL-10 (assay ID: Mm01288386), IL-1β (assay ID: Mm00434228), TNF-α (assay ID: Mm00443258), TGF-β1 (assay ID: Mm01268596), MMP2 (assay ID: Mm00439498), LYZ1 (assay ID: Mm01228256), Caspase-3 (assay ID: Mm01195085) and GAPDH (Glyceraldehyde-3-phosphate dehydrogenase, assay ID: Mm99999915) was used as an endogenous reference gene. Three experimental replicates were performed for each sample. PCR was run at 50 °C for 2 min, 95 °C for 10 min, and 40 cycles (95 °C for 15 s, 60 °C for 1 min).

**Central Cornea Thickness Analysis**

Both eyeballs were removed at the time of euthanasia and prepared for FFPE procedure. The eyeball blocks were cut until reaching the center; afterwards, serial 7 µm thickness sections were obtained and stained with H&E. Using Volocity software, 200× images were acquired for the cornea. With the aid of ImageJ software, the total thickness of the cornea (epithelium+ connective tissue) and the epithelium alone were measured centrally. Group average was calculated and represented in µm.

## Statistical Analysis

To determine statistical significance, we used one-way ANOVA test ($p < 0.05$) by GraphPad Prims version 7 was performed for control, MSCs and MSCsE groups only. The data generated from ICR control was not subjected to any statistical analysis.

## Results

### MSCs and MSCsE both Preserved Salivary and Lacrimal Gland Functions, Preserved Specialized Cells, and Upregulated Key Genes in the Gland Restoration

SFR (Saliva Flow Rate) and TFR (Tear Flow Rate) are objective measurements of the glandular function and are important tools for the evaluation of the treatment success. SFR and TFR were measured at five consecutive time points: pre-treatment at week 0 (8-week-old) then 4, 8, 12, and 16 weeks post-treatment. Upon analysis of the SFR, the untreated control NOD mice showed a steady deterioration in SFR that reached its lowest level at week 16 (24-week-old). The MSCs-/MSCsE-treated groups showed higher SFRs than the control group at all time points and comparable to that of the ICR group. Statistical analysis revealed that SFR levels were significantly higher at 4, 12, and 16 weeks post-treatment with 75–100% preservation of the function in comparison to the highest level recorded at 4 weeks post-treatment (Figure 1A). Both treated groups showed a significant increase in TFR (which represents the lacrimal gland function) at week 4 post-treatment. Afterwards, TFR declined significantly for MSCs but was maintained for the MSCsE. However, TFR for both treatments was significantly higher than the control group and comparable to ICR group especially the MSCsE-treated group (Figure 1B).

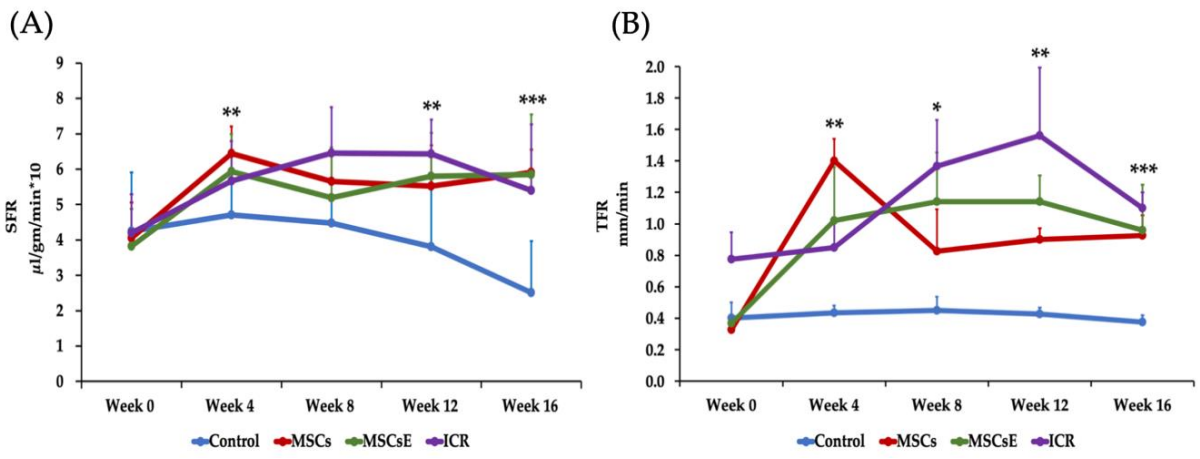


**Figure 1.** Salivary and lacrimal glands function represented as SFR (Salivary Flow Rate) and TFR (Tear Flow Rate), respectively. SFR and TFR were assessed pre-treatment at week 0 (8-week-old) then 4, 8, 12, and 16 weeks post-treatment. (**A**) SFR was determined by volume of saliva/min/gm body weight (multiplied by 10 for simplicity). Control group showed a continuous decrease of SFR (lost almost 47% of SFR at week 16 in comparison to the highest reached level, week 4), whereas MSCs-/MSCsE-treated groups maintained a significantly higher SFR (maintained almost 75–100% of SFR at week 4) than the control, their results were comparable to each other, and to the ICR group, (n = 5–12). (**B**) TFR was determined by length of wetted phenol red thread in mm/min. Control group showed a continuous decrease of TFR, whereas MSCs-/MSCsE-treated groups maintained significantly higher TFRs that are comparable to each other and to the wild type ICR group. *$p \leq 0.05$; **$p \leq 0.01$; ***$p \leq 0.001$, n = 3–6. All data were presented as mean ± S.D. Control: saline-treated; MSCs: Mesenchymal stromal cells; MSCsE: Mesenchymal stromal cells extract. Copyright (2019) MDPI.

To further explain this preservation of function, we assessed multiple markers, genes, and factors involved in the saliva/tear formation, secretion, and glandular regeneration. Immunofluorescence staining was used to evaluate the expression of special cell subpopulations in both submandibular and lacrimal glands. We found significantly higher cells positive for AQP5 (Aquaporin 5, a marker for water channel protein to identify acinar cells in submandibular glands and acinar/ductal cells

in lacrimal glands), AQP4 (Aquaporin 4, a marker for water channel in acinar and ductal cells for both glands), α-SMA (alpha Smooth Muscle Actin, a marker for myoepithelial cells), CK5 (Cytokeratin 5, a marker for ductal/progenitor cells), and c-Kit (a marker for stem/progenitor cells) in the MSCs-/MSCsE-treated groups than the control group (Figure 2 A–D).

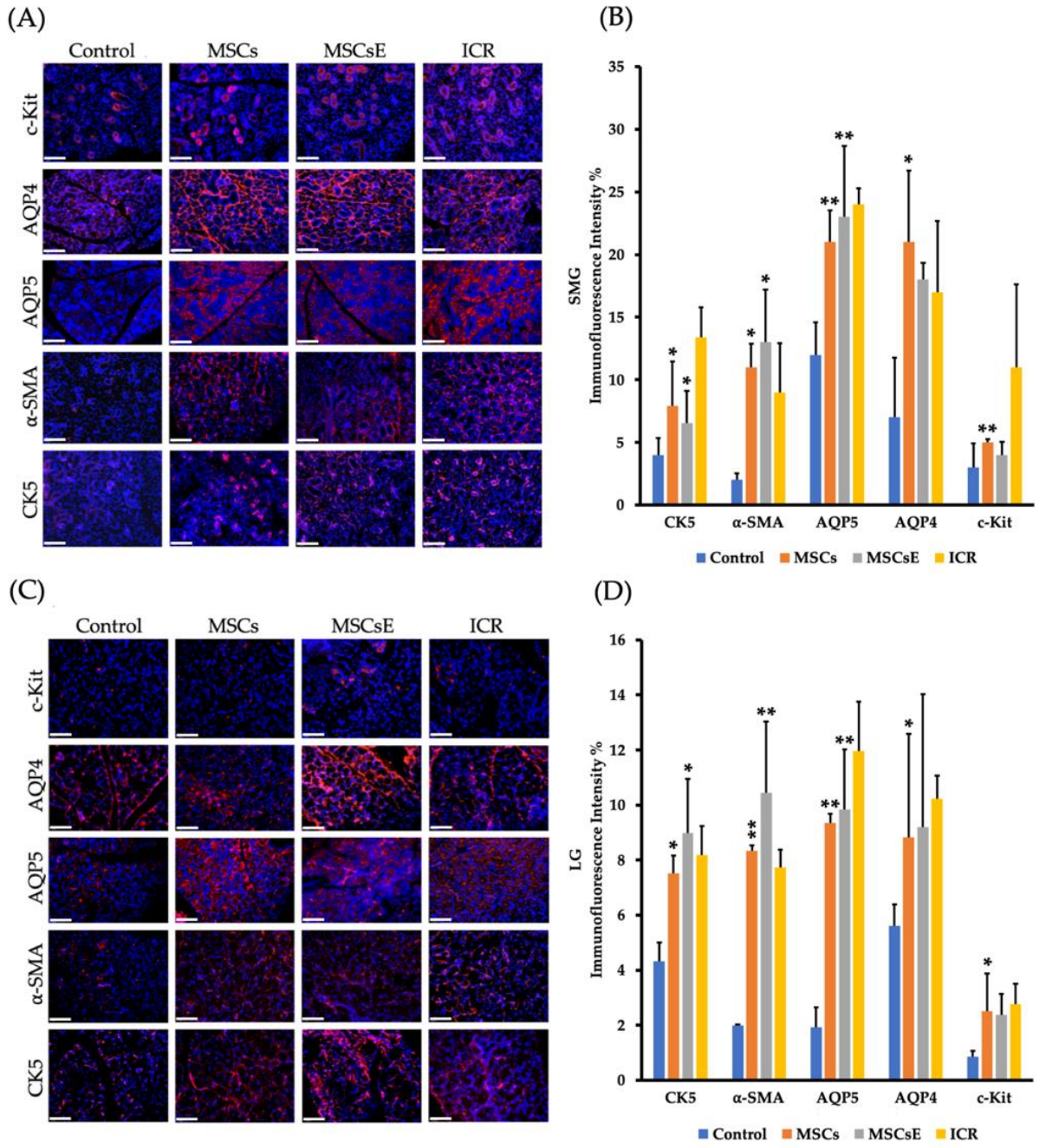


**Figure 2.** Special cell subpopulations in submandibular (SMG) and lacrimal glands (LG) were evaluated by immunofluorescence staining at 16 weeks post-treatment. (A,C) SMG/LG immunofluorescence staining, respectively, positive for AQP5 (marker for water channel protein expressed by acinar cells in SMG and acinar/ductal cells in LG), α-SMA (marker for myoepithelial cells), AQP4 (marker for acinar and ductal cells), CK5 (marker for ductal/progenitor cells), and c-Kit (marker for stem/progenitor cells) were tested in frozen sections, scale bar = 148 μm. (B,D)

Quantification of protein immunofluorescence expression levels in submandibular/lacrimal glands, respectively, from 4–6 random fields/glands by Image J software. MSCs-/MSCsE-treated groups showed higher intensities for all the tested markers when compared with the control group. All images were randomly taken at 200× magnification. *$p \leq 0.05$; **$p \leq 0.01$, $n = 3–6$. All data were presented as mean ± S.D. Control: saline-treated; MSCs: Mesenchymal stromal cells; MSCsE: Mesenchymal stromal cells extract. Copyright (2019) MDPI. Note, a correction was sent to the journal regarding the graph B (SMG intensity analysis).

**MSCs/MSCsE Treatments Promoted Proliferation, Elevated Systemic EGF Levels, and Modified Specific Key Genes in Glands Function, Proliferation, Regeneration, and Apoptosis**

We hypothesized that the trophic and regenerative effects of MSCs/MSCsE treatments are part of the mechanisms that have been implemented. Therefore, proliferation rate, gene analysis, and EGF levels were assessed. Our results showed that the treated groups demonstrated higher proliferation rates and serum EGF (Epidermal Growth Factor) levels, upregulation of several key factors in glandular function/regeneration, and decreased apoptosis. Cell proliferation was evaluated by immunofluorescence staining using the nuclear protein Ki-67 antibody (exclusively expressed in proliferating cells [73]) in salivary and lacrimal glands at 16 weeks post-treatment. Proliferation rates were significantly higher in MSCs-/MSCsE-treated groups (for both glands) compared to control group (Figure 3 A, B). Serum EGF levels were evaluated using ELISA. We found that the treatments have successfully contributed and /or induced more EGF secretion in the treated groups, especially MSCsE treatment (Figure 3C). Most tested genes for glandular function/regeneration were also upregulated for both glands in the MSCs-/MSCsE-treated groups when compared to control group (Figure 3D). In the submandibular gland, EGF, FGF2 (Fibroblast Growth Factor 2), AQP5, BMP7 (Bone Morphogenetic Protein 7) genes were all upregulated in the MSCs-/MSCsE-treated groups when compared to the control group. FGF2 gene was ~2.5 and 3 folds higher in MSCs-/MSCsE-treated groups, respectively. The AQP5 gene was upregulated 2.5 folds, which matched its protein expression results obtained by immunofluorescence analysis. However, MMP2 (Matrix Metalloprotienase-2) gene expression gave contradictory results in the MSCs-/MSCsE-treated groups; it was down-regulated in the submandibular glands and upregulated in the lacrimal glands in the MSCs-/MSCsE-treated groups (Figure 3D). CASP3 (Caspase-3) a key gene in the

apoptosis process was lower in both treated groups especially in the MSCsE. Gene analysis of lacrimal gland tissue showed significantly higher expression levels for EGF, AQP5, LYZ1 (lysozyme), BMP7, and MMP2 in MSCs-/MSCsE-treated groups compared to the control group (Figure 3D).

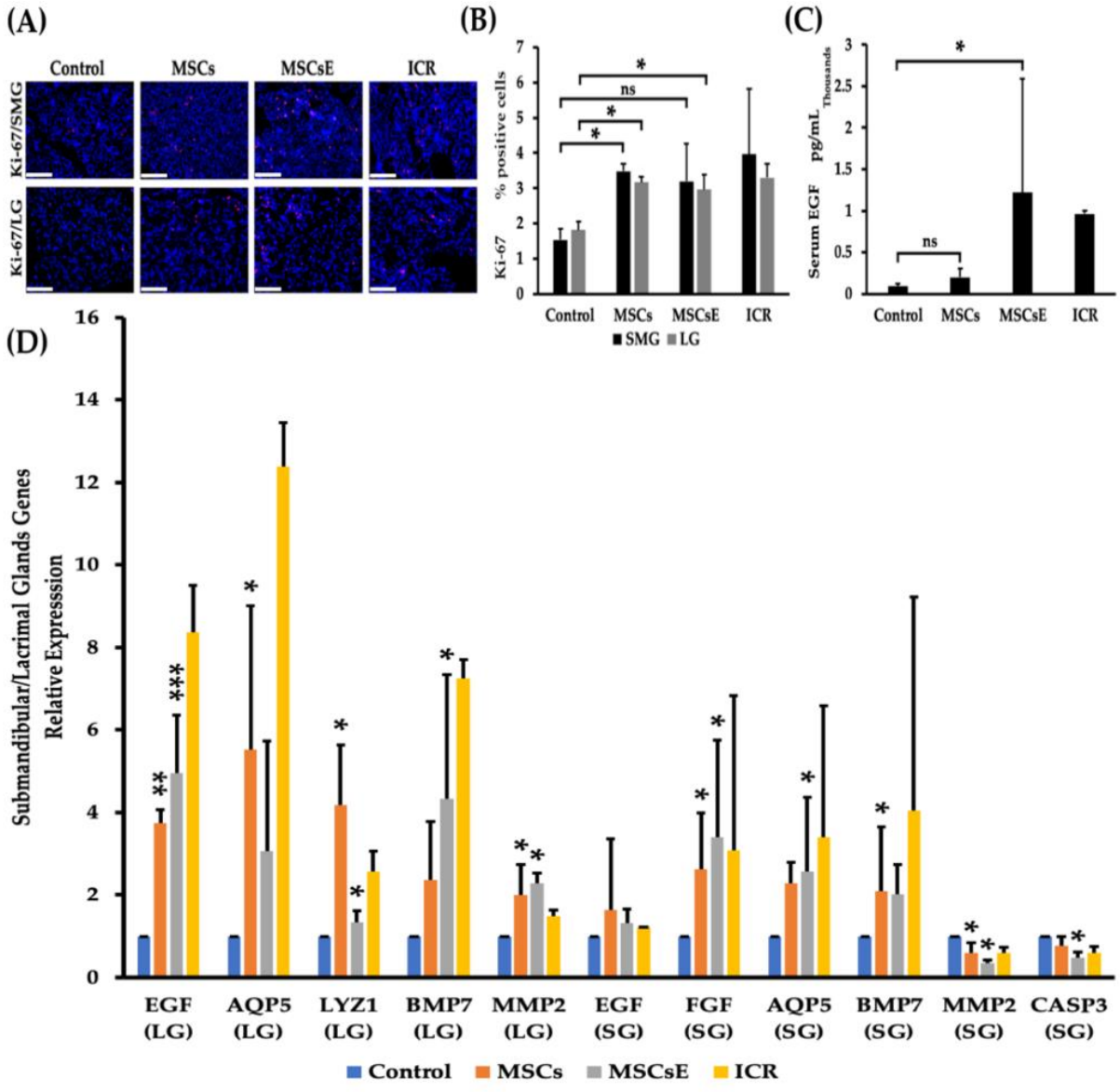


**Figure 3.** Proliferation rates, serum EGF levels, and gene expression levels of key genes at 16 weeks post-treatment. (A) Immunofluorescence staining of submandibular (SMG) (upper panel) and lacrimal (LG) (lower panel) glands for proliferation protein Ki-67 (red), nuclei were stained with DAPI (blue), (B) Proliferation rate (Ki-67-positive cells %) for

submandibular and lacrimal glands was calculated using random 200× magnified images (acquired by Volocity software) using Image J software. Two examiners independently analyzed images in a blind manner. MSCs/MSCsE treatments promoted tissue proliferation in the glands significantly higher than the control group and their rates were comparable to the ICR group. (C) Serum levels of EGF measured by ELISA. Both treatments elevated EGF levels in comparison to the control group, but only MSCsE treatment induced a significantly higher level. (D) Relative expression of key genes for tissue function, repair, regeneration, and apoptosis were analyzed by quantitative RT-PCR in lacrimal (LG) and submandibular (SG) glands. Gene expression levels in the lacrimal gland were significantly higher in MSCs-/MSCsE-treated groups than that of the control for AQP5, EGF, LYZ1, MMP2, and BMP7. Gene expression levels in the submandibular gland were significantly higher in MSCs-/MSCsE-treated groups than that of the control for AQP5, EGF, FGF2, and BMP7; and significantly lower for MMP2 and CASP3. Y-axis shows the relative expression of the gene compared to GAPDH; three experimental replicates were conducted for each sample. Scale bar = 74 μm, *$p < 0.05$, **$p < 0.01$; ***$p < 0.001$, n= 3–6. All data were presented as mean ± S.D. Control: saline-treated; MSCs: Mesenchymal stromal cells; MSCsE: Mesenchymal stromal cells extract. Copyright (2019) MDPI.

**MSCs and MSCsE Protected the Cornea Integrity by Preserving its Epithelial Thickness**

SS patients suffer from corneal thinning as a result of desiccation [74]. Therefore, preservation of the tear secretion will save the cornea from losing its thickness; hence, vision is maintained. We measured the total central corneal thickness and its epithelium alone using serial H&E stained sections (Figure 4A). Total central corneal thickness revealed higher thickness in the treated groups but not statistically significant; however, the epithelial thickness was significantly higher in MSCs-/MSCsE-treated groups when compared to the control (Figure 4).

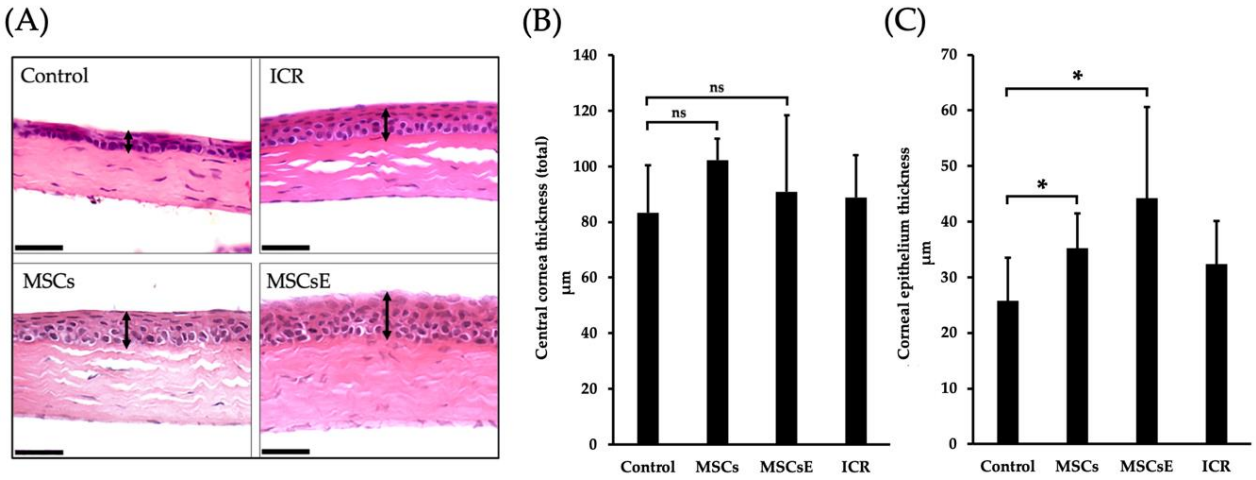


**Figure 4.** Thickness of the central cornea (total) and the corneal epithelium at 16 weeks post-treatment. (A) H&E stained images of the cornea, arrowheads represent the epithelial thickness. (B) Analysis of the total cornea thickness. (C) Analysis of corneal epithelium thickness. Images of H&E stained sections of the cornea were obtained using Volocity software then Image J was used to assess the thickness. The MSCs-/MSCsE-treated groups showed a significantly higher corneal epithelial thickness. Scale bar = 74 µm, *$p \leq 0.05$, n = 3–6. All data were presented as mean ± S.D. Control: saline-treated; MSCs: Mesenchymal stromal cells; MSCsE: Mesenchymal stromal cells extract. Copyright (2019) MDPI.

**MSCs/MSCsE Immunomodulatory and Immunosuppressive Functions Were Evidenced by A Decrease in Lymphocytic Influx, A Selective Suppression of B Cells, An Upregulation of IL-10 Secretion and Its mRNA, A Down-Regulation of Gene Expression of Inflammatory Cytokines, and A Lower Levels of Anti-SSA/Ro Autoantibodies**

The efficiency of a therapy against autoimmune diseases relies on its ability to control the immune dysregulation by targeting the pathogenic cells, while leaving the rest of the immune system intact and re-establishing peripheral tolerance [75]. We have assessed the severity of the lymphocytic infiltration by histopathological analysis of serial H&E stained sections of both salivary and lacrimal glands. Results were represented as focus score (number of lymphocytic infiltrate/4 $mm^2$, where a focus is an aggregate of ≥ 50 lymphocytes) and focus area (size of the lymphocytic

infiltrate ($\mu m^{2)}$). MSCs-/MSCsE-treated groups showed lower focus score in comparison to the control group; however, due to the small sample size, this difference was not statistically significant.; however, the focus area revealed significantly smaller foci in MSCs-/MSCsE-treated groups (Figure 5 A–C). Immunohistochemical analysis of the lymphocytic composition of the glandular infiltrates for B220 (a pan B cell marker in mice), BAFF (B cell Activating Factor) and FoxP3 (Forkhead box P3, a marker for $T_{reg}$) showed significant differences between MSCs-/MSCsE-treated groups and control groups in both lacrimal and submandibular glands (Figure 5 D–H). In the submandibular and lacrimal glands: $B220^{+}$ B and $BAFF^{+}$ cells were significantly lower in MSCs-/MSCsE-treated groups compared to the control (Figure 5 F, G). FoxP3-rich $T_{reg}$ percentage was significantly higher in MSCs-/MSCsE-treated groups (Figure 5 H).

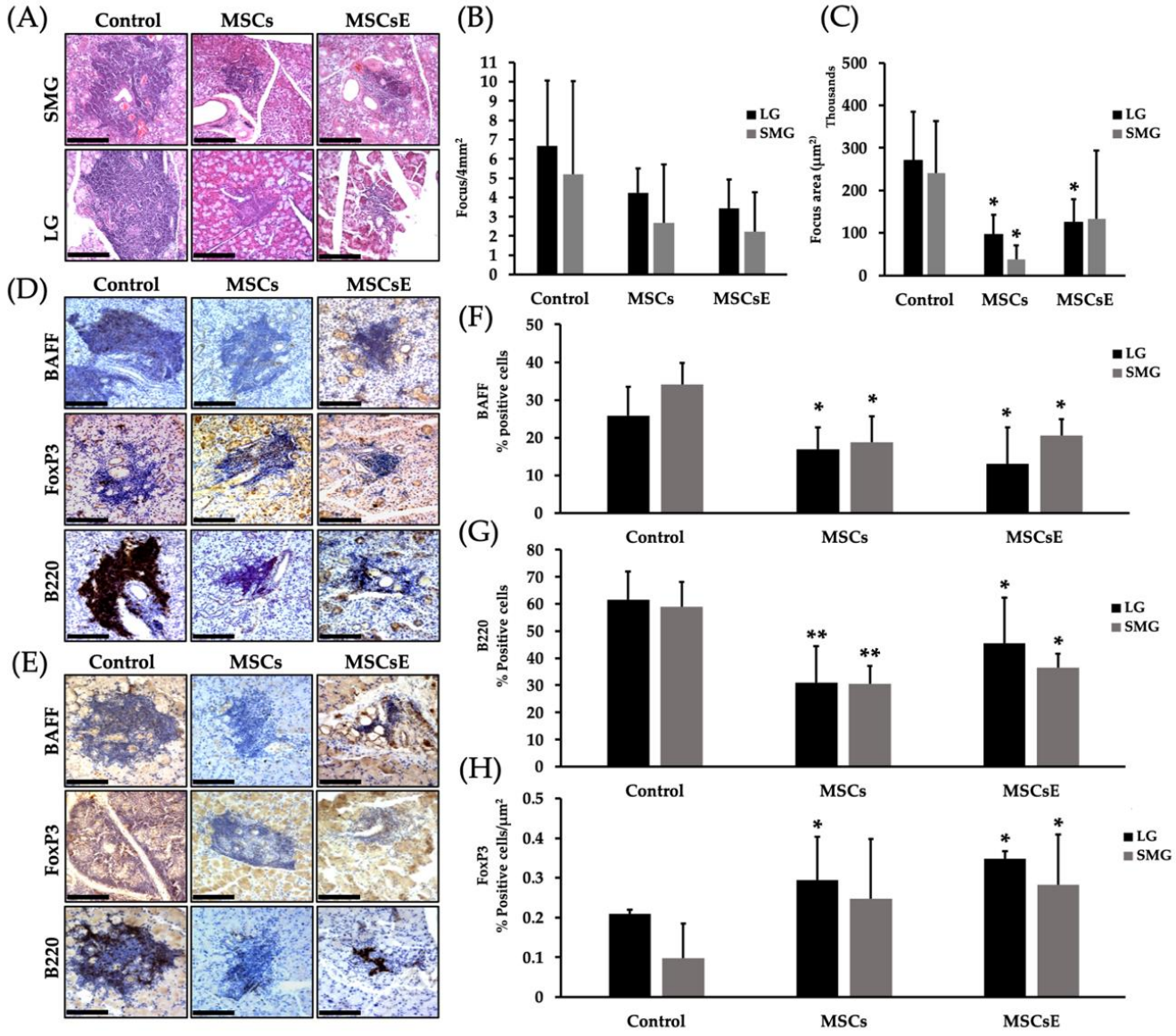


**Figure 5.** Focus score focus area and lymphocytes composition analysis in the submandibular (SMG) and lacrimal (LG) glands at 16 weeks post-treatment. (A) H&E stained images of lymphocytic infiltrates in the submandibular glands (upper panel) and lacrimal glands (lower panel). (B) Focus score analysis (number of lymphocytic infiltrates/4 $mm^2$) using serial H&E stained sections, cut at different levels, under the light microscope. The analysis revealed a lower score for the treated groups but was not statistically significant. (C) Focus area (in $\mu m^2$) was calculated by Image J software using H&E images (400×/200×) acquired by Volocity software. Treated groups showed significantly smaller focus areas. Immunohistochemical staining of lymphocytic infiltrate for B220 (a pan B cell marker in mice), BAFF (B cells activating factor),

and FoxP3 (forkhead box P3, $T_{reg}$ marker) in submandibular glands (D) and lacrimal glands (E). (F, G) Quantification of protein expression for BAFF and B220, respectively. The positive signals were measured using Image J software then divided by the size of the lymphocytic infiltrate (focus area). The results were represented as % of signal intensity. (H) Quantification of $FoxP3^+$ $T_{reg}$ cells. Positive cells were counted in each lymphocytic infiltrate then divided by the focus area (cell/ $\mu m^2$) using Image J software. MSCs/MSCsE groups exhibited a significantly higher FoxP3+ and lower B220+ and BAFF+ cells in the lymphocytic infiltrates when compared to the control group. All images were taken at 200× magnification. Scale bar = 148 μm, *$p \leq 0.05$; **$p \leq 0.01$, n = 3–6. All data were presented as mean ± S.D. Control: saline-treated; MSCs: Mesenchymal stromal cells; MSCsE: Mesenchymal stromal cells extract. Copyright (2019) MDPI.

Serum levels of anti-SSA/Ro and IL-10 were assessed by ELISA. Our results showed lower anti-SSA/Ro (Figure 6A); however, for anti-SSB/La, there was no difference detected between the groups (data not shown). IL-10 levels were expressed significantly higher in the MSCs-/MSCsE-treated groups whilst the control was very low (Figure 6B). Quantitative RT-PCR analysis for anti-/pro-inflammatory cytokines/factors genes in the submandibular and lacrimal glands showed higher gene expression levels for IL-10 and lower levels for TNF-α (Tumor Necrosis Factor alpha) in both glands, whereas TGF-β (Tumor Growth Factor beta), and IL-1β expression levels were downregulated in the lacrimal glands of the treated groups in comparison to control (Figures 6C).

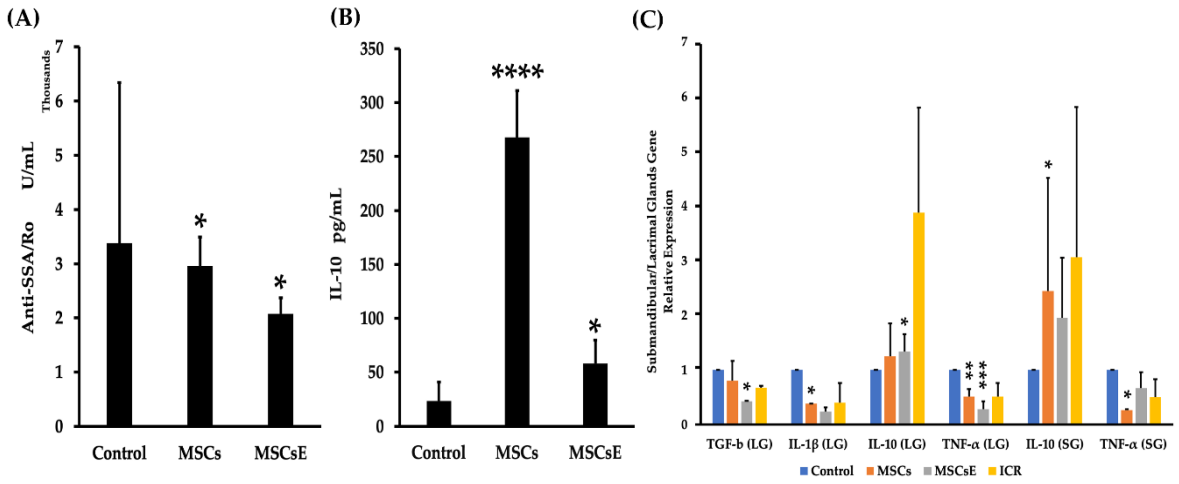


**Figure 6.** Serum levels of anti-SSA/Ro autoantibodies and IL-10, and gene expression levels of anti-/pro-inflammatory cytokines/factors at 16 weeks post-treatment. (A) Serum levels of anti-SSA/Ro autoantibodies (assessed by ELISA) for MSCs-/MSCsE-treated groups exhibited significantly lower levels in comparison to the control group. (B) Serum levels of IL-10 (assessed by ELISA) for MSCs-/MSCsE-treated groups were significantly higher than the control group. (C) Gene expression levels for anti-/pro-inflammatory cytokines/factors were measured using quantitative RT-PCR in the submandibular and lacrimal glands. MSCs/MSCsE treatments upregulated IL-10 and down-regulated TNF-α gene expressions in both glands, and down-regulated TGF-β, IL-1β in lacrimal glands. Y-axis shows the relative expression of the gene compared to GAPDH; three experimental replicates were conducted for each sample. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ****$p \leq 0.0001$, n= 4–6. All data were presented as mean ± S.D. Control: saline-treated; MSCs: Mesenchymal stromal cells; MSCsE: Mesenchymal stromal cells extract. Copyright (2019) MDPI.

## Discussion

The findings of our study were:

(1) MSCs/MSCsE treatments were successful in preserving the exocrine function of salivary and lacrimal glands in female NOD mice.

(2) Specialized cell subpopulations were preserved in the MSCs-/MSCsE-treated groups along with higher proliferation rates and higher EGF serum levels.

(3) MSCs/MSCsE treatments upregulated expression levels of multiple genes responsible for tissue regeneration, proliferation, and saliva/tears secretion, such as EGF, FGF2, LYZ1 and AQP5 genes and lowered CASP3 a gene involved in the apoptosis cascade.
(4) MSCs/MSCsE treatments promoted the formation of extracellular matrix by upregulation of BMP7 gene expression and prevented fibrosis by down-regulation of TGF-β1 gene expression.
(5) MSCs/MSCsE treatments preserved the corneal integrity by maintaining the epithelial thickness.
(6) Peripheral tolerance in salivary/lacrimal tissues was somewhat restored in MSCs-/MSCsE-treated groups; evidenced by less lymphocytic infiltration (less and smaller foci), selective suppression against B cells, inhibition of anti-SSA/Ro autoantibodies production, and down-regulated levels of pro-inflammatory genes like TNF-α, TGF-β1, and IL-1β.
(7) MSCs/MSCsE treatments influenced immunomodulation via inducing more T-regulatory cells peripherally and upregulation of IL-10.

We have reported in previous studies that BM cells and compact bone derived MSCs have successfully preserved the salivary gland function when injected into female NOD mice [49,76]. Other researchers have also reported the effectiveness of BM-derived MSCs in treating SS in NOD mice [50]. We have also reported that treatment with bone marrow cell extract (BM Soup) has preserved the salivary gland function, upregulated the expression of certain critical proteins and genes in female NOD mice [77]. This indicates that the active protein ingredients from BM, including the MSCs subpopulation in it, were preserved and employed successfully when extracted and injected into NOD mice. Hence, combining both principles into MSCsE (mesenchymal stromal cells extract) is a unique, safe and a practical treatment modality. The MSCs population in BM is quite small, 0.0017–0.0201% [78]; therefore, their expansion will enable us to enrich our MSCs population pool and accordingly, enrich the extract with more therapeutic proteins [70]. Previous reports have emphasized on the fact that MSCs exert their therapeutic and immunoregulatory capacity via secreting soluble factors in a paracrine mode [79,80]. However, MSCs possess a unique self-renewal and unlimited proliferation capacity that can be unpredictable in vivo [68]. In addition, results from MSCs utilization in clinical trials have been inconsistent and the success rates were variable [80]. It was found that the efficacy of these cells is vastly affected by their ability to sense the environment in which they exist [80]. In conclusion, MSCs cell therapy

although promising and has been used extensively, but several external and internal factors affect and limit their use. Hence, the development of a safer cell-free biological therapy that compromises the therapeutic capacities of MSCs proteins and avoids their potential risks was our goal. Additionally, the concept of formulating cells into extract is more practical in terms of storage and transfer [81].

The progression of Sjögren's Syndrome-like disease (SSLD) in NOD mice is divided into three phases. Phase 1 (initiation of glandular pathology) extends from 0–8 weeks of age. It is characterized by cellular disruption in the exocrine glands and initiation of the immune dysregulation. Phase 2 (onset of autoimmunity) extends from 8–16 weeks of age. At this stage, lymphocytic infiltration and autoantibodies formation start. Phase 3 (onset of clinical disease) starts around 16 weeks onwards. In this phase, the secretory loss is very prominent and worsens with time [82]. In our study, we designed the timing and frequency of the treatments to serve several purposes. Regarding the timing, we injected MSCs/MSCsE at 8 weeks of age which is critical in SSLD development in NOD mice. At this age, phase two of SSLD, lymphocytic infiltration and autoantibodies production take place [82]. Therefore, the treatments will combat the immune dysregulation just around the time it starts glandular infiltration and formation of antibodies against its antigens. Moreover, we hypothesized that the MSCsE will reinstate the peripheral tolerance same as the parental cells, MSCs, hoping to ameliorate or at least slow down the disease progression toward the glandular dysfunction (phase three). We tried to choose a time point that is realistic to the pathogenesis timing in SS patients. Yet, it would be more rational to test the treatment at an earlier age in NOD mice, at birth for example (phase one); however, the exact initiation of SS in humans, equivalent to phase 1, is simply unknown to us and it is extremely difficult to investigate. As per the frequency, we aimed at keeping the concentration of MSCs/MSCsE in the blood as high as possible during this phase to allow for a continuous immunomodulation and immunosuppression effects of the treatment while the immune system is actively attacking the glandular tissues. In addition, several studies have reported that MSCs exert a short-lived paracrine effect and this might apply to their extract as well; therefore, repetitive and extended treatment injections are preferred to keep their therapeutic functions for as long as possible [83,84].

MSCs are well documented for promoting tissue repair in addition to their immunosuppression and immunoregulation capacities [60,80,85]. MSCs-/MSCsE-treated groups showed higher SFR/TFR, higher protein intensity for AQP5, AQP4, CK5, α-SMA, and c-Kit, markers for acinar, ductal, myoepithelial, and progenitor/stem cells populations, respectively, in the submandibular and lacrimal glands. Proliferation, detected by Ki-67 antibody, was also upregulated and accompanied by higher serum EGF level, upregulated gene expression of EGF (submandibular and lacrimal glands), FGF2 (submandibular glands), BMP7 (submandibular and lacrimal glands), LYZ1 (lacrimal glands) and MMP2 (lacrimal glands) and down-regulated MMP2 (submandibular glands) and CASP3 (Caspase-3) in the submandibular glands. AQP5 and AQP4 are water channels that are critically involved in the formation of saliva and tears. AQP5 is located at the apical membrane of acinar cells in salivary glands, whereas in the lacrimal glands, it is located apically in the acinar and ductal cells [86,87]. AQP4 is located at the basolateral membrane of acinar cells in salivary glands and laterally in acinar cells of the lacrimal glands [88]. Several studies have reported a defective localization of AQP5 in SS patients and SS mouse models [86]. NOD mice express AQP5 weakly in the lacrimal glands but not the ICR mice, and similar results were found in SS patients [87,89–91]. In the salivary glands, AQP5 tends to be primarily located basolaterally instead of the normal apical location. As assumed, we have found that in the submandibular and lacrimal glands of MSCs-/MSCsE-treated groups the apical expression of AQP5 was upregulated as measured by the immunofluorescence staining, whereas in the control group, AQP5 was expressed partially at the apical surface with very low intensity, and the same applies to AQP4. The upregulation of AQPs in the treated mice explains the preservation of the SFR and TFR. Moreover, gene analysis results have confirmed our immunofluorescence staining results. In both glands, AQP5 gene expression was comparable to ICR group, especially in submandibular glands. We have also investigated the expression of several salivary and lacrimal glands markers involved in regeneration and proliferation, including CK5, c-Kit, and Ki-67. CK5 (cytokeratin 5) is an intermediate filament that is widely expressed at birth and considered a marker for ductal/progenitor cells, and $CK5^+$ cells are considered a reservoir for the gland regeneration [92]. C-Kit or CD117 (Type III receptor tyrosine kinase) is a marker for stem/progenitor cells in salivary and lacrimal glands [93–95]. Ki-67 is a nuclear protein used for detecting actively proliferating cells [96]. MSCs-/MSCsE-treated groups expressed higher $CK5^+$, c-$Kit^+$, and Ki-$67^+$ cells than the control group but slightly less than the ICR group. Lysozyme, secreted by the acinar cells, is a

bacteriolytic enzyme responsible for direct defense against bacteria [97,98]. Lysozyme along with lipocalin and lactoferrin compose 80% of the tear proteins [98]. We measured the lysozyme mRNA gene transcripts to evaluate the health and activity of the lacrimal gland. Our results showed an upregulation of lysozyme gene; 4 folds in the MSCs-treated and almost 1.3 in the MSCsE-treated groups, which supports the effectiveness of these treatments in reviving the lacrimal gland. Caspase-3 which is encoded by CASP3 gene, plays an important role in the execution phase of cell apoptosis. Our results showed a down-regulation of CASP3 (submandibular gland) in both treated groups, especially MSCsE, indicating a lower apoptosis. We believe that the general tissue restoration/preservation, the upregulated proliferation, and the downregulated apoptosis are in fact the function of systemic increase of EGF protein and the local upregulation of EGF and FGF2 gene expression levels.

SS patients suffer from dry eyes (keratoconjunctivitis sicca) due to tear secretion loss from lacrimal glands. The chronic dryness leads to loss of the corneal epithelium, erosions, and a possible perforation if left unmanaged [99,100]. Dry eyes patients displayed thinner central cornea in comparison to healthy subjects [101]. Preservation of the tear secretion is crucial for the health of the ocular apparatus and most importantly, the cornea. Our treatment has successfully preserved the TFR which led to the preservation of the corneal epithelium from the damaging effect of dryness.

The percentage of B cells in the lymphocytic infiltrates is very crucial for SS patients [102]. Their ratio is higher in advanced cases and in patients with higher focus score. Several studies have reported the ability of MSCs in suppressing B cells, preventing their differentiation, and decreasing their secretion of autoantibodies [67,103]. In our study, we assessed the percentage of B cells in the glandular infiltration by measuring the intensities of $B220^{+}$ (a pan B cell marker in mice) B cells and the intensity of BAFF using immunohistochemical staining. We also measured the serum levels of the autoantibodies anti-SSA by ELISA. BAFF is important for maturation and homeostasis of B cells; however, uncontrolled secretion leads to autoimmunity [104]. Excess BAFF will enable autoreactive B cells to overcome apoptotic signals in negative selection. It is expressed at high levels in several autoimmune diseases, including SS. A positive correlation was found between the levels of BAFF and autoantibodies especially anti-SSA in SS patients [105].

Our analysis showed a significantly lower intensities for $B220^+$ B cells and BAFF in the submandibular and lacrimal glands foci of the MSCs-/MSCsE-treated groups. BAFF is produced by several immune cells, including T lymphocytes and dendritic cells [106]. We believe that the immunosuppressive action of MSCs/MSCsE on these cells has resulted in less production of several factors including BAFF. The reduction of BAFF expression and/or blocking its action have led to the reduction of B cells survival signals. The later eventually steered the reduction in autoantibodies secretion by plasma cells [107]. In conclusion, because we achieved comparable results from both treatments, we believe that the extract contains enough factors and cytokines that are necessary and efficient for the immunomodulation needed in SS.

TNF-α and IL-1β genes were upregulated in SS patients and NOD mice [108,109]. Activated macrophage secretion of TNF-α and IL-1β was attenuated by MSCs [110]. We have previously reported that MSCs down-regulated TNF-α gene expression in treated NOD mice [49]. Our results showed a down-regulation of gene expression for the pro-inflammatory cytokines: TNF-α, and IL-1β in the MSCs-/MSCsE-treated groups in comparison to control group. However, MSCsE-treated group showed lower expression than MSCs treated group. We believe that the protein composition in the extract was more efficient in the suppression mechanism, probably, due to the direct bioavailability of the proteins in more significant quantities than what the MSCs can secret to achieve the same results.

Several studies, including ours, have reported that MSCs-based treatment has increased the percentage of $FoxP3^+$-$T_{reg}$ [49,85]. $FoxP3^+$ $T_{reg}$ cells are essential in self-tolerance, tissue repair, and proliferation [111,112]. $T_{reg}$ enforces immune suppression either by direct effect on antigen presenting cells like dendritic cells, or via anti-inflammatory cytokines secretion, such as IL-10 and TGF-β1 [113,114]. IL-10 is a potent immunosuppressive cytokine that can act through different channels to block immune dysregulation, such as inhibition of inflammatory cytokines TNF-α and IL-1β, antigen presentation and immune cells proliferation [115,116]. IL-10 is secreted by various T cell populations most importantly $FoxP3^+$-$T_{reg}$, it is also secreted by MSCs to target specific cells including $T_{reg}$ [117]. Our results showed a significant increase in the percentage of $FoxP3^+$-$T_{reg}$ within the lymphocytic infiltration in the submandibular and lacrimal glands of MSCs-/MSCsE-treated groups in comparison to the control group. We have also found an

upregulation of IL-10 serum levels and its mRNA, down-regulation of TNF-α and IL-1β mRNAs in submandibular and lacrimal glands. Aggarwal et al. 2005 have reported that hMSCs have induced a more anti-inflammatory and tolerogenic environment [118]. They found that hMSCs have negatively influenced DC1 secretion of TNF-α, prompted DC2 to secrete more IL-10, and caused an increased in $T_{reg}$ cell number. Thus, the high increase in IL-10 and its mRNAs was orchestrated by MSCs. MSCs-secreted IL-10 has positively influenced $T_{reg}$ which in its turn secreted more that has led to immune tolerance induction via a cascade of regulatory steps. Although IL-10 serum levels in MSCsE-treated group was significantly higher than that of the control but it was lower than the MSCs-treated group levels. We think that this difference is due to the constant release of IL-10 from the MSCs via a paracrine mode from their engraftment site. However, our quantitative RT-PCR results showed lower levels of TGF-β1 in MSCs and even much lower in MSCsE-treated group in comparison to the control group. TGF-β play an important role in the salivary gland morphogenesis, extracellular matrix deposition, and controls the immune homeostasis as well [119,120]. Mice that overexpress TGF-β suffered hyposalivation due to the excessive deposition of fibrous tissue in the gland [119]. The therapeutic level achieved by our treatments has played its role in the anti-inflammatory aspect; orchestrated by inducing IL-10 from DC2 and $T_{reg}$ and prevented the over formation of extracellular matrix and eventual fibrosis that might have occurred if excessive TGF-β1 was secreted. MSCs/MSCsE have delivered a balanced treatment that managed the immune dysregulation and promoted tissue restoration and regeneration. In agreement with our finding, Park et al. have reported that conditioned media from human umbilical cord blood-MSC down-regulated TGF-β1 and upregulated BMP7 levels in renal epithelial cells [121].

## Conclusion

This study reveals the therapeutic benefits of MSCsE in treating SSLD in NOD female mice. Our study showed very promising results that are comparable to MSCs treatment. The safety, bioavailability, convenience of use, and transference are all advantages that MSCsE can offer in comparison to MSCs. However, further investigations are required to assess further possible mechanism of action. In addition, the extract comprises many constituents, most of which are proteins; therefore, more exploration on its composition will be beneficial.

**Author contributions:** This study was designed by G.A., Y.L., and S.D.T., G.A., O.E., and M.O.B. conducted the experiments; G.A. ran the data analysis and drafted the manuscript. S.D.T. edited and directed final version of the manuscript.

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**Conflicts of Interest:** The authors declare no conflicts of interest.

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# Mesenchymal Stem Cells Extract (MSCsE)-Based Therapy Alleviates Xerostomia and Keratoconjunctivitis Sicca in Sjogren's Syndrome-Like Disease

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**Abstract:** Sjogren's syndrome (SS) is an autoimmune disease that manifests primarily in salivary and lacrimal glands leading to dry mouth and eyes. Unfortunately, there is no cure for SS due to its complex etiopathogenesis. Mesenchymal stem cells (MSCs) were successfully tested for SS, but some risks and limitations remained for their clinical use. This study combined cell- and biologic-based therapies by utilizing the MSCs extract (MSCsE) to treat SS-like disease in NOD mice. We found that MSCsE and MSCs therapies were successful and comparable in preserving salivary and lacrimal glands function in NOD mice when compared to control group. Cells positive for AQP5, AQP4, α-SMA, CK5, and c-Kit were preserved. Gene expression of AQP5, EGF, FGF2, BMP7, LYZ1 and IL-10 were upregulated, and downregulated for TNF-α, TGF-β1, MMP2, CASP3, and IL-1β. The proliferation rate of the glands and serum levels of EGF were also higher. Cornea integrity and epithelial thickness were maintained due to tear flow rate preservation. Peripheral tolerance was re-established, as indicated by lower lymphocytic infiltration and anti-SS-A antibodies, less BAFF secretion, higher serum IL-10 levels and FoxP3$^+$ $T_{reg}$ cells, and selective inhibition of B220$^+$ B cells. These promising results opened new venues for a safer and more convenient combined biologic- and cell-based therapy.

**Keywords:** Sjogren's syndrome (ss); autoimmune diseases; biologic therapy; bone marrow; cell extract; lacrimal gland; mesenchymal stem cells (MSCs); non-obese diabetic mice (NOD); salivary glands; submandibular glands

## 1. Introduction

Sjogren's syndrome (SS) is a common progressive autoimmune disease that affects females predominantly [1–3]. The prevalence of SS is variable worldwide; ranging from 0.1% to 0.72% of the population [4–11]. SS progresses slowly and patients exhibit clinical symptoms years after the disease onset [12]. The immune system targets epithelial tissues, infiltrates it with lymphocytes, and later forms autoantibodies against glands antigens [3,13–16]. The aberrant immune dysregulation leads to the destruction of epithelial tissues, especially salivary and lacrimal glands, and to several extra-glandular manifestations. The secretory function of the glands diminishes gradually resulting in dryness of the mouth (xerostomia), eyes (keratoconjunctivitis sicca), and organs containing exocrine glands, such as the nose and vagina [17–20]. The current SS management is symptomatic-based to alleviate the dryness severity and complications [21,22]. However, patients with systemic involvement

## Chapter Five

# Allogenic Bone Marrow Cells Extract (BMCE) Alleviated Sjögren's-Like Disease in NOD Mice

## Preface

In chapter four, we have shown that MSCsE was efficient as a treatment for SS-like in NOD mice. In this chapter, we assessed whether BMCE (as a BM-derived cell extract that did not go under cell culture manipulation) is efficient in managing SS-like in the lacrimal and salivary glands. Only half of NOD female mice show lacrimal gland involvement; therefore, it seems that there is a different susceptibility for both glands in females, suggesting, probably, a different pathogenesis, or simply SS heterogeneity. Additionally, we have tested the safety of BMCE as a treatment modality, which could also serve as the safety of cell extract in general, via assessing the histopathology of several organs and evaluating a number of blood biochemistry markers. Our results show that BMCE was successful in halting the immune dysregulation via inhibiting inflammatory cytokines, inhibiting B, and lowering autoantibodies. BMCE also exerted a trophic/regenerative role on the affected glands via protecting the specialized cells and promoting proliferation. The safety of BMCE was evident (limited to the follow-up period of 16 weeks) by the absence of organs pathology and the normality of the blood biochemistry tests.

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# Allogenic Bone Marrow Cells Extract (BMCE) Alleviated Sjögren's-Like Disease in NOD Mice

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## Abstract

**Background aims**. Sjögren's Syndrome (SS) is a common autoimmune disease that manifests in exocrine glands and affects middle-aged women mainly. It is a significantly debilitating disease and leads lymphoma in some patients. Due to its complexity, no treatment has achieved satisfying results. In mice, bone marrow transplantation (BMT) has successfully treated SS; however, BMT requires a prior myeloablative conditioning. **Methods**. In this study, we used a less immunogenic form of BM called "BM cell extract (BMCE)" with its secretomes from C57BL/6 mice. BMCE was injected into 8-week-old NOD female mice which were followed for 16 weeks posttreatment. Saliva and tear flow rates were assessed periodically. At the end of the study, mice were sacrificed, and various tests were performed. **Results**. Treated mice showed significantly higher salivary/tear flow rates up to 16 weeks post-treatment with preserved specialized glandular cells. Glandular inflammation was decreased, especially B cells. Serum levels of IFN-γ, TNF-α, IL-6 and anti-SSA/SSB autoantibodies were lower, and higher for EGF and IL-10. Gene expression of TNF-α, TGF-β1, MMP2, CASP3 and IL-1β genes were downregulated and up-regulated for AQP5, EGF, FGF2, BMP7, LYZ1 and IL-10. Corneal thickness, and CK5/AQP5 expressions were preserved. Mice were healthy during the follow up period, and a histopathology assessment for liver, kidneys, spleen, and pancreas showed no pathological signs. BUN Urea, creatinine, total bilirubin, ALT, AST, and GGT blood levels were within the normal range in treated mice, and LSR was higher.

**Conclusion**. BMCE is a safe treatment modality with dual therapeutic actions: regenerative and anti-inflammatory.

**Keyword:** Sjögren's Syndrome, salivary glands, autoantibodies, Bone marrow, cell extract, biologic therapy, IL-1Rα

## Introduction

SS is a common and chronic autoimmune disease with a worldwide prevalence of 0.03% - 2.8% [1, 2]. Middle aged females, in their forties-sixties, are the most affected group with a ratio of 9:1 compared to males [3]. It is a slowly progressive disease characterized by lymphocytic infiltration in exocrine glands [4]. The disease starts many years before patients experience any symptoms, such as dry mouth/eyes, and seek medical help. [5]. Patients often experience several debilitating extra-glandular symptoms, such as fatigue, depression, anxiety, and skin; pulmonary; renal; neurological; and circulatory manifestations, which worsen their quality of life [6-10]. Moreover, most importantly, SS patients develop lymphomas, especially in the salivary glands, which were reported to be 44 times higher in SS patients than in the general population, [11, 12].

Presently, no drug has proven to cure or induce remission, and the current management is directed toward symptoms palliation and prevention of complications [13]. Dryness (sicca) symptoms are managed by educating the patient, modifying the environment, eliminating any drug that might aggravate the dryness, prescribing artificial tears, and secretagogues if the patient has residual secretory tissues [13-15]. Systemic manifestations are usually managed with glucocorticoids and occasional immunosuppressants [14]. Unfortunately, the progress in developing an efficient treatment for SS is challenging and lacks behind other autoimmune diseases treatments. Patients suffer dryness and numerous systemic symptoms; hence, treating both is of an equal importance to patients. However, many of biologic therapies tested in clinical trials showed some promise in systemic symptoms management, but were underwhelming in restoring exocrine glands function [10]. Unfortunately, there is a huge gap between the current management in clinics and what is recommended based on results of double-blind placebo-controlled trials [10].

The non-obese diabetic (NOD) mouse is has been used for testing new therapies [16-22]. This model recapitulates many of the human SS features, making it suitable for drug testing. Lymphocytes infiltrate the exocrine glands (salivary and lacrimal) and a subsequent gradual deterioration in the glandular function occurs [23-26]. All female NOD mice show lymphocytic infiltrations in the submandibular glands (SMG) and around 50% of them have lacrimal glands (LG) involvement accompanied with saliva and tears flow deterioration [27]. On the contrary, NOD males show more involvement of the lacrimal than the salivary glands [28]. Additionally, NOD mice exhibit hypergammaglobulinemia due to autoantibodies raised against nuclear, intracellular, and membranous targets [29-31]. Anti-SSA/Ro and anti-SSB/La are two important autoantibodies that are used as a diagnostic feature in human, and are present in NOD mice [27].

BM is one of the largest organs and it houses various stem and progenitor cells, which are essential for hematopoiesis and regeneration [32]. BM transplant (BMT) has been widely used for the management of various conditions, such as aplastic anemia, blood cancers, and rheumatic arthritis [33-35]. However, in SS, only BM-derived MSCs have been tested in the treatment of SS patients [36, 37]. Our group and others have reported that BM and BM-derived cells efficiently alleviate SS-like disease in NOD mice [16, 37]. BM exerted a dual therapeutic action; it reduced the inflammation and preserved the glandular secretory function [16, 18, 19, 37]. However, although stem cells therapy has offered promising results in the field of autoimmune diseases treatment, post-infusion complications are a huge drawback. Complications ranging from insomnia to death have been documented in patients who have received MSCs and HSCs [38]. Some complications are unavoidable in some cases, such as infections and allergies; however, cancers and GvHD are strongly related to cellular therapies [39, 40].

We have reported previously that BM and BMCE treatments were both effective in alleviating dry mouth in NOD mice [16, 41, 42]. However, we did not assess the effect of BMCE on lacrimal gland function which we have assessed in this study. In addition, we have evaluated the safety of the treatment systemically by examining various tissues and running several blood tests. Lastly, we show here that BMCE has successfully decreased the glandular inflammation and has preserved the exocrine functions in the treated mice for up to 16 weeks post-treatment.

## Materials and methods

### Animals

All experimental procedures were performed following the guidelines imposed by the Canadian Council on Animal Care. Our protocol (2007-5330) was approved by the University Animal Care Committee (UACC) at McGill University. **Recipient:** 8-week-old female NOD mice (H-$2^{g7}$) purchased from Taconic Farms (Germantown, NY) were randomized into two groups: 1) Treatment group, mice were treated with bone marrow cell extract (BMCE), n = 12, and 2) Control group, mice were treated with normal saline, n =12. **Donors:** 8-week-old male C57BL/6 (H-$2^{d}$) purchased from Jackson Laboratory (Bar Harbor, ME, USA). Mice were kept at the animal facility according to the animal care rules and regulations. **Wild type control:** 8-week-old ICR (Institute of Cancer Research) mice were purchased from Taconic Farms (Germantown, NY). ICR is an outbred strain from which NOD subline was derived [43][37]. This strain was used as a wild type and SS-free control and received no treatment. Saliva and tear flow rates were measured at the same time as the treated and control NOD groups (n=6).

### Blood Sugar Monitoring

At 12 weeks of age, fasting blood sugar was monitored for all mice once weekly using Accu-Check® (Roche) system. Mice with borderline blood glucose (150–250 mg/dL) were checked twice weekly. Diabetic mice (> 250 mg/dL) were examined daily with administration of Insulin injections subcutaneously, if needed. Mice were evaluated after the insulin injection for any hypoglycemia, if evidenced, 1 mL of 10% dextrose was administered subcutaneously with close monitoring.

### Bone Marrow Cell Extract (BMCE) Preparation and Transplantation

BM was collected from 8-week-old male C57BL/6 mice. Briefly, Mice were euthanized according to the animal ethics protocol of McGill university. Mice were disinfected thoroughly with 70% ethanol then femurs and tibiae were surgically removed [44]. The isolated bones were placed inside a sterile 1 mL pipette tip that was cut (about 2 cm in length from the tip) and adapted inside a sterile 1.5 mL Eppendorf tube. The Eppendorf tubes were then placed in 4°C centrifuge at a speed of 4040 x g for 30 seconds. At the end of the cycle, the tubes were transferred to the laminar flow

hood where the bones and the pipette tips were discarded, and the cellular pellets were all collected together, resuspended in normal saline, and kept on ice for further processing. The cell number was assessed and adjusted to 1.0 x $10^7$ cells/100 µL which will be the volume of a single treatment. The reconstituted bone marrow cells were placed in 1.5 mL sterilized Eppendorf tubes and placed in liquid nitrogen until frozen. Afterwards, the tubes were thawed then transferred back to the liquid nitrogen. This cycle was repeated three times to ensure a complete rupture of BM cells. At the end of the third cycle, the tubes were thawed and placed in the centrifuge at 4°C at 4545 x g speed for 30 min. At the end of the centrifugation cycle, a pellet was formed which was composed of BM cells membranes and nuclei fragments, and the supernatant (BMCE), which was transferred to new tubes for an immediate use or storage in -80°C freezer.

Treated mice received 100 µL of BMCE (extract of 1.0 x10 $^7$ cells) once a week via tail vein for four consecutive weeks starting at week 8 of age. BMCE was kept on ice during the procedures, warmed up and mixed well just before the injection. Control mice received an equal volume of normal saline (100 µL) injections at the same time/frequency as the treated group.

**Secretory Function of the Salivary and Lacrimal Glands (Saliva Flow Rate: SFR, Tear Flow Rate: TFR)**

Secretory function of the salivary glands, SFR, was measured by inducing mild gas anesthesia in NOD mice using 1.5-3% isoflurane, 5% halothane and 1 L/min oxygen. Saliva secretion was stimulated by using 1.0 mg pilocarpine/kg body weight administered subcutaneously. Whole saliva was obtained from the oral cavity by a micropipette placed into a pre-weighed 0.5 mL microcentrifuge tube at the corner of the animal mouth. The collected saliva in the first 5 minutes was discarded and a new micropipette/microcentrifuge was placed to collect saliva for 10 minutes. Saliva volume was determined gravimetrically then stored in -80°C freezer. Tear flow rate was measured at the same time saliva was to reduce the discomfort and stress. After 10 minutes of the pilocarpine injection, phenol red threads were placed gently with the aid of fine tip tweezers in the medial canthus of both eyes and were left for 5 minutes; any secretion in the previous 10 minutes were first removed before the final measurement was recorded. The thread was measured by a ruler to the approximate mm and then placed in a sterilized PBS containing tube and stored in -20°C freezer. The readings of both eyes were averaged, then a group average was calculated.

**Serum Preparation and Analysis**

Shortly after the animals were euthanized, the blood was drawn via cardiac puncture. Collected blood was left to clot for 15 minutes at room temperature, then centrifuged at 1212 x g for 8 minutes. Serum levels of EGF (ab100679, Abcam), Anti-SSA/Ro (5710, Alpha Diagnostics), anti-SSB/La (5810, Alpha Diagnostics), IFN-γ (MIF00, R&D systems), TNF-α (MTA00B, R&D systems), IL-6 (ab222503, abcam), and IL-10 (ab46103, abcam) were measured using ELISA assay.

**Focus Score, Focus Area, and Acinar Area Assessments**

Focus score is defined as the number of focal lymphocytic infiltrations (at least 50 inflammatory cells) per 4 $mm^2$ of glandular tissue. Focus score was examined using multiple H&E stained histological sections (cut at 80 µm apart) with the aid of an eyepiece grid. Focus area, which is defined as the size of the lymphocytic infiltration area in $\mu m^2$. Images were first acquired using Volocity software, then analysed using Fiji software. An average was calculated for focus score and focus area (unit is $\mu m^2$) per mouse, then per group. Acinar area assessment was performed using formalin-fixed paraffin-embedded (FFPE) histological sections that were stained with periodic acid chief stain. Four-six 400X random bright field images were taken from the stained sections for each mouse using Volocity software. Acinar area was calculated by measuring the area occupied by acinar cells relative to the total field area using Fiji software. Acinar area is represented as a percentage (%), n=4-6 per group.

**Immunohistochemistry**

Salivary gland sections (FFPE) were blocked for indigenous peroxidases by using fresh 3% $H_2O_2$ after performing antigen retrieval with acetic acid pH 6. Unspecific primary antibody binding was blocked with 1% BSA and 10% normal goat serum in PBS for one hour at room temperature. The primary antibodies, B220 (550286, BD biosciences), FoxP3 (14-5773, eBiosceince), BAFF (11021244, Enzo Life Sciences) were applied to the salivary and lacrimal gland sections and incubated overnight in 4°C refrigerator. Polyclonal rabbit anti-rat secondary antibody was applied for 1 hour at room temperature. The sections were then counterstained with hematoxylin stain for one minute. 200-400X magnification images were taken for the lymphocytic infiltrates using

Volocity software. Further analysis of the percentage of positive cells was performed using Fiji software, n=4-6 per group.

**Immunofluorescence**

Frozen salivary gland sections (fixed in formalin during processing and saturated gradually with 10-30% sucrose) were blocked with 1% BSA and 10% normal donkey serum in PBS for one hour at room temperature, after performing antigen retrieval with acetic acid pH 6. The primary antibodies AQP5 (ab78486, abcam), AQP4 (ab9512, abcam), CK5 (PRB-160P, Covance), α-SMA (ab7817, Abcam), and Ki-67 (9129S, Cell Signaling Technology) were applied on the sections and kept overnight in 4°C refrigerator. Polyclonal goat anti-mouse or goat anti-rabbit flourophore-conjugated secondary antibodies were applied to the corresponding sections and kept for 1 hour at room temperature. Finally, 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI) (d1306, Invitrogen) was added for 2 min. Images were taken from 4-6 random fields per section and the signal intensity was analyzed at 200X magnification using Volocity software. An average for each mouse then a group average were calculated, n=4-6 per group.

**Central Cornea Thickness and Immunofluorescence Analysis**

Both eyeballs were removed at the time of euthanasia and prepared for FFPE procedure. The eyeball blocks were reduced to reach the center, then serial 7 µm thickness sections (cut at 80 µm apart) were obtained. The sections were stained with H&E, then images were acquired using Volocity software at 200X magnification. Determination of the thickness was performed using Fiji software. The total thickness of the cornea (epithelium+ connective tissue) and the corneal epithelium alone were measured centrally. Mouse average then group average were calculated and represented as µm, n=4-6 per group. For immunofluorescence staining, FFPE cornea sections were treated the same as sections processed in the immunohistochemistry protocol mentioned previously. Primary antibody for CK5 and AQP5 were applied for 24 hours in 4°C refrigerator. Polyclonal goat anti-mouse or goat anti-rabbit fluorophore-conjugated secondary antibodies were applied for 1 hour at room temperature. Finally, DAPI (d1306, Invitrogen) was added for 2 min. Signal intensity was analyzed from 4-6 200X magnified random fields per stained section, using Volocity software. Mouse average then group average were calculated, n=4-6 per group.

**Quantitative real-time PCR**

Total RNA extraction was performed with PureLink RNA Mini kit (Thermofisher:12183018A). High-Capacity cDNA Reverse Transcription kit ((Thermofisher:4368814) was utilized to create cDNA strands. Triplicate quantitative RT-PCR assays were performed by Step One Plus (Life Technologies) in TaqMan Universal Master Mix II (4440040, Applied Biosystem, Foster City, Canada). The probes used were for EGF (assay ID: Mm00438696), AQP5 (assay ID: Mm00437578), BMP7 (assay ID: Mm00432102), FGF2 (assay ID: Mm00433287), MMP2 (assay ID: Mm00439498), LYZ1 (assay ID: Mm01228256), CASP3 (assay ID: Mm01228256), IL-10 (assay ID: Mm01288386), IL-1β (assay ID: Mm00434228), TNF-α (assay ID: Mm00443258), TGF-β1 (assay ID: Mm01268596), and GAPDH. Glyceraldehyde-3-phosphate dehydrogenase (assay ID: Mm99999915) was used as an endogenous reference gene. PCR was run at 50°C for 2 min, 95 °C for 10 min, and 40 cycles [95 °C for 15 s, 60 °C for 1 min], n=3-4 per group.

**Histopathology and Serum Biochemistry Assessments**

kidneys, pancreas, spleen, and liver were collected at the time of the euthanasia from all BMCE- and normal saline-treated groups. The tissues were processed for FFPE procedure. The organs were later sectioned and stained with H&E. The samples were assessed first by our lab, then sent to the Comparative medicine and animal resource center at McGill university for confirmation of the assessment. Serum analysis for: BUN Urea, creatinine, total bilirubin, ALT (ALanine Transferase), AST (ASpartate Transferase), and GGT (Gamma-Glutamyl Transferase) were processed by Comparative medicine and animal resource center for biochemistry testing via colorimetric/rate assay (Ortho Vitros 350).

**Statistical Analysis:**

To determine statistical differences ($P < 0.05$), t-Test by GraphPad Prims version 9 was used. The saline-treated control and BMCE-treated were compared (comparison is between treated vs nontreated only). The data generated from ICR control was not subjected to any statistical analysis.

## Results

### BMCE Maintained the Salivary and Lacrimal Glands Function, Protected Specialized Cell Subpopulations, and Promoted/Preserved Glandular Tissue Proliferation

SFR and TFR are two objective measures for evaluating glands function. In SS, SFR and TFR deterioration/loss are two major complications that compromise patients' quality of life. Hence, preserving glandular function or re-establishing them is an essential requirement for a satisfying management. We have measured SFR and TFR for control, BMCE, and ICR at week 0 (week 8 of age, pre-treatment), then 8- and 16-weeks post-treatment. At week 0, all groups had comparable SFR and TFR results. At week 8 posttreatment (16 weeks of age), SFR of BMCE group has increased significantly compared to control group (saline-treated), whereas the ICR group showed the highest increase (Figure 1-A). Regarding TFR, the control and BMCE groups showed comparable rates at week 0, then post-treatment, a slight increase was shown in the control group, but a significantly higher rate was seen in BMCE group. TFR in the control group was deteriorating between week 8 and week 16 post-treatment but not as fast as the SFR. BMCE group maintained its TFR that was reached at week 8 post-treatment (Figure 1-B). Acinar area was assessed by calculating the area occupied by acinar cells divided by the total surface area in the examined section. It represents the percentage of the cells that actually initiates the formation of saliva/tears and they are targeted mainly by the immune attack. Our data shows that the acinar area analysis was higher in the BMCE group compared to the control (Figure 1-C). The general regenerative capacity of the glands was assessed by evaluating serum EGF level (ELISA) and the glandular proliferation rate (immunofluorescence staining for Ki-67 antibody; an antibody that is expressed exclusively by actively proliferating cells). EGF was statistically higher in BMCE group than the control but less than the ICR group (Figure 1-D). Both glands showed significantly higher proliferation rates than the control group hut less than that of the ICR (Figure E and F). Gene expression analysis using quantitative rt-PCR for EGF, AQP5, FGF, LYZ1, BMP7, MMP2 genes were higher and lower for CASP3 in the treated group; however, not all changes were significant (Figure 1-J). Immunofluorescence staining and intensity analysis of specialized subpopulations; positive for AQP5 (Aquaporin 5, water channel protein, a marker to identify acinar cells in SMG and acinar/ductal cells in LG), AQP4 (Aquaporin 4, water channels, a marker in acinar and ductal cells for both glands), α-SMA (alpha smooth muscle actin, a marker for myoepithelial cells in both

glands), CK5 (cytokeratin 5, a marker for ductal/progenitor cells in both glands), and c-Kit (a marker for stem/progenitor cells) were all higher in submandibular and lacrimal glands of BMCE group (with variable significance) in comparison to the control group (Figure 2 A-D).

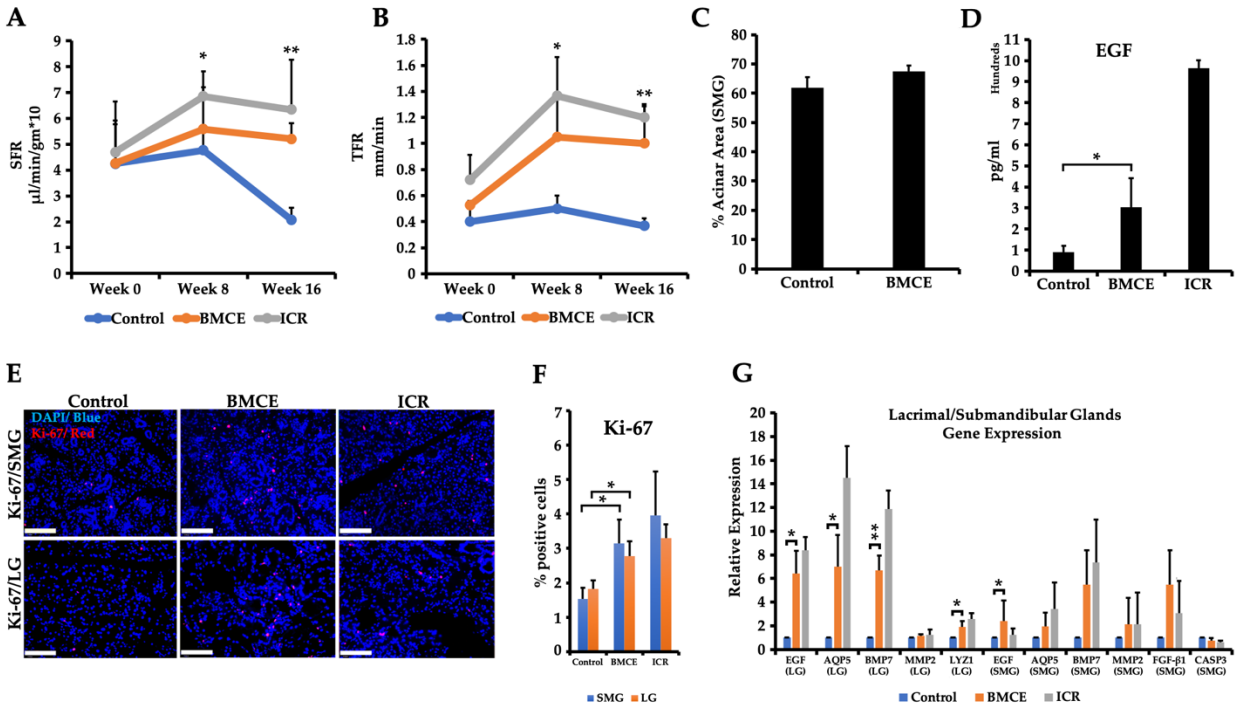
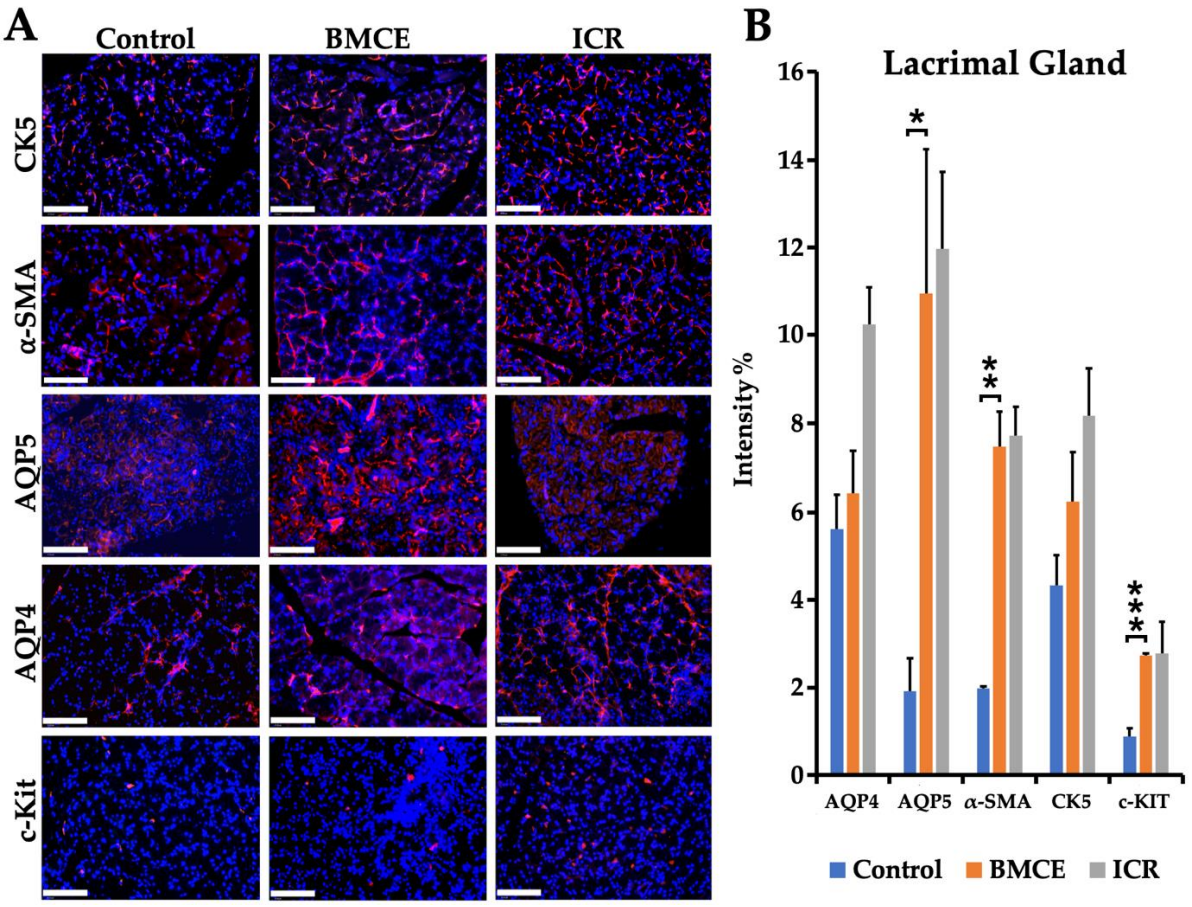



**Figure 1**. Effect of BMCE on salivary/lacrimal glands function, proliferation, and regeneration at 16 weeks post-treatment. (A) Saliva flow rate (SFR). The control group showed a continuous decrease of SFR (lost >50% of SFR in comparison to the highest reached level, week 8), whereas BMCE group maintained a significantly higher SFR (almost 90% of SFR at week 8) than the control group but slightly lower than the ICR group, n=5-12 mice per group. (B) TFR (Tear Flow rate). The control group showed a continuous decrease of TFR after week 8 post-treatment. BMCE group maintained a significantly higher TFR which is comparable to ICR group TFR. (C) Percentage of acinar area in submandibular gland (SMG) was higher than that of the control group. (D) Serum EGF level measured by ELISA is significantly higher for the BMCE group. (E) Immunofluorescence staining for Ki-67 (positive cells stain red, nuclei stained blue with DAPI in SMG (upper panel) and LG (lower panel). (F) Proliferation rate was assessed by counting the

percentage of positive Ki-67. (G) Relative expression of key genes in regeneration (AQP5, EGF, LYZ1, MMP2, FGF-ß1, and BMP7) and apoptosis (CASP3) measured by quantitative rt-PCR, Y-axis shows the relative expression of genes compared to GAPDH. Scale bar = 111 μm, n=3-6, *P≤0.05; **P≤0.01. All data were presented as mean ± S.D. All data were presented as mean ± S.D. Control: saline-treated; BMCE: Bone Marrow Cells Extract; ICR: SS-free wild type.



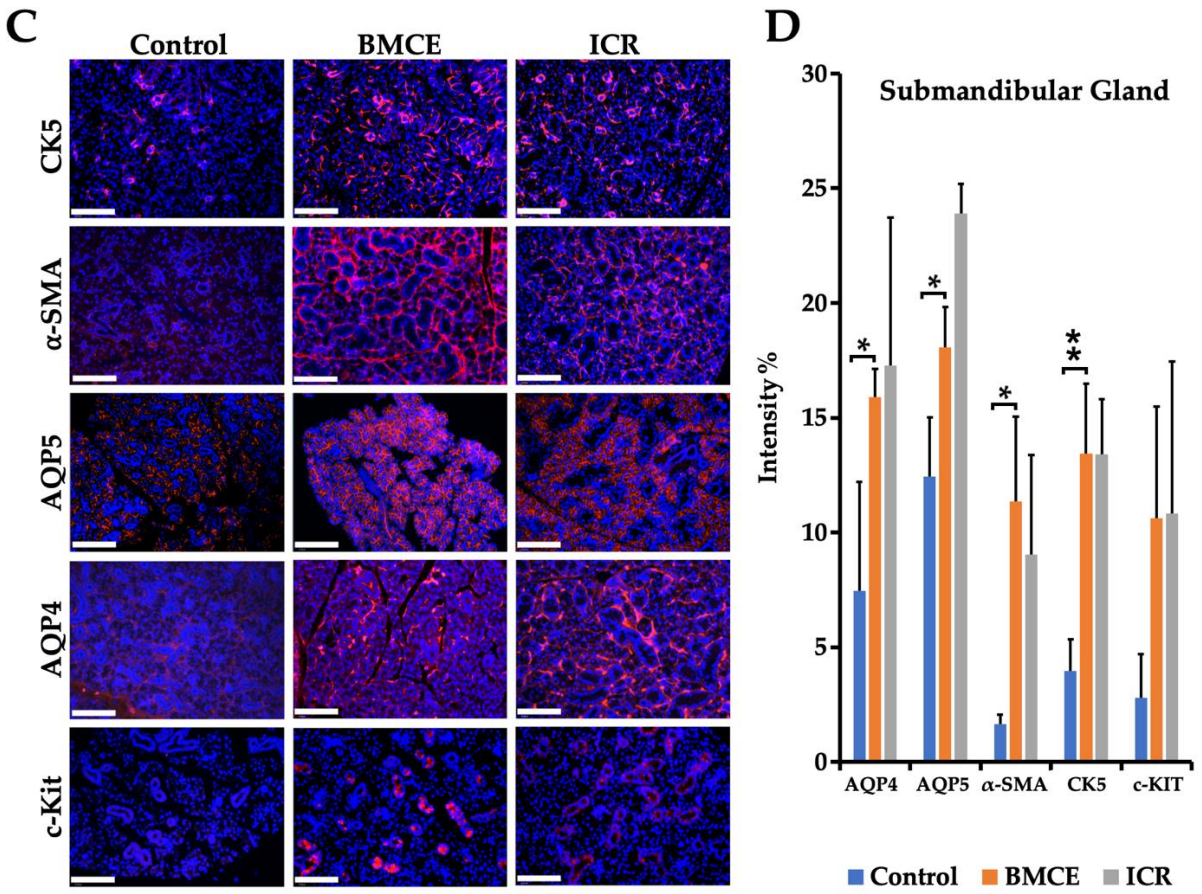



**Figure 2**. Specialized cell subpopulations in lacrimal (LG) and Submandibular glands (SMG) at 16 weeks posttreatment. (A, C) Immunofluorescence staining for AQP5 (a marker for water channel protein found in acinar cells of SMG and in acinar/ductal cells of LG), AQP4 (marker for acinar and ductal cells in LG, α-SMA (marker for myoepithelial cells), c-Kit (marker for stem/progenitor cells), and CK5 (marker for ductal/progenitor). (B, D) Quantification of the immunofluorescence signal intensity relative to the whole tissue section from 4-6 random fields/ glands by Fiji software. BMCE group showed higher intensities (variable significance) than the control group and comparable to ICR group for all tested markers. Scale bar = 111 μm, n=3-6, *P≤0.05; **P≤0.01; ***P≤0.001. All data were presented as mean ± S.D. Control: saline-treated; BMCE: Bone Marrow Cells Extract; ICR: SS-free wild-type.

**Cornea Thickness, Structure, and AQP5 Expression Were Preserved**

Cornea is subjected to thinning as a result of desiccation. SS Patients are at high risk of corneal perforation, especially when the dryness is not managed appropriately, and doctors' instructions are not well followed [45, 46]. Therefore, we hypothesized that the preservation of TFR in treated mice protected the corneal surface from abrasion and thinning. Our results showed a thicker total central cornea and its epithelial (significantly higher) component in BMCE group. The maintenance of the tears and the expression of AQP5 in the corneal epithelium participated in the maintenance of the corneal integrity and thickness from the damaging effect of the dryness. AQP5 immunofluorescence staining was significantly upregulated in BMCE group in comparison to the control group. Furthermore, we assessed the structure of the cornea by assessing CK5 (cytokeratin 5). CK5 is expressed widely by corneal epithelium and its intensity reflect the state of integrity in BMCE group. Both markers showed an increased expression in the BMCE group in comparison to the control group (Figure 3).

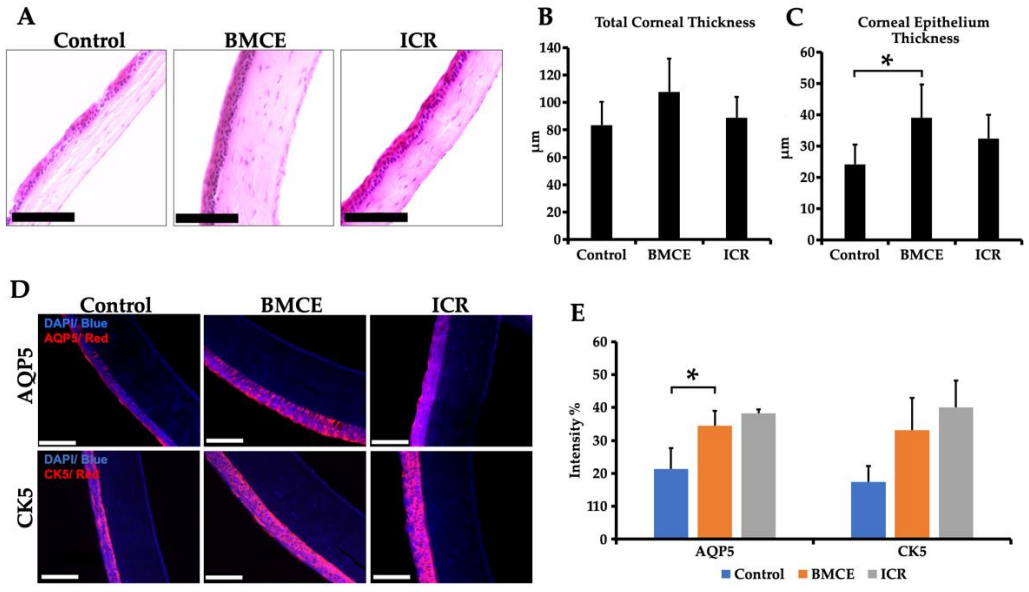


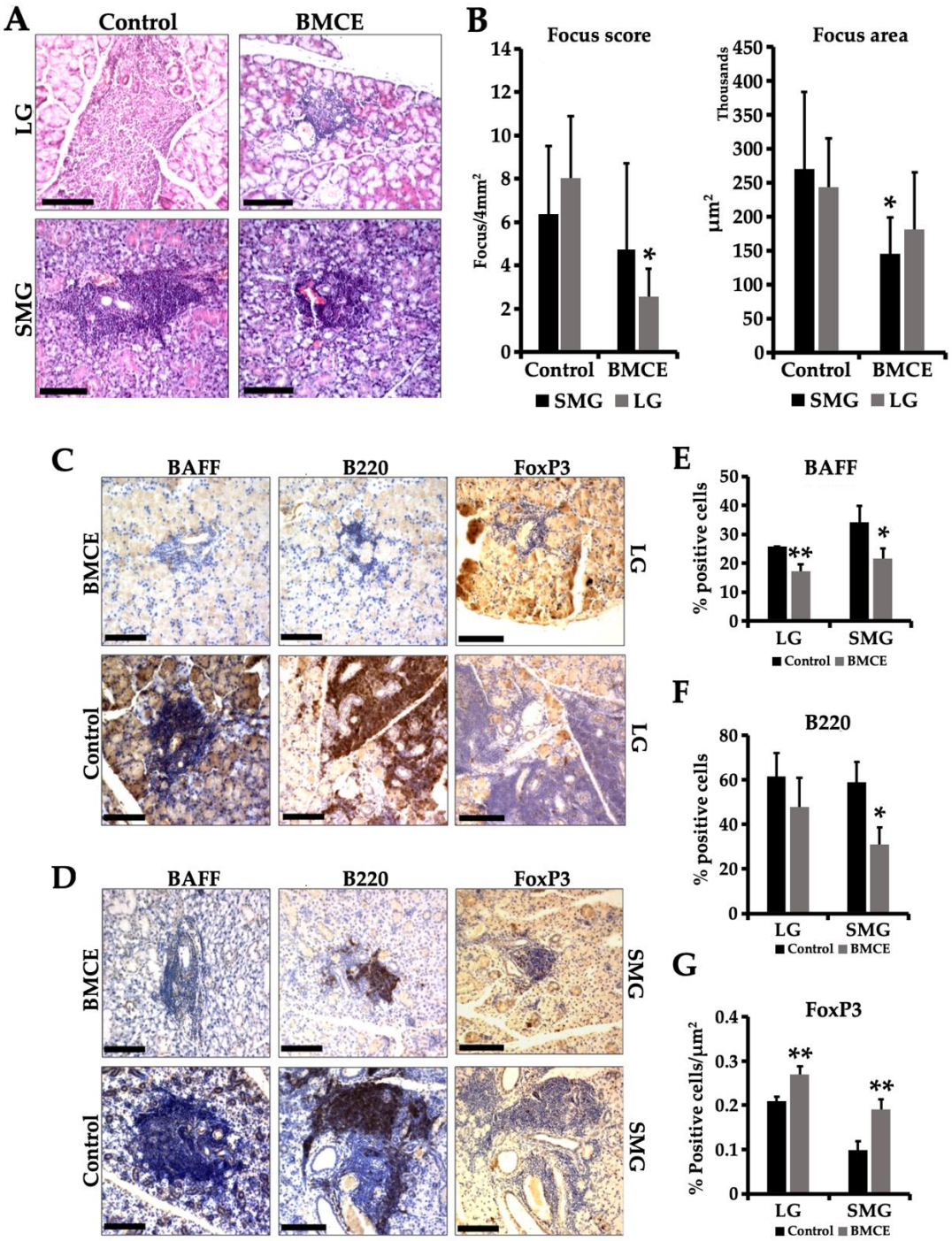

**Figure 3**. Effect of BMCE therapy on cornea at 16 weeks post-treatment. (A) H&E images of the cornea. (B) Total central cornea thickness assessment shows higher thickness but not significant for the BMCE group in comparison to control group. (C) Corneal epithelial thickness shows a significantly higher thickness in BMCE group. (D) Immunofluorescence staining of the cornea for

AQP5 (upper panel) and CK5 (lower panel). (E) Quantification of the immunofluorescence signal intensities for AQP5 and CK5. Treated group showed higher intensities (significant for AQP5) than the control but lower than that of ICR, n= 4-5 mic, *P≤0.05, scale bar = 111 μm. All data were presented as mean ± S.D. Control: saline-treated; BMCE: bone marrow cells extract; ICR: SS-free wild type.

**BMCE Reduced Glandular Inflammation, and Induced Immunoregulatory and Immunosuppression State**

Lymphocytic infiltration in the salivary/lacrimal glands and other epithelial tissues is a hallmark in SS. The inflammation increases with age, and it is accompanied with a continuous production of inflammatory cytokines and autoantibodies. We have assessed the inflammation level in BMCE and control groups. The assessment included: focus score, focus area, lymphocytic composition of lymphocytic infiltrates, serum level for IL-10; IL-6; IFN-γ; TNF-α, key genes in inflammation/antiinflammation, and serum levels of autoantibodies anti-SSA/Ro and anti-SSB/La. BMCE group showed lower focus score in both glands but only significant in lacrimal gland (LG, *P <0.05) and smaller focus area in both glands which was only significant in submandibular gland (SMG, *P <0.05) in comparison to control group (Figure 3 A, B). Immunohistochemical staining was used to identify the percentage of different types of lymphocytes in glandular lymphocytic infiltrates. We tested for BAFF (survival factor for B cells), B220 (a pan B cell marker in mice), FoxP3 (Forkhead box Protein 3, expressed by $T_{reg}$) markers (Figure 4 C, G). Our data showed a significantly lower expression for BAFF (SMG, *P <0.05; LG, **P <0.01), B220 (SMG, *P <0.05) and, but a higher expression for FoxP3 (SMG; LG, **P <0.01) in BMCE group (Figure 4 E-G). Autoantibodies are an important hallmark of SS; it is significant for establishing SS diagnosis. Their serum level evaluated by ELISA revealed a lower level of anti-SSA/Ro and anti-SSB/La) in BMCE group (*P <0.05, Figure 3 H, I). IL-10 is an anti-inflammatory cytokine which is elevated in an immunosuppression state. It is secreted mainly by $T_{reg}$, MSCs and other cells. IL-6, TNF-α and IFN-γ are all inflammatory cytokines which are elevated in SS patients and NOD mice. Serum analysis of BMCE group revealed an upregulated IL-10 level (*P <0.05, Figure 3 J) and a downregulated level for IL-6, TNF-α and IFN-γ (IFN-γ **P <0.01, Figure 3 K-M). Quantitative rt-PCR was used to assess the gene expression of IL-10, TNF-α, IL-1β, and TGF-β1. BMCE group

expressed downregulation of TNF-α, IL1β, and TGF-β1 genes, and an upregulation of IL-10 gene (Figure 3 N).



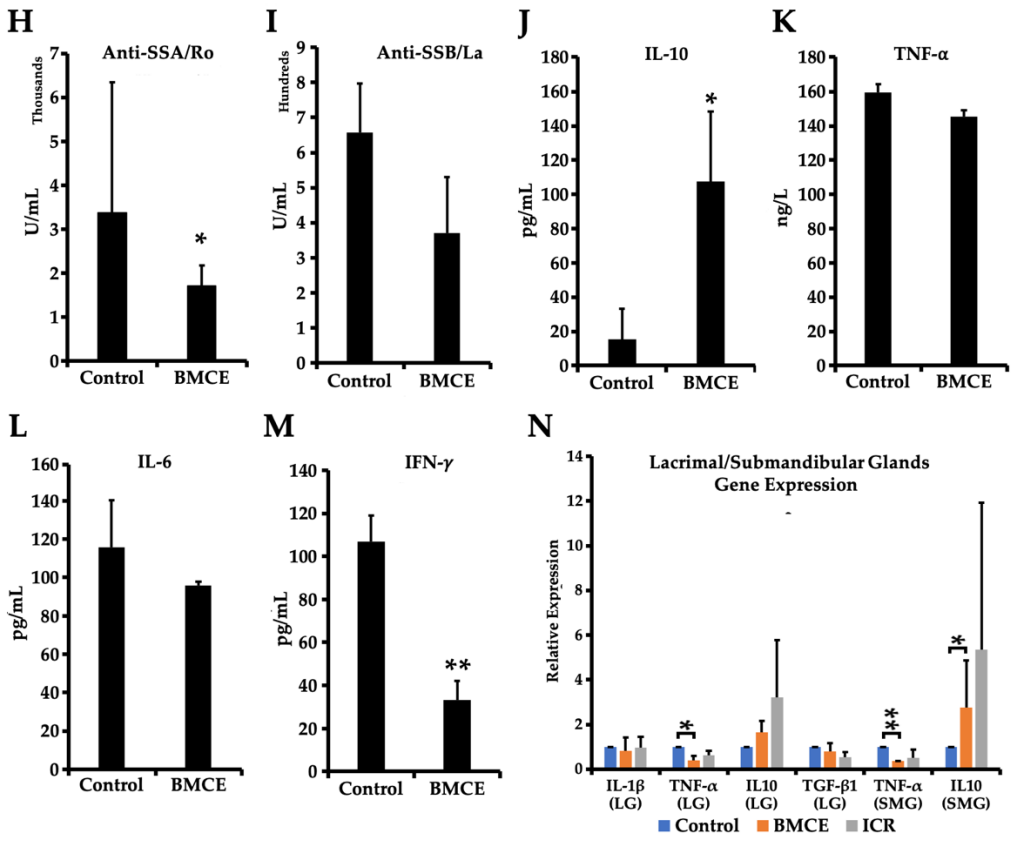



**Figure 4.** BMCE anti-inflammatory effect assessment at 16 weeks post-treatment. (A) H&E stained images of lymphocytic infiltrates in SMGs (upper panel) and LGs (lower panel). (B) Focus score and focus area. Focus score analysis (number of lymphocytic infiltrates/4mm$^2$) using several H&E stained sections, cut at 80 µm apart, under the light microscope. The analysis revealed a lower score for both glands in BMCE group but only significant in lacrimal gland (*P <0.05). Focus area (µm$^2$) was calculated by Fiji software using H&E images (400X/200X) acquired by Volocity software. BMCE group showed significantly smaller focus area in both glands but was only significant in the submandibular gland (*P <0.05). (C, D) Immunohistochemical staining for lacrimal and submandibular glands, respectively. Lymphocytic infiltrate percentage for BAFF (B cells activating factor), B220 (a pan B cell marker in mice), and FoxP3 (forkhead box P3, $T_{reg}$ marker) were assessed. Quantification of the immunohistochemical signals for (E) BAFF, for (F) B220, and for (G) FoxP3 from foci detected in the field using Fiji software. Serum was evaluated for the following: (H) Anti-SSA/Ro autoantibodies, (I) anti-SSB/La autoantibodies, (J) IL-10, (K) TNF-α, (L) IL-6, and (M) IFN-γ. (N) Relative expression of selected key genes in inflammation and antiinflammation measured by quantitative rt-PCR, Y-axis shows the relative expression of

the genes compared to GAPDH, three experimental replicates were conducted for each sample. *P <0.05; **P <0.01, n= 4-6 mice. All data were presented as mean ± S.D. Control: saline-treated; BMCE: Bone Marrow Cells Extract, ICR: SS-free wild type.

**BMCE group Showed no Signs of Toxicity**

The safety of drugs is crucial to their usefulness and expansion. The BMCE safety was assessed by 1) immediate post-injection observation of the treated mice for any abnormal signs/symptoms and weight monitoring during the 16 weeks follow up, 2) histopathological assessment for the liver, kidneys, spleen, and pancreas, and 3) serum biochemistry tests for liver and kidney function. All mice in BMCE group showed no abnormal behaviours post-treatment, weight loss, or adverse reaction at the injection site in comparison to control group. Histopathology assessment revealed normal tissue structure in BMCE group which is comparable to control group. Liver and kidney functions/toxicities were assessed by evaluating serum levels of ALT, AST, BUN, creatinine, total bilirubin, GGT, and LSR (ALT/AST). BMCE group showed serum level that were within the normal range for all the evaluated markers, except for GGT which was not detected in any of the examined groups (<10 U/L) (Figure 4). However, LSR value was higher for BMCE group (Figure 4).

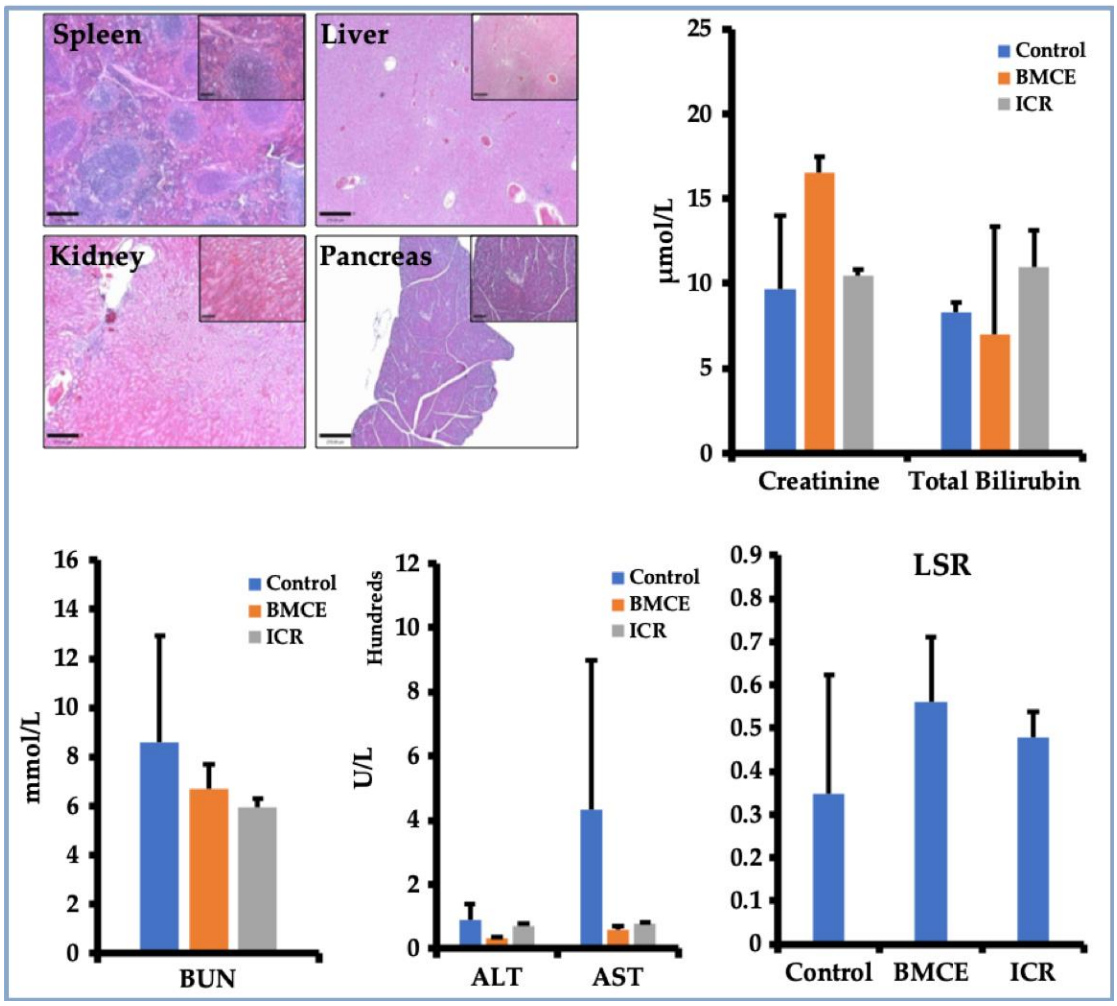



**Figure 5.** Assessment of BMCE safety. (A) H&E images of liver, spleen, kidney, and pancreas of BMCE group. All examined tissues were normal and no signs of toxicity was evident. (B-D) Serum biochemistry analysis for BUN (Blood Urea Nitrogen, Normal Range (NR): 6.4-10.4 mmol/L), creatinine (NR: 18-71 μmol/L), total bilirubin (NR: 2-15 μmol/L), ALT (NR: 28-132 U/L), and AST (NR: 59-247 U/L). The results for all groups were within the normal range for the treated and the control. There was no difference detected between BMCE group except for the ALT and AST levels (*P<0.05; n= 3–4 mice). (E) LSR (ALT/AST ratio) as an indicator of liver injury was higher for BMCE than the control. Scale bar = 76 μm. All data were presented as mean ± S.D. Control: saline-treated; BMCE: Bone Marrow Cells Extract; ICR: wild type control.

**Discussion**

In summary, BMCE therapy showed the following outcomes:1) preserved salivary/lacrimal glands functions, preserved glands stroma, and promoted cell proliferation and tissue regeneration, 2) preserved the cornea structure and thickness, and 3) reduced the glandular inflammation, down-regulated inflammatory and up-regulated anti-inflammatory cytokines, and reduced autoantibodies production.

Previously, we have reported the efficiency of BM in managing SS-like disease in NOD mice; however, treated mice were subjected to prior myeloablative conditioning to avoid GvHD complications [16, 41]. In a more recent study, we have tested BMCE (BM Soup) for the same purpose and it has successfully alleviated SS-like disease without the need for myeloablative conditioning [42]. However, we have assessed the therapeutic effect of BMCE on mice salivary glands only, but we did not assess BMCE therapeutic effect on lacrimal glands and glandular immune suppression, or BMCE systemic safety. Additionally, here, we have tested BMCE with some modifications from our previous methodology, including the extract preparation and the timing of injections. We have also assessed a number of inflammatory/anti-inflammatory cytokines and the lymphocytic composition of the infiltrates. The safety of BMCE treatment was evaluated by examining histological sections of several organs and blood chemistry tests for key proteins and enzymes.

Aiming to preserve the extracellular proteins, BM was collected following a procedure that allowed us to preserve the secretome and the soluble factors around cells without any dilution or loss during the extraction process. Secretome constitutes 13-20% of the total proteome in humans [47, 48]. Previously, we separated the BM from long bones by the flushing method; the cells were forced outside the marrow by inserting normal saline-filled syringe needles in the BM cavity, then the cells were retrieved from the large amount of fluid used. Here, BM was separated by a centrifuge force without adding any fluids. This method, we assumed, preserved most of the soluble factors secreted in the BM extracellular matrix (ECM), the secretome. Although we did not run any analysis to identify the secretome composition, several other groups did [48-51]. The secretome is produced in the BM by BM stromal cells (fibroblasts, endothelial cells, osteoblasts,

reticular cells and the osteocytes) to support the mesenchymal, hematopoietic and other progenitor cells [49]. Therefore, we believe that this unique composition of proteins, factors, and cytokines has augmented the therapeutic function of the BMCE.

BM was successfully tested in the management of SS in NOD mice; however, the use of BM in humans is a complex procedure and might cause more damage rather than curing the disease. In BM transplant (BMT) HLA-mismatched donors are usually avoided due to the high chances of severe GvHD which is very difficult to manage [33]. However, the well HLA-matched donors which seem to be less problematic after transplant still carry the risk of GvHD development. Donor T cells tend to target minor histocompatibility antigens (MiHAs) which are polymorphic genes, presented via MHC as processed antigens. However, these T receptors with high affinity to MiHAs are of low frequency. Nevertheless, in a pro-inflammatory environment, this subset of T cells might be activated to initiate GvHD [33]. Both types are associated with the risk of tumor formation when used as therapies in patients for different conditions. Hence, in SS, the chances of GvHD are high, favored by the inflammatory state of the SS disease. Therefore, the conversion of BM to BMCE achieves several goals, such as 1) eliminating the need to MHC-matching and myeloablation, 2) reducing the chances of GvHD, and 3) minimizing the chances of direct tumor formation by the donor cells.

We have reported that treating NOD female mice with BMCE has reduced the inflammation, preserved salivary glands function [42]. Moreover, several proteins associated with salivary glands biology, regeneration and repair were upregulated in treated mice. Proteins associated with inflammation were down regulated in the treated group in comparison to the control group [42]. In a separate study, we have characterized BMCE composition via protein microarray assay and found several factors related to tissue repair and regeneration, including MMP8, 9; FGF-1; HGF; OPN; SDF-1, and the immune regulation factors/cytokines IL-1Ra and IL-16 [52]. Although the composition of the BMCE is much richer in proteins than the used kit could detect, but we believe that the presence of IL-1Ra, IL-16, HGF, and FGF has played a major role in the therapeutic task of BMCE. OPN and SDF-1 are two factors that have been found at significant levels in SS patients

and animal models, and their presence in the extract, we believe, has not participated nor minimized the therapeutic effect of BMCE.

IL-1 family is a group of 11 cytokines that have pleotropic actions, such as regulation of the basal metabolic rate, blood glucose levels, blood pressure, iron metabolism, bone remodeling, neuroendocrine system, and carcinogenesis [53-60]. IL-1 is composed of three isoforms, two pro-inflammatory: IL-1β and IL-1α, and an anti-inflammatory, IL-1Ra. IL-1Ra is a naturally produced antagonist used by the immune system to neutralize the effect of IL-1α and IL-1β [61-63]. However, its binding to IL-1R is associated with no further intracellular signaling. It is believed that the imbalance of IL-1/IL-1Ra could promote inflammation in the oral cavity [64]. High levels of IL-1 and low levels of IL-1Ra were found in SS patients' saliva and it was suggested that this imbalance forms a major component of the pro-inflammatory composition [64, 65]. Moreover, higher levels of IL-1α and IL-1β were found in the tears of SS patients [66]. The previous findings strongly support the correlation between IL-1 and SS; hence, blocking IL-1 might be a reasonable approach for treating SS. As previously mentioned, we have detected significant quantities of IL-1Ra in the BMCE and we believe that it participated, partially and possibly synergistically, in the immunosuppressive effect of BMCE. On the other hand, in BMCE group, we detected downregulation of IL-1β gene expression in lacrimal gland tissue, upregulated IL-10 gene in the glandular tissues, and upregulated IL-10 cytokine in the serum. IL-10 was shown to control IL-1β activity and induce shedding of IL-1R [67]. Therefore, we believe that the elevation of IL-10 induced by MBCE treatment, has negatively affected the expression of IL-1β and upregulated IL-1Ra secretion [68, 69].

IL-16 is a pleotropic, natural and soluble cytokine produced by several cell types, such as immune cells, e.g. T cells and eosinophils and non-immune cells, e.g. fibroblasts and epithelial cells [70]. When IL-16 binds to CD4, it promotes the production of more IL-2Rα subunit, but no production of IL-2 [70]. IL-16 was thought to be a pro-inflammatory cytokine due its role in activating CD4+ T cells; however, its immunosuppressive role was evident by several studies [71-73]. Treatments with IL-16 has been found to diminish the asthma-associated inflammation, and to completely attenuate antigen-induced airway hyper-sensitivity mediated by Th2 T cells [71]. A study which

is the most interesting and relevant to our work reported that treatment of RA with IL-16 has led to a significant decrease of several inflammatory cytokines, including IL-1, IFN-γ, and TNF-α [72]. Moreover, an in vitro study found that IL-16 has a positive role in immunosuppression and or immunoregulation [73]. In addition, another study reported that adding IL-16 to CD4+ T cells up-regulates the expression of FoxP3 gene four folds [73]. At inflammation sites, IL-16 may preferentially induce a migratory response from existing $T_{reg}$ cells and via the induction of de novo generation of FoxP3 [73]. In view of the foregoing, we believe that IL-16 in BMCE has played an immunosuppressive role which is supported by the downregulation of IL-1β gene and serum IFN-γ/TNF-α and the upregulation of $FoxP3^{rich}$ $T_{reg}$ in salivary and lacrimal glands.

IL-6 has a hormone-like function that affects several biological processes, such as vascular diseases, lipid metabolism, insulin resistance, mitochondrial activities, neuroendocrine system and neuropsychological behavior [16, 74]. It is secreted by almost all stromal and immune cells, and is activated by several factors and pathways, including TNF-α and IL-1β. BMCE group exhibited lower level of IL-6; additionally, TNF-α and IL-1β showed lower levels in their serum. Thus, the low level of IL-6 was due to the low levels of TNF-α and IL-1β, which were originally lowered by the increased level of IL-10.

Serum EGF level and its gene expression in both glands were significantly elevated in BMCE group in comparison to the control group and was comparable to that of ICR group. When EGF binds to its receptors, it induces proliferation and produces anti-apoptogenic survival signals; therefore, the higher proliferation of the glands and the preservation of key cell subpopulations were all due the trophic effect of systemic EGF and possibly the BMCE content of HGF, and FGF-1. We believe this upregulation is due to the direct effect of the regenerative capacity of BMCE treatment on salivary glands and the preservation of the glands structure, including the ductal cells which secret EGF. The gene analysis of both glands revealed an upregulation of several genes involved in tissue regeneration, including FGF-β1, BMP7, and genes related to glandular function, including AQP5 and LYZ1. The increased expression of AQP5 and LYZ1 were expected due to the increase of the salivary and tear flow rates which reflects the preservation/regeneration of the glandular tissue.

Assessment of the inflammation severity in the glands revealed lower focus score and small foci in BMCE group. The immunohistochemical characterization of the lymphocytic infiltrates revealed a downregulation of B220+ B cells, less BAFF expression, and an increase in FoxP3$^{rich}$ $T_{reg}$ cell count. On the other hand, serum analysis of BMCE group revealed higher IL-10 and lower IL-6, IFN-γ, and TNF-α levels, in addition to lower levels of anti-SSA/SSB autoantibodies. These findings strongly indicate an immunosuppressive role of BMCE therapy. $T_{reg}$ is well documented for being an integral pillar in immunosuppression/immunoregulation and maintaining peripheral tolerance by secreting anti-inflammatory cytokines (e.g. IL-10) and by inhibiting abnormal actions of antigen presenting cells (APCs) [75, 76]. Diminished $T_{reg}$ was reported in SS patients which means their upregulation is necessary for managing the inflammation [77]. Although we did not assess the direct interaction between $T_{reg}$ and dendritic cells, but we think that the higher $T_{reg}$ percentage has reduced the exaggerated antigen presentation by dendritic cells in treated NOD mice. On the other hand, IL-10 level in the blood and its glandular gene expression were all upregulated in BMCE group in comparison to control group. BAFF is a crucial survival factor for B cells and its overexpression is linked to immortal B cells which escape apoptotic signals. BMCE group showed a significantly less expression of BAFF and lower cell count of B cells (B220+) in the lymphocytic infiltrates.

Tears are essential to sustain the health of the ocular surfaces, particularly the cornea. Damage to the lacrimal glands in SS due to the lymphocytic infiltration leads to dryness of the ocular surfaces, which causes recurrent infections, scarring of the cornea, and a possible perforation if left unmanaged [78, 79]. BMCE treatment preserved the TFR which consequently led to the preservation of the corneal surface. Corneal AQP5 was found to be critical in the maintenance of the corneal transparency and consequently protecting vision [78, 80]. We found an upregulated/preserved expression of corneal AQP5 in BMCE group, which suggest that AQP5 participation in the maintenance of the corneal surface integrity via maintaining the flow of water. We assessed the expression of CK5 which is a microtubule protein that is expressed by all corneal epithelium layers. BMCE group exhibited a higher intensity of CK5 protein expression which indicates the preserved corneal thickness.

Drug safety is fundamental in expanding the use of new drugs. Cell therapy has been associated with numerous pathologies that can leave devastating side effects, including tumors and GvHD. On the other hand, biologic therapies have been linked to several complications as well, such as opportunistic infections, depression, cardiovascular complications, viral infection, neurological complications, and malignancy [81-87]. Alanine aminotransferase (ALT) and aspartate aminotransferase (AST) are the most abundant enzymes in the blood which are synthesized by the liver, AST is also synthesized by heart and muscle tissues [88]. Their serum levels are commonly evaluated for liver function [89]. Serum ALT activity has been used as an inflammatory marker for different liver injuries including tumors [88]. ALT, AST, and γ-Glutamyl transferase (GGT) are all kidney function markers (leakage) which are used to assess kidney injury [90]. Our results showed that AST and ALT are both within the normal range, whereas GGT was not significant to be detected in any sample (<10 U/L). However, AST in control group was much higher than the ULN for AST, >400 U/L (NR: 59-247) and was closer to the ULN for ALT, whereas BMCE group showed values that were closer to the LLN. Blood urea nitrogen (BUN) is formed in the liver as an end-product for protein metabolism [91]. Creatine is produced mainly by liver, stored in muscles, and excreted by kidneys as creatinine. Serum creatinine (SCr) is a commonly used marker for glomerular function and the simplest test for kidney function [92]. Bilirubin is a by-product for heme metabolism, it is also considered antioxidant; however, hyperbilirubinaemia is a sign of drug-induced liver injury. Our results showed that BMCE group was within the normal range; however, its level in the control group was lower than the normal range. For BUN and total bilirubin, both groups (control, BMCE) were within the normal range. We also assessed LSR (ALT/AST ratio, as a prognostic value [93]) which is an indicator for liver injury [88]; surprisingly, control group showed lower level than that of BMCE and ICR groups due to the high AST level. We conclude from the previous results that BMCE did not induce any pathological changes, on the contrary, BMCE group will have better prognosis due to the lower level of AST, which is an important marker for life expectancy [94].

Unlike treating other autoimmune diseases; a successful SS treatment must resolve the immune dysregulation and induce regeneration of the lost tissues or protect and preserve the remaining

tissues from further damage. Unfortunately, the current available treatments and the agents tested in clinical trials are all designed to target/correct the uncontrolled immune reaction, but none is considering the regenerative need for the salivary and lacrimal glands. Therefore, the cell-based therapies and their extracts with their unique composition of immune factors/cytokines and regenerative/trophic molecules, have much to offer to SS patients than a single immune agent might have. The extract is composed of various proteins at a healthy composition and concentration that we think is the key to resolve SS immune dysregulation.

## Conclusion

BMCE is an efficient and a safe treatment for SS in NOD mice when administered before overt disease starts. It exerted its therapeutic efficacy at the cryoprotective/regenerative and immunosuppression/immunoregulatory levels. However, further evaluation of its effectiveness at an advanced disease stage is required. We also believe that a promising management of SS can be executed by combining BMCE with biologic therapy to achieve an optimum immunosuppression and glandular regeneration.

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Cytotherapy

# Allogeneic Bone Marrow Cells Extract (BMCE) Alleviated Sjogren's-Like Disease in NOD Mice.

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| Abstract: | Background aims . Sjogren's Syndrome (SS) is a common autoimmune disease that manifests in exocrine glands and affects middle-aged women mainly. It is a significantly debilitating disease and might cause lymphoma in some patients. Due to its complexity, no treatment has achieved satisfying results. In mice, bone marrow transplantation (BMT) has successfully treated SS; however, BMT requires a prior myeloablative conditioning. Methods . In this study, we used a less immunogenic form of BM called "BM cell extract (BMCE)" with its secretomes from C57BL/6 mice. BMCE was injected into 8-week-old NOD female mice, then followed for 16 weeks posttreatment. Saliva and tears were collected periodically. Results . Treated mice showed significantly higher salivary/tear flow rates up to 16 weeks post-treatment with preserved specialized glandular cells. Glandular inflammation was decreased, especially B cells. Serum levels of IFN-γ, TNF-α, IL-6 and anti-SSA/SSB were lower, and higher for EGF and IL-10. Gene expression of TNF-α, TGF-β1, MMP2, CASP3 and IL-1β genes were downregulated and up-regulated for AQP5, EGF, FGF2, BMP7, LYZ1 and IL-10. Corneal thickness, and CK5/AQP5 expressions were preserved. Mice were healthy during the follow up period and histopathology assessment of liver, kidneys, spleen, and pancreas showed no pathological signs. BUN Urea, creatinine, total bilirubin, ALT, AST, and GGTblood levels were within the normal range in treated mice, and LSR was higher. Conclusion . BMCE is a safe treatment modality with dual therapeutic actions: regenerative and anti-inflammatory. |

# Chapter Six

## Conclusion

Sjögren's Syndrome is a complex disease with numerous complications. Patients' quality of life is significantly compromised, and no drug has been able to cure or induce remission. Unfortunately, biologic therapies, which have been approved for other autoimmune diseases, did not achieve satisfactory outcomes in SS patients. On the other hand, stem cells therapy has proven their competencies in managing various conditions including autoimmune diseases. Many studies reported that cells exert their effect via paracrine signaling and can even do so remotely. Additionally, numerous studies reported that cell extract and cell secretomes have therapeutic capacities. Previously, we have tested the efficacy of BM and MSCs in alleviating SS in NOD mice; thus, we decided to test whether their extracts have therapeutic potentials.

In chapter four, we show that MSCsE was effective in alleviating SS in a comparable competency to MSCs. MSCsE exerted an anti-inflammatory/immunoregulatory and trophic/regenerative effects systemically and locally in the damaged exocrine glands. Salivary and lacrimal glands function was preserved when the treatment was administered before the overt disease stage starts. It hindered the immune attack significantly, evidenced by smaller and less inflammatory foci containing significantly less B cells which are a critical player in SS pathogenesis.

In chapter five, we have tested the efficacy and safety of BMCE in alleviating SS in lacrimal and salivary glands. The treated mice showed preserved secretory function and reduced inflammation (locally and systemically) comparable to the previous results accomplished in MSCsE. Based on the follow up period (16 weeks post treatment) and the investigations used, we could not detect any treatment-related pathology or abnormality. The tested organs and the blood biochemistry tests were all within the normal range. However, a longer follow up period and a comprehensive tissue evaluation is required to ascertain its long-term safety in the future.

Both cell extracts (BMCE, MSCsE) are composed of different proteins (different cells composition); yet, we believe that each therapy exerted its therapeutic effect via different pathogenic channels. Similar to our findings, has been seen in clinical trials in terms of efficacy of different agents. In each clinical trial, there was a small number of patients who showed improvement; therefore, it would be more logical to combine more than one agent at same time. Alternatively, a single therapeutic agent, e.g. anti-BAFF, would not treat all patients in the same efficacy. Therefore, a therapy like BM-derived cells extract would more efficiently manage SS ; yet, it also be enriched with one or more of the biologic agents, particularly severe cases. What makes BM-derived cells extract unique is its composition of a naturally constituted level of anti-inflammatory cytokines combined with growth factors. Most of the tested biologic agents showed no glandular improvement; whereas, our therapies were able to preserve and rescue the glandular function in addition to the anti-inflammatory effect.

When compared to cell therapy, cell extract is a more convenient treatment modality. We have shown in our lab that BMCE can be dried, reconstituted, and injected when needed with similar

efficiency to the fresh one. On the other hand, cells are sensitive when handled in-vitro and might behave differently under different in-vivo conditions, and not yield the anticipated therapeutic outcomes. We may not achieve the same efficacy from these cells in different patient with different disease activity. Not to mention all the side effects associated with cell therapy that we have covered previously.

The next question that would rise is: can we test these therapies in SS patients? autogenous and allogenic MSCs are now licenced in many places for various applications. Biologic agents are also approved in the management of different autoimmune diseases. However, both treatment modalities have been extensively evaluated, particularly their safety and side effects. Therefore, although we have assessed the safety of BMCE treatment, 16 weeks posttreatment, we still need to evaluate the acute reaction to the treatment, a histopathology assessment for longer posttreatment period, and a more comprehensive assessment of other organs as well.

**Future directions**

The disease goes unnoticed for many years before patients seek medical help. It silently attacks and damages the exocrine glands and other organs. Our therapies have been effective when administered at an early stage of the disease; however, to be more clinically applicable, we need to test their efficacy at an advanced stage in the same model and possibly in other models. We also need to verify the pathogenic channels that the treatments have exerted their effects in an in vitro study, although it is a challenging task, but we believe we could anticipate a number of effective agents and evaluate them. This might also help us answer some of the unanswered pathogenesis questions; working reversibly.

## Appendix

**Additional articles published by the candidate during her Ph.D. studies.**

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Full length article

# Comparative adsorption profiles of basal lamina proteome and gingival cells onto dental and titanium surfaces

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ABSTRACT

Titanium (Ti) dental implants are susceptible to bacterial infections and failure due to lack of proper epithelial seal. Epithelial cells establish a strong epithelial seal around natural teeth by the deposition of basal lamina (BL) proteins that adsorb on the tooth surface. This seal can even be re-established onto cementum or dentin following injury or periodontal therapy. However, it is unclear how tooth surfaces promote this cell attachment and protein adsorption. Understanding the interactions between BL proteins and epithelial cells with dentin and Ti will facilitate the development of implant surfaces that promote the formation of an epithelial seal and improve the success of periodontal therapy and wound healing on natural teeth. To study these interactions, we used a surface proteomic approach to decipher the adsorption profile of BL proteins onto Ti and dentin, and correlated these adsorption profiles with *in vitro* interactions of human gingival fibroblasts and epithelial cells. Results showed that dentin adsorbed higher amounts of key BL proteins, particularly laminin and nidogen-1, and promoted more favorable interactions with epithelial cells than Ti. Next, dentin specimens were deproteinized or partially demineralized to determine if its mineral or protein component was responsible for BL adsorption and cell attachment. Deproteinized (mineral-rich) and partially demineralized (protein-rich) dentin specimens revealed BL proteins (i.e. laminin and nidogen-1) and epithelial cells interact preferentially with dentinal proteins rather than dentin mineral. These findings suggest that, unlike Ti, dentin and, in particular, dentinal proteins have a selective affinity to BL proteins that enhance epithelial cell attachment.

**Statement of Significance**

It is remains unclear why natural teeth, unlike titanium dental implants, promote the formation of an epithelial seal that protects them against the external environment. This study used a surface screening approach to analyze the adsorption of proteins produced by epithelial tissues onto tooth-dentin and titanium surfaces, and correlate it with the behaviour of cells. This study shows that tooth-dentin, in particular its proteins, has a higher selective affinity to certain adhesion proteins, and subsequently allows more favourable interactions with epithelial cells than titanium. This knowledge could help in developing new approaches for re-establishing and maintaining the epithelial seal around teeth, and could pave the way for developing implants with surfaces that allow the formation of a true epithelial seal.


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Full length article

# Biomaterial surface proteomic signature determines interaction with epithelial cells

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ABSTRACT

Cells interact with biomaterials indirectly through extracellular matrix (ECM) proteins adsorbed onto their surface. Accordingly, it could be hypothesized that the surface proteomic signature of a biomaterial might determine its interaction with cells. Here, we present a surface proteomic approach to test this hypothesis in the specific case of biomaterial-epithelial cell interactions. In particular, we determined the surface proteomic signature of different biomaterials exposed to the ECM of epithelial cells (basal lamina). We revealed that the biomaterial surface chemistry determines the surface proteomic profile, and subsequently the interaction with epithelial cells. In addition, we found that biomaterials with surface chemistries closer to that of percutaneous tissues, such as aminated PMMA and aminated PDLLA, promoted higher selective adsorption of key basal lamina proteins (laminins, nidogen-1) and subsequently improved their interactions with epithelial cells. These findings suggest that mimicking the surface chemistry of natural percutaneous tissues can improve biomaterial-epithelial integration, and thus provide a rationale for the design of improved biomaterial surfaces for skin regeneration and percutaneous medical devices.

**Statement of Significance**

Failure of most biomaterials originates from the inability to predict and control the influence of their surface properties on biological phenomena, particularly protein adsorption, and cellular behaviour, which subsequently results in unfavourable host response. Here, we introduce a surface-proteomic screening approach using a label-free mass spectrometry technique to decipher the adsorption profile of extracellular matrix (ECM) proteins on different biomaterials, and correlate it with cellular behaviour. We demonstrated that the way a biomaterial selectively interacts with specific ECM proteins of a given tissue seems to determine the interactions between the cells of that tissue and biomaterials. Accordingly, this approach can potentially revolutionize the screening methods for investigating the protein-cell-biomaterial interactions and pave the way for deeper understanding of these interactions.



## 1. Introduction

Biomaterials represent a significant portion of modern healthcare with numerous clinical applications ranging from vascular stents, catheters, and degradable grafts to orthopaedic and dental implants [1]. Upon contact with a biomaterial, cells and tissues of the human body respond in a manner dictated by the biomaterial surface properties [2,3]. Surface topography and mechanical stiffness govern cellular response and have been key in the development of the field of biomaterials [4–7]. Despite this improved understanding, the mechanisms through which surface properties, particularly surface chemistry, regulate host responses still remain to be fully elucidated [2,8].

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RESEARCH ARTICLE

WILEY

# Cell culture of differentiated human salivary epithelial cells in a serum-free and scalable suspension system: The salivary functional units model

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## Abstract

Saliva aids in digestion, lubrication, and protection of the oral cavity against dental caries and oropharyngeal infections. Reduced salivary secretion, below an adequate level to sustain normal oral functions, is unfortunately experienced by head and neck cancer patients treated with radiotherapy and by patients with Sjögren's syndrome. No disease-modifying therapies exist to date to address salivary gland hypofunction (xerostomia, dry mouth) because pharmacotherapies are limited by the need for residual secretory acinar cells, which are lost at the time of diagnosis, whereas novel platforms such as cell therapies are yet immature for clinical applications. Autologous salivary gland primary cells have clinical utility as personalized cell therapies, if they could be cultured to a therapeutically useful mass while maintaining their in vivo phenotype. Here, we devised a serum-free scalable suspension culture system that grows partially digested human salivary tissue filtrates composing of acinar and ductal cells attached to their native extracellular matrix components while retaining their 3D in vivo spatial organization; we have coined these salivary spheroids as *salivary functional units* (SFU). The proposed SFU culture system was sub-optimal, but we have found that the cells could still survive and grow into larger salivary spheroids through cell proliferation and aggregation for 5 to 10 days within the oxygen diffusion rates in vitro. In summary, by using a less disruptive cell isolation procedure as the starting point for primary cell culture of human salivary epithelial cells, we demonstrated that aggregates of cells remained proliferative and continued to express acinar and ductal cell-specific markers.

**KEYWORDS**

dry mouth syndrome, human primary epithelial cells, salivary acinar cells, salivary functional units, salivary gland regeneration, serum-free culture, suspension culture, xerostomia

  

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ORIGINAL PAPER

# Broccoli extract improves chemotherapeutic drug efficacy against head–neck squamous cell carcinomas

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## Abstract

The efficacy of cisplatin (CIS) and 5-fluorouracil (5-FU) against squamous cell carcinomas of the head and neck (SCCHN) remains restricted due to their severe toxic side effects on non-cancer (normal) tissues. Recently, the broccoli extract sulforaphane (SF) was successfully tested as a combination therapy to target cancer cells. However, the effect of lower doses of CIS or 5-FU combined with SF on SCCHN remained unknown. This study tested the chemotherapeutic efficacies of SF combined with much lower doses of CIS or 5-FU against SCCHN cells aiming to reduce cytotoxicity to normal cells. Titrations of SF standalone or in combination with CIS and 5-FU were tested on SCCHN human cell lines (SCC12 and SCC38) and non-cancerous human cells (fibroblasts, gingival, and salivary cells). Concentrations of SF tested were comparable to those found in the plasma following ingestion of fresh broccoli sprouts. The treatment effects on cell viability, proliferation, DNA damage, apoptosis, and gene expression were measured. SF reduced SCCHN cell viability in a time- and dose-dependent manner. SF-combined treatment increased the cytotoxic activity of CIS by twofolds and of 5-FU by tenfolds against SCCHN, with no effect on non-cancerous cells. SF-combined treatment inhibited SCCHN cell clonogenicity and post-treatment DNA repair. SF increased SCCHN apoptosis and this mechanism was due to a down-regulation of BCL2 and up-regulation of BAX, leading to an up-regulation of Caspase3. In conclusion, combining SF with low doses of CIS or 5-FU increased cytotoxicity against SCCHN cells, while having minimal effects on normal cells.

**Keywords** Head and Neck cancer · Carcinoma, squamous cell · Sulforaphane · Drug therapy · Apoptosis · DNA damage

## Introduction

Squamous cell carcinoma of the head and neck (SCCHN) is one of the most prevalent malignant neoplasms of the upper aerodigestive tract. SCCHN is now the seventh most common cancer worldwide, with over 500,000 new cases diagnosed and 380,000 deaths annually which is nearly 4.6% of all cancer cases [1, 2]. Despite the improvements in treatment modalities, the 5-year survival rate for SCCHN patients has remained unchanged at about 50% over the past 30 years [3, 4] as 40–60% of SCCHN survivors suffer from relapse in the form of recurrences or metastases [5, 6].

Resistance to standard surgical, radiation, and chemical therapies continues to be a limiting factor in the treatment of SCCHN. One major factor in cancer treatment failure is because the efficacy of current standard chemotherapy, such as cisplatin (CIS) and 5-fluorouracil (5-FU), is restricted partly due to their severe toxic side effects. CIS forms DNA adducts which lead to induction of apoptosis in cancer cells

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ORIGINAL ARTICLE



ORIGINAL ARTICLE

# Cancer stem cells enrichment with surface markers CD271 and CD44 in human head and neck squamous cell carcinomas

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## Abstract

Head and neck squamous cell carcinoma (HNSCC) has a poor 5-year survival rate of 50%. One potential reason for treatment failure is the presence of cancer stem cells (CSCs). Several cell markers, particularly CD44, have been used to isolate CSCs. However, isolating a pure population of CSC in HNSCC still remains a challenging task. Recent findings show that normal oral stem cells were isolated using CD271 as a marker. Thus, we investigated the combined use of CD271 and CD44 to isolate an enriched subpopulation of CSCs, followed by their characterization *in vitro*, *in vivo*, and in patients' tissue samples. Fluorescent-activated cell sorting was used to isolate CD44+/CD271+ and CD44+/CD271– from two human HNSCC cell lines. Cell growth and self-renewal were measured with MTT and sphere/colony formation assays. Treatment-resistance was tested against chemotherapy (cisplatin and 5-fluorouracil) and ionizing radiation. Self-renewal, resistance, and stemness-related genes expression were measured with qRT-PCR. *In vivo* tumorigenicity was tested with an orthotopic immunodeficient mouse model of oral cancer. Finally, we examined the co-localization of CD44+/CD271+ in patients' tissue samples. We found that CD271+ cells were a subpopulation of CD44+ cells in human HNSCC cell lines and tissues. CD44+/CD271+ cells exhibited higher cell proliferation, sphere/colony formation, chemo- and radio-resistance, upregulation of CSCs-related genes, and *in vivo* tumorigenicity when compared to CD44+/CD271– or the parental cell line. These cell markers showed increased expression in patients with the increase of the tumor stage. In conclusion, using both CD44 and CD271 allowed the isolation of CSCs from HNSCC. These enriched CSCs will be more relevant in future treatment and HNSCC progression studies.

## Introduction

Cancer is the second leading cause of death in the USA and the first cause in Canada, as it is responsible for over 30% of all deaths annually (1,2). Head and neck squamous cell carcinoma (HNSCC) is the seventh most common cancer worldwide, as it accounts for over 580,000 new diagnosed cases in 2018 (3). In Canada, 5850 new cancer patients were diagnosed with HNSCC and it was responsible for 1690 deaths in 2017 (1). Despite recent advances for diagnosis and cancer treatment, the current prognosis for HNSCC is poor due to relapse in the form of local recurrence or metastasis. The 5-year survival rate has remained approximately 50% for the last three decades (4).

One reason for cancer treatment failure is considered to be related to the presence of a subpopulation of cells in the tumor called "cancer stem cells" (CSCs), which are suggested to have tumor-initiating potential combined with the ability of self-renewal and multilineage differentiation (5). Acute myeloid

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