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Treg-recruiting microspheres prevent inflammation in a murine model of dry eye disease

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Abstract

Dry eye disease (DED) is a common ocular disorder affecting millions of individuals worldwide. The pathology of DED involves the infiltration of CD4⁺ lymphocytes, leading to tear film instability and destructive inflammation. In the healthy steady state, a population of immunosuppressive T-cells called regulatory T-cells (Treg) regulates proliferation of immune cells that would otherwise lead to a disruption of immunological homeostasis. For this reason, it has been suggested that Tregs could restore the immunological imbalance in DED. To this end, one possible approach would be to recruit the body's own, endogenous Tregs in order to enrich them at the site of inflammation and tissue destruction. Previously, we have demonstrated a reduction of inflammation and disease symptoms in models of periodontitis corresponding to recruitment of endogenous Tregs, which was accomplished by local placement of controlled release systems that sustain a gradient of the chemokine CCL22, referred to here as Treg-recruiting microspheres. Given that DED is characterized by a pro-inflammatory environment resulting in local tissue destruction, we hypothesized that the controlled release of CCL22 could also recruit Tregs to the ocular surface potentially mediating inflammation and symptoms of DED. Indeed, data suggest that Treg-recruiting microspheres are capable of overcoming the immunological imbalance of Tregs and CD4⁺ IFN- γ ⁺ cells in the lacrimal gland. Administration of Treg-recruiting microspheres effectively mitigated the symptoms of DED as measured through a number of

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outcomes such as tear clearance, goblet cells density and corneal epithelial integrity, suggesting that recruitment of endogenous Treg can mitigate inflammation associated with DED.

Keywords

Dry eye disease; CCL22; Treg-recruiting microspheres; Drug delivery; Tregs

1. Introduction

Dry eye disease (DED) is a multifactorial ocular condition, characterized by inflammation of the ocular surface and tear film instability, affecting as many as 1 in 5 individuals globally [1–4]. Recent studies have revealed that inflammation plays a prominent role in the pathology of the disorder [5,6]. Specifically, the pathology of DED is now known to be a result of infiltration of pathogenic pro-inflammatory CD4⁺ T cells, causing a breakdown in immunological homeostasis and compromise of the lacrimal functional unit, which includes the cornea, conjunctiva, lacrimal glands, meibomian glands, and the inter-connecting innervation [5,6]. As T cells proliferate in ocular tissues, they secrete pro-inflammatory cytokines, such as IFN- γ [7,8], which inhibit naturally suppressive immune cells known as regulatory T cells (Tregs). Ultimately, this leads to a shift in the immunological balance between tissue-protective functional Tregs and tissue-destructive pro-inflammatory (effector) T cells [6]. For this reason, it has been suggested that increasing the local number of these immunosuppressive cells (re-establishing their local presence and influence over the tissue microenvironment) could represent a potential form of treatment for DED. For instance, adoptive transfer of *ex vivo* expanded Tregs into mice with a form of DED suppresses inflammation [9,10]. Due to the immunosuppressive properties of Tregs, the application of *ex vivo* cell expansion and transfer has been proposed as a method of therapeutic modulation in order to enhance the numbers of Tregs [11]. However, the advantages of *ex vivo* cell therapy may be somewhat diminished due to regulatory hurdles, and the difficulty of maintaining the phenotypical fidelity of Tregs, which can differentiate into either non-functional or even conventional effector T cells [12].

An alternative approach to enhance local Treg populations could be chemotactic recruitment of endogenous Tregs to the ocular tissue [13]. Macrophage-derived C-C motif chemokine ligand 22 (CCL22) is known to promote chemotactic migration of cells that express the corresponding chemokine receptor CCR4 [14–16]. Notably, since Tregs express higher levels of CCR4 than other cells [17], CCL22 can be used for preferential recruitment of endogenous Tregs [18,19]. Originally, Curiel et al. demonstrated that ovarian tumors can suppress anti-tumor immune responses, in part, by secreting CCL22 to recruit tumor-protective Tregs [20]. Subsequent studies demonstrated that cells/tissues that are attacked by the immune system (*e.g.* islet allografts or skin melanocytes in patients with vitiligo) can be protected by gene therapy to attract suppressive Tregs by producing CCL22 [21,22]. Our group previously demonstrated that an alternative method to gene therapy such as the sustained release of CCL22 from biodegradable polymeric poly (lactic-co-glycolic acid) (PLGA) microspheres (Treg-recruiting microspheres) can mimic cellular secretion and establishment of a gradient of CCL22 synthetically *in vivo* [18,19]. This leads to recruitment

of Treg *in situ* and a marked reduction of symptoms in models of a disease of local, destructive inflammation (periodontal disease) [18,19]. Given that DED is also characterized as a disorder of local, destructive inflammation, we hypothesized that this approach could recruit Tregs in order to reduce inflammation associated with DED.

Herein, we provide evidence that Treg recruitment, through local release of CCL22 in the lacrimal gland, effectively reduces inflammation in a model of DED. The findings from this study illustrate that the controlled release of CCL22 can influence endogenous Treg and, in turn, prevent destruction of ocular surface tissue.

2. Materials and methods

2.1. Fabrication of microspheres

Poly (lactic-*co*-glycolic) acid (PLGA) microspheres encapsulating recombinant mouse CCL22 (R & D Systems, Minneapolis MN) were formulated according to a previously reported water-oil-water double emulsion technique [19]. Briefly, 200 mg of Poly (D, L-lactide-*co*-glycolide) PLGA polymer (acid terminated, 50:50 lactide:glycolide, molecular weight 7000–17,000) (viscosity: 0.16–0.24 dL/g, 0.1% (w/v) in chloroform) (Sigma Aldrich, St. Louis, MO) was dissolved in 4 mL of dichloromethane and mixed *via* vortexing. Then 200 μ L of an aqueous solution containing 5 μ g of recombinant mouse CCL22 (R & D Systems, Minneapolis, MN) and 2 mg of BSA (Sigma Aldrich, St. Louis, MO) with 15 mmol NaCl was pipetted into the mixture of polymer and dichloromethane (Sigma Aldrich, MO). The first water-in-oil emulsion was prepared by sonicating the polymer with CCL22 solution at 25% amplitude for a period of 10 s. Then the second water-oil emulsion was created by homogenizing (L4RT-A, Silverson) the first water-oil emulsion with 60 mL 2% (wt./vol) polyvinyl alcohol (molecular weight ~25,000 g/mol, 98 mol% hydrolyzed; PolySciences) for a period of 60 s at 3000 rpms. The homogenized solution was then mixed with 1% polyvinyl alcohol and placed onto a stir plate for 3 h in order for the dichloromethane to evaporate. The microspheres were then collected and washed four times *via* centrifuge with deionized water in order to remove any remaining residual polyvinyl alcohol. Lastly, the microspheres were placed in 5 mL of deionized water, frozen in liquid nitrogen, and lyophilized for 72 h (VirTis BenchTop K freeze dryer). The microspheres were then stored at -20°C until use.

2.2. Characterization of microspheres

The morphology of the Treg-recruiting microspheres was characterized using scanning electron microscopy (JEOL, JSM-6330F, Peabody, MA) and volume impedance measurements were performed on a Beckman Coulter Counter (Multisizer-3, Beckman Coulter, Fullerton, CA). *In vitro* release was determined by incubating 10 mg of Treg-recruiting microspheres in 1 mL of phosphate buffered saline (PBS) with 1% BSA, placed on a rotator at 37°C . Release media (supernatant) was sampled periodically. Specifically, triplicates of each release sample were centrifuged at 3500 rpm at 4°C . After centrifuging 800 μ L of the supernatant was removed and placed into an Eppendorf tube and then replaced by 800 μ L of new PBS with 1% BSA (Leaving the remaining 200 μ L for a total volume of 1 mL). The CCL22 concentrations were quantified using an enzyme-linked immunosorbent

assay (ELISA) (R & D Systems, Minneapolis, MN). The encapsulation efficiency was completed by dissolving 10 mg of microspheres in 200 μ l of dichloromethane (DCM) (Sigma Aldrich, MI). Subsequently, 200 μ l of PBS was added to the dissolved microspheres and centrifuged. The remaining aqueous supernant was isolated and the concentration of protein was quantified using an enzyme-linked immunosorbent assay (ELISA) (R & D Systems, Minneapolis, MN).

2.3. Experimental DED model and treatments

Female Balb/c mice aged 6–8 weeks were used in this study (8 mice per group) (Charles Rivers Laboratories, Wilmington, MA). All animal experiments were approved by the Institutional Animal Care and Use Committee, University of Pittsburgh. To induce DED, mice were anesthetized, and 10 mg/mL of Concanavalin A (ConA) (Sigma Aldrich, St. Louis, MO) of saline was injected (30 μ l) into each lacrimal gland with a 28.5 gauge needle under a dissecting microscope (Olympus SZX10, Waltham, MA) [23,24]. DED treatments included Blank (empty) or Treg-recruiting microspheres (25 mg/mL), which were mixed with ConA or saline and injected into the lacrimal glands. Soluble CCL22 was injected with ConA at a concentration of (62.5 ng/1 mL) (volume of 30 μ l injected). For controls intended to probe the necessity of Treg action, Treg function was inhibited *in vivo* using anti-GITR (DTA-1) (BioXCell, Lebanon, NH), which was injected intraperitoneally (500 μ g per mouse) five days prior to the injection of ConA and Treg-recruiting microspheres [19].

2.4. Measurement of tear production

Tear production was measured with phenol red cotton threads (Oasis Medical, San Dimas, CA). Thread was placed in the lateral canthus of the eye for a period of 60 s, and wetting was measured in millimeters using a dissecting microscope (Olympus SZX10, Waltham, MA) [23].

2.5. Corneal permeability

To evaluate the corneal epithelial layer, fluorescein stain (1 μ L of 1% solution) was applied to the conjunctival sac and 5 μ l of saline was used to wash off any excess dye. The surface of the cornea was evaluated using a dissecting microscope with a fluorescent excitation lamp (Olympus SZX10, Waltham, MA). Eyes were evaluated in a masked fashion by an independent ophthalmologist, and scored 0 for no staining, score 1 for a quarter of staining, score of 2 for less than a half, score of 3 for half, and 4 for more than half of the eye.

2.6. Histopathology

At the end of the study, eyeballs were exenterated then harvested and fixed in formalin for 24 h. Eyes were sectioned 5 μ m thick and stained with Periodic Acid Schiff (PAS) to identify goblet cells in the conjunctiva. The histology section images were scanned using a Zeiss Axio Scan Z1 (Thornwood, NY). Numbers of goblet cells were quantified using Panoramic Viewer software (3D HISTECH Ltd.) of Zeiss Axio Scanned slides at 20 $\times$.

2.7. Immunophenotyping analysis by flow cytometry

Lacrimal glands, regional draining cervical lymph nodes (CLN) were harvested from the experimental murine groups at the end of the study, and single cell suspensions were prepared. In order to increase total cell counts multiple tissue samples were combined. Cells were stained with the following fluorescent conjugated antibodies: anti-CD4 eFluor450 (RM4–5), anti-CD25 APC-Cy7 (PC61), anti-FoxP3 PE (FJK-16s) (eBioscience, San Diego, CA and BD Bioscience, San Jose, CA). For intracellular cytokine staining, the cells were placed in a 96-well plate overnight in cell culture media with Cell Stimulation Cocktail (plus protein transport inhibitor, eBioscience) and stained with anti-IFN- γ FITC (XMG 1.2) (eBioscience, San Diego, CA). Absolute counting beads were used for the lacrimal gland (ThermoFisher Scientific, Waltham, MA). Stained cells were analyzed using FlowJO (Ashland, OR).

2.8. Statistical analysis

Data are expressed as mean $\pm$ standard deviation. Comparisons between multiple treatment groups were performed using one-way ANOVA, followed by Bonferroni multiple comparisons, and $p < 0.05$ was considered statistically significant. Statistical tests were performed using GraphPad Prism Software 6.0, San Diego, CA.

3. Results

3.1. Characterization of Treg-recruiting microspheres

We first sought to engineer a Treg-recruiting MS formulation that sustained the release of the chemokine, CCL22 in a relatively linear fashion such that they could potentially be used to produce a biological gradient *in vivo*. A double emulsion-evaporation technique was used to encapsulate CCL22 (hydrophilic protein with high water solubility) into poly (lactic- α -glycolic) acid (PLGA-polymer, which is hydrophobic with a low water solubility and high solubility in dichloromethane) to produce microspheres [18,19]. Treg-recruiting MS were then characterized to determine size, surface morphology, and release kinetics. Specifically, a Coulter Counter was used to determine the average size of the microspheres, which were approximately 16 μm (Fig. 1C). The Coulter Counter software guided the selection of an optimal concentration range for the sample in order to obtain a size distribution. Moreover, this microsphere size was designed to avoid cellular uptake by macrophages and other phagocytes [25]. The microspheres were also designed to contain surface pores, which ensured minimal burst release of CCL22 from the system [18]. This can be accomplished through adjustment of the osmolality difference between the inner and outer aqueous phases as described previously by Jhunjhunwala et al. [18] Surface porosity was confirmed using scanning electron microscopy (SEM) (Fig. 1A, B) [18]. Additionally, an enzyme linked immunosorbent assay (ELISA) was performed to determine protein loading and measure release from the microspheres over time (Fig. 1D). Lastly, in order to determine the encapsulation efficiency an ELISA was completed, and the total amount of CCL22 encapsulated in the microspheres was approximately 4.66 ng $\pm$ 0.5 (18.64% encapsulation efficiency).

3.2. Treg-recruiting microspheres prevent decrease in tear production, corneal staining, and goblet cell depletion

To assess whether Treg-recruiting microspheres could prevent clinical evaluations associated with DED, tear production, corneal fluorescein staining, and goblet cell density were used individually as outcomes [26]. Concanavalin A (ConA-T cell mitogen) served to establish DED (Fig. 1), which causes T cell proliferation resulting in the clinical signs associated with DED [27]. Saline administration (in place of ConA) served as a negative control [27]. As expected, ConA-treated mice (diseased) exhibited significantly reduced tear secretion, relative to saline (non-diseased). In treatment groups (“treatment” here is referred to application of the formulation at the same time as the establishment of the disease), microspheres (0.5 mg/30 μ l) were injected locally into the lacrimal gland (Fig. 2). The treatment group significantly prevented tear production loss as compared to the diseased mice. Specifically, the wetting of thread (measured in millimeters) for the treatment group was 4.75 ± 0.317 mm. Moreover, the thread wetting for the diseased-with-blank-microsphere group was 3.500 ± 0.503 mm and the diseased was 3.446 ± 0.320 mm. Overall, blank microspheres did not worsen the signs of DED compared to the untreated, disease group (Fig. 3A).

The integrity of the corneal epithelial barrier *via* fluorescein staining was also examined as an indicator of disease severity. As seen in Fig. 3, Treg-recruiting microsphere administration appears to maintain the integrity of the corneal epithelium. Consistent with an intact/un-damaged cornea, no staining was observed on the corneas of the treatment group and non-diseased mice after instillation of fluorescein (Figs. 2B, C). The corneal fluorescein scoring for the diseased was 1.656 ± 1.347 as compared to the saline, which had an average staining score of 0.5625 ± 0.629 and saline with blank microspheres of 0.4375 ± 0.512 corneal fluorescein staining score. In contrast, corneal permeability is known to cause a decrease in the rate of fluorescein elimination in DED patients compared to healthy individuals [28]. Upon examination, central punctate fluorescein staining was observed on corneas of the diseased, diseased with blank MP treatment, and diseased with soluble CCL22 groups (Fig. 3C).

Histological sections of the ocular tissue were also observed to make record of any differences in treated *vs.* untreated groups in our model of DED. After one week, eyes were exenterated, and the conjunctiva was stained with Periodic Acid Schiff (PAS) to identify goblet cells (appearing as bright purple/pink cells) interspersed throughout the stratified squamous cells of the conjunctiva. As a loss of goblet cell density occurs, tear film composition changes correlating to severity of conjunctival disease such as DED [29]. The diseased mice, with or without administration of blank microspheres and diseased with soluble CCL22 exhibited a significant decrease in goblet cell density compared to the non-diseased control group (saline and saline with blank microspheres. Specifically, the soluble CCL22 (bolus) was approximately 45 ± 8.417 goblet cells while the diseased with blank microspheres was 41.625 ± 11.363 , and diseased 36.375 ± 5.343 total numbers of goblet cells counted in the conjunctiva. Administration of Treg-recruiting microspheres diminished goblet cell depletion to levels comparable of those observed in non-diseased mice (Fig. 4).

3.3. Treatment with Treg-recruiting microspheres decreases the frequency of CD4⁺ T cells in regional draining lymph nodes

To determine whether attenuation of DED symptoms by Treg-recruiting microspheres was due to local cellular changes in the regional lymphoid tissue, a phenotypic analysis was performed of T-cell populations in the cervical lymph nodes (CLN). CLN from diseased mice exhibited a significantly greater percentage of CD4⁺ T cells compared with non-diseased mice, both with and without administration of blank microspheres (Fig. 5A). In order to further assess phenotypic expression of CD4⁺ T cell population expression of the intracellular cytokine, IFN- γ was investigated. Accordingly, our results suggest that there is a significant difference between the diseased-with-blank microspheres, non-diseased, soluble CCL22 and the treatment group (Fig. 5B). Interestingly, there was also a decrease in the percentage of Tregs for the treatment group (as compared to diseased-with-blank microspheres), but no significant difference in activated T effectors and Tregs with the soluble CCL22 in the draining lymph nodes as compared to the treatment group (Fig. 5C and D). Additionally, the diseased mice exhibited significantly higher levels of CD4⁺CD25⁺Foxp3⁻ in the CLN compared with non-diseased mice (Fig. 5C); this was reduced in Treg-recruiting microsphere groups as compared to diseased mice treated with blank microspheres.

3.4. Treatment with Treg-recruiting microspheres reduces infiltration of CD4⁺ IFN- γ ⁺ T cells in the lacrimal gland

To determine whether Treg-recruiting microspheres alter T-cell populations in the lacrimal gland, lacrimal glands were harvested and flow cytometry was performed to analyze T-cell populations. Specifically, frequencies of pro-inflammatory CD4⁺ IFN- γ ⁺ (Th1) T cells and CD4⁺ FoxP3⁺ Tregs were examined in the gland. We observed significantly greater proportions of IFN- γ -producing CD4⁺ T cells (Fig. 6A) and significantly lower proportions of FoxP3⁺ Tregs (Fig. 6B) in the lacrimal glands of diseased mice, diseased-with-blank microspheres, and soluble CCL22 as compared to the treatment group (Treg recruiting microspheres). Notably, the treatment group exhibited a significant reduction in the frequency of IFN- γ ⁺ cells with a concomitant increase in the frequency of FoxP3⁺ Tregs (Fig. 6A and B), relative to untreated diseased mice. The CD4⁺ IFN- γ ⁺ (Th1) T cells and CD4⁺ FoxP3⁺ Tregs in the treatment group were similar to the saline and saline-with-blank microspheres (Fig. 6A and B). Ultimately, shifts in the pro-inflammatory cytokine, IFN- γ ⁺, and anti-inflammatory Treg populations contributed to a two-fold increase in the Treg/Th1-type ratio in the lacrimal gland of the treatment group relative to diseased, and was significantly higher for the soluble CCL22 and blank microspheres (Fig. 6C).

3.5. Administration of anti-GITR reverses the effect of regulatory T-cell recruiting formulations

To confirm that the observed suppressive effects were due to Tregs, a glucocorticoid-induced tumor necrosis factor receptor (GITR) agonistic antibody (Anti-GITR) was administered 5 days before injection of treatment. Anti-GITR is thought to inhibit the ability of Tregs to suppress other T cells (non-Tregs) [30]. We compared the two groups ConA and Treg-recruiting microspheres of Figs. 4 and 5 with the anti-GITR. As expected, anti-GITR

injected before treatment completely eliminated the symptom-reducing effects of Treg-recruiting microspheres, with tear production and punctate staining of the cornea comparable to (or a lower degree than) that of the diseased mice (Figs. 7A, B, C, D). There was also a significant decrease of the number of goblet cells in the anti-GITR + Treg-recruiting MS as compared to the treatment group. (Figs. 7 D, E). Additionally, mice treated with anti-GITR before treatment did display a significantly greater percentage of total CD4⁺ T cells as compared to the treatment (Fig. 7F). In addition, there was no significant difference of activated effector T cells and comparable numbers of IFN- γ ⁺ Th1-type cells in the CLN of the anti-GITR + Treg-recruiting MS, relative to the diseased mice (Fig. 7F). Moreover, the lacrimal gland tissue of mice treated with anti-GITR exhibited similar frequencies of CD4⁺ IFN- γ ⁺ T cells, as compared to diseased mice and a significantly lower percentage of CD4⁺FoxP3⁺ as compared to the Treg-recruiting MS group (Fig. 7G). Overall, the administration of anti-GITR worsened signs of disease and significantly increased Th1 cells in the lacrimal gland.

4. Discussion

DED is an ocular disorder initiated by aberrant inflammation, ultimately leading to ocular surface damage, tear film instability, and inflammation of the lacrimal gland [6,8,31–34]. Given the current understanding of the inflammatory basis of DED, there are several different existing models that can be used to examine immune mediated inflammation in DED. Some of these murine models include mice exposed to a controlled environment chamber, injection of scopolamine hydrobromide with desiccating stress, and injection of either botulinum toxin B or ConA into the lacrimal gland [23,24,28,35–37]. Our study utilized ConA, a T-cell mitogen, in order to induce inflammation in an experimental murine model of DED (Fig. 2) [23,24,27]. Although this murine model does not in every way replicate the natural induction of DED, this particular animal model was utilized because it results in lymphocyte mediated destructive inflammation, a hallmark of DED [7,8,31,35,38]. Moreover, this model replicates CD4⁺ T cell inflammation as seen in humans with DED, which enabled us to investigate the effects of a Treg-recruiting microsphere formulation engineered to balance such destructive inflammation [39].

The formulation used in this study was designed to balance inflammation through local recruitment of Tregs. This chemotactic recruitment is reliant upon a sustained gradient of CCL22, a molecule that binds to the T cell surface receptor, CCR4 inducing directional migration of Tregs [40–42]. The intended chemotactic gradient will ultimately be sensitive to the amount of concentration released, with higher amounts of release having the downside of saturating directional receptor binding and decreasing chemotaxis of leukocytes thus the gradient of the microspheres is critical for migration [41]. As Tregs migrate to a local area of inflammation, the immunological homeostatic balance is altered, resulting in reduction of inflammation (19). We observed that a bolus injection of soluble CCL22 into the lacrimal gland did not prevent clinical signs associated with ConA-induced DED (Figs. 3–4). This may be the result of the rapid diffusion of the soluble CCL22 from the lacrimal gland [40–42]. Interestingly, however, we did observe a significant decrease in overall CD4⁺ T cells in the cervical lymph nodes (CLN) (Fig. 5). Since soluble CCL22 injected into the lacrimal gland could drain to the CLN, this result may be attributed to a transient gradient-

independent effects of CCL22 on T-cell activation in the CLN [43]. The lack of therapeutic efficacy with soluble CCL22, however, supports previous studies that suggest that a sustained gradient of chemokine (here CCL22 originating from the lacrimal gland) is required for recruitment of lymphocytes due to the short *in vivo* half-lives of proteins and chemokines (in some cases on the order of 30 min) (here Tregs) [15,40–42,44–47]. As further confirmation, we observed Treg-recruiting microspheres at one week remaining in the lacrimal gland when cells were counted for flow cytometry (data not shown), which suggests that the microspheres remain in the lacrimal gland and maintain a sustained gradient of CCL22 to attract Tregs to the tissue.

In regard to the clinical signs of DED, we first investigated if the Treg-recruiting microspheres could preserve the health of ocular surface. Inflammation seen in DED can affect the integrity of the cornea, which can lead to ocular irritation and an increase risk of infections [2]. Therefore, in this study we examined the integrity of the cornea [48]. Our data suggest that there was a significant reduction of punctate staining on the corneal surface of the treatment group suggesting that the treatment led to maintenance of ocular surface homeostasis (Figs. 3B,C) [49,50]. By contrast, a disruption of the ocular surface can be due to an unstable tear film, which can contribute to inflammation of the ocular surface environment as seen in the diseased, diseased with blank microspheres and soluble CCL22 group (Figs. 3B,C) [51]. Notably, administration of Treg recruiting microspheres maintained significantly higher levels of tear production as compared to the diseased groups (Fig. 3). This is consistent with a previous finding that suggested that a reduction of tear production can result in corneal epithelial defects, potentially linking these two results [43]. Overall, the data suggest that Treg-recruiting microspheres aid in preserving corneal health, and preventing the loss of tear production.

Since goblet cells are involved in the production of mucin (a component of tear fluid) [52] we also investigated goblet cell density in our study. Histological sections revealed that the treatment was able to markedly decrease the attenuation of goblet cells (Fig. 4). This maintenance of goblet cells may be associated with lower levels of the Th1 cytokine (IFN- γ) secreted by effector T cells, which were lower in Treg recruiting microsphere treated mice (Fig. 6) [29,49,52]. Notably, it has been suggested that altered ratios IFN- γ /IL-13 cytokines are associated with goblet cell loss in the gut mucosa, which could be related to loss of goblet cells in the conjunctiva.

Since previous reports have suggested a correlation to the frequency of CD4⁺ IFN- γ ⁺ cells and goblet cell density, the phenotypic expression of T cells were examined to identify the percentage of cells in the regional draining lymph nodes [29,49,52]. To the best of our knowledge, previous reports utilizing this particular model have not investigated the phenotypic expression of T cells in the regional draining lymph nodes, which serve as critical sites for the induction of ocular surface immune responses [31,38,43]. Our data suggest that the administration of ConA possessed a significantly greater percentage of CD4⁺ T cell populations in the cervical lymph nodes (CLN), which might be due to ConA increasing the proliferation of CD4⁺ T cells [53]. Interestingly, populations of pro-inflammatory Th1 cells in the CLN of diseased mice and soluble CCL22 were observed to be significantly higher than the saline and saline-with-blank-microsphere control groups

(Fig. 5B). This is not unexpected, since mice challenged with ConA have been previously reported to possess an increased IFN- γ production [27]. Interestingly, treatment with Treg-recruiting microspheres is associated with a significant decrease of Th1-type cells when compared to administration of blank microspheres and soluble CCL22 (Fig. 5B). This is consistent with previous findings by our group and others that report how sustained local concentrations of CCL22 can decrease IFN- γ levels [19,22]. Moreover, Saban et al. demonstrated that the Th-1 type immune response can be induced in the regional lymph nodes causing ocular surface inflammation in DED [54]. Therefore, we hypothesize that the recruitment of Tregs may decrease the CD4⁺ IFN- γ response in the CLN ultimately leading to the prevention of ocular surface inflammation.

To further examine the effects of Tregs, resident T-cell populations were examined in the lacrimal gland [55]. Specifically, as immune cells migrate from the lymph nodes to the lacrimal gland, these cells secrete pro-inflammatory cytokines, perpetuating inflammation and negatively affecting function of the gland in DED [56]. Notably, the data suggest that the treatment with Treg recruiting MS decreases the percentage of CD4⁺ IFN- γ ⁺ T cells and concomitantly increases CD4⁺ FoxP3⁺ Treg populations, which may prevent inflammation mediated tissue destruction. This is consistent with other reports suggesting that Tregs can mediate a tissue-protective function by suppressing effector T-cells (Figs. 6A, C) [6,57,58]. Overall, the data suggests that Treg-recruiting microspheres generate a favorable increase in the ratio of Tregs to pro-inflammatory IFN- γ ⁺ Th1 cells, which suggests an explanation for the observations of suppressed ocular inflammation, goblet cell maintenance, and restored tear film production.

Finally, in order to demonstrate that the effect observed with Treg-recruiting microspheres was due to Tregs suppressing ocular inflammation, an agonistic monoclonal antibody against GITR was utilized to inhibit the suppressive effects of Tregs on effector T cells [59]. Anti-GITR is thought to modulate Tregs through directly abrogating their suppressive function while co-stimulating other conventional T cells [30,60]. In humans, it has been suggested that the intensity of GITR expression on Tregs directly corresponds to the suppressive ability of regulatory T-cells [61]. The ability of anti-GITR to suppress Treg function and worsen evaluations of DED here is consistent with recent work suggesting that the upregulation of GITR ligand expressed on retinal pigment epithelial cells in the eye *via* pro-inflammatory cytokines plays an integral role in ocular immunity health [61]. Consistent with the diseased, diseased-with-blank microspheres, and soluble CCL22 treated mice, there were also increased percentages of CD4⁺ IFN- γ ⁺ cells, and decreases in the percentages of Tregs in the lacrimal gland of mice treated with the Treg-recruiting microspheres and anti-GITR. Overall, the data suggests that anti-GITR reversed the beneficial effects of preventing clinical signs and inflammation associated with DED, likely by affecting the suppressive function of Tregs (Fig. 7) [51].

Even though the data suggest that the Treg-recruiting microspheres were able to prevent signs and reduce inflammation in DED, there are several limitations of this particular study of note. For instance, the double-emulsion fabrication technique utilized can result in protein denaturation in the water/oil interface, which may explain the low encapsulation efficiency and potential protein damage that was not able to be detected using an ELISA [62].

Although, the double emulsion technique is well established and has been utilized in many reports to encapsulate proteins, other techniques have shown to increase encapsulation efficiency and protein activity [63]. Future work could include fabricating the microspheres with an alternative process by Reinhold et al. that does not result in some protein damage and can enhance the encapsulation efficiency of the protein [64]. Additionally, we may need to examine residual polymer accumulation by examining histological sections of the tissue in order to identify whether the bio-degradation of PLGA over time could potentially cause granulation of tissue development [25]. Future work should be geared toward examining this potential polymer accumulation and confirm that the recruitment of Tregs to the lacrimal gland can overcome pre-established inflammation. We hypothesize that, if optimized, Treg-recruiting formulations could be effective as a potential therapeutic since it has been previously demonstrated that Tregs indeed play an integral role in resolving established inflammation associated with DED [9]. One particular point of focus could be the ability of recruited Tregs to overcome any pre-existing ocular surface tissue destruction, which may serve to catalyze inflammation and disease [65,66]. However, and notably, our previous data using Treg-recruiting microspheres in a murine model of periodontitis started a pronounced reversal of the characteristic destructive tissue microenvironment toward expression of markers that would best characterize a regenerative microenvironment [19]. Ultimately, however, further studies would be required to determine if the same shift in expression in the ocular microenvironment would result from treatment with Treg-recruiting formulations. It is also important to consider the method of administration for any potential therapeutic strategy. Although, injections (such as the ones into the lacrimal glands used herein) could be applicable given that injections in the eye and lacrimal gland have become more common in clinical studies and treatments [67–70], less invasive strategies (which are still effective) would (all things being equal) be more attractive [71,72].

5. Conclusion

In summary, our study collectively suggests that the administration of Treg-recruiting microspheres effectively prevents destructive inflammation in an experimental murine model of DED. Specifically, the sustained release of the chemokine CCL22 in the lacrimal gland prevented a loss of tear production, corneal fluorescein staining and goblet cell depletion, which resulted in the decrease of signs associated with DED. This resolution of inflammation and signs of DED is likely due to the increase of Tregs and decrease of Th1 cells in the lacrimal gland. Future studies will focus upon extending this proof-in-principle toward exploration of therapeutics that recruit the body's own endogenous Tregs to resolve inflammation in DED.

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Fig. 1. Characterization of engineered porous microspheres loaded with the chemokine CCL22 and blank microspheres. (A) Representative SEM image of porous blank microspheres (1000×) (B) Representative SEM image of porous microspheres with CCL22 encapsulated (1000×) (C) A representative particle size distribution obtained using a Coulter Counter shows the Treg-recruiting microspheres. (D) Release Kinetics of CCL22 (Treg-recruiting microspheres) from porous microspheres is shown ($n = 3$).

Experimental Timeline

Fig. 2.

Treg-recruiting MS for preventing dry eye disease (DED) in mice. At Day 0, ConA and Treg-recruiting microspheres (MS) were injected into the lacrimal glands. One week after the disease induction and administration of the Treg-recruiting microspheres, the prevention of signs associated with DED was evaluated using a phenol red thread test, corneal fluorescein staining, histology, and flow cytometry analysis.

Fig. 3.

Treg-recruiting MS prevent DED symptoms in mice. (A) Phenol red thread wetting measured in millimeters for aqueous tear production ($n = 8$) shown as mean $\pm$ S.D. (B) A clinical score of the ocular surface staining scored on a scale of (0–4) ($n = 8$) shown as mean $\pm$ S.D. (C) Representative images of corneal fluorescein staining show the integrity of the epithelial layer of the cornea. Green staining on the cornea is a positive indication of disease ($n = 8$). * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$.

Fig. 4.

Treg-recruiting MS prevent reduction of goblet cell density in the conjunctiva.

Representative images of histological sections of the eyes (20×) were examined to identify differences in the Treg-recruiting MS group compared to the diseased groups (100 μm scale bar). Goblet cells shown are the pink/purple cells located in the conjunctiva labeled with arrows and the groups are shown as mean ± S.D. **p* < 0.05; ***p* < 0.01; ****p* < 0.001;

*****p* < 0.0001.

Fig. 5.

Treg-recruiting MS suppress T effector cells in the regional draining lymph nodes. (A) Data from flow cytometry performed on the regional draining lymph nodes in order to examine overall CD4⁺ T cell percentages ($n = 8$) shown as mean $\pm$ S.D. (B) IFN- γ cytokine expression was examined in the regional draining lymph nodes ($n = 8$) shown as mean $\pm$ S.D. (C) Activated T effector percentages were analyzed using flow cytometry ($n = 8$) shown as mean $\pm$ S.D. (D) FoxP3⁺ Tregs analysis on the regional draining lymph nodes using flow cytometry ($n = 8$) shown as mean $\pm$ S.D. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$.

Fig. 6.

Treg-recruiting MS enhance anti-inflammatory responses in the lacrimal gland. (A) IFN- γ cytokine expression was analyzed in the lacrimal gland ($n = 8$) shown as mean $\pm$ S.D. (B) Analysis of intracellular staining for Tregs ($n = 8$) shown as mean $\pm$ S.D. (C) The ratio of CD4 $^+$ FoxP3 $^+$ /CD4 $^+$ IFN- γ^+ cells in the lacrimal gland ($n = 8$) shown as mean $\pm$ S.D. * p 0.05; ** p 0.01; *** p 0.001; **** p 0.0001.

Fig. 7.

Administration of anti-GITR reverses the prevention signs associated with DED by Treg-recruiting MS. (A) Quantification of corneal fluorescein staining was determined by a masked independent ophthalmologist on a scale of (0–4) ($n = 8$) shown as mean $\pm$ S.D. (B) Representative images of corneal fluorescein staining shows “fluorescein” punctate staining located on the cornea is an indication of disease ($n = 8$) shown as mean $\pm$ S.D. (C) Aqueous tear production was quantified using phenol red threads (D) Goblet cells located in the conjunctiva were counted using Pannaromic Viewer software and data is shown as mean $\pm$ S.D. (E) Representative histology images of the conjunctiva (20 $\times$) indicates arrows pointing to the pink/purple goblet cells ($n = 8$). (F) Data from flow cytometry was performed on the regional draining lymph nodes to examine percentages of CD4⁺ T cells, activated effectors FoxP3⁺ CD25⁺ (%CD4⁺), and IFN- γ ⁺ (%CD4⁺) cells ($n = 8$) shown as mean $\pm$ S.D. (G) Cytokine expression (IFN- γ) and (FoxP3⁺) Treg percentages were analyzed in the lacrimal gland using flow cytometry ($n = 8$) shown as mean $\pm$ S.D. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$. (Anti-GITR was injected intraperitoneally 5 days prior to ConA and Treg-recruiting MS) (Note: all groups include ConA).