Published in final edited form as:

Sci Transl Med. 2018 July 25; 10(451): . doi:10.1126/scitranslmed.aas9164.

Neutrophils cause obstruction of eyelid sebaceous glands in inflammatory eye disease in mice

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Abstract

Meibomian glands (MGs) are sebaceous glands of the eyelid margin that secrete lipids needed to avert tear evaporation and to help maintain ocular surface homeostasis. Obstruction of MGs or other forms of MG dysfunction can promote chronic diseases of the ocular surface. Although chronic eyelid inflammation, such as allergic eye disease, is an associated risk factor for obstructive MG dysfunction, it is not clear whether inflammatory processes contribute to the pathophysiology of MG obstruction. We show that polymorphonuclear neutrophils (PMNs) promoted MG obstruction in a chronic inflammatory model of allergic eye disease in mice. Analysis of leukocytes in tears of patients with MG dysfunction showed an increase in PMN numbers compared to healthy subjects. Moreover, PMN numbers in tears positively correlated with clinical severity of MG dysfunction. Our findings point to a role for PMNs in the pathogenesis and progression of MG dysfunction.

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Author contributions: N.J.R.: experimental performance, data analysis and interpretation for all experiments, manuscript preparation, and critical reading; C.Y.: experimental performance, data analysis and interpretation for all experiments, manuscript preparation, and critical reading; R.M.: experimental performance, manuscript preparation, and critical reading; C.M.K. and R.L.R.: experimental performance, data analysis and interpretation for mouse meibography, and critical reading of manuscript; J.K.: experimental performance and data interpretation for flow cytometry data; A.L.: experimental performance and interpretation of human specimens and critical reading of manuscript; V.L.P.: data interpretation of human specimens, manuscript preparation, and critical reading of manuscript; A.S.M.: experimental performance and data interpretation for Th17 mouse experiments and critical reading of manuscript; P.K.G.: experimental performance and data interpretation for human specimens, manuscript preparation, and critical reading of manuscript; D.R.S.: principal investigator of this study, secured funding, and involved in all aspects of this study.

Competing interests: The authors declare that they have no competing interests.

Data and materials availability: All data associated with this study can be found in the paper or the Supplementary Materials.

INTRODUCTION

The pathogenesis of meibomian gland dysfunction (MGD) is poorly understood (1), although it is one of the most common conditions encountered in ophthalmology clinic today (2, 3). These modified sebaceous glands of the eyelid margin provide the specialized lipids via holocrine secretion to the tear film, which are needed to avert tear evaporation and help maintain ocular surface homeostasis (4–9). Hence, terminal gland obstruction due to hyperkeratinization of the orifice (10–13), or other chronic diffuse abnormalities of MGs that cause qualitative/quantitative changes in glandular secretion, can have deleterious effects to the homeostasis and integrity of ocular surface (14–16). These effects may lead to increased evaporation, hyperosmolarity, and tear film instability, as well as bacterial growth on the lid margin, evaporative dry eye, inflammation, and damage of the ocular surface (17). In addition, MGD can have negative effects on patient quality of life (18–20). However, the etiology of MGD remains poorly understood.

One of the risk factors associated with obstructive MGD is the presence of chronic, eyelid inflammation (blepharitis) (3, 4, 21–23). Here, we focus on chronic allergic eye disease (AED) [atopic keratoconjunctivitis (AKC)], which is one of the classically recognized forms of chronic blepharitis associated with MGD (21, 22, 24, 25). Other inflammatory disease entities associated with MGD include ocular rosacea, mucous membrane pemphigoid, Sjogren's syndrome, and chronic graft-versus-host disease (3, 26–29). The study of immune responses in these settings could provide clues to help better understand the pathogenesis of obstructive MGD. However, most animal models that mimic aspects of MGD lack an underlying primary immunological condition to help tackle this problem (30–37). Moreover, only associative data regarding the role of inflammation in MGD have been obtained in the clinical setting (15, 17, 38, 39). Hence, new tools are needed to revisit the important and unresolved question of whether the immune response can contribute etiologically to obstructive MGD (40).

Here, we leveraged our established preclinical model of AED associated with chronic inflammation (41–47) and showed that these mice develop MG obstruction in a T cell-mediated fashion. Although T helper 2 (T_H2) lymphocytes were dominant in our model, we show that the T_H17-mediated response is responsible for MG obstruction. This lymphocytic response is associated with polymorphonuclear neutrophil (PMN) recruitment, which, in turn, is the downstream effector of MG obstruction. In agreement with the rodent data, we report here that patients with MGD bear an abnormally high content of PMNs in their tear fluids and showed a positive correlation between the magnitude of PMNs and clinical MGD severity. Together, our data confirm that MG obstruction has an immune-mediated component and suggest that these specific inflammatory responses of the ocular surface might be used as indicator of MGD severity. In addition, development of therapeutic strategies targeting this immune-mediated pathway might be effective for treating MGD in patients.

RESULTS

AED mice experience exacerbated and chronic ocular surface inflammation

Our first experiment directly compared the clinical responses in our model of AED (42) to a mild allergic conjunctivitis model (48). We induced AED in C57BL/6 mice by a single intraperitoneal injection of ovalbumin (OVA)/aluminum hydroxide (Alum) mixed with pertussis toxin (PTx), and then 2 weeks later, we challenged the mice with OVA eye drop instillations every day for 7 days. In the mild allergy model, mice were immunized with OVA/Alum in the absence of PTx. Clinical score was calculated by evaluating the sum of tearing, lid edema, chemosis (bulbar conjunctival edema), and conjunctival hyperemia in masked fashion (see Materials and Methods for details) (48–52).

Analysis of clinical scores showed that AED mice displayed increased disease severity compared to mice in the mild allergy model at 20 min and 6 hours after challenge (Fig. 1A). At 6 hours after challenge, whereas disease severity remained high in AED mice, the disease resolved almost completely in mice with mild allergy. We also determined the degree of leukocyte infiltration in the conjunctiva, which could explain these distinct clinical response patterns in mild allergy versus AED models. Flow cytometry analysis of conjunctiva revealed a higher frequency of total leukocytes (CD45⁺) and eosinophils (Siglec F⁺) in AED mice, as compared to the mild model (Fig. 1, B and C). Together, these data led us to conclude that AED mice had increased and chronic clinical disease associated with enhanced leukocytic infiltration levels including eosinophils.

In performing the clinical analysis, we observed that AED mice consistently presented obstructed MGs, as evidenced by grossly visible plugging of the gland orifices at the posterior eyelid margin (Fig. 1D). Mice with AED also developed other manifestations of ocular surface disease, albeit to a lower frequency and/or persistence. These manifestations included corneal epitheliopathy, anterior marginal blepharitis, and mucoid discharge (fig. S1, A and B). Conversely, in mild allergy setting, we found no evidence of plugging (Fig. 1D) or any other aforementioned manifestations (fig. S1, A and B). Hence, we concluded that the development of MG obstruction was unique to the AED model.

MG obstruction in AED model is a key manifestation

Because plugging of the MG orifice is a pathognomonic sign of underlying MGD (14, 21), our findings suggested the possibility of underlying MGD in AED mice. To address this question, we leveraged the clinically used infrared meibography of the eyelids (fig. S2A) (53). Using this system, we detected a significant (full, $P < 0.001$; superior, $P = 0.0010$; inferior, $P = 0.0086$) reduction in the MG areas (in square millimeters) in AED (Fig. 2A) compared to naive animals, indicating that morphological changes of MGs occurred in AED mice with orifice obstruction. To better understand these changes, we next performed histological analysis of inferior eyelids, where our meibography data detected the biggest reduction in MG area. Our findings revealed substantial alterations of the MGs in AED mice, which included differences in acinar morphology, frank dilation of the central duct, and thickening of the central duct epithelium (Fig. 2B and fig. S2B). Together, these data

suggest that orifice obstruction coincided with MG alterations, including a reduction in the overall area of MGs in AED mice.

T_H17 cells play a central role in mediating MG obstruction

We next assessed whether AED mice had increased T cell responses, which could help explain chronic blepharitis seen in these mice and, in turn, may be linked with obstruction of their MGs. We therefore assessed T_H1, T_H2, and T_H17 cell frequencies in the draining lymph nodes (LNs) of both mouse models. We found the presence of a robust T_H17 response (CD4⁺IL-17⁺) in AED mice, which was completely absent in the mild model (Fig. 3A). In contrast, T_H2 responses (CD4⁺IL-4⁺; IL-5⁺ and IL-13⁺) were evident in both mild and AED mice. The presence of T_H2 response was expected because both are allergy models, albeit T_H2 frequencies in AED mice were two to four times higher than in mild allergy mice (fig. S3A). Mice with AED also had slightly higher T_H1 frequencies (CD4⁺IFN- γ ⁺) when compared to their mild allergy counterparts (fig. S3A). Together, the data showed that AED mice had increased T_H cell responses as compared to the mild model, with a uniquely present and robust T_H17 response that was absent in mild model.

Given the robust T_H17 response we found in AED mice, we then asked whether T_H17 cells could play a role in the pathogenesis of MG obstruction. We first examined the role of interleukin-17a (IL-17a) in this process by inducing AED in *Il17a*^{-/-} mice (54, 55). Consistent with their genotype, we confirmed that IL-17a-producing T cells were absent in the LNs of these *Il17a*^{-/-} mice with AED (Fig. 3B). Moreover, whereas wild-type (WT) mice showed MG obstruction after AED induction, *Il17a*^{-/-} mice did not show MG obstruction, as indicated by the absence of plugging at the lid margin (Fig. 3C). Hence, we concluded that IL-17a in AED mice was a key player in mediating the development of MG obstruction.

To address the role of T_H17 cells in IL-17a-mediated development of MG obstruction, we tested the effects of reducing T_H17 cell frequency on MG obstruction by blocking IL-23 (p19) in the AED setting (56, 57). This cytokine is key in T_H17 cell stabilization (58, 59), and thus, its blockade in WT mice ought to result in a reduction of T_H17 cell frequencies. As predicted, when IL-23 was neutralized in AED (during the sensitization phase only), we found a >50% reduction of T_H17 frequencies in the LNs of AED mice (Fig. 3D). Moreover, IL-23 blockade protected AED mice from the development of MG obstruction, as identified by the reduction of clinical plugging at the lid margin (Fig. 3E). These data suggest that T_H17 cells were necessary to mediate MG obstruction.

To more directly test the role of T_H17 cells, we performed adoptive transfer experiments to determine whether MG obstruction could be induced in mild allergy model. To accomplish this task, we generated in vitro polarized T_H17 cells (OTII) (60, 61) and adoptively transferred them into mice after induction of mild allergy. We subsequently challenged these mice with OVA and analyzed the MGs. As controls, in addition to the nontransfer group, we generated in vitro polarized T_H2 cells (60, 61) and transferred these cells into another cohort of mild allergy mice, which were subsequently challenged with OVA. As expected, adoptive transfer of T_H17 cells resulted in increased frequencies of T_H17 cells in the draining LNs of mild allergy mice, whereas T_H2 cells (or no transfer) did not (Fig. 3F). Mild allergy mice

that received T_H17 cells developed MG obstruction, as clinically evidenced by orifice plugging at the eyelids, whereas the control groups did not (Fig. 3G). Together, these data suggest that T_H17 cells were necessary and sufficient to mediate the development MG obstruction in allergy models of different severity.

T_H17 causes MG obstruction by recruiting PMNs

The role of T_H17 cells in the recruitment and activation of PMNs has been previously described (62). Therefore, we next asked whether this T_H17-PMN axis might be relevant in the T_H17-mediated obstruction of MGs in our system. We first showed that the conjunctivae of AED mice were infiltrated with PMNs and that antibody blockade of IL-23 in AED mice resulted in a significant ($P = 0.0037$) reduction in conjunctival PMN infiltration (Fig. 4A). Blockade with IL-17a antibody produced the same result (fig. S4). We also asked whether mild allergy mice adoptively transferred with T_H17 cells, which leads to MG obstruction, had increased PMN infiltration. Our data showed that these mice had increased PMN infiltration, whereas mice transferred with T_H2 cells (or no transfer) did not (Fig. 4B). Hence, together, we showed that the T_H17-mediated MG obstruction in AED was associated with PMN infiltration in the conjunctiva.

We next determined whether PMNs were responsible for causing MG obstruction. To address this question, we depleted PMNs *in vivo* by treating AED mice with an antibody targeting a lymphocyte antigen 6 complex locus G6D (Ly6G), a protein specifically expressed by PMNs (63). Ly6G antibody or the isotype control was only given during the OVA challenge phase. This depletion strategy resulted in an absence of PMNs in the conjunctiva of AED mice (Fig. 4C). PMN depletion protected AED mice from the development of MG obstruction (Fig. 4D). Thus, our data suggest that in our model of AED, T_H17 cells recruited PMNs that, in turn, mediated the development of MG obstruction.

PMN cell number of patient tears positively correlates with MGD severity

To confirm the observations from our preclinical mouse model, we leveraged the use of flow cytometry to analyze leukocytes in tear fluids of MGD patients. Because we identified PMNs as the downstream effectors of MG obstruction in our model, we sought to determine PMN numbers in MGD patient tear fluids compared to non-MGD controls. Tears, like fluids at other mucosal sites, harbor immune cells (64–66) and can be used to ascertain information about immune activity of ocular surface tissues without the need of invasive procedures. In a pilot experiment, we showed that PMNs were detectable in MGD subjects, as shown in AKC and adult blepharo-keratoconjunctivitis (BKC) subjects (fig. S5). We therefore performed a larger controlled study to address whether MGD patients had increased numbers of PMNs in tear fluids sampled from 64 subjects (MGD, $n = 50$; non-MGD controls, $n = 14$; table S1). Flow cytometry was used to enumerate total tear leukocytes (CD45⁺), myeloid cells (CD45⁺CD11b⁺), and PMNs (CD45⁺CD11b⁺- CD14⁻ CD15⁺CD16⁺Side-Scatter^{hi}) (67) on a per-subject basis (Fig. 5A). We reported the distinct presence of increased total leukocytes, myeloid cells, and PMNs in tear fluids of MGD patients compared to non-MGD control group (Fig. 5B). These results supported the idea that leukocytes, myeloid cells, and PMNs are increased in tear fluid in MGD patients.

Of our total MGD subjects examined, 12 of 50 had a diagnosed underlying immune disease affecting the ocular surface, including allergy, ocular rosacea, or Sjögren's syndrome (table S1). Statistical analysis revealed that the presence of underlying immune disease was a significant factor in the number of tear leukocytes ($P < 0.001$), myeloid cells ($P < 0.001$), and PMNs ($P < 0.001$) seen in our MGD subjects (Fig. 5C). Thus, we stratified our MGD subjects into those that had "MGD only" and those that had MGD with immune disease, "MGD + ImD." Average numbers of tear immune cells in both groups remained greater than the control (Fig. 5C). This stratification revealed that 15 of 38 of MGD only subjects and 8 of 12 of MGD + ImD showed identifiable PMN presence in tear fluid, compared to 4 of 14 in the control group (Fig. 5, C and D). Given our mouse findings involving PMNs, we further analyzed PMN⁺ patients to determine whether a possible correlation between PMN number and MGD severity existed. We therefore evaluated the correlation between leukocytes and myeloid cell numbers considering the whole subject population (PMN⁺ and PMN⁻ subjects). MGD severity was measured as meibum viscosity index using a previously described 0 to 4 scale, where 4 is the most severe phenotype (22). Subjects were stratified into MGD only and MGD + ImD groups, (fig. S6, A and B). Among PMN⁺ subjects, we found moderate to strong positive correlations ($r_s = 0.5$ and $r_s = 0.75$, respectively) between total leukocytes or myeloid cells and MGD severity. Similar results were obtained when considering together PMN⁺ and PMN⁻ subjects (fig. S6, C and D). In PMN⁺ subjects within MGD only and MGD + ImD groups, we found a strong positive correlation between tear PMN quantities and MGD severity scores (Fig. 5, E and F). The correlation was lost when all the patients were considered together (Fig. 5, E and F). In summary, PMN⁺ subjects had a positive correlation between MGD severity and total tear leukocytes, myeloid cells, or PMNs in MGD only subjects, as well as in MGD + ImD subjects.

DISCUSSION

Here, we demonstrated that AED mice develop MG obstruction, with evidence of underlying MG defects. We provided direct evidence to conclude that MG obstruction in this mouse model is immune-mediated. Moreover, we were also able to reveal a link between the AED animal model and MGD in humans. Together, our results point to a role for the immune response in the etiology in certain forms of obstructive MGD.

Several lines of evidence indicating the presence of MGD in AED mice were shown in our study. In mice, AED induced consistent and definitive plugging of their MG orifice, whereas mild allergy did not. This observation is consistent with the clinical picture in chronic ocular allergy. Unlike in seasonal allergic conjunctivitis, patients with AKC suffer from recurrent MGD (24, 25, 68). Furthermore, in line with clinical data supporting the idea that plugging of the MG orifice is a pathognomonic sign of underlying MGD (14, 21), mice with AED had cellular and morphological changes of the glands that are congruent with the histopathology of MGD. Such changes included the presence of hypertrophy in MG central duct epithelium and frank dilation of the MG central duct (10–13, 16, 39). Finally, we observed via infrared meibography the presence of reduced MG area in AED, which is consistent with results reported in patients with MGD (8, 69, 70).

We uncovered that AED in mice is a T cell-mediated entity, which supports the idea of an immune-driven form of MGD. More specifically, we show that T_H17 lymphocytes mediate this process, meaning that MGD in AED is uniquely caused by a specific T_H17-type inflammation in our mouse model. Our conclusion is supported by the current finding that T_H2 responses were present in both mild allergy and AED models, whereas evidence of T_H17 responses was restricted to AED model. In our study, we further showed that AED in *Il17a*^{-/-} mice (54, 55) did not result in MG obstruction. Likewise, blockade of IL-23 (56, 57), which resulted in a reduction of T_H17 cells, also protected AED mice from developing MG obstruction. Finally, we showed that adoptive transfer of T_H17 cells (60, 61) was able to drive the development of MG obstruction in mild allergy mice. Hence, these data together mark T_H17 cells as a specific mediator of MG obstruction in AED. The fact that MG obstruction was absent in mild allergy model rules out a role of the allergen per se as a factor. Likewise, results from T_H17 pathway inhibition or PMN depletion in AED corroborate this conclusion. In support of our findings, elevated tear IL-17 levels were previously reported in MGD patients, although the study found no correlation with dry eye clinical scores (71). Conversely, in a different study, Tan *et al.* did find a correlation between IL-17 and dry eye severity; in this case, however, MGD was not assessed (72). Lee *et al.* also found elevated IL-17 levels in patient tears but excluded patients who demonstrated two or more morphologic changes in MGs (including vascular dilation, acinar atrophy, or orifice metaplasia on the posterior lid margin patients) (73).

With respect to PMNs in AED, we showed that these granulocytes are the downstream effectors in causing MG obstruction. Evidence that led to this conclusion are multiple. Infiltration of PMNs in the conjunctiva of AED mice was reduced with IL-23 blockade. Congruent with the lung data in endotoxin-induced airway inflammation (74, 75), our finding suggests that T_H17 cells are involved in PMN recruitment to the conjunctiva. Moreover, reduced PMN infiltration in IL-23 blockade prevented AED mice from developing MG obstruction. Likewise, blockade of IL-17a also reduced PMN infiltration in the conjunctiva of AED mice. In addition, we demonstrated that T_H17 adoptive transfer in mild allergy setting resulted in recruitment of PMNs to the conjunctiva, which was correlated with the development of MG obstruction. Last, we observed that PMN depletion (63) prevented AED mice from developing MG obstruction. Together, these data suggest that T_H17 responses in AED are involved in the recruitment of PMNs to the conjunctiva, which, in turn, cause MG obstruction in these mice.

The presence of tear PMNs in patients revealed a possible link between our findings in AED and MGD in humans. More specifically, we discovered an identifiable PMN population in the tear fluid of ~67% of MGD subjects with underlying immune disease (Sjogren's syndrome, allergy, or rosacea) and in 40% of MGD patients without immune disease. The relevance of PMNs in ocular surface inflammatory diseases is consistent with previous work by others showing the presence of PMN-derived factors in tears of these types of patients (76, 77). Our findings show that PMN⁺ subjects had a strong positive correlation ($r_s = 0.75$) between the number of tear PMNs and MGD clinical severity. These data, in conjunction with our mouse work, would lead us to hypothesize a causative role for PMNs in these patients. An alternative hypothesis is that immune cell recruitment is the consequence of MGD (as opposed to cause), which may also be at play with respect to non-PMNs given that

tear leukocytes or myeloid cells also showed a positive correlation with MGD severity. However, the specificity by which tear PMN numbers strongly correlated with MGD severity in PMN⁺ subjects, in conjunction with our mouse findings, suggests that for a subpopulation of MGD patients, PMN recruitment might play a causative role in disease development.

Although our study uncovered an immune-mediated form of obstructive MGD in mice and suggests that this pathway might also be critical in human disease, not all forms of MGD have an inflammatory etiology. There are other animal models that mimic aspects of MGD where the primary defect is not immunologic, such as in conditional β -epithelial Na⁺ channel (β ENaC) knockout, global *Elovl4* mutation, age-related MG atrophy, and other mouse models (30–37). Moreover, patients may present MGD without frank anterior blepharitis, such as in posterior MG disease. Conversely, not all forms of ocular surface inflammation result in obstructive MGD. The desiccating stress model of dry eye disease (DED) is one such example (78). These mice have a T_H17 response (79), with downstream goblet cell hyperplasia (80) and dacryoadenitis (81), but not frank obstruction of MGs (82). Last, IL-17 has also been shown to play a role in maintaining ocular surface homeostasis in mice, as recently demonstrated by St Leger *et al.* (83). Hence, more work is needed to understand why T_H17 cell responses in various conditions may or may not lead to MGD.

In summary, we identified the first inducible model in AED that mimics the clinical situation of MG obstruction with underlying chronic blepharitis. We were also able to provide direct evidence of an immune etiology for MGD in this AED mouse model, with key links to the described disease pathway in humans. Specifically, we demonstrated that PMNs cause MG obstruction in a T_H17-mediated fashion in mice. Likewise, we identified MGD patients with an abnormally high PMN content in tears, and we showed that the magnitude of such PMN quantities correlated with MGD severity in patients. Hence, our results suggest that PMNs might contribute to MGD development in certain forms of obstructive MGD.

MATERIALS AND METHODS

Study design

For mouse studies, objectives were to determine whether obstruction of MGs seen in the AED model is immune-mediated and, if so, identify the immune cell population(s) and pathway that drives the disease phenotype. Mice were housed in a specific pathogen-free environment at Duke University and managed according to Association for Research in Vision and Ophthalmology Statement for the Use of Animals in Ophthalmic and Vision Research. Sample sizes for experimental groups and their respective controls, as well as experimental replicates, are indicated in the figure legends. Allergic responses and MG obstruction were examined clinically in a masked fashion. T cell responses and immune infiltration of the conjunctival tissues were measured by flow cytometry, whereas tissue morphology was assessed by meibography and histopathology. For antibody blocking experiments or adoptive transfer assays, allergy-induced mice were randomized into treated and control groups. Systemic antibody blockade of IL-17a, IL-23, or Ly6G was compared to isotype control antibody-treated mice. Mice that were adoptively transferred were compared

to nontransferred controls. Effects of these interventions were assessed at the cellular, histopathological, and clinical levels, as further detailed in the Supplementary Materials.

For human studies, objectives were to determine whether tear fluids sampled from MGD patients had increased numbers of leukocytes, with an emphasis on PMNs, and to determine whether MGD severity correlates with tear leukocyte quantity. The study protocol to assess immune cells in patients via flow cytometry with MGD and controls was approved by the Duke School of Medicine Institutional Review Board (IRB). After explanation of the purpose and the possible risks of the study, written informed consent was obtained from all participants. Fifty MGD subjects and 14 controls participated in this study. MGD subjects also had concomitant DED. All patients were 18 years old. Inclusion criteria required a previous or new diagnosis of MGD, whereas controls should not have had a history of MGD or DED. Exclusion criteria included any use of systemic anticoagulative or immunosuppressive drugs within weeks of participation or history of ocular surgery in the previous 3 months before participation. Patients with glaucoma were not included in our analysis. Patients unable and willing or unwilling to give consent or those who cannot read/speak English were excluded from participation as well. For tear cytology, protocol was approved by the IRB and adhered to the Declaration of Helsinki. A written informed consent was obtained from all subjects. Tear leukocyte number and phenotype were determined by flow cytometry, whereas MGD severity was analyzed by qualitative scoring of expressed meibum, as further detailed in the Supplementary Materials. Raw data for all the experiments are provided in table S2.

Statistical analysis

Graphical data presented throughout are expressed as the means $\pm$ SEMs. Data normality and homogeneity of variance were assessed using Kolmogorov-Smirnov test and Levene's test, respectively. Two-group comparisons were analyzed using unpaired Student's two-tailed *t* test or Mann-Whitney *U* test. Comparisons of three groups and multiple-factor comparisons were analyzed using ANOVA with Tukey's post hoc test or Kruskal-Wallis test with Conover-Inman test. Parametric/nonparametric statistical analyses used were based on normality and homogeneity of variance. A *P* value of <0.05 was considered statistically significant. Spearman's rank correlation was used to calculate correlations between immune cell numbers and MGD scores. Best-fit lines were drawn showing the data trend. Statistical graphs were generated using GraphPad Prism version 7.00.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments:

We thank J. Jester (University of California, Irvine) for helpful discussions regarding MGD. we thank L. Espandar (University of Pittsburgh). At Duke University, we thank A. Kuo, G. Vora, and C. Betancurt for help and guidance with clinical aspects. We also thank A. Garcia-Turner and L. Greene for guidance with IRB. We also thank Y. Hao and E. Reyes for technical support with tissue sections and analysis.

Funding: This study was funded by R01EY021798 (D.R.S.), P30EY005722 (D.R.S.), F32EY025557 (N.J.R.), and Miracles in Sight.

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Fig. 1. MG orifice obstruction in AED model is a key disease manifestation.

(A) Clinical disease was scored at 20 min (immediate hypersensitivity) and 6 hours after allergen challenge (late phase/chronic response) in AED and the mild allergy model ($n = 20$ mice per group). (B) Representative flow cytometry histograms showing total leukocyte (CD45⁺) numbers in conjunctiva. Quantification is shown on the right. (C) Representative flow cytometry scatterplots showing eosinophil (CD45⁺Siglec F⁺) numbers in conjunctiva. Quantification is shown on the right ($n = 5$ per group). (D) Representative images of orifice plugging from mild allergy and AED mice. Incidence of eyes with MG plugs is graphed ($n =$

5 per group). Data are expressed as means $\pm$ SEM from at least three independent experiments. Differences in clinical scores were determined by two-way analysis of variance (ANOVA) with Tukey's post hoc test. One-way ANOVA and unpaired Student's two-tailed t tests were performed to compare the cell numbers and MG plug percentages, respectively. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

Fig. 2. AED model exhibits meibographical and histological evidence of underlying MGD. (A) Representative image of binarized keratograph of eyelids (superior and inferior) with corresponding area calculation (in square millimeters). On the right, scatterplots of full, superior, and inferior lid area measurements of MGs are shown ($n = 10$ eyelids per group). Data are expressed as means $\pm$ SEM. Unpaired Student's two-tailed t tests were used. ** $P < 0.01$, *** $P < 0.001$. (B) Representative serial sections of eyelids from naive and AED at anatomically matched locations (orifice, terminal duct, and central duct). Black double-

headed arrows indicate central duct size. Images are representative of $n = 3$ plugged MGs in each mouse, with at least three mice per group.

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Fig. 3. T_H17 responses generated in AED mice play a central role in mediating MG obstruction. (A) Representative flow cytometry scatterplots (left) and quantification (right) of cervical LN T_H17 cells (naive, $n = 3$; mild, $n = 12$; AED, $n = 11$ mice). (B and C) Representative flow cytometry data of LN T_H17 cells in *IL17a*^{-/-} mice with AED in (B) (WT, $n = 5$; *IL17a*^{-/-}, $n = 4$ mice), and corresponding eyelid data shown as number of plugs per eye in (C) (WT, $n = 28$; *IL17a*^{-/-}, $n = 12$ eyelids). (D and E) Flow cytometry data of LN T_H17 cells (untreated, $n = 22$; isotype, $n = 6$; anti-IL-23, $n = 16$ mice) in (D) and corresponding eyelid data (untreated, $n = 22$; isotype, $n = 6$; anti-IL-23, $n = 16$ eyelids) in (E) after anti-IL-23 blockade or isotype control (100 μ g) given during sensitization period only. (F and G) Flow cytometry data of LN T_H17 cells (mild only, $n = 4$; +T_H2, $n = 5$; +T_H17, $n = 4$ mice) from mild allergy mice in (F) and corresponding eyelid data (mild only, $n = 8$; +T_H2, $n = 8$; +T_H17, $n = 10$ eyelids) in (G) after adoptive transfer of in vitro generated T_H2 or T_H17 cells. Data are expressed as means $\pm$ SEM from at least two independent experiments. One-way ANOVA was performed for comparisons among three groups, and unpaired Student's two-tailed t tests were used for comparisons between two groups. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

Fig. 4. TH17 cells are associated with PMN recruitment to the conjunctiva in AED, and these PMNs cause MG obstruction.

(A) Representative flow cytometry scatterplots of PMN in conjunctiva and quantifications of PMN counts on the right (untreated, $n = 5$; anti-IL-23, $n = 6$ mice), after anti-IL-23 blockade or isotype control given during sensitization period only. (B) Quantifications of conjunctival PMN counts after adoptive transfer of in vitro generated TH2 or TH17 cells to mild allergy model (mild only, $n = 4$; +TH2, $n = 7$; +TH17, $n = 6$ mice). (C and D) Flow cytometry data of PMNs in conjunctiva of AED mice (untreated, $n = 3$; isotype, $n = 4$; anti-Ly6G, $n = 6$ mice) and corresponding eyelid data (untreated, $n = 18$; isotype, $n = 15$; anti-Ly6G, $n = 28$ eyelids) after anti-Ly6G blockade or isotype control. Data are expressed as means $\pm$ SEM from at least two independent experiments. One-way ANOVA was performed for comparisons among three groups, and unpaired Student's two-tailed t tests were used for comparisons between two groups. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

Fig. 5. Increased PMN quantity in patient tear fluids strongly correlates with clinical severity of MGD.

(A) Flow cytometry analysis of tear collections and gating strategy for leukocytes (CD45⁺), myeloid cells (CD45⁺CD11b⁺), and PMNs (CD45⁺CD11b⁺CD14⁻CD15⁺CD16⁺SSc^{hi}). (B) Bar graphs show increased total leukocytes, myeloid cells, and PMNs in tear fluids of MGD subjects, as compared to controls (no MGD). (C) Stratification data of MGD subjects into those with MGD only or MGD with underlying immune disease (MGD only or MGD + ImD, respectively). Red dashed box indicates PMNs⁺ cases. See table S1. (D) Pie charts

show the break-down of PMN⁺ cases in both MGD groups (MGD only, $n = 38$; MGD + ImD, $n = 12$). (E and F) Correlation analyses between tear PMN numbers and MGD severity in PMN⁺ subjects. Severity is based on masked scoring on a 0 to 4 scale for expressed meibum quality (0 = healthy, clear, and normal meibum; 4 = obstructed, opaque, and thick meibum). Data were collected from $n = 50$ MGD patients and $n = 14$ controls. Data of dot plots are expressed as means $\pm$ SEM. Mann-Whitney U tests were used for two-group comparisons, and Kruskal-Wallis test with Conover-Inman tests were used for three-group comparisons. $*P < 0.05$, $***P < 0.001$. Spearman's rank correlation was used.