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의학박사 학위논문

**Effects of Subconjunctival  
Administration of Anti-High  
Mobility Group Box 1 on Dry Eye in  
a Mouse Model of Sjögren's  
Syndrome**

쇼그렌 증후군 마우스 모델에서 결막하 투여된  
항HMGB1이 건성안에 미치는 영향

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**Ph.D. Dissertation**

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# Abstract

**Purpose:** Extracellular high mobility group box 1 (HMGB1) acts as a damage associated molecular pattern molecule through the Toll-like receptor (TLR) to promote autoreactive B cell activation, which may be involved in the pathogenesis of Sjögren's syndrome. Moreover, extracellularly secreted HMGB1 is reported to be involved in the activation of T helper (Th) 17 cells during inflammatory disease, and may also be involved in interleukin (IL)-17 or IL-22 secretion in innate lymphoid cells (ILCs) which have important functions in innate and adaptive immunity. Considering that TLR9 abundantly expressed on B cells and plasmacytoid dendritic cells and main role of reactive B cells and plasma cells that secrete autoantibodies in the pathogenesis of Sjögren's syndrome together, we hypothesized that chronic epithelial damage to the cornea or lacrimal gland may contribute to trigger the vicious cycle of inflammation by secreting HMGB1 as a danger signal. Therefore, this study was aimed to investigate the effect of subconjunctival administration of anti-HMGB1 antibodies on dry eye in a mouse model of Sjögren's syndrome via evaluation of changes in clinical manifestations of dry eye and immunologic responses.

**Methods:** NOD.B10.*H2<sup>b</sup>* mice were used as primary Sjögren's syndrome model, and to investigate inhibitory effect of triggering by HMGB1 in early stage of inflammatory cascades, ten weeks-old mice were chosen based on previous preliminary data. Twenty-four mice were divided into 4 groups and anti-HMGB1 antibodies (0.02, 0.2 and 2 µg/0.1 cc) or phosphate buffered

saline (PBS) containing chicken immunoglobulin Y (0.2 µg/0.1 cc) were injected subconjunctivally twice a week for two consecutive weeks.

To evaluate changes in clinical manifestations, tear volume and corneal staining scores were measured and compared between before- and after-treatment. Goblet cell density was counted in periodic acid Schiff (PAS) stained forniceal conjunctiva.

For the evaluation of changes in immunologic responses, flow cytometry was performed to evaluate the changes in BrdU<sup>+</sup> cells, IL-17-, IL-10-, or IFNγ-secreting cells, functional B cells, and IL-22 secreting group 3 ILCs in cervical lymph nodes. The level of anti-SSA in plasma and IL-22 in intraorbital glands were also measured using enzyme-linked immunosorbent assay. Inflammatory foci score (>50 cells/focus) was measured in extraorbital glands after CD3 and B220 immunohistochemical staining.

**Results:** Injection of 2 µg or 0.02 µg anti-HMGB1 antibodies significantly attenuated corneal epithelial erosions ( $p < 0.05$ ). Tear volume significantly increased in groups treated with 2 µg or 0.02 µg anti-HMGB1 antibodies ( $p < 0.05$ ). Although statistical significance was failed to be shown, clinical manifestation of corneal epithelial disruption and tear secretion tended to be improved in the 0.2 µg anti-HMGB1 antibodies treated group. Goblet cell density was increased in 0.2 µg and 2 µg anti-HMGB1-treated-mice with marginal significance.

The percentage and number of BrdU<sup>+</sup>CD3<sup>+</sup>T and BrdU<sup>+</sup>B220<sup>+</sup>B cells in the draining lymph nodes of treated mice were not significantly altered compared to that in controls. For effector T cell responses, the percentage of Th17 cells

and cytotoxic T cells that secrete IL-17 were not different and IFN $\gamma$ -secreting cells were not changed following treatment. For functional B cell responses, no significant changes were found in the percentage of plasma cells or regulatory B cells in the draining lymph nodes. Level of anti-SSA in plasma was not significantly changed. The infiltrating focus of CD3<sup>+</sup>T cells was very similar to that of B220<sup>+</sup>B cells in extraorbital gland and scores of inflammatory foci were not different among the groups. Unexpectedly, the percentage of group 3 ILCs was significantly increased in the draining lymph nodes ( $p < 0.05$ ), and the expression of IL-22 was significantly increased in the intraorbital glands ( $p < 0.05$ ) after administration of 2  $\mu$ g anti-HMGB1.

**Conclusion:** Subconjunctival administration of anti-HMGB1 improved clinical manifestations of dry eye in NOD.B10.*H2<sup>b</sup>* mice. The improvement of dry eye may involve an increase of group 3 ILCs, rather than modulation of B cells or plasma cells, as shown in the present study using a mouse model of Sjögren's syndrome.

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**Keyword:** cornea; dry eye; Sjögren's syndrome; HMGB1; innate lymphoid cell

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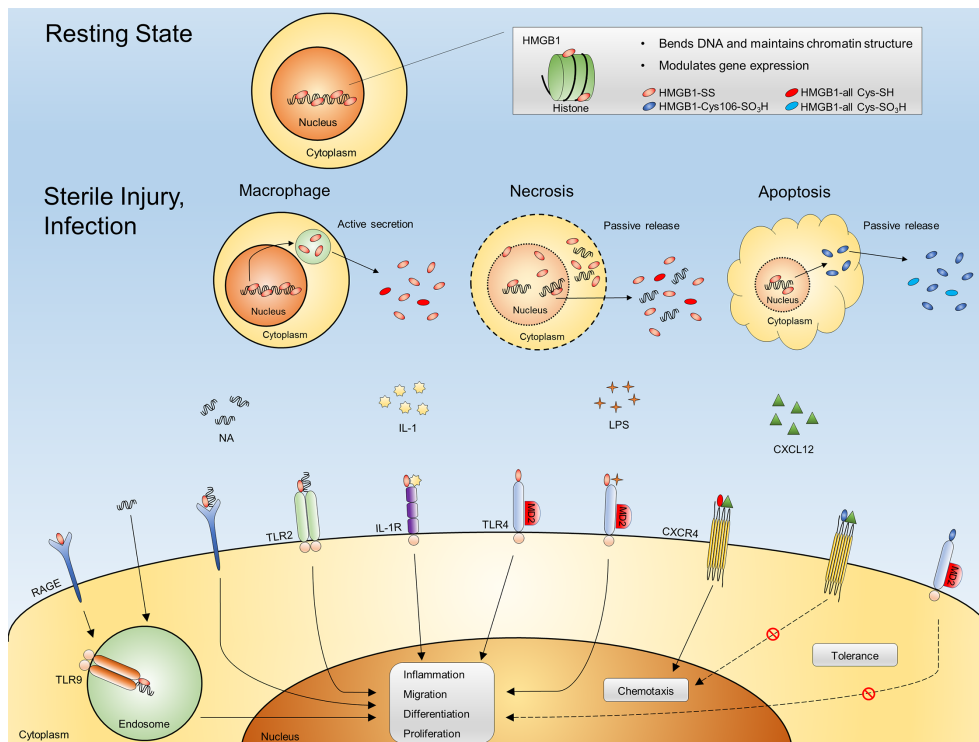
# Chapter 1. Introduction

## 1.1. Study Background

Sjögren's syndrome represents one of the most devastating examples of autoimmune dry eye, which is involved in multiple pathological mechanisms and causes severe discomfort and visual disturbance. Many studies have shown the importance of type I interferon secreted by plasmacytoid dendritic cells, B cell responses, extracellular high-mobility group box 1 (HMGB1) and IL-17 pathways in Sjögren's syndrome [1-3]. In Sjögren's syndrome, initiation of inflammation in glandular tissue, by triggering factors such as viral infection, activates plasmacytoid dendritic cells and leads to produce interferon (IFN)  $\alpha$ , which consequently activate B cells and T cells by inducing cytokine secretion by epithelial cell. Sequentially, activated T cells induce tissue damage via secretion of IFN $\gamma$  and cytokines and B cell activating factor produced by damaged epithelial cells and dendritic cells further promote B cell activation and differentiation into autoantibody producing plasma cells. Immune complex containing nucleic acid associated autoantigen released by damaged cells activate dendritic cells to produce IFN $\alpha$  and establish vicious cycle of immune activation [1]. Current animal studies show that NOD.B10.H2<sup>b</sup> mice are an excellent model of primary Sjögren's syndrome, and B cells, plasma cells, or T helper (Th) 17 cells are involved in the important pathogenic mechanisms in this mouse model [4-6].

HMGB1 is one of the most abundant non-histone nuclear proteins that contributes to chromatin stabilization, and contains two folded helical DNA-binding motifs, called A and B boxes. However, HMGB1 also has specific roles outside cells as a dual-function protein. In the resting state, HMGB1 localizes to the nucleus, where it causes DNA bending and enhances the interaction of other proteins with DNA and their transcriptional activities. However, under conditions such as sterile

injury or infection, HMGB1 is released either actively or passively into the extracellular space where it has distinctly different roles as a cytokine (Fig 1). HMGB1 has three conserved redox-sensitive cysteines (C23, C45, C106), two located at positions 23 and 45 in the A box and one at position 106 in the B box, and modification of these cysteines determines the bioactivity of extracellular HMGB1 [7]. Extracellular HMGB1, which is passively released from necrotic cells or actively secreted by macrophages and dendritic cells, is a crucial cytokine that mediates the response to infection, injury, and inflammation, including autoimmune diseases such as Sjögren's syndrome [2, 3, 8]. Necrosis- and pyroptosis-induced extracellular HMGB1 is usually in a disulfide-bonded form (between cysteine 23 and cysteine 45) that acts as a damage associated molecular pattern (DAMP) molecule through TLR 4, TLR 2, RAGE-TLR9, or IL-1R to promote dendritic cell maturation and autoreactive B cell activation. Extracellular HMGB1, in which all cysteines are reduced, binds to CXCL12 and acts through CXCR4 to cause cellular chemotaxis (Fig 1) [9-12]. In addition, extracellular HMGB1 is reported to be involved in the activation of Th17 cells during inflammatory disease [13], and may also be involved in IL-17 or IL-22 secretion in innate lymphoid cells (ILCs). ILCs are known to coordinate or limit immune responses during autoimmune disease, depending on environmental factors [14]. Conversely, apoptosis-induced extracellular HMGB1, in which all cysteines are oxidized, or cysteine 106 is oxidized, does not exhibit pro-inflammatory or chemotactic activities [7].



**Fig 1. Intracellular and extracellular roles of HMGB1 protein.** In the resting state, HMGB1 localizes to the nucleus, where it causes DNA bending and enhances the interaction of other proteins with DNA and their transcriptional activities. However, under conditions such as sterile injury or infection, HMGB1 is released either actively or passively into the extracellular space where it has distinctly different roles as a cytokine. The disulfide-bonded form of HMGB1, the usual form of extracellular HMGB1, elicits an inflammatory response, including dendritic cell maturation or autoreactive B cell activation, through specific receptors such as RAGE, TLR2, TLR4, or TLR9. Meanwhile, cysteine all-reduced HMGB1 does not have proinflammatory properties but behaves as a chemotactic cytokine through CXCR4. However, the cysteine all-oxidized form of HMGB1, which is produced during apoptotic cell death, loses the ability to induce inflammation and chemotaxis and gives rise to tolerance.

HMGB1 = high mobility group box 1; HMGB1-SS = disulfide-bonded form of HMGB1; HMGB1-all Cys-SH = cysteine all-reduced HMGB1 (cysteines at positions 23, 45, and 106 of HMGB1 express a thiol group); HMGB1-Cys106-SO<sub>3</sub>H = cysteine 106-oxidized HMGB1 (cysteine at position 106 of HMGB1 expresses

a sulfonic acid group); HMGB1-all Cys-SO<sub>3</sub>H = cysteine all-oxidized HMGB1 (cysteines at positions 23, 45, and 106 of HMGB1 express a sulfonic acid group); NA = nucleic acid; IL-1 = interleukin-1; LPS = lipopolysaccharide; CXCL12 = C-X-C motif chemokine ligand 12; RAGE = receptor for advanced glycation end products; TLR2 = toll-like receptor type 2; IL-1R = interleukin-1 receptor; TLR4 = toll-like receptor type 4; MD2 = lymphocyte antigen 96; CXCR4 = C-X-C motif chemokine receptor type 4; TLR9 = toll-like receptor type 9.

Innate lymphoid cells (ILCs) have emerged as a new type of immune cell with important functions in innate and adaptive immunity [14, 15]. Like natural killer (NK) cells, ILCs belong to the lymphoid lineage; however, unlike T and B cells, they lack antigen-receptors (T cell receptor or B cell receptor). Instead of antigen stimulation, ILCs promptly respond to multiple cell-derived factors, such as cytokines and eicosanoids, which are produced by other cells in response to pathogen-associated molecular patterns and DAMP, leading to their designation as innate immune cells. ILCs are found in various tissues including the mucosa, lymphoid tissue, liver, skin, and fat, and they have been classified into three different subpopulations, which resemble Th subsets, according to their distinct patterns of cytokine production and transcription factor. Group 1 ILCs, which are comparable to Th1 cells, consist of conventional NK cells and ILCs that secrete type 1 cytokine IFN $\gamma$  and express the transcription factor T-bet. Group 2 ILCs, which resembles Th2 cells, produce type 2 cytokines IL-4, IL-5, or IL-13, and express the transcription factors ROR- $\alpha$ , Gata3, and T cell factor (TCF)-1. Group 3 ILCs (ILC3s), which are phenotypically similar to Th17 cells, include fetal lymphoid tissue-inducer (LTI) cells, and adult ILCs that either express or lack the natural cytotoxicity receptor (NCR, Nkp46) (NCR<sup>+</sup>ILC3s and NCR<sup>-</sup>ILC3s, respectively). ILC3s produce the Th17-type cytokine, IL-17 or IL-22, and express the transcription factor ROR- $\gamma$ t. The function of ILCs in various tissues is described in Table 1, but it is not yet fully understood. For

instances, ILC3s are known to promote epithelial wound healing and maintain epithelial barrier function in the intestine. ILCs play functional plasticity in response to environmental changes such as conversion between each types of ILCs in human. Furthermore, different ILC subsets localize in distinct tissue niches and receive tissue-derived signals on different type of inflammation, which allows them to acquire diverse phenotypes with specialized effector capacities [16].

**Table 1. Functional characteristics of innate lymphoid cells (ILCs).**

Type	ILC1	ILC2	ILC3
<b>Cells</b>	NK, ILC1	ILC2	LTi, NCR <sup>+</sup> ILC3, NCR <sup>-</sup> ILC3
<b>Transcription factor</b>	T-bet	ROR- $\alpha$ , Gata3, TCF-1	ROR- $\gamma$ t
<b>Tissue signal</b>	IL-12, IL-15, IL-18	IL-25, IL-33, TSLP	IL-1 $\beta$ , IL-23
<b>Effector cytokine</b>	IFN $\gamma$	IL-4, IL-5, IL-13	IL-17, IL-22, GM-CSF
<b>Function</b>	Macrophage activation Oxygen radicals	Mucus production Macrophage activation Tissue repair Vasodilation	Epithelial survival Anti-microbial peptide

## 1.2. Purpose of Research

In Sjögren's syndrome, the conjunctival, corneal, and lacrimal epithelial cells are damaged during inflammation, and some of them may undergo necrosis to release extracellular HMGB1. Considering abundantly expressed TLR9 on B cells and plasmacytoid dendritic cells and main role of reactive B cells and plasma cells that secrete autoantibodies in the pathogenesis of Sjögren's syndrome together [1, 17], we hypothesized that chronic epithelial damage to the cornea or lacrimal gland may contribute to trigger the vicious cycle of inflammation leading to establishment of a continuous pathological condition by secreting HMGB1 extracellularly as a danger signal. Therefore, we investigated whether treatment with an anti-HMGB1 antibody could (1) improve the clinical manifestations of dry eye, (2) affect the immunologic responses in extraorbital glands, plasma, and draining lymph node, and (3) affect immunologic responses of ILC3s that might be involved in modification of epithelial wound healing in NOD.B10.*H2<sup>b</sup>* mice.



## **Chapter 2. Materials and Methods**

### **2.1. Animals**

All procedures used in this study strictly adhered to the ARVO statement regarding the Use of Animals in Ophthalmic and Vision Research. The experimental protocol was approved by the Ethics Committee at Seoul National University Hospital Biomedical Research Institute (IACUC No. 13-0162). Bilateral treatment was justified by the fact that this procedure was not visually disabling and that breeding of NOD.B10.*H2<sup>b</sup>* mice was limited, according to ARVO Statement for the Use of Animals in Ophthalmic and Vision Research, and was approved by the Ethics Committee. NOD.B10.*H2<sup>b</sup>* mice were purchased from Jackson Laboratories (Bar Harbor, ME, USA). All mice were bred in a specific pathogen-free environment and maintained at 22-24°C, relative humidity 55 ± 5%, with alternating 14/10 hour light/dark cycles (light-on 6 AM; light-off 8 PM) with free access to water and food at the mouse facility at the Biomedical Research Institute of Seoul National University Hospital. Overall health was monitored twice a week (weight and hair loss). Phenol red thread tests and corneal dye staining were performed following an intraperitoneal injection of zoletil (10 mg/kg) and xylazine (14 mg/kg), and all efforts were made to minimize suffering. Euthanasia was performed using compressed CO<sub>2</sub> gas, according to the American Veterinary Medical Association Guidelines for the Euthanasia of Animals: 2013 Edition.

### **2.2. Mouse model of Sjögren's syndrome**

Ten week-old male NOD. B10.*H2<sup>b</sup>* mice were used as an autoimmune dry eye model, because NOD.B10.*H2<sup>b</sup>* mice serve as a model for studying primary Sjögren's syndrome without development of diabetes [5, 6, 18, 19]. To investigate inhibitory effect of triggering by HMGB1 in early stage of inflammatory cascades, 10

week-old mice were chosen based on previous preliminary data. A time course of clinical manifestations and inflammatory changes in the draining lymph nodes and intraorbital glands was evaluated between 10 and 16 weeks in BALB/C (n = 28), B6 (n = 32) and NOD.B10.H2<sup>b</sup> mice (n = 34). Clinical dry eye and early inflammation was confirmed to have begun by 10 weeks (S1 Fig). For the current study, 24 male NOD. B10.H2<sup>b</sup> mice were divided into treatment (n = 18) and control groups (n = 6).

### **2.3. Anti-HMGB1 treatment**

A chicken anti-HMGB1 polyclonal blocking antibody (product number: 326052233, SHINO-TEST Corporation, Kanagawa, Japan; 0.02, 0.2 and 2 µg/0.1 cc, n = 6 for each concentration) was injected subconjunctivally into both eyes twice a week for 2 weeks. A subconjunctival injection of the same volume of phosphate buffered saline (PBS) containing 0.2 µg chicken Ig Y (Product Code: 326058471; SHINO-TEST Corporation, Kanagawa, Japan) served as a control. Three mice died during the experiments (two 0.02 µg anti-HMGB1-treated mice and one 0.2 µg anti-HMGB1-treated mouse) probably because of anesthetic overdose. Thereafter, we reduced the anesthetic dose to 20 µl.

### **2.4. BrdU proliferation analysis**

To evaluate proliferative changes in T and B cells, mice were given 0.8 mg/ml BrdU (BD Pharmingen<sup>TM</sup>, San Diego, CA) in their drinking water for 10 days, and the water was changed every two days.

### **2.5. Phenol red thread test for tear volume measurement**

To evaluate tear production, phenol red-impregnated cotton threads (FCI Ophthalmics, Pembroke, MA, USA) were applied to the lateral canthus for 60 seconds, and wetting of the thread was measured in millimeters. This was performed

under anesthesia with zoletil (10 mg/kg) and xylazine (14 mg/kg) (n = 10-12). Twenty-four mice were included in the phenol red thread test for 10-11 weeks, and 21 mice were included in the test for 12 weeks and in the final analysis after sacrifice.

## **2.6. Corneal dye staining**

To evaluate the degree of corneal epithelial defects, one drop of 3% Lissamine Green B (Sigma-Aldrich, St. Louis, MO, USA) was administered to the inferior lateral conjunctival sac. This was convenient because there was no need for cobalt light excitation [20]. The corneal surface was observed (n = 10-12), and dye staining of the cornea was scored in a blinded assay as follows: a score of 0 indicated no punctuate staining; 1 indicated less than one third of the cornea was stained; 2 indicated two thirds or less was stained; and 3 indicated more than two thirds was stained. Twenty-four mice were included in the corneal dye staining test for 10-11 weeks, and 21 mice were included in the test for 12 weeks, and in the final analysis after sacrifice.

## **2.7. Periodic acid Schiff (PAS) staining for identification of goblet cells**

The whole eyeball including the superior and inferior forniceal conjunctiva was excised and fixed in formalin. Tissues were cut into 4- $\mu$ m-thick sections through the superior and inferior conjunctival fornices, and subjected to PAS staining. The total number of PAS-stained cells in the superior and inferior fornices of each eye was counted by two observers in a blind study (n = 16-24). Cell counts were averaged to determine goblet cell density in each group.

## 2.8. Histopathology

The extraorbital glands were excised and fixed in formalin. Samples were cut into 4  $\mu$ m sections and subjected to hematoxylin-eosin, CD3 (for T cells), or B220 staining (an isoform of CD45 and pan B cell marker). For CD3 and B220 immunohistochemical staining, rabbit anti-mouse CD3 (ab16669, Abcam, Cambridge, MA) and rat anti-mouse B220 (ab64100, Abcam, Cambridge, MA) primary antibodies were used. Anti-rabbit IgG HRP-linked antibody (#7074, Cell Signaling Technology, Danvers, MA) and goat anti-rat IgG H&L (HRP) (ab97057, Abcam, Cambridge, MA) were used as secondary antibodies for CD3 and B220 staining, respectively. The total number of inflammatory foci was counted in the CD3-stained slides (n = 7-9). A score of 1 was given when the focus contained greater than 50 CD3<sup>+</sup> T lymphocytes [21].

## 2.9. Flow cytometry

Draining cervical lymph nodes were minced between the frosted ends of two glass slides in RPMI media (WelGENE, Daegu, Korea) containing 10% FBS and 1% penicillin–streptomycin. Cell suspensions were collected and incubated for 30 minutes at 4°C with fluorescein-conjugated anti-mouse antibodies: CD3 (T cells), CD4 (helper T cells, monocytes and dendritic cells), CD8 (cytotoxic T cells, natural killer, and dendritic cells), CD19 (B cells and follicular dendritic cells), B220 (B cells), CD138 (plasma cells), Nkp46 (ILCs and natural killer cells), CD45 (all leukocytes). For IFN $\gamma$ , IL-10, and IL-17A intracellular staining, cells were stimulated for 5 hours with 50 ng/ml phorbol myristate acetate and 1  $\mu$ g/ml ionomycin in the presence of GolgiPlug (BD Pharmingen<sup>TM</sup>, San Diego, CA). For IL-22 intracellular staining, cells were stimulated for 5 hours with 10 ng/ml IL-1 $\beta$  and 10 ng/ml IL-23 in the presence of GolgiStop (BD Pharmingen<sup>TM</sup>, San Diego, CA). Non-specific staining was blocked using purified 2.4G2 Ab (BD Fc Block<sup>TM</sup>). Color conjugation combinations and gating

strategies were as follows: (1) IFN $\gamma$ , and IL-17A secreting cells; CD3-PerCP cy5.5 (eBioscience, 145-2C11), CD4-APC (eBioscience, GK1.5), CD8-PE cy7 (eBioscience, 53-6.7), B220-APC cy7 (eBioscience, RA3-6B2), IL-17A-PE (BD Pharmingen<sup>TM</sup>, TC11-18H10), IFN $\gamma$ -FITC (eBioscience, XMG1.2); (2) Plasma cells; CD3-PerCP cy5.5 (eBioscience, 145-2C11), B220-APC cy7 (eBioscience, RA3-6B2), CD138-APC (BD Pharmingen<sup>TM</sup>, 281-2); (3) IL-10 secreting B cells; CD3-PE (eBioscience, 145-2C11), CD19-PerCP cy5.5 (eBioscience, eBio 1D3), B220-APC cy7 (eBioscience, RA3-6B2), IL-10-FITC (eBioscience, JES5-16E3); (4) ILC3s; CD3-PerCP cy5.5 (eBioscience, 145-2C11), B220-FITC (eBioscience, RA3-6B2), Nkp46-APC (eBioscience, 29A1.4), CD45-APC cy7 (eBioscience, 30-F11), IL-22-PE (eBioscience, 1H8PWSR). Isotype controls were as follows: rat IgG2a,k for CD138-APC; rat IgG1,k for IL-17A-PE, IFN $\gamma$ -FITC and IL-22-PE; rat IgG2b,k for IL-10-FITC.

Cells were assayed using a FACSCanto flow cytometer (BD BioSciences, Mountain View, CA). Data were analyzed using Flowjo software (Tree Star, Ashland, OR) (n = 4-6).

For BrdU (BD Pharmingen<sup>TM</sup>, San Diego, CA) staining, a BrdU FITC cell cycle assay was performed (n = 4-6). During the acquisition preview, gates were adjusted in the FSC-A vs. SSC-A plot, and the DNA 7-AAD-A voltage was adjusted to place the mean of the singlet peak (G0/G1) at 50,000 on the histogram. In addition, cell cycle gates were adjusted as needed to encompass the G0/G1, S, and G2/M populations (S2 Fig).

To evaluate changes in ILC3s, cells were negatively gated with anti-CD3-PerCP and anti-B220-FITC antibodies, then positively gated with anti-CD45-APC cy7 and anti-IL-22-PE antibodies in the presence of anti-Nkp46-APC. Subpopulations of CD3<sup>-</sup>B220<sup>-</sup>CD45<sup>+</sup>Nkp46<sup>+</sup>IL-22<sup>hi</sup> ILC3 cells (NCR<sup>+</sup> ILC3s) and CD3<sup>-</sup>B220<sup>-</sup>CD45<sup>+</sup>Nkp46<sup>-</sup>IL-22<sup>hi</sup> ILC3s cells (NCR<sup>-</sup> ILC3s) were subsequently gated (S2 Fig). Fold changes in ILC3 percentage compared to controls were measured (n

= 4-6).

## **2.10. Enzyme-linked immunosorbent assay**

Blood (800 µl – 1 ml) was obtained from the heart at the time of sacrifice. Plasma was collected after centrifugation at 2,500 rpm for 10 minutes, and used to measure the concentration of mouse anti-SSA (Ro-60, Signosis Inc. Santa Clara, CA) using an ELISA kit (Number EA-5202) according to the manufacturer's protocol. Anti-SSA in the plasma was analyzed in PBS-treated mice (n = 6), in anti-HMGB1-treated mice (n = 12), and in the positive control provided with the kit (n = 2).

Intraorbital glands were minced into small pieces, and sonicated in PRO-PREP Protein Extraction Solution (Intron Biotechnology, Seongnam, Korea) on ice. The supernatant was collected after centrifugation at 12,000 rpm for 20 minutes, and assayed for IL-22 by ELISA, according to the manufacturer's protocol (R&D Systems, Minneapolis, MN). Each sample was assayed twice (n = 8-12).

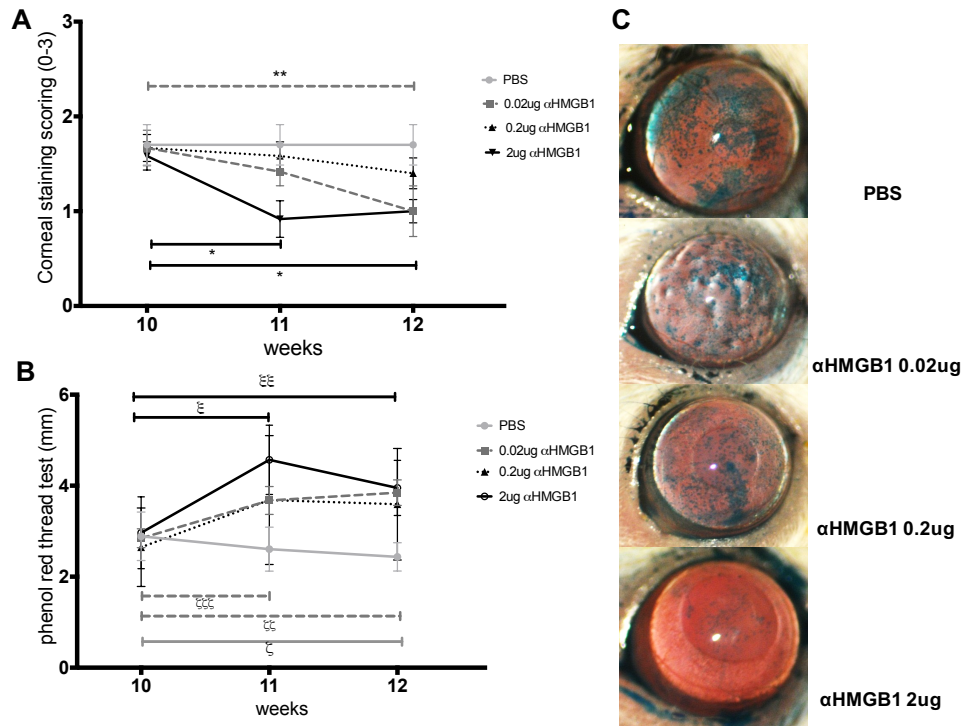
## **2.11. Statistical analysis**

GraphPad Software (GraphPad Prism, Inc., La Jolla, CA, USA) was used for statistical tests. To compare means from more than two groups, data were analyzed using the Kruskal-Wallis test. To compare means of two groups, data were analyzed using the Mann-Whitney test. To compare changes in ocular staining or tear secretion over time (baseline vs. post-treatment), which can be observed in vivo in living animals, data were analyzed using the Wilcoxon matched-pairs signed rank test. Data are presented as mean  $\pm$  standard error (SE). Differences were considered significant at  $p < 0.05$ .

## Chapter 3. Results

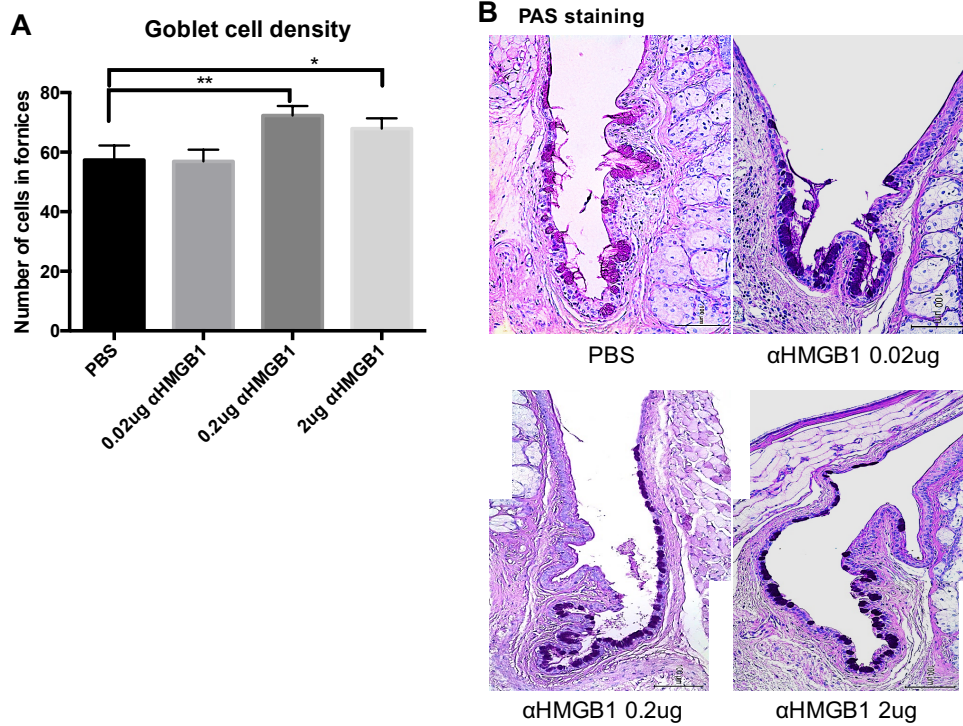
### 3.1. Effect of anti-HMGB1 treatment on the clinical manifestation of dry eye

First, we looked at the effect of anti-HMGB1 treatment on the clinical manifestations of dry eye in NOD.B10.*H2<sup>b</sup>* mice (Fig 2). The representative photos in panel C show the surface changes, and the ocular staining score was significantly decreased in 2 µg anti-HMGB1-treated mice over time, compared with the pre-treatment baseline level (Fig 2A, C; 10 weeks vs. 11 weeks,  $p = 0.016$ ; 10 weeks vs. 12 weeks,  $p = 0.016$ ). The score was also decreased in 0.02 µg anti-HMGB1-treated mice compared to the pre-treatment baseline score (10 weeks vs. 12 weeks,  $p = 0.031$ ). The phenol red thread test (Fig 2B) showed that tear secretion was significantly reduced over time in PBS-treated control mice compared to the baseline (10 weeks vs. 12 weeks,  $p = 0.002$ ). This was expected because of progression of the disease. On the other hand, tear secretion was significantly increased in 0.02 µg anti-HMGB1-treated mice (10 weeks vs. 11 weeks,  $p = 0.027$ ; 10 weeks vs. 12 weeks,  $p = 0.008$ ) and in 2 µg anti-HMGB1-treated mice (10 weeks vs. 11 weeks,  $p < 0.001$ ; 10 weeks vs. 12 weeks,  $p = 0.034$ ) compared to the pre-treatment baseline. In 0.2 µg anti-HMGB1-treated mice, ocular staining tended to be diminished and tear secretion tended to be increased, although neither were statistically significant. Goblet cell density was increased in 0.2 µg anti-HMGB1-treated ( $p = 0.007$  vs. PBS) and 2 µg anti-HMGB1-treated mice ( $p = 0.057$  vs. PBS; marginal significance) 2 weeks after treatment (Fig 3).



**Fig 2. Ocular staining score and tear secretion in NOD.B10 mice after anti-HMGB1 treatment.** (A) Decreased ocular staining score in 2  $\mu$ g anti-HMGB1-treated mice (10 weeks vs. 11 weeks, \*  $p = 0.016$ ; 10 weeks vs. 12 weeks, \*  $p = 0.016$ ) and in 0.02  $\mu$ g anti-HMGB1-treated mice (10 weeks vs. 12 weeks, \*\*  $p = 0.031$ ) compared to the baseline score. (B) Reduced tear secretion in PBS-treated control mice (10 weeks vs. 12 weeks,  $p = 0.002$ ). Increased tear secretion in 0.02  $\mu$ g anti-HMGB1-treated mice (10 weeks vs. 11 weeks,  $p = 0.027$ ; 10 weeks vs. 12 weeks,  $p = 0.008$ ) and in 2  $\mu$ g anti-HMGB1-treated mice (10 weeks vs. 11 weeks,  $p < 0.001$ ; 10 weeks vs. 12 weeks,  $p = 0.034$ ) compared to the pre-treatment baseline. (C) Representative images of ocular staining scoring after treatment at 12 weeks. Data are presented as mean  $\pm$  standard error.



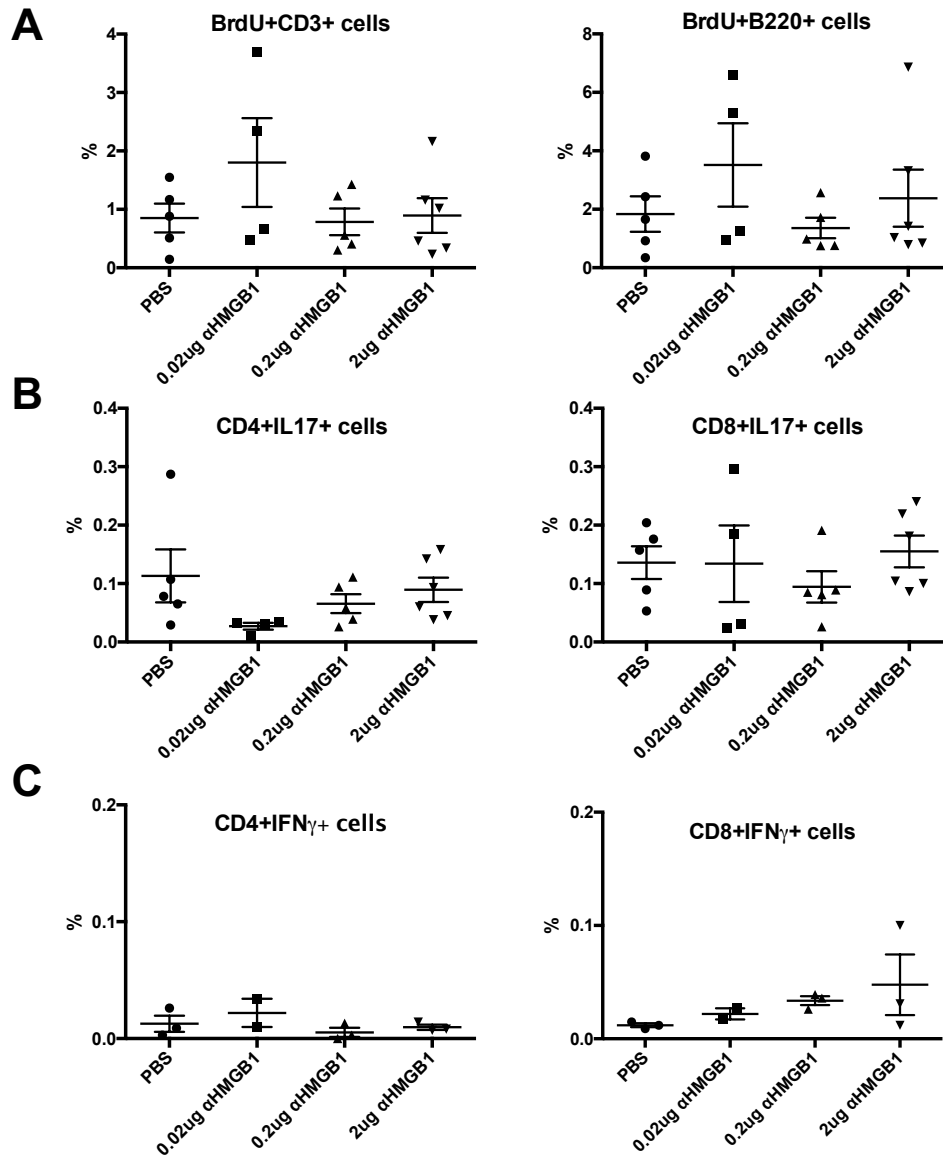


**Fig 3. Goblet cell density in NOD.B10.H2<sup>b</sup> mice conjunctiva after anti-HMGB1 treatment.** (A) Increased goblet cell density in 0.2 μg anti-HMGB1- and 2 μg anti-HMGB1-treated mice (\*\*  $p = 0.007$ , \* $p = 0.057$ ; marginal significance). (B) Representative images of PAS staining of the forniceal conjunctiva (x200). Data are presented as mean  $\pm$  standard error. Scale bar indicates 100 μm.

### 3.2. Changes in immunologic responses in the extraorbital glands, plasma, and draining lymph nodes

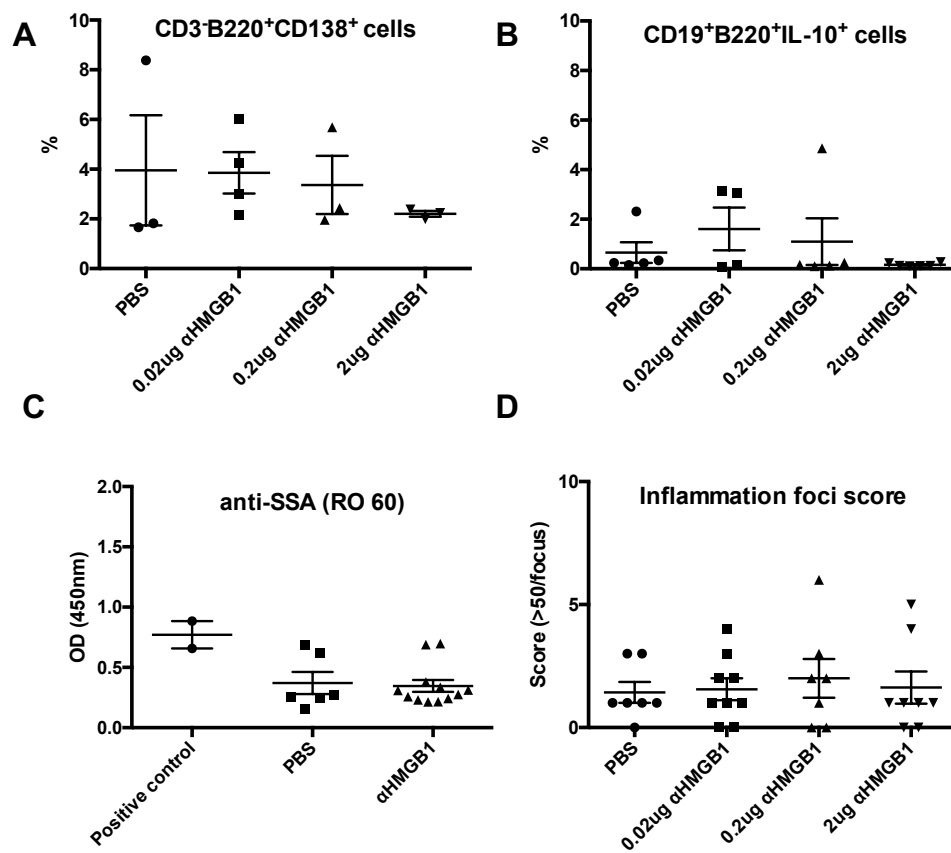
Next, we evaluated changes in immunologic responses in the extraorbital glands, plasma, and draining lymph nodes 2 weeks after treatment (Fig 4-5). The percentage and number of BrdU<sup>+</sup>CD3<sup>+</sup> T and BrdU<sup>+</sup>B220<sup>+</sup> B cells (S phase, S2 Fig) in the draining lymph nodes of treated mice were not significantly altered compared to that in controls (Fig 4A). For effector T cell responses, the percentage of Th17

cells (CD3<sup>+</sup>CD4<sup>+</sup>IL-17<sup>hi</sup>) and Tc17 cells (cytotoxic T cells that secrete IL-17; CD3<sup>+</sup>CD8<sup>+</sup>IL-17<sup>hi</sup>) did not show any significant changes (Fig 4B). IFN $\gamma$ -secreting T cells (CD3<sup>+</sup>CD4<sup>+</sup>IFN $\gamma$ <sup>hi</sup> or CD3<sup>+</sup>CD8<sup>+</sup>IFN $\gamma$ <sup>hi</sup>) did not change following treatment (Fig 4C). For functional B cell responses, no significant changes were found in the percentage of plasma cells (CD3<sup>+</sup>B220<sup>+</sup>CD138<sup>+</sup> cells) or IL-10-secreting B regulatory cells (CD3<sup>+</sup>CD19<sup>+</sup>B220<sup>+</sup>IL-10<sup>hi</sup>) in the draining lymph nodes (Fig 5A and B). Levels of anti-SSA did not significantly decrease in the plasma (Fig 5C). The infiltrating focus of CD3<sup>+</sup> T cells was very similar to that of B220<sup>+</sup> B cells (S3 Fig). Inflammatory foci scores in the extraorbital glands were not different among the groups (Fig 5D).



**Fig 4. Changes in percentage of adaptive immune cells in draining lymph nodes of NOD.B10.H2<sup>b</sup> mice after anti-HMGB1 treatment.** (A) BrdU staining showing no significant proliferative changes in CD3<sup>+</sup> T cells or B220<sup>+</sup> B cells. (B) Percentage of Th17 (CD3<sup>+</sup>CD4<sup>+</sup>IL-17<sup>hi</sup>) cells and Tc17 (CD3<sup>+</sup>CD8<sup>+</sup>IL-17<sup>hi</sup>) cells were not altered after treatment. (C) IFN $\gamma$ -secreting T cells (CD3<sup>+</sup>CD4<sup>+</sup>IFN $\gamma$ <sup>hi</sup> or CD3<sup>+</sup>CD8<sup>+</sup>IFN $\gamma$ <sup>hi</sup>) were not affected by treatment. Data are presented as mean  $\pm$  standard error. (Th17, T helper cells secreting IL-17; Tc17, cytotoxic T cells secreting

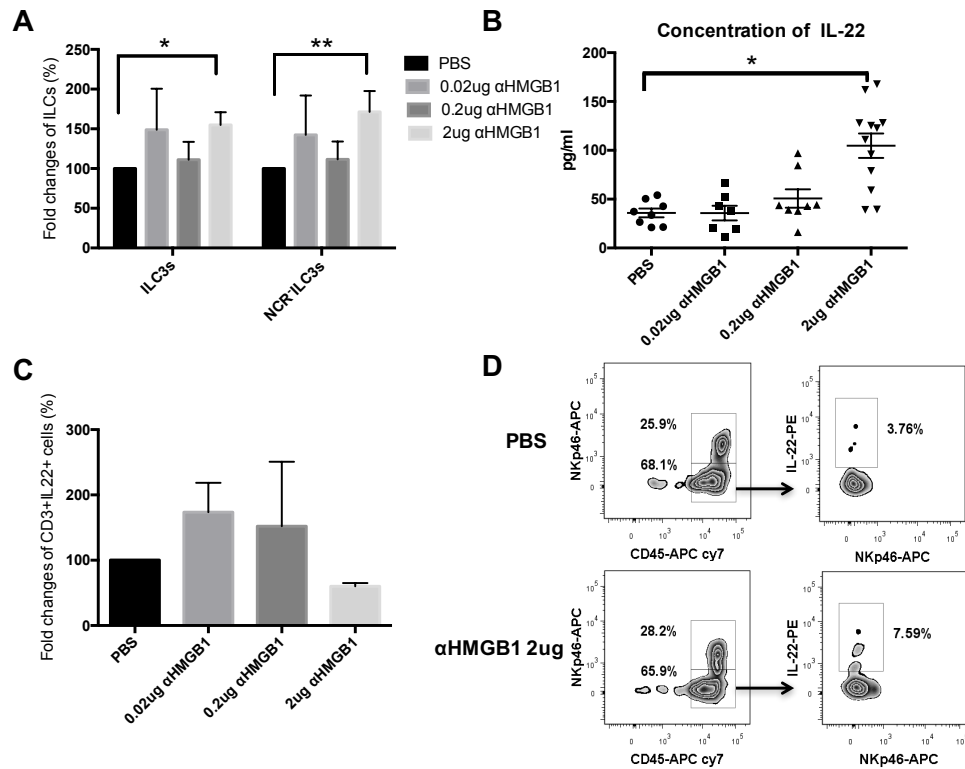
IL-17).



**Fig 5. Changes in functional B cells in cervical lymph nodes, anti-SSA antibodies in serum, and inflammation foci scores in extra orbital lacrimal glands in NOD.B10.H2<sup>b</sup> mice after anti-HMGB1 treatment.** (A) No significant change in percentage of plasma cells (CD3<sup>+</sup>B220<sup>+</sup>CD138<sup>+</sup> cells). (B) No change in IL-10-secreting B regulatory cells (CD3<sup>+</sup>CD19<sup>+</sup>B220<sup>+</sup>IL-10<sup>hi</sup>). (C) No change in the level of anti-SSA (RO 60) antibodies after treatment. (D) No significant changes in inflammatory foci scores (> 50 lymphocytes/focus) among all groups. Data are presented as mean ± standard error.

### 3.3. Changes in innate lymphoid cell type 3 in the draining lymph nodes

ILC3s are known to be involved in epithelial wound healing via secretion of IL-22. Therefore, we investigated changes in ILC3s in the draining lymph nodes 2 weeks after treatment (Fig 6A and D). Fold changes in the percentage of ILC3s (NCR<sup>+</sup>ILC3s; CD3<sup>+</sup>B220<sup>-</sup>CD45<sup>+</sup>Nkp46<sup>+</sup>IL-22<sup>hi</sup> or NCR<sup>+</sup>ILC3s; CD3<sup>+</sup>B220<sup>-</sup>CD45<sup>+</sup>Nkp46<sup>+</sup>IL-22<sup>hi</sup>) were significantly increased compared to controls (PBS vs. 2 µg anti-HMGB1,  $p = 0.025$ ). NCR<sup>+</sup>ILC3s (CD3<sup>+</sup>B220<sup>-</sup>CD45<sup>+</sup>Nkp46<sup>+</sup>IL-22<sup>hi</sup>) were also increased after 2 µg anti-HMGB1 treatment (PBS vs. 2 µg anti-HMGB1,  $p = 0.014$ ). IL-22 levels were significantly increased in the intraorbital glands after 2 µg anti-HMGB1 treatment (PBS vs. 2µg anti-HMGB1,  $p = 0.025$ , Fig 6B). There are three major populations of IL-22 secreting cells, namely Th22 cells, ILC3s, and  $\gamma\delta$  T cells. The percentage of CD3<sup>+</sup>IL-22<sup>hi</sup> cells was not different among the groups, suggesting that the increase in IL-22 secretion is not related to Th22 or  $\gamma\delta$  T cells, which express CD3 (Fig 6C).



**Fig 6. Changes in innate lymphoid cells (ILCs) in draining lymph nodes and changes in IL-22 expression in intraorbital glands after anti-HMGB1 treatment.**

(A) Fold changes in ILC3 percentage (CD3<sup>+</sup>B220<sup>-</sup>CD45<sup>+</sup>IL-22<sup>hi</sup> cells; NCR<sup>+</sup> or NCR<sup>-</sup> ILC3) showing a significant increase following 2  $\mu$ g anti-HMGB1 treatment compared with control (PBS vs. 2  $\mu$ g anti-HMGB1, \* $p$  = 0.025). Fold increase in NCR<sup>-</sup> ILC3 percentage (CD3<sup>+</sup>B220<sup>-</sup>CD45<sup>+</sup>NKp46<sup>-</sup>IL-22<sup>hi</sup> cells) (PBS vs. 2  $\mu$ g anti-HMGB1, \*\* $p$  = 0.0142). (B) Increased IL-22 levels after 2  $\mu$ g anti-HMGB1 treatment (PBS vs. 2  $\mu$ g anti-HMGB1, \* $p$  = 0.025). (C) No change in percentage of CD3<sup>+</sup>IL-22<sup>hi</sup> cells (Th22 cells or  $\gamma\delta$  T cells) in draining lymph nodes. (D) Representative images of NCR<sup>-</sup> ILC3s (CD3<sup>+</sup>B220<sup>-</sup>CD45<sup>+</sup>NKp46<sup>-</sup>IL-22<sup>hi</sup> cells) in PBS- and 2  $\mu$ g anti-HMGB1-treated groups. Data are presented as mean  $\pm$  standard error. NCR, natural cytotoxicity receptor.

## Chapter 4. Discussion

This study indicates that subconjunctival administration of anti-HMGB1 attenuates the clinical manifestations of dry eye. Furthermore, the data also suggest that the improvement in dry eye in NOD.B10.*H2<sup>b</sup>* mice may involve an increase in IL-22-secreting ILC3s, rather than modulation of B or plasma cells.

The specific mechanisms that initiate inflammation in Sjögren's syndrome are not fully understood, but we considered that the responses of reactive B cells and plasma cells that secrete autoantibodies could be key to maintaining the pathological condition [1]. Mouse models have clearly demonstrated the role of B and plasma cells in this process [4, 5]. We focused on extracellular HMGB1 because we believe that the chronic epithelial cell damage to the cornea or lacrimal glands may trigger the cycle of inflammation by secreting danger signals, such as HMGB1. It is plausible to assume that extracellular HMGB1 acts as an inflammatory cytokine through TLR9 signaling on B cells in this mouse model of Sjögren's syndrome. Considering that TLR9 expression is abundant on B cells and plasmacytoid dendritic cells [17], we proposed that anti-HMGB1 treatment would reduce the effector function of B cells in these mice. However, B cell proliferation and the proportion of plasma cells, as well as anti-SSA levels, did not change in the draining lymph nodes. We did not try to administer anti-HMGB1 systemically owing to the high cost. One reason for the failure to observe B cell modulation could be that the subconjunctival injection of anti-HMGB1 did not reach the spleen, where autoreactive B cells reside, at a sufficient therapeutic level to affect already activated B or plasma cells in the draining lymph nodes. In addition, B cell proliferation and the proportion of plasma cells were not affected following subconjunctival anti-HMGB1 treatment in the spleen (unpublished data).

It is also possible that early treatment with anti-HMGB1 did not have a

measurable effect on inflammation because the epithelial damage was not sufficiently severe to secrete enough extracellular HMGB1 and induce inflammation. In addition, HMGB1 can increase Th17 cells by upregulating IL-6 or IL-23 [22-24]. Our study showed that anti-HMGB1 treatment did not affect the function of Th17 cells or Tc17 cells. Taken together, locally administered extracellular HMGB1 did not have a significant effect on adaptive immunity associated inflammation in this mouse model. We did not investigate changes in Treg cells after anti-HMGB1 treatment; therefore, further evaluation will be required to explore immune modulation following anti-HMGB1 treatment in more depth.

Despite the fact that we did not observe modulation of the effector function of B cells, the clinical manifestations of dry eye were still improved in this model. An increase in goblet cell density could indicate a protective effect due to an increase in tear secretion and a recovery of epithelial homeostasis. We therefore investigated the effect of IL-22-secreting ILC3 cells on epithelial damage in the dry eye model. This is based on the fact that ILC3 cells may play a role in epithelial homeostasis and wound healing as well as having a role in immune modulation [25-29].

ILCs are found mainly in mucosal tissues, including the intestinal tract (ILC3 and ILC2), lungs (ILC2), skin (ILC2), and tissues associated with lymphoid structures (ILC2) [30]. Emerging evidence shows that ILCs may function to maintain immune tolerance, homeostasis, or to inversely induce a T cell response by presenting foreign antigens or by secreting pro-inflammatory cytokines, depending on environmental cues and ILC subtype [14, 15, 29, 31, 32]. The circumstances that determine this bidirectional role of ILCs (immune regulation versus inflammation/autoimmunity) are still unclear. The protective function of ILC3 cells on epithelial homeostasis through IL-22 is well established in the gut, but their role in epithelial homeostasis in the eye has not been investigated. Under normal conditions, ILC3 distribution is scarce in the cervical lymph nodes (unpublished data), while



inflammatory conditions, such as the mouse model for Sjögren's syndrome (NOD.B10.*H2<sup>b</sup>*) or an experimental uveitis model, show a large number of ILC3 cells in the cervical lymph nodes (unpublished data). The increase in ILC3 cells in the draining lymph nodes and the increased IL-22 expression in the intraorbital glands after anti-HMGB1 treatment suggest a possible role for ILC3s in the improvement of dry eye in this model. Although we could not discriminate between changes in Th22 and changes in  $\gamma\delta$  T cells, the fact that the changes in CD3<sup>+</sup>IL-22<sup>hi</sup> cells, including both Th22 and  $\gamma\delta$  T cells, were not significant suggests that the increase in IL-22 may be caused mainly by ILC3s. Our study showed a higher distribution of NCR<sup>-</sup> ILC3s (Nkp46<sup>-</sup>) and an increase in NCR<sup>-</sup>ILC3s (Nkp46<sup>-</sup>) in the cervical lymph nodes compared to NCR<sup>+</sup>ILC3s (Nkp46<sup>+</sup>) following treatment. These lineage-negative NCR<sup>-</sup> ILC3s (CD3<sup>-</sup>B220<sup>-</sup>Nkp46<sup>-</sup>CD45<sup>+</sup>IL-22<sup>hi</sup> cells) are a subset of ILC3s known to effectively promote epithelial wound healing in the intestine [30, 33]. Our data suggest that NCR<sup>-</sup>ILC3s, rather than NCR<sup>+</sup>ILC3s, may function during dry eye (S4 Fig).

Many cytokines show pleiotropic effects on cellular homeostasis depending on the concentration (lower or higher than normal endogenous levels). Extracellular HMGB1 also appears to have diverse effects on different cells, depending on the concentration or redox status of cysteine. Lower levels of endogenous HMGB1 may be required for optimal wound closure [34], while a higher level of HMGB1 acts as a pro-inflammatory cytokine and does not affect epithelial wound healing [12, 34]. HMGB1 with disulfide bonded-cysteine or reduced-cysteine exhibits inflammatory activities or chemotaxis, while HMGB1 with oxidized-cysteine exhibits tolerogenic activities [7, 35, 36]. In our autoimmune dry eye model, in spite of not measuring the exact level, we believe that the level of extracellular HMGB1 is higher than endogenous levels, which then provokes inflammation. This could explain why blocking HMGB1 improves dry eye, although the exact redox status of cysteine is

not known. On preliminary experiments, clinical dry eye and early inflammation was confirmed to have begun by 10 weeks (S1 Fig). Moreover, severity of inflammation progressively increased until the last follow-up time point of 16 weeks in preliminary study. Therefore 10 week-old mice were used to evaluate effect of anti-HMGB1 antibody on attenuating inflammation in Sjögren's syndrome in the current study. It was also because irreversible destruction of lacrimal gland with the most severe inflammation may work as a bias in evaluation of the effect of blocking HMGB1 in inflammation.

The present study had several limitations. First, the number of the animals was small owing to limited breeding. We were not able to directly reveal a plausible mechanism for how anti-HMGB1 treatment could cause an increase in ILC3. Given the fact that HMGB1 can interact with Treg cells or directly with conventional T cells and modulate them [37], there are several possible direct or indirect pathways through which HMGB1 could communicate with ILCs, which have not yet been discovered. This study was also unable to provide direct evidence of a correlation between the increase in ILC3s and clinical improvement. We did not perform adoptive transfer because of technical difficulties, since the number of ILCs was very small. Therefore, further studies to investigate the exact role of ILCs are warranted. Nevertheless, to our knowledge, this is the first study to report an improvement in dry eye after anti-HMGB1 treatment and to propose a possible role for ILC3s in this mouse model. Furthermore, this improvement in primary Sjögren's syndrome disease model may shed some light on the possibility of better therapeutic effects on environmental dry eye which could have less severe inflammation than primary Sjögren's syndrome. Although the current study used subconjunctival injection to deliver drug effectively with less cost and more bioavailability, topical application of this blocking antibodies may still have similar effect in this perspective. Further studies regarding this issue and appropriate dosage should be warranted in order to

benefit clinical situation from this study.

## Chapter 5. Conclusion

In conclusion, subconjunctival administration of anti-HMGB1 improved the clinical manifestations of dry eye in NOD.B10.*H2<sup>b</sup>* mice, although the anti-HMGB1 treatment did not affect B or plasma cells. Considering the increase in group 3 ILCs in the draining lymph nodes and the increase of IL-22 expression in the intraorbital glands after anti-HMGB1 treatment, a possible role for group 3 ILCs in the improvement of dry eye in this mouse model of primary Sjögren's syndrome is suggested.

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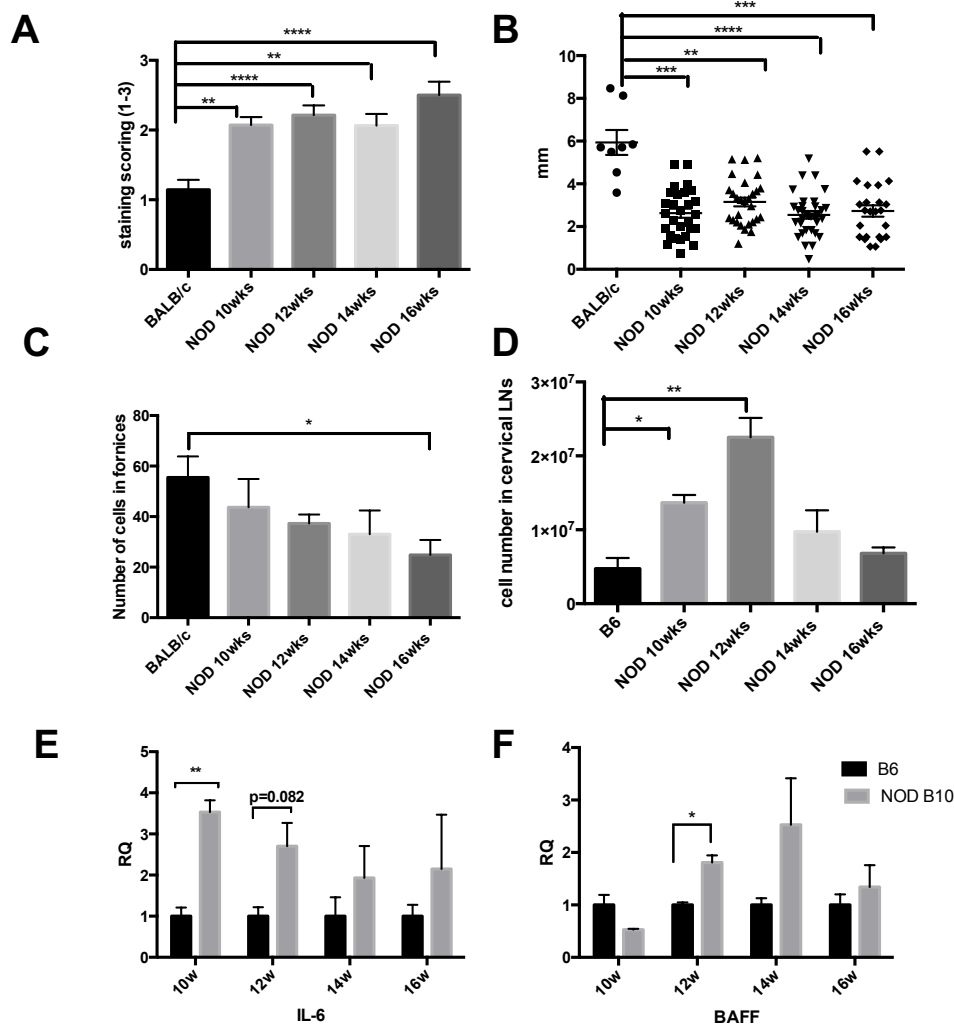
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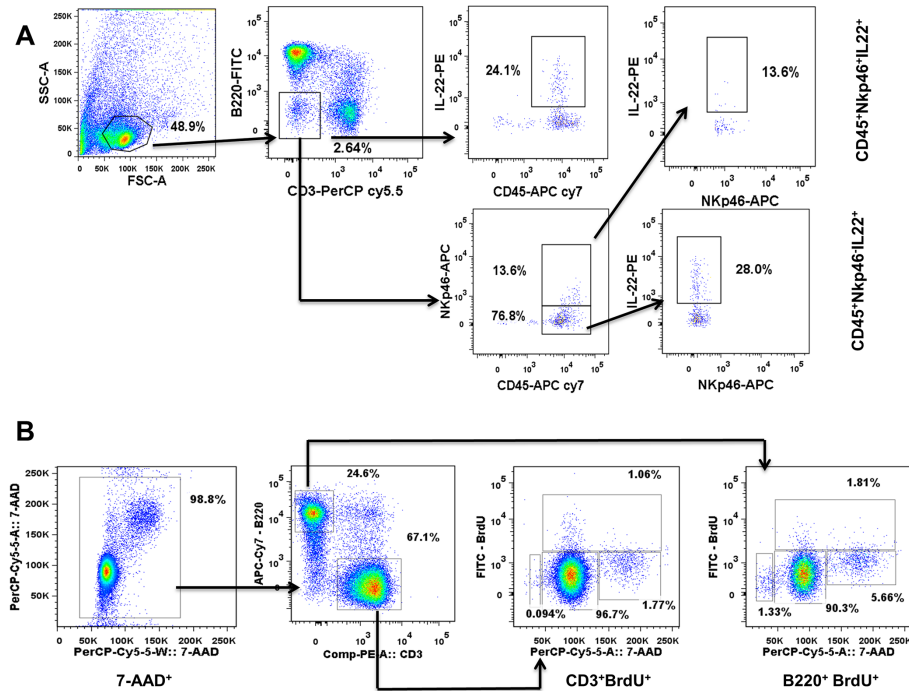


## Supporting Information

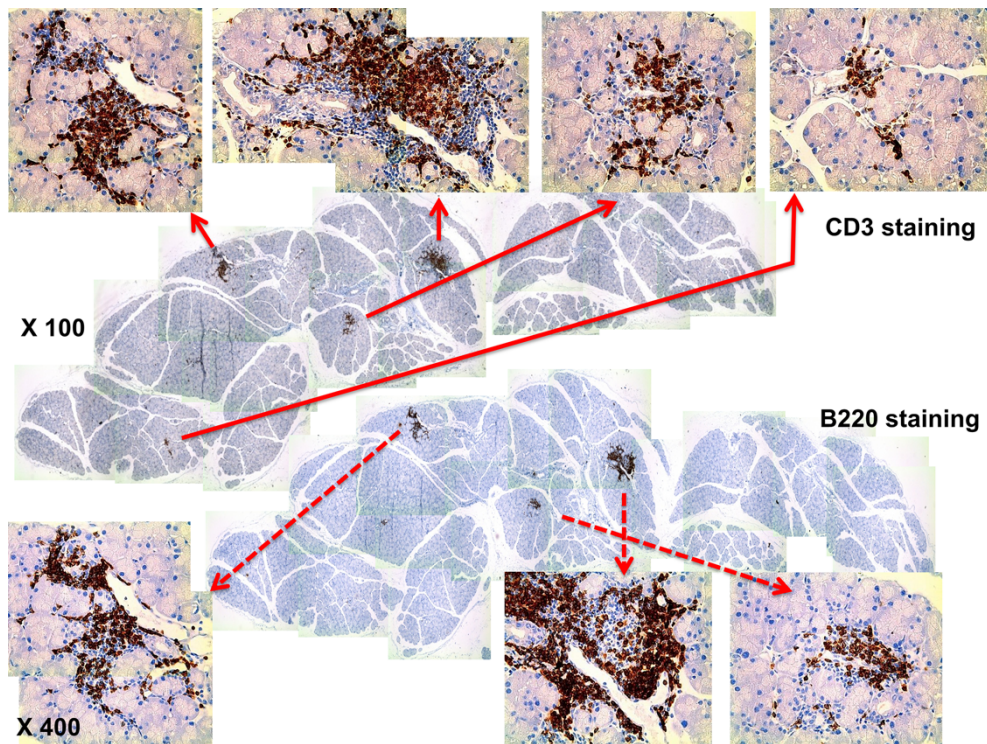


**S1 Fig. Preliminary experiments showing time course of clinical manifestation of dry eye and early inflammatory changes in cervical lymph nodes and intraorbital glands in NOD.B10.H2<sup>b</sup> mice.** Clinical manifestation of dry eye and inflammatory responses were evident by 10 weeks. (A) Significantly decreased ocular staining score in NOD.B10.H2<sup>b</sup> mice at 10 weeks compared to BALB/c mice (Kruskal-Wallis test, \*\*p < 0.01, \*\*\*\*p < 0.0001). (B) Phenol red thread test showing a significant decrease in tear secretion in NOD.B10.H2<sup>b</sup> mice at 10 weeks compared to BALB/c mice (Kruskal-Wallis test, \*\*p < 0.01, \*\*\*p < 0.001, \*\*\*\*p < 0.0001). (C) Goblet cell density in conjunctiva of NOD.B10.H2<sup>b</sup> mice showing a decrease at 16 weeks compared to BALB/c mice (Kruskal-Wallis test, \* p < 0.05). (D) Increased cell numbers in cervical lymph nodes of NOD.B10.H2<sup>b</sup> mice at 10 to 14 weeks compared

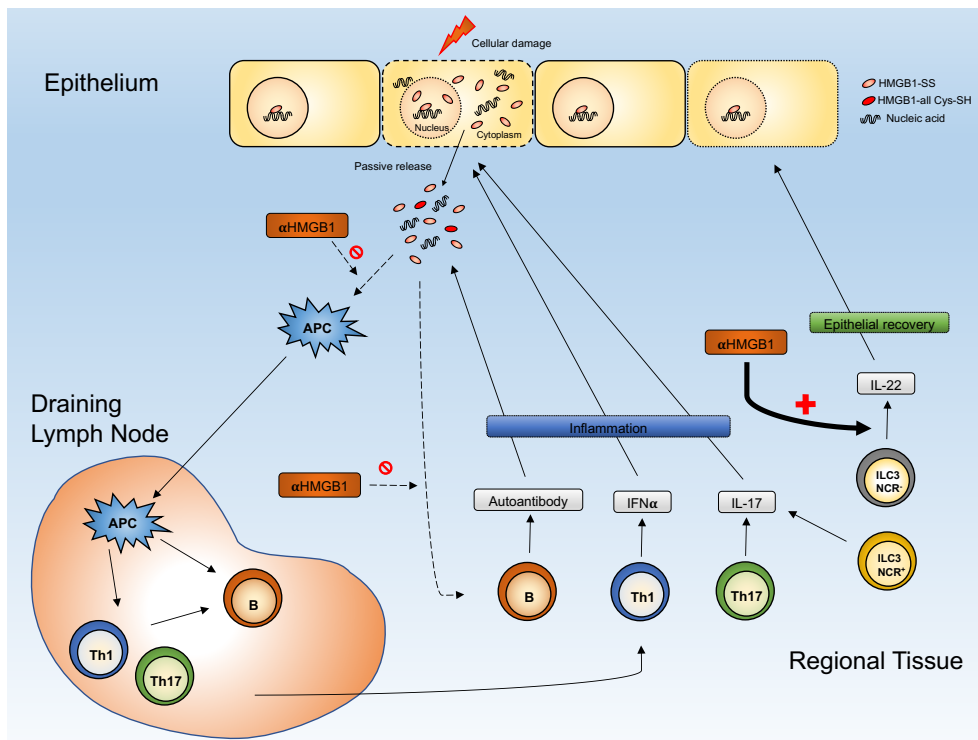
to 10 week-old B6 mice (Kruskal-Wallis test, \*  $p < 0.05$ , \*\* $p < 0.01$ ). (E-F) Increased IL-6 and BAFF levels in NOD.B10.*H2<sup>b</sup>* mice at 10-12 weeks compared to controls (one-way ANOVA, \* $p < 0.05$ , \*\* $p < 0.01$ ). RQ indicates a ratio of mRNA levels relative to controls. NOD, NOD.B10.*H2<sup>b</sup>* mice.



**S2 Fig. Sorting BrdU<sup>+</sup> cells and ILC3s.** (A) ILC3s were negatively gated for anti-CD3 and anti-B220 antibodies and positively gated for anti-CD45 and anti-IL-22 antibodies. Subpopulation of NCR<sup>+</sup> ILC3s (CD3<sup>+</sup>B220<sup>-</sup>CD45<sup>+</sup>Nkp46<sup>+</sup>IL-22<sup>hi</sup> cells) and NCR<sup>-</sup> ILC3s (CD3<sup>+</sup>B220<sup>-</sup>CD45<sup>+</sup>Nkp46<sup>+</sup>IL-22<sup>hi</sup> cells) were subsequently gated. (B) During the acquisition preview, gates were adjusted in the FSC-A vs. SSC-A plot, and the DNA 7-AAD-A voltage was adjusted to place the mean of the singlet peak (G0/G1) at 50,000 on the histogram. In addition, cell cycle gates were adjusted as needed to encompass the G0/G1, S, and G2/M populations. NCR, natural cytotoxicity receptor.



**S3 Fig. Immunohistochemistry of the extraorbital glands of NOD.B10.H2<sup>b</sup> mice.**  
 The infiltrating focus of CD3<sup>+</sup> T cells (red arrows, upper panel, x400) almost matched that of B220<sup>+</sup> B cells (red dashed arrows, lower panel, x400).



**S4 Fig. Role of anti-HMGB1 antibody in the pathogenesis of Sjögren's syndrome.** The chronic epithelial cell damage to the cornea or lacrimal glands may trigger the cycle of inflammation by secreting danger signals, such as HMGB1, in addition to known roles of B cells and T cells to contribute on vicious cycle of immune activation in Sjögren's syndrome (Dashed arrows). In the present study, considering the increase in group 3 innate lymphoid cells (ILCs) in the draining lymph nodes and the increase of interleukin (IL)-22 expression in the intraorbital glands after anti-HMGB1 treatment, a possible role for group 3 ILCs in the improvement of dry eye in this mouse model of primary Sjögren's syndrome is suggested (Bold arrow).

HMGB1 = high mobility group box 1; HMGB1-SS = disulfide-bonded form of HMGB1; HMGB1-all Cys-SH = cysteine all-reduced HMGB1 (cysteines at positions 23, 45, and 106 of HMGB1 express a thiol group); NA = nucleic acid; αHMGB1 = anti-HMGB1 antibody; APC = antigen presenting cell; Th = T helper; ILC3 = group 3 innate lymphoid cell; NCR = natural cytotoxicity receptor.

## 국문초록

# 쇼그렌 증후군 마우스 모델에서 결막하 투여된 항HMGB1이 건성안에 미치는 영향

**목적** : 세포외부에서 HMGB1 (High mobility group box 1)은 DAMP (Damage associated molecular pattern)분자로 작용하여 Toll-like 수용체 (Toll-like receptor, TLR)를 통해 자가반응성 B 세포의 활성화를 촉진하며 이러한 기전을 통해 쇼그렌 증후군의 발병기전에 관여할 수 있다. 특히 세포외로 분비된 HMGB1은 염증질환에서 인터루킨 (Interleukin, IL)-17을 분비하는 도움 T 세포 (Th17)의 활성화에 연관되어 있는 것으로 보고되고 있으며, 선천면역과 적응면역 모두에서 중요한 역할을 하는 선천 림프양 세포 (Innate lymphoid cells, ILC)가 IL-17 또는 IL-22를 분비하는 데에도 관련될 수 있다. B 세포와 형질세포에서 TLR9이 풍부하게 표현되어 있으며, 쇼그렌 증후군의 병인에 있어 자가반응성 항체를 분비하는 B 세포와 형질세포의 중요성을 고려할 때, 만성적인 각막이나 눈물샘 상피세포의 손상으로 인해 분비된 HMGB1이 위험신호로 작용하여 염증의 악순환을 시작하는데 기여할 것으로 생각하였다. 따라서 이 연구는 쇼그렌 증후군의 마우스 모델을 이용하여 항HMGB1의 결막하 투여 후 건성

안 임상 양상과 면역반응 변화를 알아봄으로써 건성안에 미치는 영향에 대해 평가하고자 하였다.

**방법** : 원발성 쇼그렌 증후군 모델로서 NOD.B10.H2<sup>b</sup> 마우스를 이용하였으며, 연쇄적 염증 반응의 초기 단계에서 HMGB1의 염증 유발에 대한 억제 효과를 평가하기 위해, 기존의 예비 실험의 결과에 근거하여 10주령의 마우스를 대상으로 하였다. 24마리의 마우스는 4군으로 나누어 각각 항HMGB1항체 (0.02, 0.2, 2 µg/0.1 cc) 또는 면역글로불린Y를 포함하는 인산염완충식염수 (Phosphate buffered saline, PBS)를 주2회씩 2주연속 결막하 주사하였다.

임상양상의 변화를 평가하기 위해, 치료 전과 후의 눈물양 및 각막염색 점수를 측정하고 비교하였다. 결막구석조직의 PAS (Periodic acid Schiff)염색을 통해 술잔세포밀도를 측정했다.

면역반응변화의 평가를 위해, 경부림프절의 유세포분석을 시행하였으며, BrdU<sup>+</sup> 세포, IL-17 분비 세포, IL-10 분비 세포, IFN $\gamma$  분비 세포, 기능성 B 세포, IL-22 분비 선천 림프양 세포 (group 3 ILCs)의 변화를 측정했다. 혈장내 항SSA 항체의 농도와 안와내눈물샘에서의 IL-22 농도는 효소결합 면역흡수법 (Enzyme linked immunosorbent assay, ELISA)로 측정하였다. 안와외부눈물샘 조직을 CD3와 B220 면역조직화학염색을 시행한 후 염증병터점수(병터당 세포수 50개 이상)를 계산하였다.

**결과** : 0.02µg과 2µg의 항HMGB1항체를 투여하였을 때 각막상피미란이 유의하게 완화되었다 ( $p<0.05$ ). 눈물량은 0.02µg과 2µg의 항HMGB1항체

를 투여한 군에서 유의하게 증가하였다 ( $p<0.05$ ). 비록 통계적 유의성은 보일 수 없었지만, 0.2 $\mu$ g의 항HMGB1항체를 투여한 군에서도 각막상피 미란과 눈물분비량에서 임상증상이 호전되는 경향을 보였다. 술잔세포밀도는 항HMGB1항체 투여 후 증가하는 경향을 보였으며, 0.2 $\mu$ g과 2 $\mu$ g의 용량을 투여한 군에서 통계적 유의성에 근접하였다.

항HMGB1항체를 투여한 군의 유출림프절에서 BrdU<sup>+</sup>CD3<sup>+</sup> 세포와 BrdU<sup>+</sup>B220<sup>+</sup> 세포의 백분율 및 숫자는 대조군에 비해 유의하게 변화하지 않았다. 효과 T 세포의 반응에 있어서, Th17 세포와 IL-17을 분비하는 세포독성 T 세포의 백분율은 차이가 없었으며, IFN $\gamma$  분비 세포도 항HMGB1항체 투여 후 변화를 보이지 않았다. 기능성 B 세포 반응에 있어서 형질세포나 조절성 B 세포의 백분율에 유의한 차이는 보이지 않았으며, 혈장내 항SSA 항체의 농도도 유의한 변화를 보이지 않았다. 안와의 부눈물샘 조직에서 CD3<sup>+</sup> T 세포의 침윤 병태는 B220<sup>+</sup> B 세포의 분포와 유사하였다. 경부림프절에서 IL-22 분비 선천 림프양 세포 (group 3 ILCs)가 유의하게 증가하였으며 ( $p<0.05$ ), 0.2 $\mu$ g의 항HMGB1항체를 투여한 군에서 안와내눈물샘에서의 IL-22 발현도 유의하게 증가하였다.

**결론** : 쇼그렌 증후군 마우스 모델을 이용한 본 연구를 통해, 항HMGB1항체의 결막하 투여가 건성안의 임상증상을 완화시키는 것을 알 수 있었으며, B 세포나 형질세포의 조절보다는 IL-22 분비 선천 림프양 세포 (group 3 ILCs)의 증가가 이러한 건성안 증상의 호전의 기전에 관여할 것으로 생각된다.

**주요어:** cornea; dry eye; Sjögren's syndrome; HMGB1; innate lymphoid cell  
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