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Autoimmune Dacryoadenitis and Keratoconjunctivitis Induced in Rabbits by Subcutaneous Injection of Autologous Lymphocytes Activated ex vivo Against Lacrimal Antigens

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Abstract

Autologous peripheral blood lymphocytes (PBL), activated in a mixed cell reaction when co-cultured with purified rabbit lacrimal epithelial cells, are known to induce a Sjögren's-like autoimmune dacryoadenitis and keratoconjunctivitis when injected directly back into the donor animal's inferior lacrimal gland (LG). This study shows that autoreactive lymphocytes injected subcutaneously in a site away from the LG is capable of inducing an autoimmune disease in a rabbit. Induced disease (ID) develops more slowly, taking 4 weeks as compared to 2 weeks in the direct injection model. Initially, both clinical symptoms and histopathology are less pronounced than in the direct injection ID model, but later the immunocytochemistry shows the same CD4⁺/CD8⁺ ratio of 4:1 for both injection methods. The finding that lymphocytes activated against lacrimal antigens can travel or home from the injection site back to the inferior and superior LG, as well as the conjunctiva, suggests that these anatomical sites may have common epitopes that induce pathogenic CD4⁺ T cells that produce a Sjögren's-like syndrome.

Keywords

Dry eye; Sjögren's syndrome; lacrimal gland; autoimmune disease

1. Introduction

Approximately 4 million Americans have a severe form of dry eye associated with Sjögren's syndrome. Sjögren's is an inflammatory autoimmune disorder characterized by lymphocytic

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infiltration of the LG [1–3]. Immunologic, genetic, hormonal, and environmental factors all play crucial roles in the manifestation of autoimmunity in the LG that results in tear film instability, blurred vision, ocular surface damage, and visual fatigue [4–7]. While lymphocytic infiltration of the LG is associated with exocrine insufficiency [8,9], the mechanisms linking the presence of lymphocytes with functional quiescence and eventual destruction of the secretory parenchyma have not been elucidated. Evidence from mouse autoimmune disease models points to roles for inflammatory mediators, such as IL-1 and TNF- α [10], while other studies implicate agonistic anti-M₃-muscarinic acetylcholine autoantibodies in a novel form of receptor desensitization [11]. In most autoimmune disorders, the infiltrates consist mainly of CD4⁺ and CD8⁺ T cells; but in Sjögren's syndrome the excess of CD4⁺ T cells over CD8⁺ T cells (about 4:1) is unusually high [12].

Animal models have been developed to elucidate the pathogenesis of dry eye syndrome and to evaluate experimental therapies. Murine models resembling secondary Sjögren's syndrome have been widely used [13–15], but several studies have been conducted in mouse and rat models with induced diseases resembling primary Sjögren's syndrome [16,17] and in dogs with spontaneous autoimmune dacryoadenitis [18].

Mircheff et al. [19] and Guo et al [20] reported that LG epithelial cells present autoantigen epitopes and induce activation and proliferation of co-cultured, autologous peripheral blood lymphocytes (PBLs). Lymphocytes that have been activated in this ex vivo model of autoimmunity adoptively transfer a robust, focal, dacryoadenitis when injected directly into the donor rabbit's remaining lacrimal tissue [21]. The infiltrates consist mostly of CD4⁺, CD8⁺T cells at a 4:1 ratio same as in Sjögren's syndrome, and CD18⁺ bone marrow-derived cells. Rabbits with autoadoptively transferred dacryoadenitis exhibit clinical signs of a bilateral ocular surface disease, which were shown in initial studies to become progressively more severe over a period of 8 weeks [22].

The present study tested the hypothesis that administration of autoreactive lymphocytes by subcutaneous injection, i.e., a protocol that avoids trauma to the lacrimal gland or ocular surface, would be sufficient to induce Sjögren's-like autoimmune dacryoadenitis and keratoconjunctivitis.

2. Materials and Methods

2.1 Animals and Immunocytochemical Reagents

Female New Zealand white rabbits, each weighing between 3.4 and 4 kg, were obtained from Irish Farms (Norco, CA). The animals were maintained and used in compliance with institutional guidelines and in accord with the Association for Research in Vision and Ophthalmology Resolution on the Use of Animals in Ophthalmic Research. Clinical examinations were performed on all eyes prior to any experimental procedures to establish baseline data and to exclude any animals with ocular defects. Schirmer strip paper was purchased from Rose Stone Enterprises (Alta Loma, CA). FUL-GLO fluorescein strips and rose bengal strips were purchased from Akorn Inc. Laboratories (Buffalo Grove, IL). Antibodies specific for rabbit CD4, CD8, and CD18 were purchased from Spring Valley Labs (Woodbine, MD) and antibodies specific for RTLA (rabbit T lymphocyte antigen) were obtained from Cedarlane Laboratories (Hornby, Ontario, Canada). Species-specific secondary antibodies were obtained from Chemicon International (Temecula, CA). ABC reagent was obtained from Vector Laboratories Inc. (Burlingame, CA).

2.2 Clinical Ocular Surface Studies

All ocular surface assessments were performed on day 0 for baseline and at 2 and 4 weeks post injection. Schirmer's tests were performed without anesthesia on both eyes of all animals. Tear film stability was evaluated by instilling fluorescein and determining the tear break up time (BUT) under examination with a slit lamp biomicroscope equipped with a blue filter [22]. Ocular surface defects were determined following rose bengal staining. A standardized grading system [22] was used to score the intensity of staining of the cornea.

2.3 Lymphocyte Activation in Mixed Cell Reaction

Left inferior LG were surgically removed under anesthesia for the isolation of purified epithelial cells (pLGEC) using the methods described by Guo et al. [20]. Peripheral blood was collected from each rabbit for isolation of lymphocytes. pLGEC and PBLs were cultured separately for 2 days. The pLGECs were then gamma irradiated (2500RAD) and co-cultured in a mixed cell reaction with an equal number of autologous PBLs for 5 days at 37°C. Mixed cell reactions in 96-well plates were used for ³H-thymidine uptake assays of PBL activation, and parallel reactions in 12-well plates were used to obtain cells for autoadoptive transfer experiments. PBLs from mixed cell reactions with stimulation indices greater than 2 were considered to contain activated lymphocytes (autoreactive lymphocytes) and were injected subcutaneously back into the neck of the donor rabbits, hereafter referred to as the ID/ systemic group (n=9). The control group of animals (n=8) also had their left inferior glands removed, but were injected with non-stimulated lymphocytes (i.e. autologous PBLs that had not been co-cultured with pLGECs). The third group received stimulated PBLs directly in the right inferior lacrimal gland (ID/direct, n=7).

2.4 Histopathology and Immunocytochemistry Studies

Rabbits were sacrificed at 4 weeks post injection. Right inferior lacrimal gland (iLG) and both superior lacrimal glands (sLGs) were removed and bisected longitudinally. One part was fixed in 10% formalin and embedded in paraffin for histology. The paraffin-embedded tissue samples were sectioned at 5 µm, de-paraffinized, and stained with hematoxylin and eosin (H&E). The other part of each gland was embedded in OCT and cryosectioned at 7 µm for immunostaining. Immunostaining was done as described previously [24]. Briefly, the sections were fixed in chilled acetone, air dried, and rehydrated in phosphate buffered saline. Blocking was done in 5% BSA for 15 minutes. The sections were then incubated at room temperature for 1 hr with the primary antibody at an appropriate dilution: mouse anti-rabbit CD4 (1:200), mouse anti-rabbit CD8 (1:200), mouse anti-rabbit CD18 (1:1000), and goat anti-rabbit RTLA (1:500). Sections were rinsed and incubated for 60 minutes with appropriate secondary antibodies. After rinsing, the sections were quenched in 0.3% H₂O₂ in 40% methanol for 15 minutes and incubated in ABC reagent for 30 minutes, rinsed 3 times and developed in a solution of 0.05% diaminobenzidine containing 0.03% H₂O₂ for 3 minutes. The sections were again rinsed, counterstained with hematoxylin, and mounted for photography. The positive cells showed an intense brown color in the blue hematoxylin background. Entire sections were scanned and analyzed with Analysis 3.0 (Olympus Soft Imaging System Lake Wood, CO), an automated cellular imaging system. This combination of a proprietary, color-based imaging technology with automated microscope provides quantitative data, including percent positive with intensity scoring and area measurement. Data collected from clinical analysis and the automated cellular imaging systems were subjected to paired t test and analysis of variance.

3. Results

3.1 Clinical Evaluation of Ocular Surface

All eyes in the three study groups were assessed initially and at 2 and 4 weeks post-injection. Clinical evaluations included tear production determined by Schirmer's test, tear BUT, and rose bengal staining. Results are presented in Fig. 1 A–C. The mean Schirmer test value for control eyes was 7.5 ± 0.5 mm (Fig. 1A). No significant changes in tear production were recorded in the ID/systemic group at 2 weeks post injection, but a 25 % decrease ($p \leq 0.05$) was observed after 4 weeks. In the ID/ direct group (Fig 1A) the tear production was impaired significantly at 2 weeks post injection and remained low even after 4 weeks. As seen in Fig. 1B, tear BUT remained constant for the control group throughout the 4 week study, whereas tear BUT for the ID/systemic group decreased from 15.3 ± 0.5 to 12.3 ± 1 by 2 weeks post-injection and to 7.6 ± 1 by 4 weeks post-injection. In ID/direct group the tear BUT decreased at 2 weeks time and did not rebound in 4 weeks.

The rose bengal staining mean baseline scores for the control and ID groups were 0.6 based on a scale of 4. The mean score for the ID/systemic group increased to 1.8 ± 1 at 2 weeks post-injection and 3.4 ± 1 at 4 weeks post-injection (Fig. 1C). The positive staining score for the ID/direct group increased to 2.5 at 2 weeks time and 3.5 at 4 weeks post injection showing a significant persistent damage in the ocular surface damage.

3.2 Lacrimal Gland and Conjunctival Histopathology

Examination of H&E-stained sections revealed that the inferior LGs from the control group (Fig. 2 A) had only rare immune cell infiltrates whereas those from the ID/direct group (Fig. 2 B) were heavily infiltrated and the immune cells were frequently concentrated around the ducts and venules. Some of these areas showed degenerating acini, but other areas appeared to be completely free of infiltrates. The ID/systemic (Fig 2 C) showed perivenular and periductal infiltrates. The size of the lymphocytic foci was comparatively smaller than in the ID/direct iLG. The sLGs from the ID/direct group also showed more infiltration than in the control group (Fig. 2 D&E). Numerous plasma cells were also present in the intra-acinar space of the superior LGs from the ID animals, and necrotic activity was evident in a few cases (not shown). The ID/systemic sLG also showed infiltrates in a similar pattern as seen in ID/direct sLG.

The conjunctiva from 1 of the 8 control animals (Fig. 3A), showed occasional lymphocytic infiltration, which was diffused and mild compared to the infiltration observed in conjunctiva from the ID/systemic group (Fig. 3B). The conjunctiva of 6 of the 9 ID/systemic animals contained lymphocytic foci within the epithelium, and multiple sites of aggregated lymphocytes were present within the venules. The conjunctiva from ID/direct had significantly more infiltration than in control group (data not shown).

3.3 Characterization of Immune Cells Infiltrating the Lacrimal glands

The infiltrates of the inferior and superior LGs of ID/systemic animals were immunopositive for CD4, CD8, RTLA, and CD18 (Fig. 4A–H, respectively). CD4⁺ cells were primarily concentrated in aggregates around ducts and venules. CD8⁺ cells, although less frequent, were found around venules in a few samples and scattered throughout the interstitial space and between the acini. RTLA-positive cells were more commonly found as foci around the venules and often appeared as clusters. CD18⁺ cells were also evident in perivenular foci. CD18⁺ indicates the presence of macrophages and plasma cells. The results of quantitative image analyses are summarized in Table 1. Cells staining intensively for CD18 were observed in both the inferior and superior LGs, but CD18⁺ cells were significantly more abundant in inferior LGs. As summarized in Table 1, both types of LGs from the ID/systemic group contained

significantly more CD4⁺, CD18⁺, and RTLA⁺ cells than the corresponding glands from the control group. In contrast, the numbers of CD8⁺ cells did not differ significantly between the groups. The focal infiltrates of immune cells were abundant in periductal and interacinar areas in ID/direct iLG. All the antigens studied increased in ID/direct group except CD8 which did not change in number from the control group. Cells immunopositive for CD4 was found enveloping the venules and ducts and also found in interacinar areas. The foci were larger for CD4, RTLA and CD18 expressing cells in the ID/direct animals (Fig 5 A–D) compared to the ID/systemic group iLG. In some instances the ductal epithelium was also immunopositive. There was variation in the size of the foci in different lobes of the ID/direct group also there was variation in the immunopositivity in different lobes of the same gland. The expression pattern of CD4, CD8, RTLA and CD18 in ID/direct sLG was very similar to that of ID/systemic sLG (data not shown)

4. Discussion

The present study, using the New Zealand White rabbit as experimental model, demonstrates that activated effector lymphocytes that have been expanded *ex vivo* in mixed cell reactions with autologous pLGEC adoptively transfer autoimmune pathologies to the inferior and superior lacrimal glands, conjunctiva, and cornea when injected subcutaneously into their original donor animals. Niederkorn et al [27] demonstrated that autoreactive effector cells generated in an *in vivo* model are potentially capable of adoptively transferring dacryoadenitis and keratoconjunctivitis to naïve recipient mice. When lacrimal exocrine function was paralyzed by systemic administration of scopolamine, continuous exposure to a low-humidity environment and regular periods of exposure to high airflow induced an expansion of autoreactive effector CD4⁺ T lymphocytes. The specific circumstances required for adoptive transfer in the murine models reveal a significant difference between the relative robustness of the immunoregulatory mechanisms of mice and rabbits. In contrast to the findings of the present study, Niederkorn et al [27] found that autoimmune pathophysiology could not be transferred to immunocompetent recipients; it could only be transferred to immunodeficient nude mice or to Balb/c mice that had been depleted of regulatory T cells by injection of anti-CD25. We believe that the fundamental difference between Niederkorn's rodent model and our model is that the rodent relies much more heavily on TGF- β than does the rabbit. The level of TGF mRNA is about 10-fold higher in rodent than in rabbit (unpublished data).

The results of our current study indicate that the presence of a sufficient number of activated effector cells abrogates normal immunoregulatory mechanisms in the rabbit, cornea, and conjunctiva. It is interesting to note the differences in the time-courses and severities of the ocular surface and pathophysiologies that arise following direct injection into the lacrimal glands versus those that occur following systemic injection [22]. A comparison of the clinical and histopathology parameters resulting from the different routes of injection is provided in Table 2. The direct injection route resulted in a more rapid decline in both tear production and tear stability compared to the systemic route. These observations are reminiscent of the prolonged time-course of salivary histopathology in SCID mice that had been injected intraperitoneally with isolated mononuclear cells from the submandibular glands of MRL/pr mice with autoimmune sialadenitis. No inflammatory lesions were detectable at 4 weeks post-injection, but Sjögren's-like lesions were present in the salivary and s after 8 weeks [28].

The tissue damage was very pronounced in the direct injection model, with a large number of lymphocytes in the periductal area infiltrating between the lobules with a reduced number of acini. But in the systemic injection model the lymphocytes were fewer in number with some intact lobules. Tissue damage caused by the initial autoimmune response might be aggravated with the large recruitment of lymphocytes, which in turn trigger the production of pro-inflammatory factors in the direct injection model. In the case of systemic injection model, the

presence of fewer infiltrating cells accounts in part for the slow progression of the disease; and the initial autoimmune response may not be sufficient to make necessary changes in the epithelial cells to perform like APCs.

Although the dacryoadenitis induced by subcutaneous injection was milder than that induced by direct injection into the lacrimal gland, in both cases the infiltrates contained far more CD4⁺ T cells than CD8⁺ T cells. This is a characteristic feature of immunohistopathology in patients with Sjögren's syndrome [12], and in the lacrimal glands of several murine models, including aly/aly and r1ΔT/r2n Mice [17,29].

Several studies have confirmed that epithelial cells can produce inflammatory cytokines [30, 31,10]. Therefore, it is possible that the greater severity of the disease that occurs after direct injection results from inflammatory mediators elicited at least partially by trauma to the tissue synergizing with the vastly greater number of activated effector cells that are delivered into the gland almost instantaneously.

In summary, autoimmune dacryoadenitis and keratoconjunctivitis can be induced in rabbits by either direct or systemic/subcutaneous injection with autologous lymphocytes activated ex vivo against lacrimal antigens. The disease induced by direct injection of autoreactive lymphocytes into the inferior lacrimal gland appears more quickly and is more severe than disease induced via a systemic injection site. The induction of disease both on the ocular surface and in the lacrimal gland via a subcutaneous injection of autoreactive lymphocytes supports our contention that (1) inflammation in the gland is not caused solely by the actual injection and (2) lymphocytes activated against lacrimal antigens can home from the injection site back to the lacrimal gland.

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References

1. Lemp MA. Report of the National Eye Institute/Industry workshop on Clinical Trials in Dry Eyes. *Clao J* 1995;21:221–232. [PubMed: 8565190]
2. Schaumberg DA, Sullivan DA, Buring JE, Dana MR. Prevalence of dry eye syndrome among US women. *Am J Ophthalmol* 2003;136:318–326. [PubMed: 12888056]
3. Schein OD, Munoz B, Tielsch JM, Bandeen-Roche K, West S. Prevalence of dry eye among the elderly. *Am J Ophthalmol* 1997;124:723–728. [PubMed: 9402817]
4. Foulks GN. The evolving treatment of dry eye. *Ophthalmol Clin North Am* 2003;16:29–35. [PubMed: 12683246]
5. Mircheff AK, Warren DW, Wood RL. Hormonal support of lacrimal function, primary lacrimal deficiency, autoimmunity, and peripheral tolerance in the Ocular Immunol. *Inflamm* 1996;4:145–172.
6. Pflugfelder SC. Antiinflammatory therapy for dry eye. *Am J Ophthalmol* 2004;137:337–342. [PubMed: 14962426]
7. Stern ME, Beuerman RW, Fox RI, Gao J, Mircheff AK, Pflugfelder SC. A unified theory of the role of the ocular surface in dry eye. *Adv Exp Med Biol* 1998;438:643–651. [PubMed: 9634949]
8. Fox RI, Kang HI. Pathogenesis of Sjogren's syndrome. *Rheum Dis Clin North Am* 1992;18:517–538. [PubMed: 1323135]
9. Tsubota K, Saito I, Miyasaka N. Granzyme A and perforin expressed in the s of patients with Sjogren's syndrome. *Am J Ophthalmol* 1994;117:120–121. [PubMed: 7904795]

10. Zoukhri D, Hodges RR, Byon D, Kublin CL. Role of proinflammatory cytokines in the impaired lacrimation associated with autoimmune xerophthalmia. *Invest Ophthalmol Vis Sci* 2002;43:1429–1436. [PubMed: 11980857]
11. Qian L, Xie J, Rose CM, Sou E, Zeng H, Hamm-Alvarez SF, Mircheff AK. Altered traffic to the lysosome in an ex vivo lacrimal acinar cell model for chronic muscarinic receptor stimulation. *Exp Eye Res* 2004;79:665–675. [PubMed: 15500825]
12. Pepose JS, Akata RF, Pflugfelder SC, Voigt W. Mononuclear cell phenotypes and immunoglobulin gene rearrangements in biopsies from patients with Sjogren's syndrome. *Ophthalmology* 1990;97:1599–1605. [PubMed: 1965021]
13. Gao J, Killeddar S, Cornelius JG, Nguyen C, Cha S, Peck AB. Sjogren's syndrome in the NOD mouse model is an interleukin-4 time-dependent, antibody isotype-specific autoimmune disease. *J Autoimmun* 2006;26:90–103. [PubMed: 16413168]
14. Theofilopoulos AN, Dixon FJ. Murine models of systemic lupus erythematosus. *Adv Immunol* 1985;37:269–390. [PubMed: 3890479]
15. Yeoman CM, Franklin CD. Assessment of sulphasalazine as a treatment modality in Sjogren's disease in NZB/NZW F1 hybrid mice. *Clin Exp Rheumatol* 1996;14:53–57. [PubMed: 8697658]
16. Liu SH, Prendergast RA, Silverstein AM. Experimental autoimmune dacryoadenitis. I. disease in the rat. *Invest Ophthalmol Vis Sci* 1987;28:270–275. [PubMed: 8591907]
17. Oak JS, Deane JA, Kharas MG, Luo J, Lane TE, Cantley LC, Fruman DA. Sjogren's syndrome-like disease in mice with T cells lacking class 1A phosphoinositide-3-kinase. *Proc Natl Acad Sci U S A* 2006;103:16882–16887. [PubMed: 17071741]
18. Kaswan RL, Salisbury MA, Ward DA. Spontaneous canine keratoconjunctivitis sicca. A useful model for human keratoconjunctivitis sicca: treatment with cyclosporine eye drops. *Arch Ophthalmol* 1989;107:1210–1216. [PubMed: 2757551]
19. Mircheff AK, Gierow JP, Lee LM, Lambert RW, Akashi RH, Hofman FM. Class II antigen expression by lacrimal epithelial cells. An updated working hypothesis for antigen presentation by epithelial cells. *Invest Ophthalmol Vis Sci* 1991;32:2302–2310. [PubMed: 1830042]
20. Guo Z, Azzarolo AM, Schechter JE, Warren DW, Wood RL, Mircheff AK, Kaslow HR. epithelial cells stimulate proliferation in autologous lymphocyte preparations. *Exp Eye Res* 2000;71:11–22. [PubMed: 10880272]
21. Guo Z, Song D, Azzarolo AM, Schechter JE, Warren DW, Wood RL, Mircheff AK, Kaslow HR. Autologous lacrimal-lymphoid mixed-cell reactions induce dacryoadenitis in rabbits. *Exp Eye Res* 2000;71:23–31. [PubMed: 10880273]
22. Zhu Z, Stevenson D, Schechter JE, Mircheff AK, Atkinson R, Trousdale MD. Lacrimal histopathology and ocular surface disease in a rabbit model of autoimmune dacryoadenitis. *Cornea* 2003;22:25–32. [PubMed: 12502944]
23. Zhu Z, Stevenson D, Schechter JE, Mircheff AK, Crow RW, Atkinson R, Ritter T, Bose S, Trousdale MD. Tumor necrosis factor inhibitor gene expression suppresses immunopathology in a rabbit model of autoimmune dacryoadenitis. *Cornea* 2003;22:343–351. [PubMed: 12792478]
24. Trousdale MD, Zhu Z, Stevenson D, Schechter JE, Ritter T, Mircheff AK. Expression of TNF inhibitor gene in the promotes recovery of tear production and tear stability and reduced immunopathology in rabbits with induced autoimmune dacryoadenitis. *J Autoimmune Dis* 2005;2:6. [PubMed: 15985164]
25. Mircheff, AK.; Wang, Y.; de Saint Jean, M.; Ding, C.; Schechter, JE. Lacrimal epithelium mediates hormonal influences on APC and lymphocyte cycles in the ocular surface system. In: Zierhut, M.; Rammensee, HG.; a. J. W. S., editors. *Antigen Presenting Cells and the Eye*. New York: Informa; 2007. p. 93-119.
26. Mircheff AK, Wang Y, Jean Mde S, Ding C, Trousdale MD, Hamm-Alvarez SF, Schechter JE. Mucosal immunity and self-tolerance in the ocular surface system. *Ocul Surf* 2005;3:182–192. [PubMed: 17131026]
27. Niederkorn JY, Stern ME, Pflugfelder SC, De Paiva CS, Corrales RM, Gao J, Siemasko K. Desiccating stress induces T cell-mediated Sjogren's Syndrome-like lacrimal keratoconjunctivitis. *J Immunol* 2006;176:3950–3957. [PubMed: 16547229]

28. Hayashi Y, Haneji N, Hamano H, Yanagi K. Transfer of Sjogren's syndrome-like autoimmune lesions into SCID mice and prevention of lesions by anti-CD4 and anti-T cell receptor antibody treatment. *Eur J Immunol* 1994;24:2826–2831. [PubMed: 7957574]
29. Takada K, Takiguchi M, Konno A, Inaba M. Spontaneous development of multiple glandular and extraglandular lesions in aged IQI/Jic mice: a model for primary Sjogren's syndrome. *Rheumatology (Oxford)* 2004;43:858–862. [PubMed: 15126673]
30. Hunger RE, Carnaud C, Vogt I, Mueller C. Male gonadal environment paradoxically promotes dacryoadenitis in nonobese diabetic mice. *J Clin Invest* 1998;101:1300–1309. [PubMed: 9502771]
31. Zoukhri D. Effect of inflammation on function. *Exp Eye Res* 2006;82:885–898. [PubMed: 16309672]

Fig. 1. Basal tear production

Fig 1A Schirmer test was performed as previously described (Zhu et al., 2003a) on 17 rabbits (Day 0) and then nonstimulated lymphocytes were injected subcutaneously in the neck of 8 rabbits (designated as control group). Dacryoadenitis was induced by subcutaneous injection with stimulated lymphocytes from the mixed cell reaction in 9 rabbits (designated as induced dacryoadenitis ID/systemic group). In the third group dacryoadenitis was induced by injecting stimulated lymphocytes from the mixed cell reaction into the contralateral inferior lacrimal gland (7 rabbits and designated as ID/direct). The left lacrimal gland of each rabbits was excised for mixed cell reaction and the other gland was not manipulated in control and ID/systemic group. The Schirmer test was repeated at 2 and 4 weeks post-injection. An asterisk indicates

a significant difference from the control group with ID/direct and ID/systemic with $p \leq .05$. P . The values were calculated using independent sample t-tests. Fig. 1B. **Tear break-up time.** Tear break-up time demonstrates tear instability (Zhu et al., 2003a). Slit-lamp examination was performed in all three groups before surgery (Day 0) and after 2 weeks and 4 weeks. The group designations and statistical analysis were the same as Fig. 1A. Fig. 1C. **Rose bengal score.** Detection of ocular surface defects due to deficiency in precorneal tear film protection was evaluated with rose bengal stain as previously described (Zhu et al., 2003a) and the intensity of staining of the medial and lateral bulbar conjunctiva and the cornea was scored. The designations for groups and statistical analysis are the same as in Fig 1A.

Fig. 2. Histopathology with H&E stained s

Fig. 2A. Inferior lacrimal gland from a control rabbit injected subcutaneously with nonstimulated lymphocytes 4 weeks post injection. Inflammatory cells were rare. Fig. 2B. Section from an ID/direct iLG at 4 weeks post-injection. Large lymphocytic foci were observed around venules and within the connective tissue around ducts. Plasma cells were also abundant. Fig. 2C. Inferior lacrimal gland section from ID/systemic group. The infiltrating cells were most commonly found around ducts and venules. The size of the lymphocytic foci were smaller compared to the ID/direct model. Fig. 2D. sLG sections from control rabbits rarely displayed immune cells compared to ID samples. Fig. 2E. Infiltrating lymphocytes were common in the sLG of ID/direct rabbits, especially around venules. Some lobules were rich in plasma cells. Fig. 2F. The infiltration in ID/systemic sLG was comparable to that of ID/direct sLG.

Fig. 3. Histopathology of conjunctiva

Fig. 3A. Infiltration was rare in the conjunctiva of control animals. Fig. 3B Conjunctiva from ID/systemic animals showed infiltrating lymphocytic foci in the epithelium and in vessels.

Fig. 4. Immunocytochemistry studies of CD4, CD8 RTLA and CD18 expression at 4 weeks post-injection

Frozen sections of inferior and superior s (iLG and sLG) from ID/systemic animals were immunostained for CD4, CD8, RTLA and CD18. Fig. 4A&B. Both iLG and sLG displayed significant numbers of CD4⁺ cells mainly around ducts and venules (see arrows). The control gland sections for both iLG and sLG showed very few dispersed CD4⁺ cells (not shown). Fig. 4C&D. Both iLG (C) and sLG from ID/systemic animals displayed CD8⁺ cells around the ducts and dispersed in interacinar spaces. Fig. 4E&F. RTLA⁺ cells were numerous in both iLG and sLG of ID animals. RTLA⁺ cells appeared in dense foci enveloping the venules, between the acini, and dispersed throughout the gland. Fig. 4G&H. Distribution of CD18⁺ cells was

similar to RTLA⁺ cells in both iLG and sLG of ID animals. CD18⁺ cells were sparse in iLG and sLG of control animals (not shown).

iLG-ID/Direct

Fig. 5. Immunocytochemistry studies of CD4, CD8, RTLA and CD18 expression at 4 weeks post-injection in iLG from ID/direct animals

Frozen sections of iLG from ID/direct animals were immunostained for CD4, CD8, RTLA and CD18. Fig 5A. iLG: CD4⁺ cells were abundant surrounding ducts and venules. Fig 5B. CD8⁺ cells were present but infrequent around ducts and venules. Fig 5C: RTLA⁺ cells were abundant in dense foci around the ducts and venules and dispersed throughout the gland. Fig. 5D: CD18⁺ cells were distributed similar to RTLA cells.

Table 1

Immunohistochemical analysis of inflammatory cells in following injection with either non-non-stimulated (control) or stimulated T lymphocytes (ID/systemic)

Tissue Marker	Tissue	Control (Mean % $\pm$ SE)	ID /systemic (Mean % $\pm$ SE)	P Value
RTLA	iLG	4.4 $\pm$ 0.6	7.4 $\pm$ 0.17	0.001
	sLG	2.3 $\pm$ 0.3	4.3 $\pm$ 0.7	0.02
CD18	iLG	0.7 $\pm$ 0.3	2.3 $\pm$ 0.4	0.004
	sLG	1.7 $\pm$ 0.4	3.7 $\pm$ 0.9	0.05
CD4	iLG	0.21 $\pm$ 0.13	0.73 $\pm$ 0.24	0.005
	sLG	0.37 $\pm$ 0.13	1.07 $\pm$ 0.34	0.07
CD8	iLG	0.09 $\pm$ 0.03	0.20 $\pm$ 0.07	0.09
	sLG	0.60 $\pm$ 0.23	0.20 $\pm$ 0.05	0.08

Inferior and superior glands were removed at 4 weeks post-injection. For each marker studied 16 sections were stained by ABC method and analyses were done in a cellular imaging system Analysis 3.0 (Olympus Soft Imaging System Lake Wood, CO). Data are shown as mean positive percentage $\pm$ SE. Both groups of rabbits received injections subcutaneously in the neck.

Table 2

Comparison of two inoculation routes for induction of autoimmune dacryoadenitis in a rabbit model

Parameter Evaluated	ID/direct	ID/systemic
Onset of detectable clinical dry eye	2w post injection	4w post injection
<u>Severity of Disease</u>		
Schirmer Test	< 50%	< 25%
Tear Break-up Time	< 60%	< 40%
Rose Bengal Score	> 2.8	> 3.5
Lymphocytes Infiltration in LGs	Fold Increase	Fold Increase
RTLA ⁺	> 7.4	> 1.7
CD18 ⁺	> 21	> 3.2
CD4 ⁺	>7.3	> 3.5

In the direct injection method, the stimulated lymphocytes from the mixed cell reaction were injected directly into the remaining gland. In the systemic injection method the stimulated lymphocytes were injected subcutaneously in the neck. The numbers represent the percent decrease or increase from the respective control group. Onset of disease was compared between the direct injection (2 weeks) vs. the subcutaneous method (4 weeks).