Lacrimal Gland Inflammation Deregulates Extracellular Matrix Remodeling and Alters Molecular Signature of Epithelial Stem/Progenitor Cells

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PURPOSE. The adult lacrimal gland (LG) is highly regenerative and is able to repair itself even after substantial damage; however, this ability to regenerate is lost with the development of dry eye conditions in chronically inflamed LGs. This study compares changes in the cell adhesion and cell matrix molecules and stem cell transcription factors in the LGs of healthy mice and of two mouse models of Sjögren's syndrome: nonobese diabetic (NOD) and MRL-lpr/lpr (MRL/lpr) mice during the early stage of inflammation.

METHODS. The LGs from 12- to 13-week-old female MRL/lpr and male NOD mice along with their respective control strains were harvested and divided into three pieces and processed for quantitative (q) RT-PCR and qRT-PCR Arrays, histology, immunohistochemistry, and Western blotting.

RESULTS. The extracellular matrix (ECM) and adhesion molecules RT²-PCR array combined with protein expression data revealed changes in the expression of integrins, matrix metalloproteinases, and other molecules, which are associated largely with invasion, attachment, and expansion of the lymphocytic cells, whereas changes in the stem cell transcription factors revealed substantial decrease in expression of transcription factors associated with epithelial stem/progenitor cell lineage.

CONCLUSIONS. We concluded that the expression of several important ECM components is significantly deregulated in the LG of two murine models of Sjögren's syndrome, suggesting an alteration of the epithelial stem/progenitor cell niche. This may result in profound effects on localization, activation, proliferation, and differentiation of the LG stem/progenitor cells and, therefore, LG regeneration.

Keywords: dry eyes, lacrimal gland, stem cell markers, extracellular matrix, gene expression

The main function of the lacrimal gland (LG) is to produce secretions that lubricate and protect the ocular surface. Aqueous tear deficiency, called dry eye, results from multifactorial disorders of the tear film. This condition has been reported to be increasing in recent years due to pollution, aging, substantial increase in the development of autoimmune diseases within the human population, and dietary and cultural changes.^{1–3}

It has been shown recently that murine LG has a remarkable ability to repair itself upon acute injury; however, LG repair fails in the case of chronic inflammation.^{4,5} The reason for lack of repair during chronic inflammation is not really clear; however, several reports suggest that permanent extracellular matrix (ECM) remodeling affects regeneration processes in diseased LGs.^{5–7} It also is possible that changes in the ECM during chronic inflammation may affect stem/progenitor cell function.⁵ However, to our knowledge the last hypothesis has not been explored.

Lacrimal gland injury/inflammation induces the process called epithelial-mesenchymal transition (EMT).^{5,8} During EMT, epithelial cells lose cell-cell attachment and polarity, and epithelial-specific markers, undergo cytoskeletal remodeling

and gain a mesenchymal phenotype.^{9,10} During the repair phase, ECM is remodeled and replaced with new proteoglycans, collagen, and elastin fibers. New ECM is invaded by proliferating and differentiating epithelial cells, dermal fibroblasts, nerves, and vascular endothelial cells from adjacent uninjured tissue and stem cells that move to the wound site.⁹ Increased degradation of ECM structures of the LG has been implicated in the pathogenesis of Sjögren's syndrome (SjS).^{11,12} Chronic inflammation maintains increased levels of different proteases, such as metalloproteinases (MMPs), elastase, and other proteolytic enzymes, which permanently obliterate components of the ECM, and change normal growth factor distribution and the number of growth factor receptors that are essential for successful repair and cell differentiation.^{11–13} Metalloproteinase-2 and -9 (gelatinases A and B) have key roles in the migration of LG epithelial cells during wound healing.^{14,15} This idea has been supported by publications showing the therapeutic effect of MMP-9 inhibition in the rabbit lacrimal gland inflammation model.^{16,17} Furthermore, elevated levels of MMP-9 and MMP-2 are strongly associated with the inflammatory cells, which invade diseased LG.^{18–21}

Other important molecules for maintaining LG acinar integrity are proteins forming intercellular contacts.^{22–24} Alteration of genes encoding interepithelial junctional complexes, such as tight junctions (TJ), adherens junctions, desmosomes, and gap junctions, affects permeability of the paracellular spaces between the LG epithelial barrier.²⁵ Therefore, loss of gap junctions or alterations in their regulation affects secretory ability of the gland and may lead to some form of dry eye condition.²⁴ Changes in the components of TJ proteins ZO-1 and occludins could be involved in LG pathogenesis in SjS or aging.^{13,26,27} In addition, E-cadherin (a component of adherens junction) is very important for LG epithelial integrity and epithelial differentiation.²⁸ Even slight downregulation of E-cadherin expression may mediate EMT in the LG.²⁸

Lacrimal gland repair and function also depend on signaling between epithelial cells and the ECM.^{29,30} Integrins are important transmembrane receptors that bridge cells with the ECM in many tissues following injury and during regeneration.³¹ Although the role of integrins in LG injury/inflammation has not been defined clearly, several publications suggest their importance for normal LG function. Thus, cell adhesion through integrins regulates secretion from LG acinar cells³² and integrins expression/localization is altered during chronic LG inflammation.³³

A variety of mouse models have been developed to study pathological conditions of LG inflammation.^{34,35} Lacrimal glands of nonobese diabetic (NOD) and MRL-lpr/lpr (MRL/lpr) mice display many features similar to LGs in patients afflicted with SjS.³⁴ Both mouse models spontaneously develop SjS-like disease phenotype, such as lymphocytic infiltrates in the salivary and LGs, and secretory dysfunction at approximately 12 to 16 weeks of age.^{12,35} However each specific mouse model does not reflect the complete disease profile of SjS in human patients. A very attractive paradigm for understanding of mechanisms of SjS is defining common markers or target molecule characteristics for inflammation in different mouse models and in patients that could be used for advanced diagnostics or intervention to treat dry eye conditions.

Although the roles of ECM in mechanisms that control LG inflammation/repair still are not completely clear, several therapeutic interventions with the potential to correct degraded ECM have been tested recently. One of the recent directions for therapy is targeting adhesion molecules.³⁶ For example, leukocyte adhesion and transmigration through the endothelial cell layer is regulated tightly by the expression of certain adhesion molecules and direct interaction between microvasculature cells and leukocytes. In addition, it has been shown that inhibition of T-cell binding to ICAM-1 decreases ocular surface inflammation in dogs.³⁶ Molecular modulation strategies that targeted integrins, ICAM-1, VCAM-1, and combined ICAM-1/VCAM-1 and ICAM-1/integrin $\alpha 4$ were used successfully to block inflammation in other tissues.³⁷ However, modulation of ECM molecules to treat dry eye conditions have not been used broadly because of lack of information about the role of ECM changes in LG stem cell function and gland regeneration. The treatment of the dry eye condition in humans and animals may have different clinical effects. For example clinical trials testing the impact of inhibition of ocular surface inflammation using cyclosporine A (Restasis) on dry eye symptoms were inconclusive, compared to dog studies, since the vehicle was almost as effective as the active drug.^{38–40} However, those studies were targeting ocular surface inflammation rather than chronic LG inflammation, and it is very unlikely that eye drops would reach this apparatus in a concentration high enough to have a significant clinical effect.

In healthy LGs, the ECM matures and governs the differentiation of the epithelial cells that restore LG acinar structure.⁴¹ In diseased glands, the ECM is remodeling constantly, and epithelial differentiation stays incomplete and LG acinar structure remains unrestored.^{9,10,12} Thus, the diseased/chronically inflamed state of the LG and other exocrine glands could be due to abnormal ECM maturation,^{32,42–44} which may affect activation or differentiation of LG stem/progenitor cells.

We compared changes in the expression of cell adhesion and cell matrix molecules and stem cell transcription factors in the LGs of two mouse models of SjS: NOD and MRL/lpr during the early stage of disease progression. Histological comparisons of LGs of 12-week-old NOD and MRL/lpr mice showed more severe changes in the LGs of NOD mice than in the LGs of the MRL/lpr mice. The LG of NOD mice had more areas with obliterated acinar structure, larger size of lymphocytic foci, and altered pattern of the ECM. The Mouse Extracellular Matrix & Adhesion Molecules RT² Profiler PCR Array and protein expression data showed that changes in ECM gene expression correlated with the severity of LG disease (such as morphological changes of the gland, size of lymphocytic foci, and distraction of the acinar epithelium) and were found in the mRNA expression of several groups of genes, such as MMPs and their inhibitors, integrins, and other molecules involved in cell-cell and cell-ECM interactions. Both NOD and MRL/lpr mice had very similar patterns of integrins expression and changes in several other ECM molecules, mostly reflecting common inflammatory changes in the LG due to formation of primary lymphocytic infiltrates. At the same time, changes in expression of stem cell transcription factors showed alterations within the LG epithelial stem/progenitor cells, suggesting that early changes due to expansion of lymphocytic infiltrations primarily affect LG epithelial stem cell potential and, as a result, LG regeneration ability.

Our study suggested that changes in the extracellular microenvironment at the early stages of LG disease progression may control LG stem/progenitor cell function and LG regeneration.

MATERIALS AND METHODS

Animals and Treatment

MRL/MpJ-Fas<lpr>/J (MRL/lpr, female, 12-week-old) and NOR/LtJ (NOD, male, 13-week-old) mice and their respective age- and sex-matched MRL/MpJ (MRL/+) and BALB/cJ control mice were purchased from the Jackson Laboratories (Bar Harbor, ME, USA). All experiments were performed in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research, and were approved by the Tufts Medical Center and the Scripps Research Institute Animal Care and Use Committees. Animals were euthanized and the exorbital LGs were harvested under a sterile laminar airflow hood and processed immediately for RNA extraction.

RNA Extraction and RNA Quality Control

Lacrimal gland tissue was homogenized for three 30-second cycles at 338g using IKA ULTRA TURRAX T8 tissue homogenizer, and RNA was extracted using the Qiagen RNeasy Mini Kit (# 74104; Qiagen, Valencia, CA, USA) according to the manufacturer's protocol. The RNA purity and quantity was analyzed using NanoDrop 1000 (Thermo Scientific, Wilmington, DE, USA). Extracted RNA underwent quality assessment on Agilent 2100 Bioanalyzer (Thermo Scientific) by visual examination of ribosomal bands and RNA Integrity Number

(RIN) calculation. The samples then were stored at -70°C until use.

Expression Profiling Using RT² Profiler PCR Array

RNA was reverse transcribed to cDNA using RT² First Strand Kit (SABiosciences, Qiagen, Valencia, CA, USA). The Mouse Extracellular Matrix & Adhesion Molecules RT² Profiler PCR Array (PAMM-013Z; SABiosciences) and the Stem Cell Transcription Factors RT²-PCR Array (PAMM-501Z; SABiosciences) were used to measure expression levels of 84 individual genes important for cell-cell and cell-matrix interactions. Fluorescent signal was captured using ABI 7300 Real-Time PCR System (Applied Biosystems, Life Technologies, Carlsbad, CA).

Analysis of Differentially Expressed Genes

The threshold cycle (Ct) for each well was determined by real-time cycler software. Statistically significant differences in mean Ct values were determined using the RT² Profiler PCR Array Data Analysis software v.3.5 (SABiosciences; available in the public domain at <http://www.sabiosciences.com/dataanalysis.php>). The difference was considered significant when there was a $P < 0.05$ and >2.0 -fold change. Genes with multiple undetermined Ct values in KC and control samples were excluded from the final analysis. Reference genes for normalization of real-time PCR data were b-actin (*ACTB*), β -2 microglobulin (*B2M*), and glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*).

Western Blot

Total protein was prepared from LGs using radioimmunoprecipitation assay (RIPA) lysis buffer and sonicated. Equal aliquots of protein were resolved by SDS-PAGE, transferred to a polyvinylidene difluoride membrane, and probed with antibodies to integrin α L/CD11a (#ab89725; Abcam, Cambridge, United Kingdom), integrin α X (Clone 2F1C10, # 60258-1-Ig; ProteinTech, Rosemont, IL, USA), integrin α E (H-260, sc-28662; Santa Cruz Biotechnology, Inc., Dallas, TX, USA), CD3 (#ab16689; Abcam), B220 (#550286; BD Pharmingen, San Jose, CA, USA) MMP3 (#ab38913; Abcam), MMP7 (ab38997; Abcam), MMP11 (clone EP1259Y, #ab52904; Abcam), Timp1 (Clone MAB9801, #151624; R&D Systems, Inc., Minneapolis, MN, USA), TIMP4 (#ab58425; Abcam), and MMP-2 or MMP-9 antibodies (RP3-MMP-2 and RP3-MMP-9, Triple Point Biologics, Inc., Forest Grove, OR, USA). β -Actin, antibody (mAbGEa; Thermo Scientific) was used as a reference. Horseradish peroxidase (HRP)-conjugated appropriate secondary antibodies and a chemiluminescent detection system was used to visualize immunoreactive protein bands. All experiments were performed in duplicates.

Histology, LG Immunostaining, and Microscopy

Paraffin sections were prepared and stained with hematoxylin and eosin (H&E) using conventional methods. Frozen sections of LGs were stained with the MMP-2 or MMP-9 rabbit polyclonal antibodies (RP3-MMP-2 and RP3-MMP-9; Triple Point Biologics, Inc.) and antibody to α -smooth muscle actin (SMA) to visualize myoepithelial cells and acini borders or with rabbit polyclonal antibody to Pax6 (PRB-278P; Covance Research Products, Inc., Denver, PA, USA) or rabbit polyclonal antibody to Oct4 (11263-1-AP; ProteinTech) and with antibody to heparin sulfate (MAB 1948, rat monoclonal clone A7L6; EMD Millipore, Darmstadt, Germany) to visualize basal membranes around the acini. The Zeiss LSM 780 laser scanning confocal microscope (LSCM; Carl Zeiss Meditec, Inc., Dublin, CA, USA)

was used to obtain the images. Images were observed using Imaris software (Bitplane Scientific Software, Zurich, Switzerland).

Statistical Analysis and Data Presentation

Statistical analyses were performed using Prism Software (GraphPad, San Diego, CA, USA). In bar graphs, data are presented as means $\pm$ SD from a representative experiment or several normalized experiments. Fold changes were calculated from the pooled differences by taking ratios of values for individual measurements in experimental clusters over the mean of the appropriate control clusters. The unpaired 2-tailed Student's *t*-test was used to determine significance ($P < 0.05$) in difference between data sets.

RESULTS

LG Inflammation in NOD and MRL/lpr Mice

Previous publications suggest that tear production is reduced substantially in the NOD and MRL/lpr mice even during early stages (in 12–13-week-old mice) of disease.^{45–48} We analyzed sections of the LGs obtained from 12- to 13-week-old NOD and MRL/lpr mice. Lacrimal glands of NOD mice were more severely affected by inflammation than the LGs of the MRL/lpr mice (Fig. 1). The LGs of NOD mice had more areas with obliterated acinar structure and a larger size of lymphocytic foci compared to MRL/lpr LGs (compare Figs 1B, 1E, 1H to Figs. 1C, 1F, and 1I). The majority of MRL/lpr LG sections (Figs. 1C, 1F, 1I) had mild to moderate degrees of periductal lymphocytic infiltrations. In these mice, we found only a few foci where the infiltrations penetrated the ductal epithelia with occasional destruction of the acini (3 LGs were studied). Conversely, in LGs of NOD mice, intense chronic inflammation was centered not only around lacrimal ducts, but also within the acinar structures. Acinar destruction (Fig. 1E, white arrow) and focal fibrosis (Fig. 1F) also were significantly elevated within and near the foci of NOD mice. In NOD but not MRL/lpr mice, we found epithelial cell debris, especially within zones of infiltration. In NOD LGs, small groups of mononuclear cells also were penetrating the spaces between acini (not shown). In contrast, areas between the inflamed foci in MRL/lpr LGs had a relatively normal appearance (not shown). Lacrimal glands obtained from the control BALB/c (for NOD mice) and MRL/+ (for MRL/lpr mice) animals of the same age and sex had normal LG structure (Figs. 1A, 1D, 1G).

Quantification of the number of large >100 and small <100 μm inflammatory foci within the 40 mm^2 area of NOD and MRL/lpr LGs confirmed our conclusion that LGs of NOD mice have significantly more large lymphocytic infiltrates than LGs of MRL/lpr mice and, therefore, they are more severely affected by inflammation (Fig. 1J).

ECM Changes in the LGs of NOD and MRL/lpr Mice

To study changes in the ECM of LGs of NOD and MRL/lpr mice compared to LGs obtained from corresponding healthy controls (see Material and Methods), we employed the Mouse Extracellular Matrix & Adhesion Molecules RT² Profiler PCR Array. In NOD LGs, we identified 25 genes for which expression significantly ($P < 0.05$) changed more than 2-fold compared to control LGs. A much larger proportion of genes (22) were upregulated versus downregulated (2; Supplementary Table S1). In LGs of MRL/lpr mice, we found changes in expression of 33 genes (Supplementary Table S2). Increased expression of several integrins was detected in the LGs of NOD and MRL/lpr mice compared to their respective

FIGURE 1. Histological sections of LGs obtained from NOD, MRL/lpr and corresponding control mice. (A, D) Lacrimal gland sections obtained from 12-week-old BALB/c mice (control for NOD) stained with H&E. (G) Lacrimal gland sections obtained from a 12-week-old MRL/lpr mouse stained with trichrome. (B, E, H) Lacrimal gland sections of a 12-week-old NOD mouse. Numerous inflammatory foci with lymphocytic infiltrates (*if*) between acini. (E) Nonobese diabetic LG inflammatory foci showing acini with interacinar cell infiltrates (*white arrows*). (C, F, I) Sections of MRL/lpr LG showing smaller size periductal inflammatory foci. (A–F) H&E staining, (G–I) trichrome. (J) quantification of inflammatory foci in the LGs of 12-week-old NOD and MRL/lpr mice. Number of large (>100 μm) and small (<100 μm) foci were counted within the section area of approximately 40 mm² of the LG of NOD and MRL/lpr mice. Statistical analysis using unpaired Student's *t*-test showed that the LG of NOD mice contained significantly more large >100 μm lymphocytic infiltrates than the LGs of MRL/lpr mice. Data are presented as means ± SD from a representative number of sections of approximately 40 mm² obtained from at least three LG's.

controls (Figs. 2A, 2B). Expression of six integrins was significantly upregulated ($P < 0.05$) in both mouse models (Figs. 2A, 2B). Integrins are used as prognostic indicators of many diseases. In addition, changes in integrin signaling may affect stem cell niches and stem cell function.^{49,50}

In both mouse models, we observed a substantial and significant increase of CD44 antigen, a marker of inflammation and lymphoproliferation in primary Sjs⁵¹ (5.4-fold increase in the LGs of NOD mice, 2.6-fold increase in the LGs of MRL/lpr mice, $P < 0.01$). Expression of the intracellular adhesion molecule-1 (ICAM-1), which recruits leukocytes into sites of inflammation, also was increased 9-fold in the LGs of NOD mice and 3-fold in the LGs of MRL/lpr mice ($P < 0.01$; Supplementary Tables S1, S2; Figs. 2A, 2B).

In addition, secreted phosphoprotein 1 (*SPPI*, Osteopontin), which can bind to some types of integrins⁵² (7.8-fold increase in NOD LGs, $P = 0.001126$; 15-fold increase in MRL/lpr LGs, $P = 0.000221$) and vascular cell adhesion protein 1 (*Vcam1*; 17.2-fold increase in NOD LGs, $P = 0.000717$; 6.1-fold increase in MRL/lpr LGs, $P = 0.000616$) were upregulated in both mouse models. Conversely, the tissue inhibitor of metalloproteinases-3 (*TIMP3*), was downregulated in the LGs of NOD and MRL/lpr mice (correspondingly, 2.7- and 2.4-fold decrease, $P < 0.05$; Supplementary Tables S1, S2). Changes in *TIMP1* and *TIMP2* expression were less than 2-fold (not shown).

Notably, we found statistically significant increases in the expression of the TGFβ-induced gene (*TGFBI*, 4-fold change, $P = 0.000757$; Supplementary Table S1) in LG of NOD mice. This gene encodes an RGD-containing protein that binds to type I, II, and IV collagens. Expression of this protein is triggered by TGFβ and it acts to inhibit cell adhesion. In the LG of NOD mice, we also found significant (3-fold, $P < 0.008199$) upregulation of platelet endothelial cell adhesion molecule-1 (*PECAM-1* or *CD31*) mRNA expression. In addition to the expression within vascular compartments, *PECAM-1* is expressed at different degrees on most leukocyte subtypes and platelets.⁵³ Expression of collagen III and IV in NOD mice was

changed but not significantly (not shown), while collagen I (*Col1a1*) mRNA expression was increased 2-fold ($P = 0.008308$). In the LGs of NOD mice, we also found significant increases in the expression of contactin-1 (*Cntn1*), elastin microfibril interfacier 1 (*Emilin1*), ectonucleoside triphosphate diphosphohydrolase-1 (*Entpd1*), tenascin C (*TnC*), and vitronectin (*Vtn*, Supplementary Table S1). However, since expression of these genes was not changed significantly in the LGs of MRL/lpr mice, we believe that they could not be proposed as players of common LG inflammation/injury mechanisms.

To verify our results on integrins mRNA expression, we tested expression of Integrin-αE and Integrin-αL (highly changed in the LG of NOD and MRL/lpr mice) proteins by Western blotting. Similar to mRNA expression, both integrin proteins were increased in the LGs of NOD and MRL/lpr mice compared to their respective control LGs (Figs. 2C, 2D). Moreover, comparable to quantitative qRT-PCR data, the level of protein expression was substantially higher in the LGs of NOD mice than in LGs of MRL/lpr mice (Figs. 2A–D).

The physiological pathways inducing normal and abnormal metabolic degradation of the ECM during tissue remodeling, regeneration, and disease consists of a family of MMPs, which are zinc-containing proteinases with distinct specificities for the individual components of the ECM. Expression of MMPs was altered in both mouse models; however, levels of MMP expression were highly variable. In the LGs of NOD mice, expression of several MMPs (MMP-7, -11, and -9) was significantly upregulated, while MMP12 expression was downregulated. At the same time, expression of several MMPs, disintegrin, and metalloproteinase with thrombospondin motifs-2 and -5 (Adams2 and 5) generally were decreased or unchanged in the LGs of MRL/lpr mice (Supplementary Table S2). *TIMP1* and 2 expressions were increased but not significantly ($P > 0.05$). We studied levels of expression of some MMP proteins by Western blot analysis using antibodies to MMP3, MMP7, and MMP11. In both mouse models of the disease, LG had significantly higher levels of MMP3, MMP7, and

FIGURE 2. Changes in integrins and *Icam1* mRNA expressions in the LGs of NOD (A) and MRL/lpr (B) mice. *Icam1*, intercellular adhesion molecule 1; *Itga2*, integrin α 2; *Itga3*, integrin α 3; *Itga4*, integrin α 4; *Itga5*, integrin α 5; *Itgae*, integrin α E, epithelial-associated; *Itgal*, integrin α L; *Itgam*, integrin α M; *Itgav*, integrin α V; *Itgax*, integrin α X; *Itgb1*, integrin beta 1 (fibronectin receptor beta); *Itgb2*, integrin beta 2; *Itgb3*, integrin beta 3; *Itgb4*, integrin beta 4. (C) Western blot showing the expression of the *Itgal* and *Itgax* in the LG of Nod and MRL/lpr mice compared to the LGs of appropriate control mice. (D) Quantification of the *Itgal* and *Itgax* protein expression (shown in [C]), data obtained from four independent experiments). Three independent arrays were performed; only significantly ($P < 0.05$) changed genes with 2-fold difference are shown. Statistically significant differences in mean Ct values were determined using the RT² Profiler PCR Array Data Analysis software v.3.5 (SABiosciences).

MMP11 expression than in corresponding control LGs, while expression of TIMP1 and 4 was almost unchanged (Figs. 3A, 3B).

Expression of MMP2 and 9 mRNAs in the LGs of NOD mice was unchanged while the expression of MMP2 and MMP9 was downregulated in the LGs of MRL/lpr mice (Supplementary Tables S1, S2). As reported previously, LG epithelial cells and lymphocytes produce MMP-2 and MMP-9 and their secretion is upregulated in SJS LGs.^{19,54} Immunostaining of LG sections showed that MMP2 and MMP9 proteins were expressed at low levels in all acinar cells of WT control LGs obtained from BALB/c (Figs. 4A, 4B) and MRL/+ (Figs. 4C, 4D) mice. NOD and MRL/lpr LGs showed increased levels of MMP2 and 9 expression within the LG and especially in and around the lymphocytic foci (Figs. 4E–I). Labeling of MMP2 was mostly detected in the acinar cells within areas with disorganized acinar structures surrounding foci (Figs. 4E, 4F, 4H). Strong MMP9 labeling was found in the cytoplasm of the infiltrating lymphocytic cells and epithelial cells around the lesions (Figs. 4G, 4I). Using Western blot analysis, we also found strong upregulation of MMP2 and 9 expression in the LG of MRL/lpr and NOD mice (Fig. 4J). Most

notably, we found upregulation of an active cleaved form of these MMPs (Fig. 4J). Expression of MMPs was increased at the protein level but not at the mRNA level.

Stem Cell/Progenitor Cell Expression Signatures Are Altered in the Diseased LG

Stem/progenitor cells are clinically important for tissue regeneration. Timely activation of a cascade of transcription programs during progenitor cell expansion and maturation is governed by lineage-specifying transcription factors. Stem/progenitor cells, in many tissues including the LG, maintain core molecular signatures.^{55–58} Whether these molecular signatures are the same in the diseased LG is not known.

We performed Transcription Factor RT-PCR profiler arrays to investigate changes in the stem cell transcription factor expression profiles in the LGs of 12- to 13-week-old NOD and MRL/lpr mice compared to the appropriate control mice of the same age. In the LGs of MRL/lpr mice, we identified 12 differentially expressed genes (Figs. 5A, 5B). Eleven genes were significantly downregulated, while only one was upregulated

FIGURE 3. Western blot analysis of MMPs and their inhibitors expressions in the extracts from LGs of NOD, MRL/lpr and control (BALB/c or MRL/+) mice. (A) MMP3, MMP7, MMP11, TIMP1 and TIMP4 protein expression in the extracts from LGs of NOD, MRL/lpr, and respective control (BALB/c or MRL/+) mice. (B) Quantification of Western blots shown in (A) (the result of three independent experiments).

FIGURE 4. Analysis of MMP2 and MMP9 expression in the LGs of NOD, MRL/lpr, and control mice. Immunohistochemistry showing expression of MMP2 or MMP9 proteins (green) in the LG of control BALB/c (A, B), MRL/+ (C, D), NOD (E-G), or MRL/lpr (H, I) mice. Myoepithelial cells were labeled with an antibody against α -smooth muscle actin (red), nuclei are labeled with DAPI (blue). (H) Western blot analyses of MMP2 and MMP9 expressions in the LG lysates from control (MRL/+ and BALB/c), MRL/lpr, and NOD mice. GAPDH was used as a loading control.

FIGURE 5. Stem Cell Transcription Factors qRT-PCR array shows that in both mouse models of SjS, inflammation alters expression of transcription factors, which are found mainly in the LG epithelium and involved in regulation of epithelial morphogenesis. (A, B) MRL/lpr compared to MRL/+; (C, D) NOD compared to BALB/c. Epithelially expressed transcription factors are: Forkhead box P1 (*Foxp1*), Iroquois related homeobox 4 (*Irx4*), Notch gene homolog 2 (*Notch2*), Paired box gene 6 (*Pax6*), Runt related transcription factor 1 (*Runx1*), Sine oculis-related homeobox 2 homolog (*Six2*), MAD homolog 2 (*Smad2*), SRY-box containing gene 6 (*Sox6*), SRY-box containing gene 9 (*Sox9*), Trans-acting transcription factor 1 (*Sp1*), Signal transducer and activator of transcription 1 (*Stat1*), and Signal transducer and activator of transcription 1 (*Stat3*). Other transcription factors (nonepithelial or unknown localization) with altered expression in NOD and/or MRL/lpr mouse LGs were Forkhead box P2 (*Foxp2*), Jun oncogene (*Jun*), Kruppel-like factor 4 (*Klf4*), Nuclear receptor subfamily 2, group F, member 2 (*Nr2f2*), Paired box gene 5 (*Pax5*), and Werner syndrome homolog 1 (*Wrrn*). Three independent arrays were performed, only significantly ($P < 0.05$) changed genes with 1.5-fold difference are shown. Statistically significant differences in mean Ct values were determined using the RT² Profiler PCR Array Data Analysis software v.3.5 (SABiosciences). Expression of Pax6 in the MRL/+ (E), MRL/lpr (F), BALB/c (G), and NOD (H) LGs. Expression of Oct4 in the MRL/+ (I), MRL/lpr (J), BALB/c (K), and NOD (L) LGs. Quantification of the number of Pax6 and Oct4 expressing cells per acinus. Z stacks images were collected and positive cells were counted within each whole acinus included in Z stack. Statistical analysis was performed as described above. Data are presented as means $\pm$ SD, $P < 0.05$.

(Figs. 5A, 5B). The expression of 18 transcription factors was altered significantly in the NOD mice. Sixteen of these genes were downregulated and the expression of 2 genes was increased. It is interesting that, although transcription factors array contains epithelially and mesenchymally expressed transcription factors, we found decreases in predominantly epithelially expressed transcription factors and factors regulat-

ing epithelial morphogenesis. The following transcription factors were downregulated in both mouse models: paired box protein-6 (*Pax6*; MRL/lpr LGs, 2.9-fold decrease, $P = 0.00782$; NOD LGs, 2.5-fold decrease, $P = 0.013728$); *Irx4* kruppel-like factor 4 (*Klf4*; MRL/lpr LGs, 2.2-fold decrease, $P = 0.043124$; NOD LGs, 2.6-fold decrease, $P = 0.014322$); SRY-box containing transcription factor and 9 (*Sox9*; MRL/lpr LGs, 2.1-

fold decrease, $P = 0.027348$; NOD LG, 1.7-fold decrease, $P = 0.001972$); and SMAD family member 2 (*Smad2*; MRL/lpr LG, 3-fold decrease, $P = 0.000452$; NOD LG, 2.7-fold decrease, $P = 0.00085$). *Pax6* regulates LG cell proliferation and branching morphogenesis and has a major role in maintenance of the adult ocular tissues.^{59,60} *Klf4* is the most abundant gene in ocular tissues, including the LG, and has important roles in cell proliferation and differentiation. *Sox9* expression is restricted to the LG epithelium.⁵⁸ *Sox9* controls the expression of heparan sulfate-synthesizing enzymes (HSSE), which are required for the synthesis and function of heparan sulfate (HS), to promote FGF and other growth factors signaling and LG morphogenesis.⁶¹ Other factors with decreased expression in MRL/lpr and NOD LGs were Iroquois homeobox protein-4 (*Irx4*; MRL/lpr LG, 2.1-fold increase, $P = 0.004942$; NOD LGs, 2-fold decrease, $P = 0.00045$), mesenchymally expressed forkhead box P2 (*Foxp2*; MRL/lpr LGs, 3.6-fold decrease, $P = 0.00017$; NOD LGs, 1.8-fold decrease, $P = 0.04716$), nuclear receptor subfamily 2 (*Nr2f2*; MRL/lpr LGs, 3.7-fold decrease, $P = 0.003041$; NOD LG, 2.2-fold decrease, $P = 0.042417$) and Wilms tumor 1 homolog (*Wt1*; MRL/lpr LGs, 2.2-fold decrease, $P = 0.000001$; NOD LG, 2.4-fold decrease, $P = 0.000001$).

Lacrimal glands of NOD mice showed significant decreases in other epithelial markers, which were not substantially changed in the LGs of MRL/lpr mice. We found not substantial but significant downregulation of Runt-related transcription factor 1 (*Runx1*; 1.4-fold downregulation, $P = 0.0348$) expression. As we showed previously, the transcription factor *Runx1* is expressed by the LG epithelial cell progenitors.⁵⁸ It regulates their proliferation and is involved in the mechanisms controlling LG regeneration.⁴⁵ We also found a decrease in Notch family transcription factor-2 expression (*Notch2*; 1.5-fold decrease, $P = 0.048417$). Notch2 functions as a receptor for membrane-bound ligands Jagged1, Jagged2, and Delta1 and controls cell-fate determination. Decreases in Notch2 expression is associated with the poorly differentiated serous epithelia.⁶² In addition, LGs of NOD mice showed decrease in expression of epithelially expressed forkhead box P1 (*Foxp1*; 2.4-fold decrease, $P = 0.02474$), POU domain, class 5, transcription factor 1 (*Pou5f1*, also known as *Oct4*, 1.9-fold decrease, $P = 0.00293$) and sine oculis-related homeobox transcription factor-2 (*Six2*; 1.49-fold decrease, $P = 0.049892$). It has been shown that *Six2* acts within a network of genes including eyeless (Pax family), eyes absent (Eya family), and dachshund (Dach family) to trigger eye tissue organogenesis.⁶³ To validate expression of some of the transcription factors, we performed immunostaining of MRL/lpr, NOD, and control mice with the Pax6 and Oct4 antibodies. *Pax6* is one of the most important regulators of LG development and regeneration.^{29,58,60,64,65} *Oct4* is an important pluripotency factor that regulates self-renewal of many cell types.^{66–69} We found a substantial decrease in number of Pax6 expressing cells in MRL/lpr (Figs. 5E–H, 5M; 1.8-fold decrease, $P < 0.05$) and NOD (3.5-fold decrease, $P < 0.05$). Although number of Oct4 expressing cells was downregulated in the LGs of MRL/lpr and NOD mouse models of SJS (Figs. 5I–M), statistically significant decrease in Oct4 expressing cells was determined only in NOD mice (6.5-fold decrease, $P < 0.05$; Fig. 5M).

Our data also showed that the expression of *Stat1* was upregulated in both mouse models of LG inflammation. The STAT signaling pathway is one of the common pathways of physiological and pathological responses in humans. We also found a significant increase in paired box protein-5 (*Pax5*; 2.1-fold increase, $P = 0.016034$) in the LGs of NOD mice. *Pax5* expression is an extremely specific marker for B cell lineage.^{70–72} Upregulation of Pax5 expression in the LGs of the NOD mice suggests intensive invasion of B-cells into the glands of this mouse model. To test this idea, we performed

immunostaining of LG sections of the NOD and MRL/lpr mice using antibodies to B220 and CD3 antigens. B220 represents a subset of mouse CD45 isoforms predominantly expressed on all B lymphocytes,^{73,74} including pro-, mature, and activated B cells, while CD3 antigen (also known as the T3 complex) is defining the T cell lineage.^{75–77} Immunostaining analysis revealed that LG inflammatory foci of the MRL/lpr mice (Figs. 6A, 6C, 6E) had small amounts of B and T types of cells, whereas LG foci of the NOD mice were predominantly composed of large numbers of B-cells (Figs. 6B, 6D, 6F). Quantification of B and T cells showed that LG foci of NOD contained approximately 81% of B cells and only 5% of T cells compared to the LG MRL/lpr mice, where B cells represent only 8% of all cells, while T cells reach 11%. This result confirms our qPCR data and suggests that increases in *Pax5* mRNA expression in NOD LG reflects intensive invasion of B-cells into the LGs and could be used as a reliable marker of LG disease progression.

DISCUSSION

Our results suggested that changes in the components of ECM reflect lymphatic involvement and modulated expression of different proteinases. Both NOD and MRL/lpr LGs showed significant and specific increases in integrins expression. Inflammation is critically dependent on integrin-mediated adhesive interactions of cells with their ECM-rich environment. In the cornea and other tissues, the leukocyte-specific $\beta 2$ -integrins allow infiltrating cell attachment to the ECM and to other epithelial cells. It associates with the *Itgal*, encoding antigen CD11A, to form lymphocyte function-associated antigen-1. One of the most important ligands for $\beta 2$ -integrins is *ICAM-1* and *VCAM1*. We found that expression levels of $\beta 2$ -integrins, *Itgal*, *ICAM-1*, and *VCAM1* were significantly increased in both mouse models of LG inflammation (see Results). Moreover, LGs of more severely affected NOD mice had higher changes in $\beta 2$ -integrins, *Itgal*, *ICAM-1*, and *VCAM1* expression. Expression of other important integrins changed in both diseased mouse models. Changes were higher in the LGs of NOD mice. Both mouse models for LG inflammation also showed changes in *Itgax* and the encoding antigen CD11c. High levels of CD11c are found on human dendritic cells, monocytes, macrophages, and neutrophils.⁷⁸ Thus, changes in the expression of integrins complex could be a diagnostic marker of LG inflammation, while the level of changes in their expression, could reflect the severity of inflammation/disease.

We also confirmed a previously reported substantial increase in CD44 (a transmembrane glycoprotein) transcript expression in the NOD LGs.⁷⁹ Moreover, CD44 expression was significantly increased in MRL/lpr LGs. Increase in CD44 isoform was found to promote lymphocyte activation, migration, and proliferation in the LGs of the SJS patients^{79,80} and inflammatory diseases of other tissues.⁸¹

There were no significant correlations between the mRNA and the protein expression of several MMPs in both mouse models. In both mouse models, levels of MMP proteins were generally upregulated while mRNA levels did not change or were downregulated. This discrepancy between mRNA and protein expressions may reflect regulation of MMPs at the translational level either by increasing mRNA translation or reducing mRNA stability. Enhanced mRNA translation was reported in response to cellular stress.⁸² Our finding corroborated with previously reported data showing no significant correlation between the mRNA and protein expression of MMP-2, MMP-9, and TIMP-1 in either cancerous or noncancerous tissue.⁸³

FIGURE 6. Lacrimal gland of NOD mice show higher number of infiltrating B cells compared to MRL/lpr LGs. Lacrimal gland sections of MRL/lpr (A, C, E) and NOD (B, D, F) mice were probed with B220 (B-cell marker, red) and CD3 (T-cell marker, green) antibodies; nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI; blue). (G) Quantification of B and T cell in infiltration foci of NOD and MRL/lpr mice. Results are presented as a ratio of labeled cells (T_{green} or B_{red}) to total cells (all DAPI⁺ labeled cells) within each focus ($n = 30$, $P < 0.05$).

We also found moderate changes in collagen expression and collagen binding growth factor TGFBI (known to be regulated by the TGF β). Although the levels of changes in collagen and regulatory growth factors expressions that can affect LG stem/progenitor cell function are not known, the impact of collagen expression changes on cellular response could be manifold. Collagen synthesis and degradation affected by inflammation may contribute to changes in cell spreading and migration and, therefore, regeneration and disease progression.^{84–86}

Epithelial cell and epithelial stem cell/progenitor cell behavior in chronic inflammation is poorly characterized. The stem cell transcription factors array showed a significant decrease in the majority of LG epithelial stem/progenitor cell markers, such as *Pax6*, *Sox6*, *Sox9*, *Six2*, *Klf4*, and *Notch2*, that control LG development and regeneration. This finding suggests that chronic inflammation decreases regenerative function of the LG. A partial loss of function of these transcription factors should delay stem cell activation and lead to a reduction of epithelial progenitor differentiation, and as a result delayed or ceased LG regeneration in these mice.

Upregulation of Stat1 mRNA level in both mouse models of LG inflammation and increased expression of Pax5 in the NOD mouse model suggests the induction of pathways connected with activation of proinflammatory stem cells. Stat1 belongs to a family of nuclear proteins mediating the action of a number of cytokines. In humans, Stat1 has a critical role in the signal transduction pathway activated by IFN- γ and regulates signaling pathways converting the IFN- γ signal into gene expression, such as *COX*, *VCAM*, and *ICAM*, which are critical players in different pathologies associated with inflammatory processes. We also showed that high levels of Pax5 expression in NOD mice were associated with expansion and differentiation of B cell lineage and appearance of larger lymphocytic foci and number of lymphocytic cells within the LGs of NOD mice compared to LGs of MRL/lpr mice.

CONCLUSIONS

In summary, our data suggested that morphological and ECM changes in the LGs of NOD and MRL/lpr mice at early stage of LG inflammation reflect common changes due to initial invasion and expansion of the lymphocytic cells. However, these initial changes in ECM expression lead to substantial alterations in epithelially-expressed stem cell transcription factors, suggesting impaired function of the epithelial cell progenitors and decreased regeneration ability of the diseased LG.

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