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REVIEW

Age-related changes in ocular mucosal tolerance: Lessons learned from gut and respiratory tract immunity

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Summary

The ocular surface is the part of the visual system directly exposed to the environment, and it comprises the cornea, the first refractive tissue layer and its surrounding structures. The ocular surface has evolved to keep the cornea smooth and wet, a prerequisite for proper sight, and also protected. To this aim, the ocular surface is a bona fide mucosal niche with an immune system capable of fighting against dangerous pathogens. However, due to the potential harmful effects of uncontrolled inflammation, the ocular surface has several mechanisms to keep the immune response in check. Specifically, the ocular surface is maintained inflammation-free and functional by a particular form of peripheral tolerance known as mucosal tolerance, markedly different from the immune privilege of intraocular structures. Remarkably, conjunctival tolerance is akin to the oral and respiratory tolerance mechanisms found in the gut and airways, respectively. And also similarly, this form of immunoregulation in the eye is affected by ageing just as it is in the digestive and respiratory tracts. With ageing comes an increased prevalence of immune-based ocular surface disorders, which could be related to an age-related impairment of conjunctival tolerance. The purpose of this review was to summarize the present knowledge of ocular mucosal tolerance and how it is affected by the ageing process in the light of the current literature on mucosal immunoregulation of the gut and airways.

KEYWORDS

ageing, conjunctival tolerance, dry eye, goblet cells, mucosal tolerance, nasal tolerance, oral tolerance, T cells

INTRODUCTION

Ocular surface disease is a broad term for dry eye and other illnesses affecting the front part of the eye such as Sjögren's syndrome (autoimmune dry eye), meibomian gland dysfunction (a disorder affecting the oil-producing glands located within the eyelids), post-refractive surgery dry eye, age-related dry eye, ocular graft-versus-host-disease and others. Additional ocular surface disorders of relevance in the elderly are ocular allergy and microbial infections. Dry eye presents with ocular irritation, gritty sensation and blurred vision. Ocular surface disease is a frequent reason to seek eye care [1], and using trade-off research techniques, severe dry eye has been compared with severe angina in terms of impact on the quality of life by affected patients [1–4]. Dry eye is one of the most common eye diseases, with a reported prevalence of 5·5%–15% worldwide [5–7]. Known risk factors for dry eye disease include ageing, contact lens wear, female sex and autoimmunity [5,8–12]. Dry eye prevalence increases with every decade in women and men, although it is more prevalent in women [5]. Because it affects more women than men, both sex and gender differences have been implicated [13], although no definitive consensus has been achieved. Both innate immunity and

Abbreviations: ACAID, anterior chamber-associated immune deviation; APC, antigen-presenting cells; DC, dendritic cell; DTH, delayed-type hypersensitivity; GC, goblet cell; M, months; OVA, ovalbumin.

adaptive immunity play a role in dry eye pathogenesis, and a vicious circle of inflammation is well established [14].

The ocular surface is the part of the visual system directly exposed to the environment, and it comprises the cornea, the first refractive tissue layer and the only transparent tissue in the body, and its surrounding structures. The ocular surface has evolved to keep the cornea smooth and wet, a prerequisite for proper sight, and protected. To this aim, the ocular surface is a bona fide mucosal niche with an immune system capable of mounting a strong response to fight against dangerous pathogens. Given the potential harmful effects of uncontrolled inflammation leading to extensive fibrosis and corneal opacification, the ocular surface has several mechanisms to keep the immune response in check to preserve corneal clarity. This regulation is part of what is collectively known as peripheral tolerance because it is how the immune system differentiates self from non-self-antigens and prevents autoimmunity [15]. Specifically, the ocular surface is maintained inflammation-free and functional by a particular form of peripheral tolerance known as mucosal tolerance [16], which is also at work in the gut and in the airways. Ageing of the immune system, or immunosenescence, has been linked to increased frequency of infections, cancer and autoimmunity in the elderly [17,18]. For a comprehensive review of how ageing affects the specific components of the ocular immune system, see Galletti and de Paiva [19]. Interestingly, a dysregulated immune response underlies many ocular surface disorders that become more prevalent in the elderly [19], suggesting mucosal tolerance in the ocular surface changes with ageing. Consistently, immunoregulation in the gut and in the airways changes as we age. Thus, the purpose of this review was to summarize the present knowledge of ocular mucosal tolerance in the context of peripheral immune tolerance of the eye and how it is affected by the ageing process. Because the eye is a unique organ that may be intricate to the immunologist unfamiliar with ocular anatomy, we will address peripheral immune tolerance mechanisms in its different sections. But since a considerable body of evidence has emerged from studies of gastrointestinal and respiratory disorders in which age-related immunoregulatory changes play a role, we will first review the literature to learn from other mucosal sites where mucosal tolerance was characterized first and to a greater extent. Our intent is to show differences and similarities in the immunoregulation of these three different mucosal sites through ageing. We also want to highlight how age-related perturbations of ocular mucosal tolerance could participate in ocular surface disease development such as dry eye.

AGEING AND MUCOSAL TOLERANCE IN THE GUT AND AIRWAYS

All mucosal sites (gut, airways and ocular surface) are exposed to the environment to a varying extent and thus need to cope with harmless and dangerous antigens of their own. The set of regulatory mechanisms by which the mucosal immune system does not react against the harmless foreign antigens it comes in contact with is known as mucosal tolerance. It is evidently not a passive phenomenon where the immune system 'does not see' an antigen but follows a co-ordinated sequence of events where the antigen 'is chosen to be ignored' by the immune system.

Oral tolerance

Because any food is a foreign entity, the gastrointestinal tract has developed ways to cope with this interaction, that is, to tolerate the non-self-antigens that are derived from food protein. This regulation is termed oral tolerance and requires peripherally induced $Foxp3^+$ Tregs [20]. Oral tolerance is crucial for optimal health and modulation of the gut immune system, as shown by mice raised with an elementary diet devoid of dietary antigens: they become more susceptible to developing food allergy upon introduction of a new antigen in the diet than mice fed a conventional diet [21]. Oral tolerance can be evidenced in laboratory animals if they are fed an antigen before parenteral immunization with the same protein. When the antigen is absorbed in the intestines, the absence of inflammation and danger signals indicates to the mucosal immune system that the antigen is not a threat, and the tolerance develops [22]. First, an antigen present in the lumen of the gastrointestinal tract needs to be captured by intraepithelial and lamina propria APCs; then, these APCs travel to the lymph nodes guided by the CCR7-CCL19/CCL21 axis, where they induce tolerogenic T cells [23]. Intestinal goblet cells have been shown to deliver antigens to $CD103^+$ lamina propria DCs [24]. These goblet cell-associated passages are critical for oral tolerance as mice devoid of goblet cells do not develop tolerance to dietary antigens [25]. For a thorough discussion of the mechanisms underlying oral tolerance, see Ref. [20] and [26].

Factors such as age, dose, frequency, route of delivery and intestinal microbiome influence oral tolerance and maintenance [27–30]. Either a single high dose or repeated smaller doses and oral administration of antigens are essential for oral tolerance development [31]. Also, intravenous or intraportal delivery of OVA does not induce the same immune response as orally administered OVA, showing that intestinal uptake of the antigen is crucial [29]. Another important factor is age. Several studies have shown that aged mice have decreased oral tolerance [22,29,32–35]. Oral OVA administration is sufficient to induce oral tolerance in young (8 weeks old) mice [22] but middle-aged and elderly (~15·5 and 19 months old, respectively) mice are refractory [34,35]. The humoral response to OVA is also impaired in aged mice. In some studies, a progressive decrease in the humoral response was

observed in mice aged 9 months or older [29,35]. In others, a lack of proper antibody levels was seen as early as 6–8 months of age [36]. Interestingly, 15-month-old mice that were orally immunized at an early age showed a comparable humoral response to young mice [37], suggesting that early vaccination is key for preserving proper antibody production in the elderly. Studies have also shown that aged mice (>20 months of age) have an exaggerated cellular and humoral response to orally administered OVA, suggesting that altered immune processes in the elderly might lead to autoimmunity and inflammation [36]. A decrease in DCs and changes in Peyer's patches architecture, seen as early as 6–8 months of age, and T-cell dysregulation observed at 24 months have been implicated as mechanisms for disrupted oral tolerance [36,38,39].

Instances when oral tolerance mechanisms fail can have mild clinical consequences, such as urticaria and skin rash, or become life-threatening situations with oral and laryngeal oedema, anaphylaxis and cardiac arrest. Common food allergens are peanuts, nuts, shellfish and cow milk proteins [40], which trigger IgE production. IgE-mediated food allergies elicit mast cells and basophils that rapidly release histamine and vasoactive factors, leading to the symptoms of hives, angio-oedema, bronchospasm and anaphylaxis. Chronic forms of oral tolerance disruption may be accompanied by vomiting, cramping, abdominal pain and diarrhoea. Besides food avoidance, oral immunotherapy (which involves induction of oral tolerance) has emerged as an option for treating certain allergies in children. For example, oral immunotherapy for peanuts entails giving small, escalating doses of peanuts to a child to increase the amount of food without triggering an allergic reaction [41].

Food allergy is a burdensome health problem. Prevalence of food allergy is highest among children, with reported rates varying between 2% and 26%, depending on the population studied and the method used to define food allergy [42,43]. High incidence and remission rates and over-reporting characterize this age group [43]. Although food allergies are more frequent in young individuals, they can occur at any age. In a nation-wide US study [44], serological prevalence and clinical prevalence were highest in children aged 1–5 years (28·1% and 4·3%, respectively) and progressively declined with age, reaching values of 13·0% and 1·3%, respectively, in the 60+ age group. In another cross-sectional study of 109 people in a Hungarian geriatric nursing home (mean age 77 years), specific IgE to food allergens was detected in 25% of residents and positive skin prick tests with food allergens correlated with chronic alcohol consumption [45]. Contrasting with children, the elderly tend to under-report food allergy symptoms and other allergies [45–53]. Despite the decrease in prevalence with age, it is evident that food allergy still represents a significant health issue in the elderly. Furthermore, food allergy in the young arises due to the immaturity of the gut mucosal immune system, and this correlates with animal studies: neonatal mice cannot develop oral tolerance to a fed antigen before 7 days of age [54]. Contrastingly, aged mice lose their ability to develop oral tolerance to newly introduced dietary antigens, as previously detailed [22,29,32–35]. Thus, food allergy in the elderly is less frequent but has a different underlying pathophysiology than in children [46]: there is loss of oral tolerance to dietary antigens instead of inability to develop oral tolerance to newly introduced dietary antigens.

Food allergy in the elderly relates to an impaired immune system (immunosenescence) and is compounded by numerous physiological changes, including a deficiency in iron, zinc and vitamin D [45–47]. Gastric atrophy and anti-acid medication have also been associated with increased food allergy in the elderly, probably because of persistence of intact food allergens due to reduced digestive enzymatic activity [55]. Also, chronic alcohol consumption is linked to gastric atrophy and hypoacidity, pancreatitis and a direct cytotoxic effect on the gastrointestinal mucosa (leading to inflammation and decreased barrier function), all of which may contribute to the increased association with food allergens in the elderly [45]. Interestingly, in the same study alcohol was not a risk factor for increased skin test positivity for respiratory allergens, suggesting that its effect is locally restricted to oral tolerance mechanisms and not to a generalized potentiation of Th2 responses [45]. However, this topic is controversial and there is inconsistency in several studies [56], warranting further research on the effect of drugs and medications on oral tolerance as we age. At any rate, as evidenced by food allergy presenting in the elderly, failure of oral tolerance in ageing is not infrequent and the underlying mechanisms deserve additional study.

Inflammatory bowel disease is another group of disorders in which oral tolerance is affected [57]. Patients with inflammatory bowel disease cannot develop oral tolerance to newly fed antigens [58], exhibit signs of active immunization against common food antigens [59] and display non-tolerant immune responses against gut microbiota [60]. Although food-specific immunity is not involved in inflammatory bowel disease pathogenesis [61], loss of gut mucosal tolerance to food (i.e. oral tolerance) reflects a generalized disruption of the regulatory steps that suppress inflammation towards harmless microbiota antigens in the gut, which is the core pathophysiological mechanism of inflammatory bowel disease [62]. In line with this, restoration of oral tolerance to a food antigen (egg protein) by intravenous administration of antigen-specific Tregs and subsequent feeding of the same antigen (meringue cakes) showed results in a clinical trial involving patients with inflammatory bowel disease [63]. Thus, inflammatory bowel disease, despite not having a higher prevalence in the elderly, still constitutes a remarkable example of how dysregulated mucosal tolerance can drive local inflammation in a mucosal site [62].

Conversely, studies have shown that oral tolerance can be used to prevent or decrease some pathological states. Administration of heat-shock proteins in experimental models of atherosclerosis either at the time of initiation or after moderate disease establishment can modify the size of atherosclerotic plaques through increased frequency of $CD4^+$ $Foxp3^+$ Tregs. Aged (18-months) $ApoE^{-/-}$ mice immunized with mycobacterial heat-shock protein 65 and subjected to a high cholesterol diet showed atherosclerosis progression. In contrast, oral administration of mycobacterial heat-shock protein 65 before immunization decreased the extension of plaques and increased the frequency of splenic Tregs [64,65].

In summary, oral tolerance was the first mucosal tolerance mechanism described [40] and is the one most studied. Several factors affect how the gut immune system handles food-derived and other antigens through the ageing process, resulting in decreased or abolished oral tolerance. Thus, ageing profoundly impacts oral immunization, food allergy and other gastrointestinal disease states. Furthermore, because of the systemic influence of the gut immune system [66,67] and the shared aspects of the mucosal immune responses, some of these observations could also apply to other mucosal sites such as the respiratory tract and the ocular surface.

Mucosal tolerance in the airways

Due to the respiratory tract's continuous exposure to airborne antigens, mucosal tolerance is highly relevant as a peripheral tolerance mechanism in the airways [68]. It was first described in 1981 [69], about 70 years later than oral tolerance [40]. More recently, its breakdown has been recognized as a key pathophysiological mechanism in allergic airway diseases such as allergic rhinitis and asthma [70]. For example, psychological stress and cigarette smoking, two environmental factors linked to asthma severity in patients, directly impair respiratory tolerance in mice [71,72]. Experimentally, both nasal tolerance and bronchial tolerance have been described in animals. Nasal instillation or inhalation of aerosolized antigens can lead to antigen presentation in cervical and peribronchial lymph nodes that drain the nasal cavity and the lower airways, respectively [73,74]. This is possible because local DCs take up antigen in the mucosal linings and then migrate relying on CCR7 guidance to the draining lymph nodes [74]. The upper and lower airways harbour 4 different DC populations: epithelial $CD103^+$ DCs (conventional DC1 or cDC1), stromal $CD11b^+$ $CD24^+$ $CD64^-$ conventional DCs (cDC2), monocyte-derived $CD11b^+$ $CD24^-$ $CD64^+$ DCs and plasmacytoid $B220^+$DCs (pDCs) [75,76]. Of these, cDC2 [76] and pDCs [77] seem to be responsible for mucosal tolerance in the lungs.

Lung immune homeostasis depends on a network of interactions between immune cells that include the airway epithelium, macrophages, neutrophils, and tissue-resident lymphocytes, among other cell types (for a thorough review, see Ref. [78]). Contrasting the studies on oral tolerance in young and aged animals (see previous section), no direct assessment of respiratory mucosal tolerance in older individuals or animals has been published. However, many studies have explored the effect of ageing on the pulmonary immune response [78]. Ageing dysregulates cytokine production in lung epithelial cells favouring pro-inflammatory interleukin-1β and interleukin-6 release [79], and conversely, DCs from aged subjects contribute to airway inflammation by activating bronchial epithelial cells [80]. Of note, aged DCs have higher nuclear factor-κB activity [81], which is inversely associated with their tolerogenic potential [82]. The latter and other observations add to the dysfunction of DCs that comes with age, which favours inflammation and loss of tolerance [83]. Also, the aged lung microenvironment leads to a reduction in tissue-resident alveolar macrophages, which are better at resolving inflammation after injury [84]. In a comparison of young and aged mice sensitized with ovalbumin (OVA) as an antigen and then challenged with the same antigen in the airways, the older mice developed less airway hyperresponsiveness but more inflammation, eosinophilia, goblet cell hyperplasia and interferon-γ [85]. Thus, just as is the case for oral tolerance, respiratory tolerance is highly likely to be affected by ageing, because many of the mechanisms that underlie the tolerant mucosal immune response are similarly changed by ageing in the gut and the airways. However, specific animal studies about the effect of ageing on respiratory tolerance are missing.

Allergic airway disease is traditionally associated with young age, but it remains highly prevalent (5%–10%) throughout life [86]. In a study of asthmatic patients over 60 years of age, 10% had first developed asthma after their 60th birthday [87]. Asthma in the elderly has distinct clinical features that have been linked to oxidative stress and inflammageing, such as increased neutrophilic infiltration and less atopy [88]. Similar considerations apply to allergic rhinitis in advanced age [89], which are probably associated with an increase in Th2 responses in the elderly [90]. In a Finnish study of 8000 respondents, incidence of allergic asthma (defined by accompanying allergic rhinitis) decreased with age, but the incidence of non-allergic asthma peaked in adulthood [91]. In another large European multicentre study, occupational exposure was a significant risk factor for the development of new-onset asthma in adults [92]. As in both studies the subjects did not experience asthma during childhood, it is tempting to speculate that changes in respiratory tolerance through ageing could be implicated in this phenomenon. In line with this, IgE sensitization to cat allergen (a known aeroallergen) was associated with the development of new-onset asthma in a cohort of aged men (mean age 61) followed for 3 years [93].

Also, in another study of elderly (60+ years) asthma patients, 33% were positive for cat-specific IgE and 53% were positive for at least one indoor allergen, that is antigens to which they were almost continuously exposed [94]. In the already mentioned study of a Hungarian geriatric home population, 40% of residents were positive for IgE specific for one or more of the 19 respiratory allergens tested, and this trait was associated with smoking [45]. In fact, smoking constitutes a risk factor for IgE sensitization in aged subjects [95]. Tobacco smoke activates several signalling pathways in bronchial epithelial cells, most prominently the nuclear factor-κB pathway, which triggers a pro-inflammatory response [96] and is directly involved in mucosal tolerance induction or abrogation in all mucosal surfaces [97].

Taken all together, the findings summarized above support the notion that ageing impairs respiratory tolerance, as it has been firmly established for oral tolerance. Specific studies are needed on the actual extent of respiratory mucosal tolerance loss with ageing and how it modulates allergic airway disorders because of the high translational impact.

PERIPHERAL IMMUNE TOLERANCE IN THE EYE: IS IT ALL THE SAME?

As Medawar's observations that allogeneic skin grafts implanted in the anterior chamber are not rejected [98], it has been clear that the eye controls the immune response within its domains. This feature, shared with the brain and the testes, has been termed immune privilege [99–102]. Unsurprisingly, immune-privileged sites are operationally defined as those where foreign tissue grafts survive indefinitely, contrasting with non-privileged sites where such grafts undergo rapid immune rejection. This property indicates the existence of active regulatory mechanisms that suppress immune responses, explaining how corneal allografts in patients do not require systemic immunosuppression to remain viable. Immune privilege could thus be interpreted as a site-specific form of peripheral immune tolerance [15], that is the set of mechanisms through which the immune system differentiates self from non-self-antigens and prevents autoimmunity. In the laboratory, the immune privilege of the eye can be evidenced by a reaction termed anterior chamber-associated immune deviation (ACAID): when an antigen, for example OVA, is injected into the anterior chamber of the eye, it sets in motion an immune response that has unique features and a systemic reach. An equivalent reaction seems to occur in patients with ocular varicella-zoster [103]. Remarkable progress has been made on the molecular and cellular basis of these observations beyond the scope of this review and to which we will refer collectively as intraocular immunology (see Ref. [104]).

Despite the stark anatomical differences, there are many unifying aspects in the immunology of the anterior and posterior segments of the eye [99], the most important of which is the already mentioned existence of 'immune privilege as the result of local tissue barriers and immunosuppressive microenvironments' [105]. A comparable posterior segment equivalent of ACAID has been described as vitreous cavity-associated immune deviation [106], and allogeneic retinal grafts placed in the vitreous cavity or the subretinal space are not rejected [107]. Recently, evidence of immune surveillance in the lens has surfaced [108]. Unfortunately, this abundance of knowledge on intraocular immunology has come with the notion that everything related to the eye also bears immune privilege, which is incorrect and does not apply to the ocular surface (Figure 1). This is a well-known fact in the clinic as limbal allografts for ocular surface reconstruction, such as other solid organ transplants, require HLA typing and systemic immunosuppression [109]. Also, subconjunctival tissue allografts in mice are quickly rejected and lead to immunization [107].

Although the ocular surface is not immune-privileged, it exhibits another form of peripheral immune tolerance shared with every other mucosal site: mucosal tolerance [16,20,110–112]. Mucosal tolerance is critical for immune homeostasis because the mucosal surfaces in the gut, airways and the eyes serve as barriers to the environment. Therefore, these sites face a dilemma: to cope with commensal microbes, food and airborne particles while at the same time to attack invading pathogens [20,111]. Through mucosal tolerance, these organs actively suppress the potential immune response against the myriad harmless antigens to which they are continuously exposed, remaining functional to absorb nutrients, exchange air or refract light rays. Contrasting with immune privilege, mucosal tolerance can be operationally defined as the active suppression of systemic immunization against a specific antigen if such antigen is administered through a mucosal surface before the immunization [20].

Mucosal tolerance in the ocular surface can be evidenced by an assay similar to that of ACAID, although the underlying immune mechanisms are different (Table 1) [16,113]. If a harmless antigen (one that does not elicit an inflammatory response in and of itself) is applied to the ocular surface of mice, it is taken up by antigen-presenting cells (APC) that migrate to the draining cervical lymph nodes, where they present it to naïve T cells [114]. In homeostatic conditions, these APCs do not sense danger-associated signals from the microenvironment along with the antigen, so they have a tolerogenic (anti-inflammatory) programme imprinted on them. Thus, when they interact with their cognate naïve T cells, the APC delivers additional signals that induce the T cells to differentiate into regulatory T cells (Treg). Very few antigen-specific Tregs are involved in this response, but they are potent enough to impact immune regulation

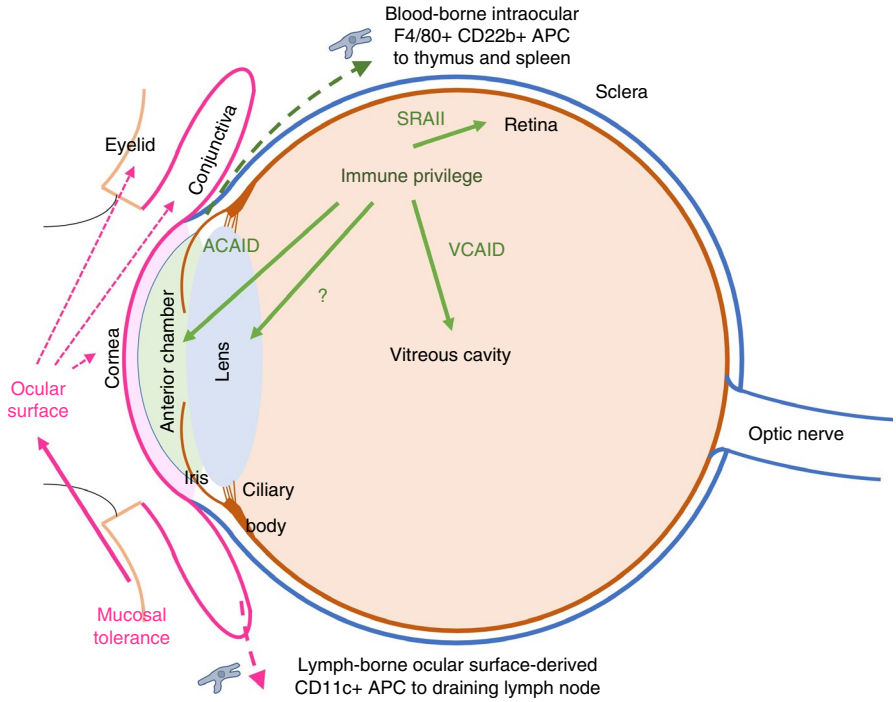

FIGURE 1 Peripheral tolerance in the eye. The eye globe is delimited by the cornea anteriorly and the sclera posteriorly. Within the eye, the lens separates the anterior chamber (green-filled) from the vitreous cavity (orange-filled). The retina lines the inner surface of the back of the eye globe, and the subretinal space is the virtual space between the neuroretina and the retinal pigment epithelium. All these tissues and structures within the eye globe are regarded as intraocular and have immune privilege, a site-specific form of peripheral tolerance with unique features such as bloodborne antigen-presenting cells (APCs) reaching the thymus and spleen. Specific descriptions in the literature of intraocular immune privilege for some intraocular structures are shown in green with their corresponding abbreviations (ACAID: anterior chamber-associated immune deviation, VCAID: vitreous chamber-associated immune deviation, SRAII: subretinal space-associated immune inhibition). By contrast, the ocular surface (pink) is a collective term for the exposed portion of the eye and comprises the cornea, the conjunctiva (the mucosal lining surrounding the cornea that extends to the inner surface of the eyelids), the eyelids, and other tissues and structures not depicted. The ocular surface is regarded as extraocular, and from an immunological viewpoint, it exhibits mucosal tolerance: a peripheral tolerance mechanism common to every mucosal lining that is based on antigen presentation in the lymph nodes and regulatory T cells

profoundly. The assay further exploits this aspect to evidence the presence of the Tregs. If these tolerized mice are then injected subcutaneously with the same antigen mixed with an adjuvant that promotes a strong response in naïve animals, the antigen-specific Tregs will suppress the process, leading to poor immunization. Thus, when the tolerized mice are later on challenged by either subcutaneous or intradermal injection of the same antigen alone, instead of a vigorous localized hypersensitivity response that peaks after two days, a small reaction (measured by swelling) develops. This challenge reaction is known as delayed-type hypersensitivity (DTH) and is akin to the purified protein derivative (PPD) skin test for tuberculosis diagnosis [115]. It involves a recall cellular response to an antigen mediated by local uptake by tissue-resident APC and presentation to effector $CD4^+$ T cells that release T helper (Th)1 cytokines, thus amplifying inflammation [37]. In tolerized mice, the previously generated Tregs suppress this reaction, hence the reduced swelling readout. It should be emphasized that the DTH assay used to evidence mucosal tolerance in immunological research is just a tool to assess the presence of either antigen-specific Tregs or effector T cells. For a thorough review of the mechanisms underlying ocular mucosal tolerance, see Galletti et al [16,19].

CONJUNCTIVAL TOLERANCE AND AGEING

As in the respiratory and intestinal mucosa, delivery (i.e. instillation) of an antigen to the ocular mucosa leads to tolerance, that is the generation of Tregs and absence of clinical inflammation signs upon subsequent administration of the antigen. Ocular mucosal (i.e. conjunctival) tolerance was first described in 1994 [121] and characterized a few years later [110], but its role in ocular pathophysiology was only addressed recently [122–124]. Ocular surface Tregs, which underlie mucosal tolerance, have been reviewed elsewhere [125].

Disruption of ocular mucosal tolerance has been described in several ocular disease models. First, it was observed after topical instillation of benzalkonium chloride

TABLE 1 Comparison between ocular mucosal tolerance and immune privilege

	Ocular mucosal tolerance	Ocular immune privilege
Anatomical location	Ocular surface	Cornea, anterior chamber, vitreous chamber, subretinal space
APCs involved	$CD11c^+$ dendritic cells [114]	$F4/80^+$ $CD11b^+$ macrophages
Route of APC exit from the eye	Lymph (CCR7-dependant chemotaxis) [116,117]	Blood [104]
Location of antigen presentation	Eye-draining lymph nodes [114]	First thymus [118], then spleen [119]
Mechanism of antigen presentation	*Lymph node*: Tolerogenic $CD11c^+$ DCs present antigen to naïve $CD4^+$ T cells	*Thymus*: $F4/80^+$ macrophages present to NKT cells through CD1d *Spleen*: $F4/80^+$ macrophages transfer antigenic peptides to marginal zone B cells; then, B cells present them on MHC I and MHC II to $CD8^+$ and $CD4^+$ T cells. NKT and γδ T cells are required.
Result of antigen presentation	Induction and expansion of antigen-specific $CD4^+$ $Foxp3^+$ Tregs	Induction and expansion of antigen-specific $CD4^+$ $Foxp3^+$ Tregs and $CD8^+$ $Foxp3^+$ $CD103^+$ Tregs [120]
Functional result	Systemic suppression of antigen-specific effector T-cell responses	Systemic suppression of antigen-specific effector T-cell responses

[122]. Benzalkonium chloride is a common preservative in eye drops, which are associated with ocular surface toxicity [126]. Mechanistically, loss of mucosal tolerance to an exogenous harmless antigen explains the increased incidence of ocular allergy, secondary dry eye and discomfort caused by this preservative [127]. Moreover, benzalkonium chloride-induced models of dry eye have been described in mice [128] and rabbits [129], with the accompanying loss of conjunctival goblet cells, corneal epithelial death and $CD4^+$ T-cell activation [130]. Perhaps unsurprisingly, conjunctival tolerance is also affected by a corneal alkali burn [131], as this model is associated with extensive ocular surface damage and epithelial disruption.

Impaired conjunctival tolerance to a harmless antigen was also reported in various models of dry eye [123,124,132–134], an autoimmune disease for which the specific antigens remain uncharacterized but that can be reproduced in naïve mice by adoptive transfer of pathogenic $CD4^+$ T cells [135–138]. In the laboratory, dry eye can be modelled by different methods. First, there are autoimmune animal strains that spontaneously develop eye and lacrimal gland alterations at young age [139–142]. Also, it can be modelled by subjecting young mice to desiccating stress, that is low humidity conditions with or without cholinergic blockade of lacrimal gland secretion [143–145]. Surgical excision of one or more of the glands that contribute to the tear film in mice also causes ocular surface desiccation and a dry eye phenotype comparable to the other methods [146]. Remarkably, loss of conjunctival tolerance is another unifying feature of all these disease models. Of note, in the induced dry eye models, mucosal tolerance to harmless antigens is impaired not immediately but after three days of desiccating stress, suggesting that there is a threshold of ocular surface damage that must be surpassed before this immunoregulatory mechanism is overcome [132,133]. Interestingly, middle-aged and elderly wild-type mice spontaneously develop dry eye disease, displaying loss of conjunctival goblet cell density and corneal barrier disruption (hallmarks of dry eye) as early as 9–12 months of age [147,148].

The putative antigens targeted by the pathogenic $CD4^+$ T cells that drive the disease in dry eye remain elusive, although some studies have implicated kallikrein proteins [149–151]. In experimental studies, this limitation is usually overcome by introducing a known harmless foreign antigen (e.g. OVA) to the ocular surface as a surrogate ocular surface-derived antigen. In an attempt to understand whether changes in conjunctival tolerance also participate in age-related dry eye, we evaluated conjunctival tolerance to OVA in mice of three different ages (2, 9 and 24 months of age; Figure 2A) following established protocols [110,122,127,132,133]. Young mice that received OVA eye drops for three consecutive days before immunization displayed less oedema in the ears (low DTH), showing that they developed mucosal tolerance to OVA (Figure 2B). Interestingly, the 9-month-old group did not show a statistical difference in ear thickness when exposed previously to OVA eye drops, that is did not develop conjunctival tolerance to OVA. The literature shows that loss of oral tolerance to OVA is already present in mice aged 6–8 months [36]. Concordant with previous studies, the elderly group (24 months of age) did not show an adequate response to immunization [18,152], making the interpretation of the effect of prior topical OVA eye drops difficult in this age group. Furthermore, an increased frequency of $CD4^+Foxp3^+$ cells in ocular draining lymph nodes has been reported, and a numerical increase in these cells might be compensating [153] for a qualitative effect in this age group. Another factor to consider is the DTH readout itself, which is used to evidence antigen-specific memory T cells. However, cutaneous immune responses (as is the case for the DTH) are dependent on adequate antigen presentation by skin APCs,

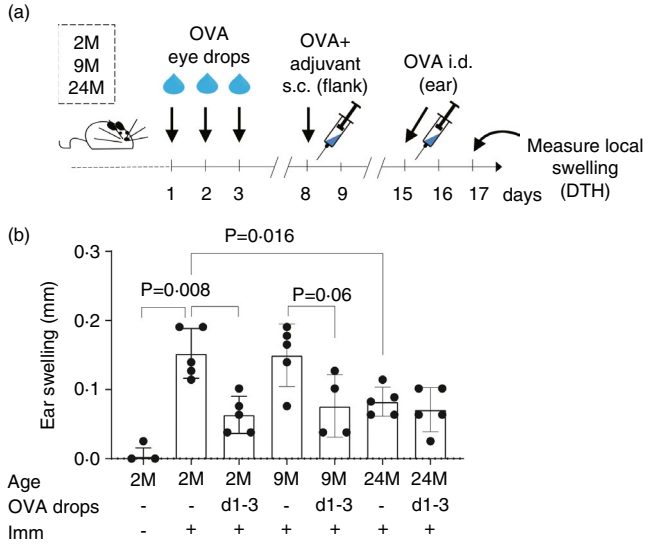

FIGURE 2 Ocular mucosal tolerance in aged mice. (a) Schematic of experimental design in mice of different ages: 2, 9 and 24 months (M). Conjunctival immune tolerance was measured by delayed-type hypersensitivity (DTH) to OVA using the following protocol: OVA eye drops were administered topically for 3 days (d1-3); then, mice were immunized (Imm) subcutaneously (s.c.) with OVA + complete Freund's adjuvant on day 8 and finally challenged with the same antigen by intradermal (i.d) ear injection (OVA in the right ear and PBS in the left ear) on day 15. Ear swelling was measured 48 h later. (b) In vivo DTH (ear swelling) measurements. Results are the difference between the antigen-injected and PBS-injected ears of mice in each group. (n = 5/group, mean ± SEM, Kruskal–Wallis followed by Dunn's multiple comparisons test). These experiments were approved by the Institutional Animal Care and Use Committees at Baylor College of Medicine

which are also decreased in the aged [154]. Altogether, these results suggest that conjunctival tolerance is impaired by ageing, as is also the case for oral tolerance.

Many changes in the aged ocular surface immune system may favour tolerance disruption (for a complete review, see Galletti and de Paiva [19]). In the gut, retinoic acid-loaded APCs participate in tolerance induction. Interestingly, a decrease in conjunctival aldehyde dehydrogenase activity (a critical step in retinoic acid metabolism) in APCs and a higher number of activated APCs are observed in the aged conjunctiva [155]. This is accompanied by an increasingly inflammatory milieu (elevated *interleukin-1β*, *MHC II*, *interferon-γ* and *interleukin-12* mRNA transcripts). Aged APCs obtained from ocular draining nodes have an activated phenotype and prime preferentially Th1 cells in antigen presentation assays in vitro [155]. Goblet cells constitute an epithelial cell subpopulation that is highly immunoregulatory in the ocular surface [156] and pivotal in mucosal tolerance induction in the gut [24,25]. Conjunctival goblet cells also experience age-related changes in humans and mice [19]. Mice deficient in conjunctival goblet cells have defective ocular mucosal tolerance [123,124] and spontaneously develop dry eye [123,157], which also suggests a mechanistic association between ocular mucosal tolerance loss and dry eye pathogenesis. Studies of conjunctival tolerance in Sjögren's syndrome-like mice are lacking in the literature. Aged lacrimal glands also display lymphocytic infiltration [147,148,158]. All the aforementioned mechanisms at work in the aged ocular surface have been linked to decreased or altered Treg generation and impaired mucosal tolerance induction in other tissues [25,123,124,159–161]. Thus, it is possible that a combination of a pro-inflammatory milieu, immunosenescence and age-related changes in APC, goblet cell loss and altered Tregs influence conjunctival tolerance. Disrupted conjunctival tolerance, in turn, may favour disease onset or progression in the elderly. In line with this, pharmacological inhibition of nuclear factor-κB activity in the ocular surface epithelium restores mucosal tolerance in two dry eye models while improving the disease phenotype, further evidence of a pathophysiological link [132,133]. Remarkably, 12- to 14-month-old mice display corneal staining phenotype and respond more slowly to topical corticosteroids when subjected to experimental dry eye using the desiccating stress model [162,163].

Thus, a breakdown in ocular mucosal tolerance to harmless antigens seems to be a constant feature in diverse ocular surface disease models (benzalkonium chloride eye drops, desiccating stress, lacrimal gland excision, mice devoid of goblet cells), and remarkably, impairment of this homeostatic mechanism also occurs with ageing. In humans, advanced age is associated with an increased prevalence of several ocular surface disorders, among which dry eye is the most prominent. However, clinical data of ocular surface disorders in the elderly do not always follow clear-cut categories, in part due to symptom overlap between presentations. For instance, allergic conjunctivitis represents a significant fraction (16%) of referrals of elderly patients for allergic disease [47], but it is under-reported or under-recognized because ocular symptoms are considered part of rhinoconjunctivitis [164]. Also, more subtle, chronic allergic reactions in the ocular surface of aged patients may be misinterpreted because of concurrent use of topical eye medications (with preservatives) and/or mistaken for dry eye [126]. Also, patients with allergic rhinoconjunctivitis have increased tear osmolarity [165], a finding implicated in dry eye pathogenesis [14,134]. Conversely, dry eye patients are more likely to be sensitized to known allergens and report symptoms typically associated with allergic rhinoconjunctivitis [166]. In addition, the diagnosis of 'elderly onset Sjögren's syndrome', a severe form of dry eye, is controversial, with some groups suggesting that the signs and symptoms are only related to ageing of the immune system, while others affirming that is it indeed autoimmunity and should be treated as such [49–53]. At any rate, dysregulated ocular mucosal tolerance underlies the corresponding animal models for all these presentations, including ageing, which underscores its pathogenic contribution. Still, much remains to be learned about

the pathogenic mechanisms specific to the aged ocular surface and its diseases.

CONCLUSIONS

The ocular surface immune system is radically different from that of inside the eye globe. Instead of immune privilege, another form of peripheral tolerance is in effect to keep inflammation in check in the outer ocular structures: mucosal tolerance. Conjunctival tolerance is akin to the mucosal tolerance mechanisms found in the gut and airways, and also similarly, this form of immunoregulation in the eye is affected by ageing just as it is in the digestive and respiratory tracts. Although the extent of the experimental evidence and clinical data for each location differs greatly, these three mucosal sites reviewed here experience dysregulatory changes with ageing that result in loss of mucosal tolerance, a highly relevant homeostatic function for mucosal health. The best case can be made for the gastrointestinal tract, where there is ample experimental and clinical data supporting the pathophysiological implications of oral tolerance loss in the elderly. In the airways, there is also extensive clinical evidence suggesting disruption of respiratory tolerance in aged subjects and there are several mechanistic studies in animal models that support this notion, but conclusive exploration of mucosal tolerance status in the airways of aged mice is lacking.

The case for the ocular surface, which is the actual purpose of this review, is further complicated by the fact that the putative autoantigens of its most prominent immune disease, dry eye, remain unidentified. There is considerable evidence of mucosal tolerance disruption in several animal models of ocular surface disease, and here we also present new data on how ageing affects experimental induction of conjunctival tolerance in mice (Figure 2). As for the gut and the respiratory tract, there are also numerous mechanistic studies on the effect of ageing on specific components of the immune response of the ocular mucosa [19]. Perhaps gut immunology, and more specifically inflammatory bowel disease, could serve as a guide to future research into the mechanisms of dry eye in the elderly, given the similarities outlined in this review and elsewhere [16] between the two mucosal sites and immune-based mucosal disorders, respectively. Thus, progress in the eye field could be made by applying current knowledge of age-related changes in other mucosal sites.

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