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Galectin-3, a Damage-Associated Molecular Pattern, in Tears of Patients with Vernal Keratoconjunctivitis

--Manuscript Draft--

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Abstract:	(Purpose) Galectin-3 is one of the damage-associated molecular patterns (DAMPs), which are released from damaged or dying cells. In this study, we investigated the concentration and source of galectin-3 in the tears of patients with vernal keratoconjunctivitis (VKC) and evaluated whether the concentration of galectin-3 in tears represents a biomarker of corneal epithelial damage. (Study Design) clinical and experimental. (Methods) We measured the concentration of galectin-3 in tear samples from 26 patients with VKC and 6 healthy controls by enzyme-linked immunosorbent assay (ELISA). The expression of galectin-3 in cultured human corneal epithelial cells (HCEs) stimulated with or without tryptase or chymase was investigated by polymerase chain reaction (PCR), ELISA, and Western blotting. We also estimated the concentration of galectin-3 in the supernatants of cultured HCEs that were induced to necrosis. Finally, we investigated whether recombinant galectin-3 induced the expression of various genes related to cell migration or the cell cycle in HCEs by using microarray analysis. (Results) High concentrations of galectin-3 were detected in the tears of patients with VKC. The concentration showed significant correlation with the severity of corneal epithelial damage. Stimulation of cultured HCEs with various concentrations of tryptase or chymase had no effect on the expression of galectin-3. However, high concentrations of galectin-3 were detected in the supernatants of necrotic HCEs. Recombinant human galectin-3 induced various cell migration- and cell cycle-related genes. (Conclusion) The concentrations of galectin-3 in the tears of patients with VKC may represent a biomarker of the severity of corneal epithelial damage.
Author Comments:	
Response to Reviewers:	Reviewer #1: 有益なアドバイスをいただき感謝いたします。先生のご質問に対しまして以下の回答をさせていただきます。ご満足いただければ幸いです。 (Major Points) 著者らは、涙液中のgalectin-3濃度が、春季カタルにおける角膜上皮障害のバイオマー

カーになることを報告しています。そして、galectin-3の由来はネクローシスを起こした角膜上皮細胞であり、マスト細胞の脱顆粒により放出される物質であるトリプターゼおよびキマーゼには影響を受けないことを培養角膜上皮細胞を使用して検討しています。これらの検討は、涙液中galectin-3レベルの臨床的意義を検証するための重要な研究結果であると考えられます。研究結果は、よくまとめられておりますが、以下のminor commentsについての再検討が必要です。

(Minor Points)
1) Statistical analysis: Figure 1bで使用した回帰式についての記載がありません。1-way ANOVA with Bonferroni correctionはFig.2で使用しているのでしょうか？それならば、結果およびFig. 2の説明の中で記載してください。

【回答】Figure 1bで使用した回帰式はGraphPad Prism (version 9), Simple linear regressionです。図中に記載しました。1-way ANOVA with Bonferroni correctionはFigure2で使用しましたが、有為差認めませんでした。図中に記載しました。

2) Table: Tableの題名を記載してください。Tableの説明は本文中で行ってください。表内で使用した略語については、欄外で説明してください。

【回答】Tableの題名を記載いたしました。Tableの説明は本文中(LL 207-215)に記載しました。表内の略語は欄外で説明しました。

3) Figure Legends:①Figure 1a, 1b, 2, and 3の題名をFigure Legendsに記載してください。また、Fig. 1a、Fig. 1bとはせず、Figure 1の中でaとbとして説明を記載してください。②Fig. 3の記述内容について、もう一度検討してください。

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High concentrations of galectin-3 detected in the supernatants of necrotic human corneal epithelial cells (HCEs), compared to that of intact HCEs.

Reviewer #2:
In this paper, authors reported the concentration of tear galectin-3 in patients with VKC and evaluated its role as a biomarker of corneal epithelial damage and found that their conclusion that significant high concentration of galectin-3 was detected in tears of VKC and the concentration was significantly correlated with the severity of corneal damage. Their conclusion that the concentrations of galectin-3 in tears of patients with VKC can be a biomarker of the severity of corneal damage seems reasonable.

1. In Discussion section, authors noted that the mechanism of increased galectin-3 in the tears of patients with VKC may be different from that in dry eye (lines 254 - 256), however considering that absence of the effect of chymase nor tryptase on the mRNA or protein expression of galectin-3 seems contradicting to their hypothesis because of their important role in the development of proliferative change in VKC (lines 249 - 254), therefore please reconsider this part of the paragraph.

【answer】
Thank you for good advice. We change the sentences in Discussion. (LL 259-266)

2. As authors stated in Discussion section, it can be accepted that "Therefore, we hypothesize that galectin-3 in the tears of patients with VKC may be produced by necrotic corneal and conjunctival cells by eosinophil toxic proteins. (lines 277 - 279)". However, form the clinical standpoint, we are interesting whether tear galectin-3 has any allergological role in the development of severe corneal damage, because its concentration was significantly correlated with the severity of corneal damage as graded scoring system. If this can be proved, we might be able to change the treatment regimen according to tear galectin-3 level. But considering that corneal plaque or shield ulcer are composed of the debris of necrotic epithelium, galectin-3 level seems reflecting only the result of necrotic process. To evaluate the pathophysiological role properly especially in severe spectrum of ACD such as VKC, more discussion

	regarding these points should be described in Discussion section. 【answer】 Thank you for good advices. We added another pathophysiological effect and sentences in Discussion. (LL 287-290)

Reviewer #1:

有益なアドバイスをいただき感謝いたします。先生のご質問に対しまして以下の回答をさせていただきます。ご満足いただければ幸いです。

(Major Points)

著者らは、涙液中の galectin-3 濃度が、春季カタルにおける角膜上皮障害のバイオマーカーになることを報告しています。そして、galectin-3 の由来はネクローシスを起こした角膜上皮細胞であり、マスト細胞の脱顆粒により放出される物質であるトリプターゼおよびキマーゼには影響を受けないことを培養角膜上皮細胞を使用して検討しています。これらの検討は、涙液中 galectin-3 レベルの臨床的意義を検証するための重要な研究結果であると考えられます。研究結果は、よくまとめられておりますが、以下の minor comments についての再検討が必要です。

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Galectin-3, a Damage-Associated Molecular Pattern, in Tears of Patients with Vernal Keratoconjunctivitis

Running title: Galetin-3 and vernal keratoconjunctivitis

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Main text: 3,042 words

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References:53 references

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Abstract

(Purpose)

Galectin-3 is one of the damage-associated molecular patterns (DAMPs), which are released from damaged or dying cells. In this study, we investigated the concentration and source of galectin-3 in the tears of patients with vernal keratoconjunctivitis (VKC) and evaluated whether the concentration of galectin-3 in tears represents a biomarker of corneal epithelial damage.

(Study Design)

clinical and experimental.

(Methods)

We measured the concentration of galectin-3 in tear samples from 26 patients with VKC and 6 healthy controls by enzyme-linked immunosorbent assay (ELISA). The expression of galectin-3 in cultured human corneal epithelial cells (HCEs) stimulated with or without tryptase or chymase was investigated by polymerase chain reaction (PCR), ELISA, and Western blotting. We also estimated the concentration of galectin-3 in the supernatants of cultured HCEs that were induced to necrosis. Finally, we investigated whether recombinant galectin-3 induced the expression of various genes related to cell migration or the cell cycle in HCEs by using microarray analysis.

(Results)

High concentrations of galectin-3 were detected in the tears of patients with VKC. The concentration

showed significant correlation with the severity of corneal epithelial damage. Stimulation of cultured HCEs with various concentrations of tryptase or chymase had no effect on the expression of galectin-3. However, high concentrations of galectin-3 were detected in the supernatants of necrotic HCEs. Recombinant human galectin-3 induced various cell migration- and cell cycle-related genes.

(Conclusion)

The concentrations of galectin-3 in the tears of patients with VKC may represent a biomarker of the severity of corneal epithelial damage.

Key words:

Galectin-3

Vernal keratoconjunctivitis

Tryptase

Chymase

DAMPs (damage-associated molecular patterns)

Introduction

Galectins are a family of soluble lectins that have a conserved carbohydrate recognition domain (CRD) and bind β-galactoside–containing glycans. [1] A total of 15 galectins have been identified in animals, and galectins are widely distributed among various type of cells and tissues [2]. All galectins share close sequence homology in their CRD but exhibit different affinities for different saccharide lignans [2].

Galectin-3 is a unique member of chimera-type galectins and has both extracellular and intracellular functions. Galectin-3 was first discovered as a protein that binds immunoglobulin E (IgE) and characterized as a 28-kDa antigen (Mac-2) on the surface of murine macrophages [2-7] Galectins are thought to mediate diverse biological processes and are involved in the regulation of cell activation, cell adhesion, differentiation, cytokine secretion, inflammation, wound healing, and apoptosis [8-12]. Galectin-3 has now been found to be related to the physiopathology of multiple diseases, such as hepatic fibrosis, pulmonary fibrosis, heart failure, Sjögren's syndrome, cancer, asthma, and dry eye [2], [13-20].

In allergic inflammation, galectin-3 plays a crucial role in the process of leukocyte trafficking and activation and cytokine production. For example, recombinant human galectin-3 can directly increase the rolling and adhesion of eosinophils from allergic donors in an α4 integrin-dependent manner [21, 22]. These activities were inhibited by specific galectin-3 monoclonal

antibodies and lactose. In mouse models of allergic rhinitis and asthma, high levels of galectin-3 mRNA and protein were detected in the nasal mucosa, lung, and bronchoalveolar lavage fluid [23-27]. Mouse models with atopic dermatitis also had increased galectin-3 protein expression in the skin [28]. These reports show that galectin-3 expression is upregulated in allergy-related diseases.

In the field of ophthalmology, Hrdličková-Cela E et al found that tears harvested from the eyes of patients with ocular surface inflammation (such as adenoviral conjunctivitis and corneal degeneration) contained high concentrations of galectin-3 [29]. In contrast with tears from patients with ocular surface inflammation, tears from healthy volunteers contained no galectin-3 [29]. Recently, Andrade FEC et al found that the expression of galectin-3 proteins was constantly detected in the nucleus and cytoplasm of conjunctival epithelial cells in healthy individuals [30]. They also showed that galectin-3 levels in conjunctival epithelial cells were markedly higher in patients with keratoconus than in healthy controls [30]. It is well known that patients with keratoconus frequently have allergic and atopic diseases, such as hay fever, asthma, and atopic dermatitis. The same group recently observed strong galectin-3 expression in the nucleus and cytoplasm of epithelial cells obtained from the bulbar conjunctival epithelium of patients with VKC [31]. They also found a marked increase in galectin-3 in conjunctival tissues in murine experimental allergic conjunctivitis models [31].

Despite the above research, the relation between the concentration of galectin-3 in tears

and the severity of corneal epithelial damage in patients with VKC remains unknown. Furthermore, the source and functions of galectin-3 in the tears of these patients has not been investigated. Therefore, in this study we focused on the concentration, source, and functions of galectin-3 in the tears of patients with VKC.

Material and Methods

Human samples

After obtaining written informed consent, we obtained tears from 26 patients with VKC (18 men and 8 women; mean age, 11.4 years) and 6 healthy controls (3 men and 3 women; mean age, 24.2 years). All procedures were approved by the ethics committees of Juntendo University School of Medicine (approval NO. 2019244), and the study was conducted in accordance with the tenets of the Declaration of Helsinki. This study is part of a larger observational study that was approved by the institutional review board and will be conducted from 2020 to 2022. This study included 26 consecutive patients with VKC who were treated at the Department of Ophthalmology Juntendo University, Juntendo and Urayasu Hospital, Tokyo and Chiba, Japan, and 6 healthy volunteers who did not have allergic conjunctivitis or a history of wearing contact lenses. When tear samples were collected from 26 patients, the treatment of eye drops to these patients was A; 0.1% tacrolimus, B; 0.1% cyclosporine, C: histamine H_1 receptor antagonist, D:0.1% betamethasone, E: 0.1% fluorometholone.

Tear sampling

Tears were sampled from the affected eye in unilateral cases or from the more severely affected eye in bilateral cases. Tears were sampled using the Schirmer I method with filter paper (Schirmer Tear Production Measuring Strips; Showa Yakuhin Kako, Tokyo, Japan), and the Schirmer strips were stored at −20°C until further analysis. The Schirmer strips were thawed and eluted overnight at room temperature using 0.5 M NaCl and 0.5% Tween 20 containing 0.05 M phosphate-buffered solution (pH 7.2). The amount of tears obtained was calculated by considering 1 mm of a wet Schirmer strip to contain 1 μl of tears. Thus, the end concentration of the eluted solution corresponded to a 20-fold dilution of the original tear sample.

Clinical evaluation criteria of corneal epithelial damage

Corneal epithelial damages were graded for severity and the clinical evaluation criteria were made depending on the guideline in allergic conjunctival diseases of Japan Ocular Allergic Society [32]. We divided patients into 4 groups on the basis of the score for the severity of corneal epithelial damage: 0, no damage; 1, superficial epitheliopathy; 2, pseudoexfoliative epitheliopathy; 3, persistent erosion, shield ulcer, and corneal plaque.

Culture of human corneal epithelial cells

Human corneal epithelial cells (HCEs) were purchased from Science Cell Research Laboratories (Carlsbad, CA, USA). Frozen cells were warmed in a water bath at 37 ℃, and the thawed cell pellet

was washed in 5 mL of medium and centrifuged at 1200 rpm for 3 minutes at room temperature. Then, HCEs were gently resuspended in approximately 1 mL of Defined Keratinocyte serum-free medium (Defined Keratinocyte SFM; Thermo Fisher Scientific Inc, Japan) with growth supplements that eliminated the requirement for bovine pituitary extract. The medium was changed every 2 to 3 days. After the cells reached 75% to 90% confluence, subculture was performed.

Real-time polymerase chain reaction

HCEs in the third passage were prepared for real-time polymerase chain reaction (PCR). HCEs were incubated in Defined Keratinocyte SFM without growth supplements for 24 hours before the experiments were performed. The cells were stimulated with various concentrations (2, 20, 200 ng/mL) of human mast cell tryptase (Sigma-Aldrich, USA) or chymase (Sigma-Aldrich, USA) for 24 hours. Then, RNA was extracted with an RNeasy Mini Kit (QIAGEN, Hilden, Germany) according to the manufacturer's protocol. cDNA was synthesized from 750 ng of total RNA with PrimeScript RT Master Mix (Takara, Shiga, Japan). Quantitative PCR was then performed with a SYBR Premix Ex Taq II (TliRNaseH Plus; Takara) with an Applied Biosystems 7900HT thermocycler and analyzed by Sequence Detection System 2.4 (Applied Biosystems, Foster city, CA, USA). The primer of galectin-3 is 5'-GTTATCTGGGTCTGGAAACC and 5'-TCTGTTTGCATTGGGCTTCACC, glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was

used as the reference gene.

Enzyme-linked immunosorbent assay

The concentrations of galectin-3 in tears from patients with VKC and healthy controls were measured with an enzyme-linked immunosorbent assay (ELISA) kit (R&D systems, USA) according to the manufacturer's protocol. The detection limit of this kit was 0.085 ng/ml. HCEs were stimulated with or without various concentrations of tryptase or chymase for 24 hours before measuring the concentrations of galectin-3 in the supernatants.

Inducing HCEs to necrosis

Necrosis was induced by 3 cycles of freezing and thawing, in accordance with our previous report [33] . HCEs cultured with Defined Keratinocyte SFM were frozen at -80 ℃ for 20 minutes and thawed at 37℃ for 20 minutes for 3 cycles. The supernatant obtained from the necrotic HCEs was used for further analysis.

Western blotting for galectin-3

HCEs in the third passage were incubated in Defined Keratinocyte SFM without growth supplements for 24 hours. Then, cells were treated with or without various concentrations of

chymase or tryptase for 24 hours before the experiments were performed. Total cell extracts from cultured HCEs were obtained by lysis in radioimmunoprecipitation assay buffer (50 mM Tris-HCL, pH 7.5, 5 mM EDTA, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% sodium dodecylsulphate [SDS], 1% nonidet P-40, and 150 mM NaCl) containing a serine protease inhibitor (phenylmethylsulfonyl fluoride) (Roche, Molecular Biochemicals, Mannheim, Germany). After centrifugation to remove cell debris, supernatants were subjected to sodium dodecyl sulfate-polyacrylamide (SDS-PAGE) gel electrophoresis with NuPAGE 4% to 12% Bis-Tris Gels (Life Technologies, Gaithersburg, MD, USA) and Laemmli buffer (4% SDS, 10% 2-mercaptoethanol, 20% glycerol, 0.004% bromophenol blue, and 0.125M Tris-HCl). Proteins were subsequently electrotransferred to an Immobilon-P Transfer Membrane (Millipore, Darmstadt, Germany). The membrane was blocked in 2% ECL Prime Blocking Agents (Amersham Pharmacia Biotech, Piscataway, NJ, USA)/TBS with 0.1% Tween-20 (TBS-T) for 1 hour at room temperature, then incubated with anti-galectin-3 antibody (abcam, UK) overnight at 4 ℃. Working concentrations of anti-galectin-3 antibody in phosphate buffered saline T (PBS-T) with 5% skim milk were added at 1:1000 dilution for anti-galectin-3 or 1:5000 dilution for GAPDH (Cell Signaling Technology, MA, USA). After 5 washes in TBS-T, the blots were incubated with the secondary antibodies (horseradish peroxidase-conjugated anti-mouse or anti-rabbit IgG) at 1:2000 dilution. An ECL detection system (Amersham Pharmacia Biotech, Piscataway, NJ, USA) was used to visualize the target proteins on

23-1 Galectin-3, (The concentrations and source)

the blots according to the manufacturer's instructions. Exposure time ranged from 5 seconds to 1 minute, depending on the protein. A digital imaging system, Amersham Imager 600 (GE Healthcare, NJ, USA), was used to record and analyze images of the membranes.

DNA microarray analysis

HCEs in the third passage were incubated in Defined Keratinocyte SFM without growth supplements for 24 hours before the experiments were performed. The cells were also stimulated for 24 hours with 10 μg/mL of recombinant human galectin-3 (abcam, UK), then RNA was extracted with an RNeasy Mini kit (QIAGEN) according to the manufacturer's protocol. Gene expression microarray analysis with the Human Gene 2.0 ST Array (Affymetrix, Thermo Fisher Scientific, Waltham, MA, USA) containing 53 617 probes of characterized human genes was consigned to Filgen Inc., (Nagoya, Japan). Predictive enrichment analysis was performed on sets of genes exhibiting an expression change (fold change > 2 but < 0.5). Pathway analysis was carried out by using the "KEGG pathway function." Data are presented as heatmap and MA plots generated with MeV (http://mev.tm4.org) and Prism 8 software (GraphPad, La Jolla, CA).

Statistical analysis

All results are expressed as the mean and SD. Data were analyzed with GraphPad Prism software

(GraphPad, La Jolla, CA). Data were compared between 2 groups by a 2-tailed student's *t* test and between multiple groups by 1-way analysis of variance with Bonferroni correction. Differences of $P < 0.05$ were considered statistically significant.

Results

The concentrations of galectin-3 in the tear and severity of corneal epithelial damage of VKC patients (Table)

Table shows sex, age, concentrations of galectin-3 in the tear, severity of corneal epithelial damage and the treatment of vernal keratoconjunctivitis (VKC) patients. We divided patients into 4 groups on the basis of the score for the severity of corneal epithelial damage: 0, no damage; 1, superficial epitheliopathy; 2, pseudoexfoliative epitheliopathy; 3, persistent erosion, shield ulcer, and corneal plaque. When tear samples were collected from 26 patients, the treatment of eye drops to these patients was A; 0.1% tacrolimus, B; 0.1% cyclosporine, C: histamine H_1 receptor antagonist, D:0.1% betamethasone, E: 0.1% fluorometholone.

Correlation between the concentrations of galectin-3 in the tear and the severity of corneal epithelial damage (Figure 1a・b)

High concentrations of galectin-3 were detected in the tears of patients with VKC, compared to that of controls. Galectin-3 was not detected in the tear of all healthy controls (Fig 1a). The concentrations of galectin-3 in tears showed a significant correlation with the severity of corneal

222 epithelial damage (Figure 1b).

223

224 **The mRNA and protein expression of galectin-3 in cultured HCEs stimulated with or without**

225 **tryptase or chymase (Figure 2)**

226 Adding various concentrations (2, 20, and 200 ng/mL) of tryptase or chymase to the culture medium

227 did not affect the expression level of galectin-3 in HCEs at the mRNA (Fig 2-a,b) or protein level

228 (Fig 2-c, d, e, f).

229

230 **The concentrations of galectin-3 in the supernatants of necrotic HCEs (Figure 3)**

231 High concentrations of galectin-3 were detected in the supernatant of necrotic corneal epithelial cells

232 (HCEs), compared to that of intact HCEs

233

234 **Global gene expression analysis of HCEs stimulated with recombinant galectin-3 (Figure 4)**

235 To investigate how galectin-3 affects HCEs, global gene expression in HCEs stimulated with or

236 without recombinant galectin-3 was analyzed by microarray. HCEs were incubated with recombinant

237 human galectin-3 protein (10 μg/mL) (Abcam, Cambridge, UK) for 24 hours, after which RNA was

238 extracted for microarray analysis. In HCEs treated with galectin-3, 18 genes (19 probes) were

239 upregulated 2-fold or more (log2 FC > 1) and 35 genes (61 probes) were downregulated 0.5-fold or

less (log2 FC < -1) (Fig 4-a). The most upregulated genes in HCEs treated with galectin-3 were histone cluster 1 H3b, histone cluster 1 H2be, and matrix metalloproteinases 1, which had 3.98-, 2.93-, and 2.77-fold changes in their expression ratios, respectively (Fig 4-b). The top 10 genes that were upregulated in HCEs treated with galectin-3 included several histone cluster genes. KEGG pathway analysis showed that genes associated with the cell cycle, mitosis, cell cycle checkpoints, and M phase pathways were enriched in the upregulated genes (Fig 4-c).

Discussion

In this study, we estimated the concentrations of galectin-3 in the tears of 26 patients with VKC and detected elevated concentrations in all but 4 of the patients. The concentrations of galectin-3 in tears showed a significant correlation with the severity of corneal epithelial damage.

Uchino Y. et al found that the concentration of galectin-3 protein was significantly higher in tears of patients with dry eye than in tears of healthy individuals [20]. Western blotting identified an intact galectin-3 band (~28.0 kDa) in tear samples from healthy individuals, but 50% of the patient samples were characterized by the additional presence of a partially degraded form (~25.4 kDa) [20] . However, in our Western blotting experiments we did not detect the partially degraded form of galectin-3 in the tears of patients with VKC (data not shown). Previous reports showed that the collagen-like domain of galectin-3 is susceptible to rapid and efficient cleavage by

matrixmetalloprotease-2 (MMP-2) and MMP-9 [34, 35]. Therefore, we also examined the effects of chymase and tryptase on galactin-3 produced by HCEs. Tryptase and chymase did not degrade galectin-3 from the intact form to the cleared form. Chymase and tryptase are released from degranulated mast cells and have strong protease activity. Our previous studies revealed that the tears of patients with VKC show high concentrations and activity levels of chymase and tryptase [36]. In particular, the concentrations and activity levels of chymase in the tears of patients correlated with the severity of corneal epithelial damage [36]. Therefore, high concentrations of chymase may induce corneal epithelial cells to necrosis, resulting high amounts of galectin-3 in the tear of VKC patients. In the present study, high amounts of galectin-3 proteins were detected in the supernatants of necrotic HCEs. Recent studies showed that when cells die via accidental necrosis or via regulated necrosis (necroptosis), secondary necrosis (late apoptosis), or both, their cytosolic and nuclear contents are released into the extracellular space [37-39]. Damaged or dying cells release endogenous damage-associated molecular patterns (DAMPs), which are capable of eliciting inflammatory responses, analogous to the immune response that is triggered by bacteria and viruses [40]. Galectin-3 is one of DAMPs. DAMPs can be subdivided into 3 broad categories: (1) intracellular molecules released by dying cells (ex: S100 proteins, HMGB1, IL-1α, galectin-3 and heat shock protein 60・70・72); (2) leaderless proteins secreted by professional immune cells (ex: HMGB1, IL-β, galectin-3 and uric acid); and (3) components of the extracellular matrix. Like other

members of the galectin family, galectin-3 does not possess a secretion signal peptide that would direct transport through the classical endoplasmic reticulum-Golgi apparatus secretory pathway. In this study, we detected high amounts of galectin-3 in the supernatants of necrotic HCEs.

In patients with VKC, a large number of eosinophils infiltrate the giant papillae and are degranulated to release toxic proteins. Eosinophils store 4 toxic proteins in their specific granules: 2 ribonucleases (ECP: eosinophil cationic protein and EDN: eosinophil-derived neurotoxin), a peroxidase (EPO: eosinophil peroxidase and MBP: Major basic protein). MBP is believed to exert its toxic effect by disrupting the membranes of parasites and bacteria. MBP has also been reported to show toxicity toward host cells, such as branchial epithelial cells in asthma and as keratinocytes in atopic dermatitis [41-44]. Several reports indicated that high concentrations of MBP and ECP were detected in the tears of patients with VKC [45-47]. These eosinophil toxic proteins in tears may induce corneal epithelial damage in VKC. Pseudoexfoliative epitheliopathy is a characteristic in VKC patients. However, the mechanism of this epitheliopathy is not well known. The possibility is that the debris of necrotic epithelial cells attached corneal surface via galectin-3 and mucins. Because galectin-3 interacts membrane associated mucins in corneal and conjunctival surface. Therefore, we hypothesize that galectin-3 in the tears of patients with VKC may be produced by necrotic corneal and conjunctival cells by eosinophil toxic proteins.

Many reports revealed that exogeneous galectin-3 enhanced cell migration in various corneal wound healing models [48-52]. Re-epithelization of corneal wounds was significantly slower in galectin-3 knockout mice, and exogeneous galectin-3 accelerated epithelial wound healing in rodent cornea [48-51] . Recently, Fujii A et al found that exogenous galectin-3 enhanced wound healing in monkey corneal epithelium [52] . The gene homology of galectin-3 between humans and monkey, mouse, and rat was 95%, 87%, and 83%, respectively. The group also detected relatively high levels of galectin-3 in corneal epithelium but negligible galectin-3 in tear fluid [52] . In contrast, in this study we detected high concentrations of galectin-3 in the tears of patients with VKC. Furthermore, we found that recombinant galectin-3 strongly induced the expression of genes related to the cell cycle, mitosis, cell cycle checkpoints, M phase, and MMP. The production and activation of MMP is required for cell motility at the leading edge of migrating epithelium during re-epithelialization [53] . Therefore, high concentrations of galectin-3 in the tears of patients with VKC may have a crucial role in the re-epithelization of corneal epithelial cells that have been damaged by eosinophils toxic proteins. In conclusion, the concentrations of galectin-3 in the tears of patients with VKC may represent a biomarker of the severity of corneal epithelial damage.

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Figure Legends

Fig. 1

(a) The concentrations of galectin-3 in the tear of VKC patients and healthy controls.
Each of the points represents the concentration of galectin-3 in the 26 patients and 6 healthy controls. The concentration of galectin-3 was higher in the tears of patients with VKC than in the tears of healthy controls. (2-tailed student's t test)

(b) The correlation between the concentrations of galectin-3 in the tear and the severity of corneal epithelial damage of VKC patients.
The concentrations of galectin-3 was significant correlated with the severity of corneal epithelial damage of VKC patients. (GraphPad Prism (version 9) Simple linear regression)

Fig. 2

The influences of tryptase and chymase in the expression of galectin-3 of cultured human corneal epithelial cells.
The mRNA and protein expression of galectin-3 in cultured human corneal epithelial cells (HCEs) treated with tryptase (a, c, e) and chymase (b, d, f). Real-time polymerase chain reaction showed that the mRNA expression levels of galectin-3 in HCEs treated with tryptase (a) and chymase (b) were not changed. Western blotting analysis showed that the protein expression of galectin-3 was not changed in cultured HCEs treated with tryptase (c) and chymase (d). Enzyme-linked immunosorbent assay (ELISA) showed that concentrations of galectin-3 were not changed in the supernatants of HCEs treated with tryptase (e) and chymase (f). (One-way ANOVA with Bonferroni's multiple-comparison test)

Fig. 3

The concentrations of galectin-3 in the supernatants of necrotic corneal epithelial cells.

High concentrations of galectin-3 detected in the supernatants of necrotic human corneal epithelial cells (HCEs), compared to that of intact HCEs. (2-tailed student's t test)

Fig. 4

Global gene expression analysis of human cultured corneal epithelial cells stimulated with galectin-3.

After 10 μg/mL of recombinant human galectin-3 or vehicle was added to cultures of HCEs for 24 hours, the comprehensive gene expressions were analyzed by microarray. (a) MA plot depicting the change in gene expression between control vehicle-treated and galectin-3–treated samples as the log2-fold change on the Y-axis and the log of mean of expression counts on the X-axis. (b) The heat map shows the top 18 genes that were upregulated or bottom 35 genes that were downregulated in HCEs with galectin-3. (c) Pathway analysis was performed on sets of genes exhibiting a change in expression.

Table

Table 1. The concentrations of galectin-3 in tears and severity of corneal epithelial damage of VKC patients

VKC Sample No.	Sex	Age	Galectin-3 (ng/ml)	Severity of epithelial damage	Treatment
1	M	12	9.10	3	A+D+E
2	M	8	9.15	3	A+D+E
3	M	8	9.30	2	A+D+E
4	F	13	8.91	2	A+E
5	M	15	9.18	3	A+E
6	F	16	9.12	2	A+E
7	M	9	9.30	3	C+E
8	M	7	9.16	3	A+E
9	M	6	8.76	3	A+E
10	F	10	5.92	3	A+E
11	F	10	1.92	0	C+E
12	M	9	2.20	1	C
13	M	12	3.92	1	A
14	F	16	4.12	2	A
15	M	18	1.82	2	A
16	M	14	0.92	1	B+E
17	M	15	0.90	1	B+E
18	F	19	0.31	1	B
19	M	8	0.18	0	E
20	M	10	0.07	0	E
21	M	9	N.D	0	D
22	F	8	N.D	0	D
23	F	7	N.D	1	D+E
24	M	9	N.D	0	E
25	M	15	0.81	1	D+E
26	M	13	0.82	1	D+E

M: Male

F: Female

Figure1

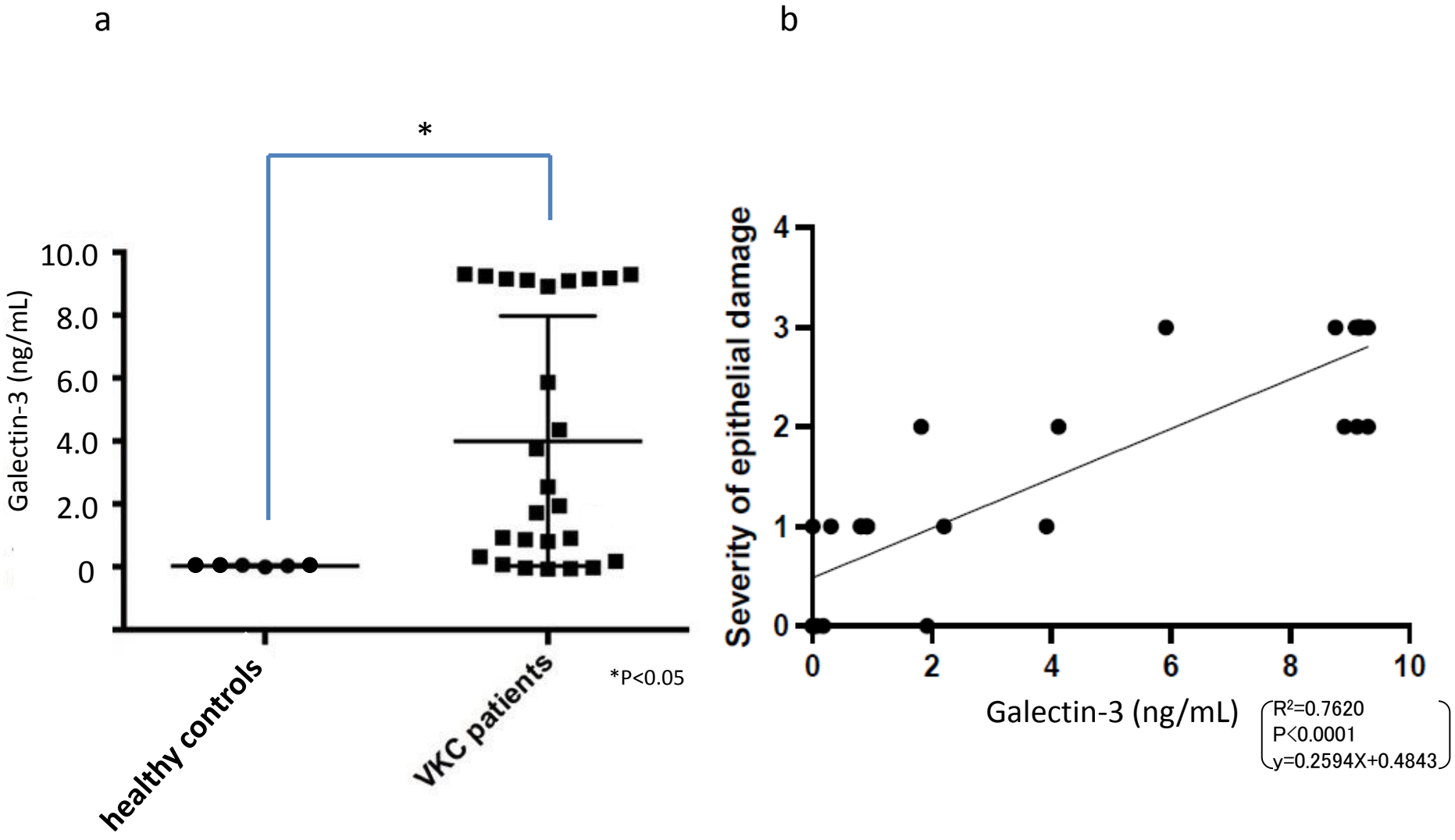

a
*
Galectin-3 (ng/mL)
10.0
8.0
6.0
4.0
2.0
0
healthy controls
VKC patients
*P<0.05
b
Severity of epithelial damage
4
3
2
1
0
0
2
4
6
8
10
Galectin-3 (ng/mL)
R²=0.7620
P<0.0001
y=0.2594X+0.4843

Figure2

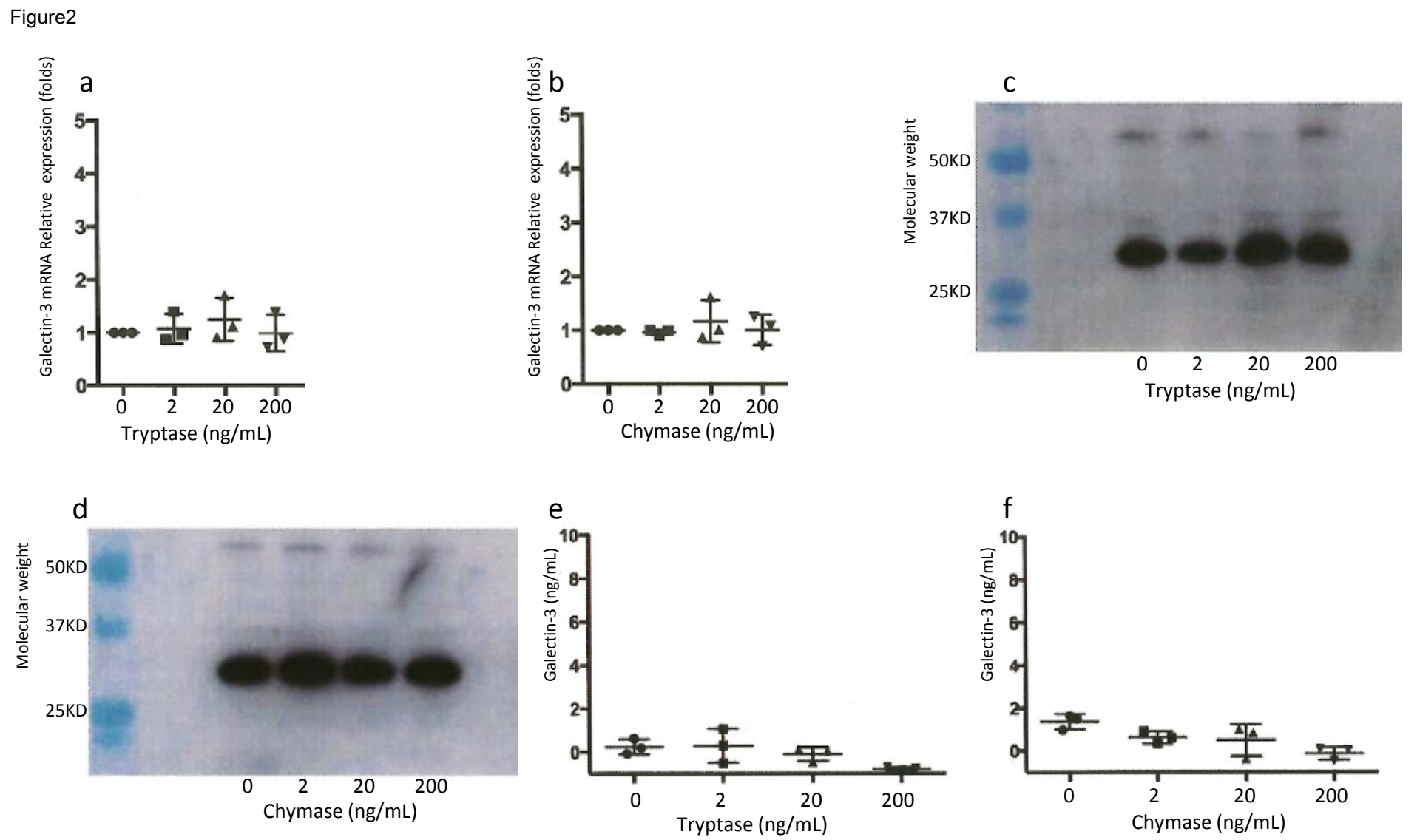

a
Galectin-3 mRNA Relative expression (folds)
0
1
2
3
4
5
0
2
20
200
Tryptase (ng/mL)
b
Galectin-3 mRNA Relative expression (folds)
0
1
2
3
4
5
0
2
20
200
Chymase (ng/mL)
c
Molecular weight
50KD
37KD
25KD
0
2
20
200
Tryptase (ng/mL)
d
Molecular weight
50KD
37KD
25KD
0
2
20
200
Chymase (ng/mL)
e
Galectin-3 (ng/mL)
0
2
4
6
8
10
0
2
20
200
Tryptase (ng/mL)
f
Galectin-3 (ng/mL)
0
2
4
6
8
10
0
2
20
200
Chymase (ng/mL)

Figure3

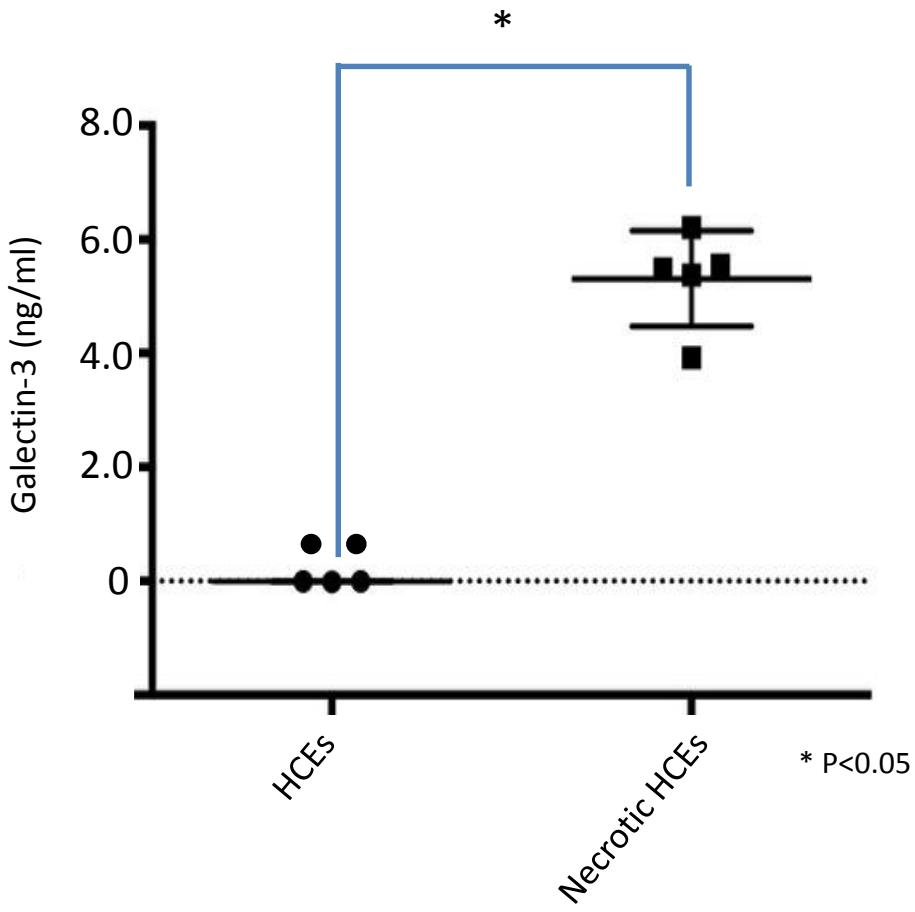
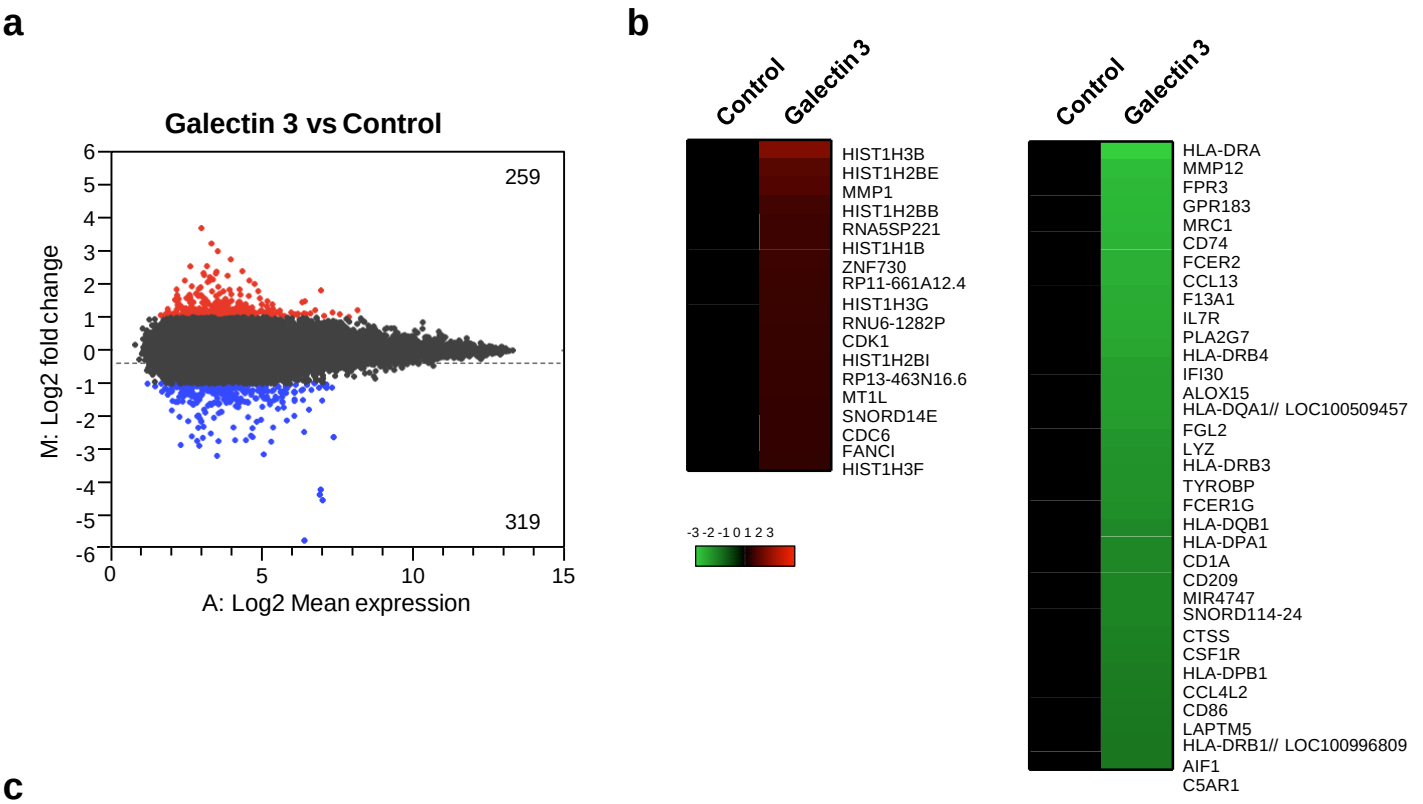


Figure4


a
Galectin 3 vs Control
259
319
M: Log2 fold change
A: Log2 Mean expression
b
Control
Galectin3
HIST1H3B
HIST1H2BE
MMP1
HIST1H2BB
RNA5SP221
HIST1H1B
ZNF730
RP11-661A12.4
HIST1H3G
RNU6-1282P
CDK1
HIST1H2BI
RP13-463N16.6
MT1L
SNORD14E
CDC6
FANCI
HIST1H3F
HLA-DRA
MMP12
FPR3
GPR183
MRC1
CD74
FCER2
CCL13
F13A1
IL7R
PLA2G7
HLA-DRB4
IFI30
ALOX15
HLA-DQA1// LOC100509457
FGL2
LYZ
HLA-DRB3
TYROBP
FCER1G
HLA-DQB1
HLA-DPA1
CD1A
CD209
MIR4747
SNORD114-24
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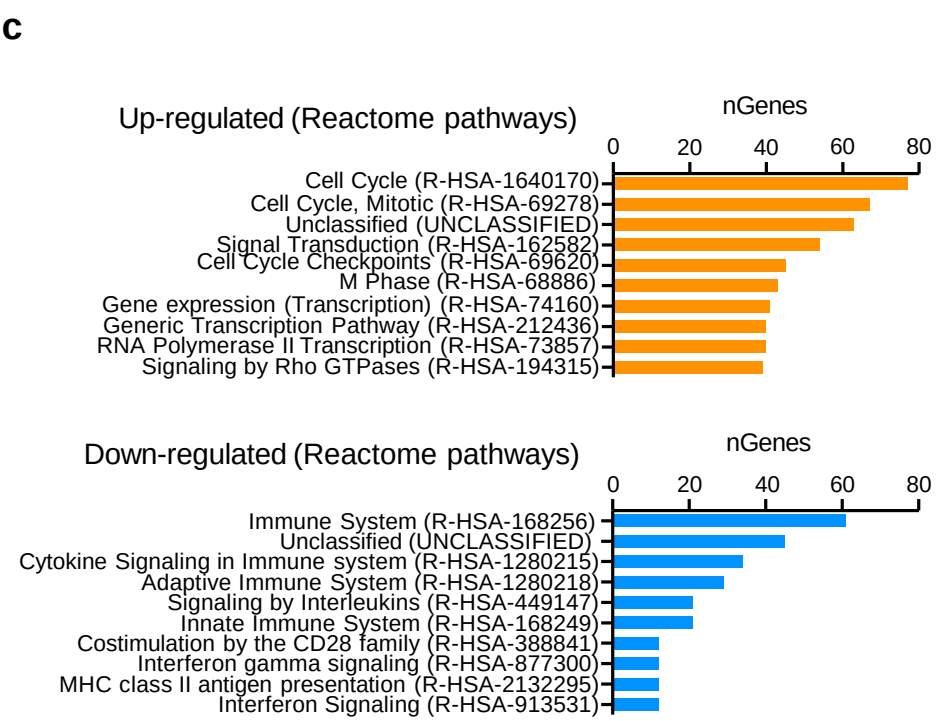
CD86
LAPTM5
HLA-DRB1// LOC100996809
AIF1
C5AR1


c
Up-regulated (Reactome pathways)
nGenes
Cell Cycle (R-HSA-1640170)
Cell Cycle, Mitotic (R-HSA-69278)
Unclassified (UNCLASSIFIED)
Signal Transduction (R-HSA-162582)
Cell Cycle Checkpoints (R-HSA-69620)
M Phase (R-HSA-68886)
Gene expression (Transcription) (R-HSA-74160)
Generic Transcription Pathway (R-HSA-212436)
RNA Polymerase II Transcription (R-HSA-73857)
Signaling by Rho GTPases (R-HSA-194315)
Down-regulated (Reactome pathways)
nGenes
Immune System (R-HSA-168256)
Unclassified (UNCLASSIFIED)
Cytokine Signaling in Immune system (R-HSA-1280215)
Adaptive Immune System (R-HSA-1280218)
Signaling by Interleukins (R-HSA-449147)
Innate Immune System (R-HSA-168249)
Costimulation by the CD28 family (R-HSA-388841)
Interferon gamma signaling (R-HSA-877300)
MHC class II antigen presentation (R-HSA-2132295)
Interferon Signaling (R-HSA-913531)

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Article entitled: Galectin-3, a Damage-Associated Molecular Pattern, in Tears of Patients with Vernal Keratoconjunctivitis

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