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Neuroimmune crosstalk in the cornea: the role of immune cells in corneal nerve maintenance during homeostasis and inflammation

Contents

Abbreviations

ACAID	Anterior Chamber-Associated Immune Deviation	MHC II	class II major histocompatibility complex
APCs	antigen-presenting cells	MMC	mitomycin C
CCR2	C-C chemokine receptor type 2	NGF	nerve growth factor
CGRP	calcitonin gene-related peptide	NK1R	neurokinin-1 receptor
CNS	central nervous system	NPY	neuropeptide Y
CNTF	ciliary neurotrophic factor	PEDF	pigment epithelial-derived factor
CXL	corneal collagen cross-linking	PK	penetrating keratoplasty
DCs	dendritic cells	POAG	primary open-angle glaucoma
DED	dry eye disease	PRK	photorefractive keratectomy
DHA	docosahexaenoic acid	SBNP	sub-basal nerve plexus
HIV	glial cell-derived neurotrophic factor	SMILE	small incision lenticule extraction
HSV	herpes simplex virus	SNT	superficial nerve terminal
HZV	herpes zoster virus	SP	substance P
IGF	insulin-like growth factor	TGF-β	transforming growth factor beta
IL	interleukin	Treg	regulatory T cell
IVCM	*in vivo* confocal microscopy	TRPA1	transient receptor potential ankyrin 1
LASEK	laser epithelial keratomileusis	TRPM8	transient receptor potential melastatin 8
LASIK	laser-assisted *in situ* keratomileusis	TRPV1	transient receptor potential vanilloid receptor 1
MANF	mesencephalic astrocyte-derived neurotrophic factor	VEGF	vascular endothelial growth factor
MDSCs	myeloid-derived suppressor cells	VIP	vasoactive intestinal peptide

1. Introduction

The cornea is one of the most densely innervated tissues in the human body, with a nerve fiber density of approximately 46 mm/mm^2 at the basal epithelium layer near the corneal apex (Marfurt et al., 2010). Corneal nerves detect endogenous and exogenous signals to maintain the homeostasis of the ocular surface by regulating tear production, modulating blink reflexes, and releasing numerous neurotropic substances (Beuerman and Schimmelpfennig, 1980; Nishida, 2005). The corneal sensory nerves are supplied by the ophthalmic branch of the trigeminal nerve. In the epithelium, they stratify to form the so-called sub-basal nerve plexus (SBNP), also termed the intraepithelial corneal basal nerves (Stepp et al., 2020), at the level of the basal epithelium. The superficial nerve terminals (SNT) arising from the SBNP terminate as free nerve endings within the apical layers of the epithelium (Figure 1).

Although the cornea lacks blood vessels and is regarded as an immune privileged tissue, there are distinct populations of resident immune cells distributed throughout the corneal epithelium and stroma, including dendritic cells, macrophages, mast cells and innate lymphoid cells (Liu and Li, 2021). In addition to these resident immune cells, inflammatory cells such as neutrophils and adaptive T cells can infiltrate into the cornea to participate in the pathology of many corneal diseases (Bron et al., 2017; Yu et al., 2022).

Neuroimmune interactions, relating to the physical and functional interplay between nerves and immune cells, have been investigated in many tissues, including the respiratory epithelium (Veres et al., 2009), meninges (Schain et al., 2018), skin (Kashem et al., 2015) and cornea (Hamrah et al., 2016; Jiao et al., 2020a). Many molecular components have been co-identified in the immune and nervous systems. For example, receptors for neurotransmitters that are found on nerves, such as acetylcholine receptors, are also expressed on DCs, macrophages and T cells, suggesting a role for neural modulation of immune responses (Kawashima et al., 2015; Wang et al., 2003). Recent studies have shown that 'anti-inflammatory' M2 macrophages, DCs and adaptive immune cells (T cells and B cells) play a pivotal role in the mechanisms involved with peripheral nerve degeneration and regeneration (Chen et al., 2015). With wider use of *in vivo* confocal microscopy (IVCM) in clinical and research settings, and the development of transgenic animal models, considerable attention has been given to understanding the interaction between corneal nerves and immune cells (Bitirgen et al., 2018a; Choi et al., 2017; Gao et al., 2016a).

Neurogenic inflammation in the cornea provides evidence for corneal neuroimmune crosstalk. Corneal nerve damage induces immune responses to promote the clearance of disrupted axons, and release of neurotrophic factors that trigger inflammation (Lasagni Vitar et al., 2022). The inflammatory response, including activation of immune cells and released cytokines, can further induce nerve damage, to propogate the corneal neuroinflammation (Launay et al., 2016). The reciprocal relationship between immune cells and sensory nerves complicates the picture of neuroimmune interactions in the cornea. Many studies have demonstrated a negative association between corneal innervation and activated immune cells (D'Onofrio et al., 2021; Xu et al., 2021). However, there are also redress regulations in the course of neurogenic inflammation and neuroinflammation, i.e., some immune cell

phenotypes, cytokines and neurotrophic factors may contribute to protecting corneal innervation (Gao et al., 2016a; Liu et al., 2018). Understanding the mechanisms underlying corneal neuroimmune crosstalk is important for developing new therapeutic strategies for corneal healing and nerve regeneration.

This review summarizes and compares findings from studies that have investigated neuroimmune crosstalk (i.e., the reciprocal interaction between immune cells and sensory nerves) in the cornea, with the goal of reframing current dogma that corneal immune cells are negatively associated with corneal nerve integrity. Clinical trial investigations of emerging therapies, and novel treatment approaches currently in pre-clinical development, for corneal nerve regeneration associated with immunomodulation, are also described.

2. Assessing corneal nerves and immune cells

2.1. In vivo confocal microscopy

In vivo confocal microscopy (IVCM), a non-invasive real-time imaging technique, has advanced the evaluation of the corneal architecture in humans (De Silva et al., 2017; Niederer and McGhee, 2010). IVCM provides high-resolution, *en face* images to enable qualitative and quantitative analyses of corneal structures, including nerves, epithelial cells and immune cells (Figure 2). Corneal SBNP characteristics have been clinically investigated in health and disease using IVCM (Chinnery et al., 2021a; Cruzat et al., 2017; Malik et al., 2003; Petropoulos et al., 2020). Moreover, IVCM has been useful to identify and assess corneal immune cell characteristics, such as their density, distribution and morphology, and, more recently, their association with corneal nerves, in a wide range of ocular and systemic diseases. Studies have reported corneal immune cell changes using IVCM in conditions such as dry eye disease (Aggarwal et al., 2020), infectious keratitis (Kwon et al., 2018) and pterygium (Wang et al., 2010), as well as systemic neurodegenerative diseases that are not typically known to impart clinically obvious ocular surface changes, such as multiple sclerosis (Bitirgen et al., 2017) and mild cognitive impairment (Dehghani et al., 2020). There are also an increasing number of studies reporting negative correlations between corneal SBNP density and intraepithelial immune cell density (Cavalcanti et al., 2018; Cruzat et al., 2011), suggesting that corneal immune cells may be involved with, or respond to, corneal neuropathy.

Although IVCM is a valuable clinical tool for assessing corneal sensory nerves and immune cells, it has some limitations. The visibility of corneal features within the image field can be affected by various factors including image quality, imaging depth and post-capture image enhancements, potentially leading to inconsistencies in the delineation of the sub-basal nerve plexus and subsequent nerve parameter measurements (Patel and McGhee, 2013). In view of these considerations, a tool was developed to assess the methodological quality of clinical studies using laser-scanning IVCM when evaluating corneal nerve parameters (De Silva et al., 2017). The tool can also be prospectively used by researchers when designing IVCM studies, to minimise potential biases when quantifying corneal parameters, such as nerve and immune cell densities.

A practical consideration with IVCM is that it can only be used to visualize nerves comprising the SBNP, but rarely the SNT; this latter nerve plexus plays an important role in corneal sensory function, particularly as related to the sensitization of nociceptors. In addition, although the dendriform cells observed using IVCM at the basal epithelium are believed to be $CD11c^+$ dendritic cells (DCs), based on *ex vivo* immunostaining data of human corneal flatmounts (Yamagami et al., 2005; Zhivov et al., 2005), their specific surface markers cannot be verified *in vivo*. Currently, phenotypic classification of *in vivo* corneal epithelial immune cells relies mostly on morphological characteristics, with immune cells without dendrites considered 'immature' DCs, and those with elongated cell processes referred to as 'mature' DCs (Chinnery et al., 2021b). Evidence for the existence of "immature" DCs without dendrites in the healthy corneal epithelium of rodents is lacking; this could be explained by inter-species differences, or, more controversially, may indicate that DCs without dendrites in human corneas represent a different cell population altogether (Loi et al., 2022). For this reason, when referring to immune cells in the human corneal epithelium more broadly, we will refer to them as "immune cells", except when describing findings from clinical studies where the authors have used the term "dendritic cells" (or "DCs").

2.2. Animal models

Animal models offer the opportunity to garner more detailed knowledge about the morphology, distribution and phenotype of corneal nerve structures and immune cell populations, owing to the capacity to perform *ex vivo* immunostaining and high-resolution confocal microscopy. This is especially critical when evaluating the apically-projecting SNT that cannot be consistently imaged using current clinical IVCM devices. Animal studies can also be used to determine the identity of corneal immune cell and nerve subpopulations, and to investigate the molecular mechanisms underlying corneal nerve repair.

Transgenic mouse models that allow for direct visualization of peripheral nerves have been reviewed recently (Yamakawa et al., 2020). The thy1-YFP transgenic mouse, a neurofluorescent murine model whereby the *thy1* gene promoter expresses yellow fluorescent protein, can be used to readily visualize corneal sensory nerves *in vivo,* although only approximately 46% of corneal nerves express the reporter protein (Yu and Rosenblatt, 2007) (Figure 3A). The transgenic $Gaba_{B1}$-GFP reporter mouse, expressing fluorescent G-protein coupled receptors for the neurotransmitter γ-aminobutyric acid, has been validated for corneal nerve visualization by labeling about 90% of $TuJ1^{+}$ (class III beta-tubulin) corneal nerve fibers (Hanack et al., 2015). The TRPM8-eYFP transgenic mouse that expresses fluorescent Transient receptor potential melastatin 8 (TRPM8) also provides a means for observing corneal cold thermoreceptor fibers (Parra et al., 2010).

In terms of transgenic models used to visualize other corneal features, such as immune cells, corneal DCs can be readily identified in CD11c reporter mice using intravital microscopy (Lee et al., 2010; Seyed-Razavi et al., 2019); when crossed with thy1-YFP mice, nerves, DCs and their physical interactions can be examined using intravital multiphoton microscopy (Fig 3B) (Jamali et al., 2020b). Transgenic reporter Cx3cr1 GFP 'knock-in' mice can also be used to visualize resident corneal epithelial $Cx3cr1^{+}$ DCs (Chinnery et al., 2008; Chinnery et al., 2015; Chinnery et al., 2007). Heterozygous $Cx3cr1^{GFP/+}$ mice have a targeted replacement of the CX3CR1 gene by GFP, enabling visualization of $Cx3cr1^{+}$ cells, which are resident to the mouse corneal stroma and epithelium (Fig 3C). $Cx3cr1^{GFP/GFP}$ (homozygous) mice also express GFP, but are functionally Cx3cr1-deficient. Interrogation of the function of Cx3cr1 signaling in the mouse cornea revealed that Cx3cr1-homozygous mice, lack resident

epithelial DCs in the healthy cornea (Fig 3D-F) (Chinnery 2007). In these mice, stromal macrophages appear phenotypically normal, but there is a striking loss of intraepithelial DCs (Fig 3E and F). Other transgenic mouse models, such as CD11c and CD207-diptheria toxin mice, in which corneal DCs can be pharmacologically depleted either systemically or via subconjunctival injection, also provide evidence for the possible neuroprotective function of DCs during corneal homeostasis and wound healing (Choi et al., 2017; Gao et al., 2016a; Gao et al., 2011).

3. Corneal innervation

3.1. Corneal innervation anatomy and function

The outermost layer of the cornea consists of a stratified squamous, non-keratinized epithelium, innervated by a rich sensory nerve supply (Figure 1). Corneal nerves originate from the ophthalmic division of the trigeminal ganglion (cranial nerve V), travelling suprachoroidally and ultimately branching to form the limbal plexus where they penetrate the corneoscleral limbus (Al-Aqaba et al., 2010; Marfurt et al., 1989). The limbal plexus branches and enters the cornea to form the stromal nerve trunks before turning anteriorly towards the ocular surface (Muller et al., 2003). Nerve branches arising from the anterior stromal plexus divide further and run parallel between the basal epithelium and Bowman's layer, forming the so-called SBNP. The plexus of SBNP axons typically forms a distinctive whorl-like pattern approximately 2.5 mm infero-nasal to the corneal apex (Marfurt et al., 2010) (Figure 4A). Several nerve branches of the SBNP turn upward, penetrating vertically through the epithelium, and terminating just beneath the epithelial surface as the SNT (Muller et al., 1996) (Figure 4B). Using block-face scanning electron microscopy, the SBNP has recently been shown to have physical and functional interactions with the basal corneal epithelial cells (Courson et al., 2019; Parlanti et al., 2020). The SNT create large nociceptive fields, with an estimated density of about 605 nociceptive terminals per square millimeter in the central cornea of humans that can rapidly respond to mechanical, thermal and chemical stimuli (Marfurt et al., 2010).

Sensory nerves in the human cornea are either nociceptive Aδ or C-type fibers. The Aδ fibers are myelinated and have a fast conducting velocity and a relatively large diameter (1 to 5 μm), whereas the slow-conducting C fibers are unmyelinated and thinner in diameter (0.2 to

1.5 µm) (Al-Aqaba et al., 2019). Approximately 70% of corneal sensory fibers are of the polymodal subtype, most of which are unmyelinated C type nerves. Polymodal nociceptors convey sharp and sustained pain through slow-conducting C fiber activation in response to mechanical, heat and chemical stimuli, with a sustained discharge of nerve impulses (Downie et al., 2021). Polymodal nociceptors can also be activated by endogenous stimuli and inflammatory mediators (Alamri et al., 2015; MacIver and Tanelian, 1993). In addition to the sensation of pain, polymodal nociceptors contribute to reflex tear secretion caused by corneal stimulation (Acosta et al., 2004). The transient receptor potential vanilloid receptor 1 (TRPV1) plays a significant role in nociceptive transduction in polymodal receptors. It has been shown that over 90% of $TRPV1^+$ corneal afferent neurons are likely to be polymodal nociceptors in guinea pigs (Alamri et al., 2015), and TRPV1 is also expressed in intraepithelial nerve terminals in the mouse corneal epithelium (Jiao et al., 2021). Transient receptor potential ankyrin 1 (TRPA1) and acid-sensing ion channels are also involved in pain sensations transmitted by polymodal nociceptors (Bandell et al., 2004; Callejo et al., 2015). The activities of TRPV1 and TRPA1 are modulated by inflammation, which is proposed to underpin ocular discomfort in inflammatory conditions, such as allergic keratoconjunctivitis (Acosta et al., 2013).

Another 20% of corneal sensory fibers have mechanoreceptors that are sensitive to mechanical contact and generate one, or at most a few, nerve impulse(s) to convey acute sensations (Belmonte et al., 2004); these mechanoreceptors are all thinly myelinated Aδ type nerves. In contrast to polymodal nociceptors, mechano-nociceptors are responsible for immediate and sharp sensations of pain induced by insults, including touching or scratching the corneal surface (Belmonte et al., 2017). Though TRPV1 is absent from Aδ mechanoreceptors (Murata and Masuko, 2006), another mechanosensitive ion channel (PIEZO2) may contribute to the sharp sensation of pain, which has been identified in corneal afferent neurons in the trigeminal ganglion (Alamri et al., 2015). Future studies are required to confirm its expression in the intraepithelial sensory nerves of the cornea.

The remaining 10% of corneal nerve fibers are Aδ and C fibers, which have cold-sensitive nociceptors that respond to cool temperatures (less than about 33°C) at the ocular surface (Belmonte et al., 2017). It has been reported that corneal cold thermoreceptor activity is

enhanced with elevated tear osmolarity due to tear film evaporation, which may be associated with ocular discomfort in dry eye disease (Kovacs et al., 2016; Parra et al., 2010). In addition, inflammation can modulate the activity of corneal cold thermoreceptor by inhibiting impulses in response to cooling (Acosta et al., 2013). The transient receptor potential melastatin 8 (TRPM8) cation channel plays an important role in corneal cold sensation (Parra et al., 2010). Through the response to cooling and elevated osmolarity, TRPM8-dependent impulses are responsible for regulating basal tear secretion (Parra et al., 2010).

Corneal nerves are not only involved in regulating tear production and blinking reflexes to maintain a lubricated and intact corneal epithelium, but also release numerous neurotropic substances, such as substance P (SP) and calcitonin gene-related peptide (CGRP), to modulate wound repair and assist in the maintenance of ocular surface health (Beuerman and Schimmelpfennig, 1980; Nishida, 2005). In mice, an age-related decline in corneal sensory nerve density has been reported (De Silva et al., 2019; Stepp et al., 2018), and reduced SP levels have also been described in the cornea and tear film of both aged mice and humans (Marco et al., 2018). SP released from corneal C fibers has an important role in maintaining corneal nerve structure and sensitivity (Marco et al., 2018), as well as promoting corneal wound healing in synergism with insulin-like growth factor (IGF) (Nagano et al., 2003; Nishida, 2005). A clinical trial demonstrated that eye drops containing SP and IGF-derived peptides induce a rapid re-epithelialisation of persistent epithelial defects in neurotrophic keratopathy (Yamada et al., 2008). The SP receptor is not only expressed on corneal sensory nerves but also on corneal immune cells, suggesting these neurotropic substances have dual neuro- and immune-mediated actions in the cornea. Although SP plays an important role in corneal wound healing by binding to its receptor in epithelial cells and attenuating the cytokine-chemokine network (Yanai et al., 2020), it may adversely affect ocular inflammation in some conditions (Suvas, 2017). In animal models of bacterial keratitis (*Pseudomonas aeruginosa*), SP causes an elevation of levels of pro-inflammatory cytokines (Foldenauer et al., 2012; McClellan et al., 2008), and application of a SP antagonist improves disease outcomes (Hazlett et al., 2007). Excessive release of SP may further promote leukocyte infiltration and delay the apoptosis of neutrophils, leading to neurogenic inflammation (Lasagni Vitar et al., 2022; Zhou et al., 2008). In addition, SP is also reported to promote inflammatory corneal neovascularisation, with increased infiltration of $CD45^+$

leukocytes in mouse corneas after total de-epithelisation, alkali burn, or intrastromal sutures (Barbariga et al., 2018).

On the basis of an abundance of $CGRP^+$ nerves supplying the cornea, CGRP may also participate in corneal wound healing (He and Bazan, 2016). Regeneration of $CGRP^+$ nerves after corneal injury accelerated corneal epithelial healing (Cortina et al., 2012), and CGRP has been shown to enhance epithelial wound healing *in vitro* (Mikulec and Tanelian, 1996). Corneal sensory nerves release CGRP after electrical stimulation and heat or chemical irritants but not cold stimuli, indicating the release of CGRP may requires a vigorous stimulation of polymodal nerve endings (Belmonte et al., 2003). Similar to neuropeptide SP, the release of CGRP is believed to be pro-inflammatory leading to neurogenic inflammation (Belmonte et al., 2004; Lasagni Vitar et al., 2022). However, CGRP can also contribute to the resolution of corneal inflammation, as evidenced by the release of CGRP by macrophages and transformation of macrophages towards the anti-inflammatory phenotype in a mouse model of P. aeruginosa keratitis (Yuan et al., 2022).

The role of corneal sensory nerves in both maintaining epithelial maintenance and promoting wound healing is also closely linked with corneal epithelial stem cell function. In a mouse model of corneal nerve depletion induced by ophthalmic nerve electrocoagulation, limbal stem cell density was 75% lower than in control eyes, leading to impaired corneal epithelial healing (Ueno et al., 2012). The proliferative activity of corneal epithelial stem cells was 50% lower in the denervated eyes (Ueno et al., 2012). These results are consistent with the clinical observation of limbal stem cell deficiency in eyes with neurotrophic keratopathy, where patients are prone to developing persistent epithelial defects and non-healing ulcers (Delic et al., 2022; Dua et al., 2018). A recent study confirmed the dependence of corneal stem cells on corneal innervation, and demonstrated the critical role of sensory nerve TRPV4 in maintaining stemness of limbal basal cells (Okada et al., 2019). Impaired epithelial healing caused by sensory nerve damage can be rescued by TRPV4 gene introduction, which is accompanied by the recovery of stem cell function (Okada et al., 2019). Other neurotrophic factors, including nerve growth factor (NGF), glial cell-derived neurotrophic factor (GDNF) and brain-derived neurotrophic factor (BDNF), may also play an important role in maintaining corneal epithelial stem cells (Qi et al., 2007).

In addition to its well described sensory nerve supply, the cornea is also considered to receive innervation from the autonomic nervous system; this topic has been covered recently in a review by Vitar et al (2022). The mammalian cornea receives sympathetic nerve fibers that predominantly originate from superior cervical ganglion (Marfurt and Ellis, 1993). This sympathic innervation constitutes approximately 15% of the total corneal innervation in rabbits and cats (Morgan et al., 1987; Tervo and Palkama, 1978), but its contribution is considered relatively limited in humans (McDougal and Gamlin, 2015; Toivanen et al., 1987). Sympathetic nerve fibers were reported to be predominantly located in the limbal stroma (Ehinger, 1966; Marfurt and Ellis, 1993), but a recent study has shown that around 10% of the central intraepithelial nerves in rats express sympathetic nerve markers (He et al., 2021). Parasympathetic fibers are also presented in the corneal stroma of rats and cats (Marfurt et al., 1998; Morgan et al., 1987), but these findings have not been confirmed in humans. Though the function of autonomic nerves in the cornea is not well understood, they may contribute to ocular surface health, corneal wound healing responses and/or neurogenic inflammation (Jones and Marfurt, 1996; Yun et al., 2016). The autonomic innervation in the cornea also releases neuropeptides including neuropeptide Y (NPY), acetylcholine and vasoactive intestinal polypeptide (VIP), which may participate in wound healing and angiogenesis (Ekstrand et al., 2003). VIP has also been shown to regulate inflammatory responses and nerve regeneration in the cornea (Zhang et al., 2020b). Animal studies have shown that sympathetic nerve activation inhibits corneal re-epithelialization and promotes neutrophil infiltration after injury, while parasympathetic nerve activation has the opposite effect (Xue et al., 2018). Sympathetic nerves may replace corneal sensory innervation in reactivated herpes stromal keratitis, which is dependent on $CD4^+$ T cell activation (Yun et al., 2022).

3.2. Changes to corneal sensory innervation in ocular conditions

Many ocular conditions have known associations with structural and/or functional abnormalities to corneal sensory nerves, which have been thoroughly reviewed relatively recently (Al-Aqaba et al., 2019). For the purpose of the present review, we focus on conditions that have been the subject of reports relating to neuroimmune interactions in the cornea.

3.2.1. Dry eye disease

Dry eye disease (DED) is one of the most common disorders affecting the ocular surface. The latest international Dry Eye WorkShop II (DEWS II) report from the Tear Film and Ocular surface Society acknowledged that corneal neurosensory abnormalities have an etiological role in DED (Craig et al., 2017). Using IVCM, some studies have reported reduced SBNP density in patients with DED (Cox et al., 2021; Giannaccare et al., 2019; Labbe et al., 2013), while others have reported that corneal nerve density remains unaffected (Hosal et al., 2005; Zhang et al., 2005). This discrepancy in the literature might, at least in part, be explained by the studies assessing patients with different stages, subtypes and/or severities of DED. In addition, variations in IVCM imaging methods, including the device used, number of images assessed, the method of image selection, and the location and depth of the corneal imaging, may contribute to reported differences in corneal nerve parameters in different studies (De Silva et al., 2017). For example, laser-scanning IVCM enables higher resolution images of intraepithelial nerves than the slit-scanning device, which may result in differential sensitivity to detecting corneal nerves *in vivo* using the two methods (Cruzat et al., 2017).

Tear hyperosmolarity and inflammation are considered key elements of the pathophysiology of DED (Bron et al., 2017). Tear hyperosmolarity can trigger innate immune and adaptive ($CD4^+$ T cell) responses in the cornea (Stern et al., 2013), which involves neuropeptide release from damaged nerves (Figure 5). In desiccating stress-induced models of DED in mice, inhibiting neurokinin-1 receptor (NK1R), the principle SP receptor, reduced DED severity by suppressing T helper cell mediated responses (Taketani et al., 2020; Yu et al., 2020). *In vitro*, substance P can also modulate T cell activation, selectively inducing Th17 cell formation, or switching, from memory $CD4^+$ T cells to amplify inflammation (Cunin et al., 2011). Dendritic cells (DCs) play an important role in the activation of $CD4^+$ T cells (Schaumburg et al., 2011), and many studies report an increased density of DCs in patients with DED (Xu et al., 2021). In mice, hyperosmolarity can induce morphological and phenotypic maturation of corneal intraepithelial DCs within hours after topical exposure (Senthil et al., 2021). Together, these studies provide support for mechanistic links between nerve stimulation and recruitment and activation of corneal DCs, and also the immunomodulatory roles of neuropeptides in the cornea during DED.

Neuroinflammation in DED not only occurs in the cornea but can also involve the trigeminal ganglion and the trigeminal brainstem sensory complex. In a DED mouse model induced by unilateral excision of the extraorbital lacrimal gland and Harderian gland, reduced corneal intraepithelial nerves, decreased corneal mechanical hypersensitivity and increased spontaneous electrical activity of the ciliary nerve were observed (Fakih et al., 2019). Notably, increased pro-inflammatory markers were detected in the ipsilateral trigeminal ganglion and the trigeminal brainstem sensory complex (Fakih et al., 2019). Persistent ocular dryness and inflammation triggered corneal nociceptor activity that led to the development of corneal hypersensitivity and peripheral sensitization (Fakih et al., 2019). Similar results were observed in a topical benzalkonium chloride (BAK) model of ocular surface inflammation (Launay et al., 2016). The spread of inflammation from the cornea to the peripheral nervous system in experimental DED, and BAK-induced inflammation, lends further support to neuroimmune crosstalk in the cornea.

3.2.2. Neurotrophic keratopathy

Neurotrophic keratopathy (also known as neurotrophic keratitis) is defined as "a disease related to alterations in corneal nerves leading to impairment in sensory and trophic function with consequent breakdown of the corneal epithelium, affecting health and integrity of the tear film, epithelium and stroma" (Dua et al., 2018). Dua et al. recently reviewed neurotrophic keratopathy, and suggested a clinically relevant classification with three stages that relate to disease severity and prognosis: 1) Mild: epithelial changes only, without epithelial defect; 2) Moderate: epithelial defect without stromal defect; and 3) Severe: stromal involvement from corneal ulcer to lysis to perforation, with corneal hypo-aesthesia/anaesthesia (Dua et al., 2018). Many ocular and systemic diseases that involve corneal nerve impairment can induce neurotrophic keratopathy (Dua et al., 2018). One of the well-accepted theories is that impaired corneal nerves and reduced corneal sensation can result in diminished blink reflexes, reduced tear secretion and a deficit in neurotrophic support (Belmonte and Gallar, 2011; Wilson and Ambrosio, 2001). For example, corneal nerve injury is associated with reduced levels of the neurochemicals SP and acetylcholine; factors that have been shown to promote epithelial wound healing (Semeraro et al., 2014). Animal and clinical studies have demonstrated therapeutic effects with SP and IGF in

neurotrophic keratopathy (Nagano et al., 2003; Yamada et al., 2008). Therapeutic interventions using neurotrophins such as nerve growth factor (NGF) have shown great promise in treating neurotrophic keratopathy (Sacchetti and Lambiase, 2017), with recently conducted randomized, double-masked, vehicle-controlled trials confirming the efficacy of recombinant human NGF for treating neurotrophic keratopathy (Bonini et al., 2018; Pflugfelder et al., 2020).

Chronic inflammation triggered by corneal nerve disruption is also involved in the development of neurotrophic keratopathy. Disrupted tear secretion can induce tear hyperosmolarity and increased release of pro-inflammatory factors and neuropeptides, leading to neurogenic inflammation and progression of the disease (Baudouin et al., 2013; Dua et al., 2018). Actived collagenolytic enzymes in corneal inflammation can initiate stromal collagen melting, leading to the disease progressing to a moderate or severe stage (Fini et al., 1992). Studies have shown alterations to immune cell activity and cytokine release in diabetes-induced neurotrophic keratopathy (Lagali et al., 2018; Leppin et al., 2014).

3.2.3. Iatrogenic causes

Refractive surgery leads to corneal nerve degeneration as a result of transecting the sub-epithelial or anterior stromal nerves during the procedure. Several studies in animal models have explored the morphological and functional alterations to corneal nerves after experimental photorefractive keratectomy (PRK) (Bech et al., 2018; Medeiros et al., 2018). The regeneration of the SBNP starts shortly after PRK and reaches half the baseline density by day 15 in mice (Bech et al., 2018). In rabbits, nerve fibers sprout during the first month following PRK, however even after six months neither the SBNP nor the stromal nerves are fully recovered (Medeiros et al., 2018). In humans, 70%-80% of the SBNP remains absent at one month after PRK surgery and its regeneration takes much longer, with corneal nerve density only reaching half the pre-operative (baseline) levels after one year (Erie et al., 2005).

Laser-assisted *in situ* keratomileusis (LASIK) also leads to an 80% acute decrease in the density of nerves in the SBNP (Moilanen et al., 2008). Nerve regeneration can be observed in the central cornea at six months post-surgery, but it can take at least three years for the SBNP

to recover to its preoperative density, if at all (Calvillo et al., 2004; Erie et al., 2005). Importantly, it is well established that post-LASIK patients are prone to developing dry eye signs, including tear instability, decreased tear production and reduced corneal sensitivity (De Paiva et al., 2006); these clinical features have been linked to the damaged epithelial sensory nerves (Battat et al., 2001).

Laser epithelial keratomileusis (LASEK), which combines elements of LASIK and PRK, is milder in terms of its impact on SBNP density and corneal sensitivity compared to the LASIK at three and six months post-surgery; however, data at longer follow-up periods are not available (Lee et al., 2006). Recent advances in surgical procedures, such as small incision lenticule extraction (SMILE), where the refractive lenticule is extracted through a small incision without the creation of a corneal flap (Sekundo et al., 2011), aim to achieve more rapid post-operative corneal re-innervation (Denoyer et al., 2015). A recent study has reported that eyes subjected to SMILE procedures had significantly higher corneal nerve fiber density and nerve fiber length compared to those that had undergone LASIK at 4.1 years (Liu et al., 2020b); the individuals recruited in this study were of a relatively young age (mean of 25 years at the time of surgery). The extent of corneal nerve regeneration after SMILE was age-dependent, with younger patients having more rapid nerve than older individuals (Li et al., 2021a).

Cataract surgery is another common iatrogenic cause of corneal nerve damage that occurs due to full thickness sensory nerve transection in the operated eye. Reduced corneal nerve fiber number and density, and increased nerve beading, have been described from analyses using corneal IVCM during the first three months after cataract surgery (De Cilla et al., 2014; Misra et al., 2015). A recent study demonstrated that corneal SBNP parameters, including nerve fiber density, fiber length and nerve total branch density, were decreased in both eyes in patients one month after unilateral cataract surgery, suggesting a contralateral eye effect on corneal nerve degeneration (Giannaccare et al., 2020). However, after the unilateral cataract surgery, the density of corneal DCs was described to not differ in either eye relative to preoperative levels (Giannaccare et al., 2020).

Penetrating keratoplasty (PK) is a surgical procedure that involves full thickness resection of the cornea, which includes severing the corneal nerve network. Corneal nerve fibers regenerate much slower after penetrating keratoplasty compared to cataract or refractive surgery, likely due to the widespread damage to axons during the procedure or the different corneal nerve conditions before the surgeries. At 12 months after PK, no SBNP fibers were detected by IVCM in a group of 20 patients (Darwish et al., 2007). In most patients, regeneration of the corneal SBNP and stromal nerves is observed to take one to 14 years after surgery, but no correlation was observed between the SBNP and stromal nerve trunk density (Al-Aqaba et al., 2012b). This finding suggests that the regenerated SBNP likely penetrated the donor graft directly from the host, and stromal nerves did not contribute to the epithelial innervation at this stage. Following PK, corneal sub-basal innervation can still be abnormal even after 40 years (Niederer et al., 2007), with reduced corneal sensation also existing for decades (Rao et al., 1985; Richter et al., 1996). In deep anterior lamellar keratoplasty (DALK), a partial graft procedure, regeneration of the SBNP and the recovery of corneal sensation are similar to PK (Lin et al., 2014; Zhang et al., 2013). Descemet's membrane endothelial keratoplasty (DMEK) and Descemet-stripping endothelial keratoplasty (DSEK), which are less invasive surgeries, cause acute reductions in SBNP density and corneal sensation, which are predominantly due to the corneal incision and descemetorhexis during surgery; however, most corneal nerve parameters return to baseline within four to 10 months post-operatively (Ahuja et al., 2012; Bucher et al., 2014).

Corneal collagen cross-linking (CXL) is a widely used treatment, intended to attenuate the clinical progression of keratoconus. The original protocol for the procedure involves removal of the corneal epithelium (giving it the name ‘epithelium-off’ CXL), thus leading to the destruction of the SBNP (Al-Aqaba et al., 2012a). Transepithelial CXL, where the corneal epithelium is retained *in situ* (so-called ‘epithelium-on’ CXL), results in significantly less damage to the SBNP (Al-Aqaba et al., 2012a; Caporossi et al., 2012). Physical removal of the epithelium is accepted as the main cause of corneal nerve damage from CXL procedures. Corneal SBNP density returns to pre-operative (baseline) levels at 12 months after epithelium-off CXL (Jordan et al., 2014), while complete regeneration of the SBNP after epithelium-on CXL was observed within six months (Bouheraoua et al., 2014). Corneal sensitivity also showed a faster recovery with the epithelium-on CXL method, compared to epithelium-off CXL (Spadea et al., 2015). Given the lower degree of corneal nerve damage

following the ‘epithelium on’ procedure, it is interesting to note that a recent systematic review reported that there was inadequate evidence that transepithelial CXL is more efficacious than epithelium-off CXL, in terms of disease progression, visual acuity and adverse events (Ng et al., 2021).

3.2.4. Corneal infection

Ocular herpes simplex virus (HSV) infection is often characterized by mild manifestations such as blepharoconjunctivitis during primary infection, followed by long periods of latency in the trigeminal ganglion. Upon reactivation of the latent virus, recurrent infections can present as more severe corneal epithelial dendritic keratitis or a geographical corneal lesion (Lobo et al., 2019). Due to its neurotropic classification, HSV-1 pathophysiology is a topic of great interest in the ophthalmology, immunology and neuroimmunology fields. A decrease in corneal sensory nerve density and sensitivity has been described in a mouse model during primary corneal HSV infection (Chucair-Elliott et al., 2015). The virus can track in a retrograde direction, through the corneal nerve axons into the trigeminal ganglion, leading to long-term latency. Consistent with the concept of a connection between the two eyes by a retrograde neural and/or immune pathway (Lee et al., 2019), Hamrah *et al.* reported that in patients with unilateral HSV keratitis, both the infected and uninfected contralateral corneas showed similar alterations to corneal nerves, including decreased SBNP density and reduced corneal sensation (Hamrah et al., 2010). In addition, the nerve damage was evident in both the acute and chronic stages of HSV infection, suggesting that the infection can rapidly induce corneal neuropathy. A recent study revealed that the corneal SBNP in the HSV-affected eye regenerated slowly during the virus latency phase, however corneal nerve density did not reach the level of the healthy control group even after a follow-up period of 29-46 months; in addition, the nerve regeneration did not result in an improvement in corneal sensation (Moein et al., 2018). These findings suggest that corneal HSV-1 infection leads to a severe loss of corneal nerves that takes years to regenerate, and that this neuropathy involves both eyes despite an often unilateral presentation.

Corneal nerve damage also occurs in patients with non-viral infectious keratitis including bacterial, fungal and *Acanthamoeba* keratitis (Cruzat et al., 2011; Muller et al., 2015). Fungal and *Acanthamoeba* keratitis cause a profound decrease in corneal nerve parameters compared

to epithelial herpetic keratitis (Kurbanyan et al., 2012), and the degree of corneal neuropathy is reportedly more severe than for bacterial keratitis (Cruzat et al., 2011). Following antimicrobial treatment, corneal nerve regeneration occurred after the resolution of infection, but did not reach baseline levels even at six months after cessation of treatment (Muller et al., 2015). Interestingly, a reduced density of corneal nerves and the presence of subclinical corneal immune cell infiltrates was observed in the contralateral (unaffected) eyes in patients with unilateral microbial keratitis. The observed contralateral eye effect, reminiscent of a sympathetic immune response, might be one explanation for the nerve damage in the unaffected eye (Cruzat et al., 2015). In a study of tear cytokines in patients with unilateral bacterial keratitis, interleukin (IL)-1β, IL-6 and IL-8 were elevated only in affected eyes while IL-10 and IL-17a were elevated only in unaffected contralateral eyes (Yamaguchi et al., 2014). The cytokine response in affected eyes might be pathogen-specific, whereas the unaffected eyes may be exhibiting a prophylactic host defense response by producing IL-17A.

The inflammation in the unaffected eyes might occur through a brainstem pathway (Launay et al., 2016). In a non-infectious benzalkonium chloride-induced unilateral corneal injury mouse model, the corneal nerve damage elicited neuron activation and inflammation in both the ipsilateral and contralateral sensory trigeminal complex in the brainstem, characterized by activated microglial cells and a release of pro-inflammatory mediators (Launay et al., 2016). A recent study also reported increased corneal nociceptor activity, in both the ipsilateral and contralateral eyes, in animals with mild unilateral corneal inflammation induced by UV exposure, as well as increased cold thermoreceptor activity bilaterally in a chronic tear deficiency model (Luna et al., 2021). The activation of projecting neurons and altered corneal sensory nerve activity may contribute to ocular pain and inflammation in the contralateral eye (Guzman et al., 2018). These contralateral eye studies provide strong evidence of the existence of functional neuroimmune interactions in the cornea that involve higher order structures, including the trigeminal ganglion and brainstem.

3.2.5. Glaucoma

Interest in corneal neuroimmune interactions in glaucoma patients stems from evidence that long term treatment of this disease with topical therapies is known to adversely affect the

ocular surface, mostly due to the presense of preservative agents in eye drop solutions. While most studies ascribe the ocular surface changes to the use of eye drops (Martone et al., 2009; Ranno et al., 2011), evidence of corneal nerve changes in patients with untreated glaucoma also exists (Jing et al., 2021). Structural alterations to corneal nerves, including lower nerve fiber length and branch number, have been reported in patients with untreated normal-tension glaucoma (Jing et al., 2021). However, patients with untreated primary open-angle glaucoma (POAG) share similar corneal SBNP features to healthy controls, suggesting that normal-tension glaucoma might be a primary neurodegenerative disease that is distinct from POAG (Jing et al., 2021).

The presence of corneal nerve damage in individuals with medically managed glaucoma provides evidence that topical, intraocular pressure-lowering medications can negatively effect corneal nerves (Villani et al., 2016). The commonly used preservative, benzalkonium chloride, induces the nerve damage due to its known neurotoxic effects (Sarkar et al., 2012). A recent study revealed that multi-therapy glaucoma patients had worse corneal nerve fiber parameters compared to those on unpreserved mono-therapy treatment regimens (Agnifili et al., 2022). Reduced corneal nerve fiber length was also observed in patients using preserved glaucoma mono-therapy compared to a healthy control group (Agnifili et al., 2022). Fogagnolo *et al.* (2015) investigated the effect of benzalkonium chloride exposure in a clinical trial and found that latanoprost (preserved prostaglandin analog), but not tafluprost (unpreserved prostaglandin analog), induced corneal SBNP changes, including altered nerve branching and nerve beading at 12-months of follow-up. Thus, it is becoming clear that preservative-containing topical treatments used to manage glaucoma can disrupt corneal nerve homeostasis. Furthermore, long-term use of such therapies increases the density of putative dendritic cells in the corneal epithelium (Marsovszky et al., 2014; Zhivov et al., 2010). However, whether the increase in corneal immune cells is associated with the neurotoxicity, or whether it is a direct response to the preservative agent(s), is unclear.

3.3. Changes to corneal sensory innervation in systemic conditions

As a densely innervated tissue, the cornea is vulnerable to damage from primary systemic diseases and chemotherapy-induced disorders that involve peripheral neurodegeneration (Campagnolo et al., 2013; Misra et al., 2017). Several systemic conditions have been

associated with subtle changes to the corneal epithelium and sensory nerves, which makes this tissue an attractive target for the identification of imaging biomarkers that may signal early stages of diseases. Chronic inflammatory autoimmune conditions have also been associated with diffuse corneal inflammation, and altered corneal nerve structure (Villani et al., 2008; Villani et al., 2010). IVCM allows non-invasive visualization of corneal nerves and immune cells, enabling early detection, as well as assessment of the progression, of peripheral neuropathy and response to treatments (Ferrari et al., 2013; Wang et al., 2015).

3.3.1. Diabetes mellitus

Diabetes mellitus is known to affect multiple ocular tissues, including the cornea; progressive damage to corneal epithelial cells and sensory nerves defines a condition known as diabetic keratopathy (Bikbova et al., 2018). A number of clinical studies have shown that both type 1 and type 2 diabetes can negatively impact corneal innervation, with anatomical changes that include both decreased nerve fiber length and nerve fiber density (Jiang et al., 2016), and increased corneal nerve tortuosity (Ishibashi et al., 2012). Functional impairment has also been reported, including reduced corneal sensitivity to mechanical stimuli (Lv et al., 2014). Notably, decreases in corneal sensitivity are not congruent with the early morphological changes seen in the corneal sub-basal nerves (Messmer et al., 2010; Rosenberg et al., 2000), indicating IVCM may be able to detect corneal neuropathy at an early stage, before the onset of corneal sensory dysfunction.

The precise role of corneal immune cells in the early stages of diabetes-associated corneal neuropathy is unclear, with contrasting findings from mouse and human studies. Some animal and human studies suggest that diabetic corneal neuropathy (Leppin et al., 2014; Tavakoli et al., 2011) is associated with a higher density of presumed dendritic cells in the corneal epithelium. Lagali *et al.* (2018) reported that the proportion of so-called ‘mature’ dendritic cells was increased in individuals with impaired glucose tolerance and peaked within the first 10 years of a type 2 diabetes diagnosis. Others report that experimental diabetes in mice reduces corneal dendritic cell density, and that this underpins delayed corneal epithelial wound healing responses after injury (Gao et al., 2016b). It is possible that the contributions of corneal immune cells to diabetic neuropathy relates to the type, stage and/or duration of disease. Alternatively, the recruitment and/or activation of corneal intraepithelial immune

cells may be a consequence of systemic inflammation due to chronic metabolic disturbance. In this respect, evidence exists from mouse high fat diet models, considered a model of pre-type 2 diabetes, whereby higher corneal immune cell density is observed in the early phases of the dietary intervention (Jiao et al., 2020b), with corneal immune cell changes apparent weeks prior to the onset of nerve pathology (Hargrave et al., 2020).

3.3.2. Central nervous system diseases

Corneal sensory nerve alterations have also been described in central nervous system (CNS) degenerative diseases. In a clinical study of individuals with moderate to severe Parkinson's disease, the density of the corneal SBNP was significantly lower in this population compared to healthy controls, despite no measurable difference in corneal sensitivity (Misra et al., 2017). Interestingly, a positive correlation was also observed between corneal SBNP density and Addenbrooke's Cognitive Examination-Revised scores, where higher scores are indicative of better cognitive function (Misra et al., 2017). Similar results of impaired corneal nerves and an association with cognitive function have been reported in individuals with mild cognitive impairment or dementia (Ponirakis et al., 2019).

Similar to these observations in humans, a number of animal studies have recently reported corneal nerve degeneration in mouse models of dementia. In transgenic mice overexpressing human non-mutated *tau*, corneal nerve degeneration occurred prior to cognitive deficits and peripheral neuropathy (Marquez et al., 2021). A recent study from our laboratory identified corneal nerve changes in a mouse model with CNS tauopathy, which was preceded by morphological alterations to the resident corneal epithelial dendritic cells at earlier stages of the disease (Jiao et al., 2020a). In a similar study involving P301L mice, which overexpress human *tau* protein, corneal nerve loss was greater in eight month-old mice compared to controls, and impaired corneal DC responses after corneal injury were observed in three month old P301L mice compared to controls (Li et al., 2021b). These alterations to corneal neuroimmune parameters in transgenic mice during the early stages of CNS degenerative disease are supported by human data, with altered corneal immune cell morphology described in patients with mild cognitive impairment, in the absence of significant corneal nerve abnormalities (Dehghani et al., 2020). Thus. Given the close relationship between corneal immune cells and sensory nerves, future studies evaluating corneal nerve changes as a

possible early biomarker of CNS degenerative disease should also consider including corneal immune cell analyses as an additional biomarker of interest.

Severe acute respiratory syndrome coronavirus 2 is a respiratory virus that causes coronavirus disease (COVID-19). Despite initially being considered a pulmonary disease, evidence of neurological manifestations affecting both the central and peripheral nervous systems have been reported (Severo Bem Junior et al., 2020; Wan et al., 2021a). Corneal IVCM imaging has revealed that four weeks after acute COVID-19 infection, patients with ongoing neurological symptoms have a lower corneal nerve fiber density and nerve fiber length, and a higher density of corneal DCs, compared to healthy controls (Bitirgen et al., 2021). A negative correlation between the severity of longCOVID and the extent of corneal nerve fiber loss has also been described (Bitirgen et al., 2021). Barros *et al.* (2022) reported similar findings among individuals who had overcome COVID-19; these patients showed lower corneal nerve fiber density and nerve fiber length compared to age-matched controls, accompanied by a higher density of immune cells in the central cornea. Overall, the percentage of patients with DCs in the central cornea was higher in COVID-19 patients (diagnosed > 6 months prior) with lower OSDI scores (<5 out of 100) (Barros et al., 2022). In what is a rapidly evolving clinical field, it is possible that IVCM imaging of corneal neuroimmune parameters will be useful as a surrogate marker of peripheral neuropathy, and also as a non-invasive means of assessing immune cell activation in peripheral tissues following COVID-19 disease, particularly in patients experiencing persistence of symptoms known as ‘long COVID’.

3.3.3. Autoimmune diseases

Various autoimmune diseases have been demonstrated to have a negative effect on corneal nerve features. In patients with rheumatoid arthritis, IVCM imaging showed more bead-like formations along corneal nerve axons, as well as more hyperreflective (presumably activated) keratocytes in the stroma (Villani et al., 2008). Likewise, decreased corneal nerve fiber density and increased tortuosity were observed in individuals with Grave’s orbitopathy, while those with active disease showed more hyperreflective keratocytes compared to those that had an inactive phase of disease (Villani et al., 2010). It is unclear if the corneal nerve impairments occur as a primary component of those autoimmune disorders, or if they are

secondary to ocular surface pathophysiology, including local immune responses (Shaheen et al., 2014).

3.3.4. Other systemic diseases

A range of diseases that historically have been considered to not lead to corneal sequelae have been shown to manifest sub-clinical changes using IVCM imaging. For example, lower corneal nerve density and DED symptoms have been described in individuals with migraine (Kinard et al., 2015). People with migraine also experience more severe DED symptoms and ocular pain compared to those without migraine, suggesting DED symptoms in individuals with migraine may be driven by corneal nerve dysfunction (Farhangi et al., 2020). Damaged corneal nerve structure has also been described in individuals with fibromyalgia, including lower SBNP density and reduced stromal nerve thickness (Erkan Turan et al., 2018; Ramirez et al., 2015). In patients with human immunodeficiency virus (HIV) infection, corneal nerve fiber density was lower compared to controls (Kemp et al., 2017). Among these patients with HIV, lower corneal nerve density and a higher nerve tortuosity coefficient were identified in those with sensory neuropathy relative to those without (Kemp et al., 2017). However, these results need to be interpreted carefully as the patients with HIV-associated sensory neuropathy were older than those without sensory neuropathy, and age is an important factor in corneal nerve parameters (De Silva et al., 2019; Roszkowska et al., 2021).

4. Corneal immune cells

Historically, the cornea has been regarded as an immune privileged tissue due, in part, to the absence of bone marrow-derived cells, except for intraepithelial DCs at the limbus (Gillette et al., 1982; Streilein et al., 1979). Subsequent studies have revealed that corneal immune privilege involves several mechanisms, including anatomical barriers, immunoregulatory processes and an immunosuppressive microenvironment (Hori et al., 2019). Further, advances in imaging techniques and the development of transgenic mice have contributed to an understanding that there are, in fact, distinct populations of resident immune cells distributed throughout the corneal epithelium and stroma (Figure 6).

4.1. Dendritic cells (DCs)

Some studies have historically classified corneal immune cells with long dendrites as 'Langerhans cells' (Zhivov et al., 2005), which were generally assumed akin to epidermal DC populations that express Langerin (Valladeau et al., 2000). However, later data indicate that some dermal DCs also express Langerin, distinguished from the epidermal Langerhans cells by the expression of CD103 (Bursch et al., 2007). Similar to the skin, Langerin is expressed in human corneal DCs (Mayer et al., 2007), but it remains unclear whether they are related to the Langerhans cells or dermal Langerin$^+$ DCs. There is evidence that the murine corneal epithelium is endowed with a resident population of Langerhans cells, while there is also a minor population of Langerin$^+$ DCs in the stroma that are not Langerhans cells (Hattori et al., 2011). In this review, when commenting on mouse studies, we will refer to corneal intraepithelial, dendriform CD45$^+$ CD11c$^+$ cells as DCs.

Dendritic cells belong to a group of antigen-presenting cells (APCs) that typically express Class II major histocompatibility complex (MHC II) antigens. MHC II is required for the presentation of small, endocytosed peptides on the surface of APCs, in the context of co-stimulatory molecules CD80 and CD86. Presentation of antigenic peptides by APCs to naïve T-lymphocytes largely underpins the mechanism of adaptive immunity. The previously accepted dogma was that APCs were exclusively present in the peripheral cornea, but absent from the central cornea. Hamrah *et al.* (2002) earlier described that CD11c$^+$ DCs were present in both the central and peripheral corneal epithelium of BALB/c mice, with a centripetally decreasing density and that the DCs in the central and paracentral corneal epithelium of BALB/c mice were MHC II$^-$, reflecting an immature status (Hamrah et al., 2003a; Hamrah et al., 2002).

In the presence of inflammation, resident corneal DCs can undergo maturation by upregulating MHC II (Hamrah et al., 2002) and co-stimulatory markers CD80 and CD86 (Senthil et al., 2021). In more recent years, several other studies report that the majority of resident CD11c$^+$ intraepithelial DCs in both BALB/c and C57BL/6J mice co-express MHC II (Gao et al., 2016b; Jiao et al., 2019; Knickelbein et al., 2009; Leppin et al., 2014), thus it is becoming increasingly accepted that, at least in mice, resident corneal epithelial DCs are MHC-II positive in the steady state. Using transgenic Cx3cr1$^{gfp/gfp}$ knockin mice (Fig 3D&F),

which are deficient in Cx3cr1 receptor signaling, it was demonstrated that the presence of resident MHC II^+ DCs to the corneal epithelium was completely dependent on expression of the chemokine receptor Cx3cr1 (Chinnery et al., 2007). The dependence of corneal DCs on Cx3cr1 signaling was also validated using Cx3cr1 knockout mice (Chinnery et al., 2007). Cx3cr1 has also been shown to regulate the presence and sampling behaviour of resident intraepithelial immune cells to other mucosal sites, including the gut (Niess et al., 2005) and olfactory epithelium (Vukovic et al., 2010).

Similar to the reported presence of intraepithelial $CD11c^+$ MHC II^+ APCs in healthy mouse corneas (Knickelbein et al., 2014), an *ex vivo* analysis of human corneal tissue showed $CD45^+$ intraepithelial DCs that co-expressed $CD11c^+$ and HLA-DR^+, with a centripetally decreasing density (Yamagami et al., 2005). Similar expression patterns of human corneal $CD11c^+$ DCs were also reported by Mayer *et al.* (2007). Thus, due to a close overlapping profile of DC phenotype and distribution to humans, murine models serve as effective systems to further investigate the phenotypes of corneal epithelial DCs and their roles in the process of corneal disease and wound healing, including nerve regeneration.

While macrophages are the predominant immune cell subset in the anterior corneal stroma (Brissette-Storkus et al., 2002; Chinnery et al., 2008; Liu et al., 2017), a minor population of DC subsets, including $CD11c^+$ $CD11b^+$ DCs, also reside in this corneal layer (Hamrah et al., 2003c). Morphologically, stromal $CD11c^+$ DCs are indistinguishable from macrophages, however they do appear to be preferentially associated with sites of nerve branch points, where the nerves traverse the basement membrane of the epithelium (Gao et al., 2016a). Similarly, macrophages in the anterior stroma have been observed to extend cellular processes into the basal epithelium, where they appear to interact with the sub-basal nerve plexus (Supplementary Figure 1 and Supplementary Video 1; Chinnery et al., 2017a). A sparse population of plasmacytoid DCs have also been described in the anterior corneal stroma, and are thought to produce interferon-gamma and regulate T cell immunity (Ochando et al., 2006; Reizis et al., 2011; Sosnova et al., 2005). Plasmacytoid DCs contribute to immune responses against corneal HSV-1 infection by promoting survival of regulatory T cells, indicating a critical antiviral role for these cells in the cornea (Jamali et al., 2020a).

4.2. Macrophages

Macrophages also reside in the healthy cornea. Although it is well-accepted that tissue macrophages originate from the bone marrow, resident macrophages can also derive from the yolk sac (Gomez Perdiguero et al., 2015). Macrophages participate in innate immune responses by phagocytosing debris and secreting a variety of inflammatory cytokines to assist with tissue remodeling and inflammation (Gordon and Martinez-Pomares, 2017). Following nerve injury, macrophages are recruited to the injury site, contributing to the removal of axonal and myelin debris, which is the source of inhibitory regeneration signals (Chen et al., 2015). In the mouse corneal stroma, $CD45^+$ macrophages are distributed throughout the anterior and posterior stroma (Figure 6), expressing typical markers including CD11b, Iba1 and F4/80 (Chinnery et al., 2017a). Approximately 30% of corneal macrophages express MHC II molecules, which enable them to act as APCs in the cornea (Chinnery et al., 2008; Sosnova et al., 2005). In the human cornea, it has been shown that $CD45^+CD11b^+CD11c^-$ resident macrophages are localized in the posterior corneal stroma (Mayer et al., 2007).

Macrophages are usually classified into pro-inflammatory phenotype (M1) and anti-inflammatory phenotype (M2) according to their distinct cytokine expression patterns and functions (Brown et al., 2012; Gordon and Taylor, 2005). In brief, M1 macrophages are activated by lipopolysaccharide, tumor necrosis factor-α and interferon-γ, to participate in pro-inflammatory responses including anti-tumor immunity. M2 macrophages can be activated by specific cytokines, including IL-4, IL-10 and IL-13, to promote tissue repair and wound healing. Another marker, C-C chemokine receptor type 2 (CCR2), has been recently used to classify corneal macrophages in mice. Local (subconjunctival) depletion of either $CCR2^+$ or $CCR2^-$ macrophages delayed corneal wound healing after epithelial debridement, with corneal $CCR2^+$ macrophages sharing similar gene expression and function to M1 macrophages and $CCR2^-$ corneal macrophages resembling M2 macrophages (Liu et al., 2017). Based on their phenotype and gene expression, $CCR2^-$ corneal macrophages likely originate from the yolk sac, similar to $CCR2^-$ macrophages and yolk sac-derived microglia (Liu et al., 2017). Using single-cell profiling and cell fate mapping of ocular immune cells, Wieghofer *et. al.*, demonstrated that despite most of the corneal macrophages exhibiting a molecular signature similar to bone marrow-derived monocytes, including continuous replenishment from the circulation during adulthood, there is also a contribution of embryonic precursors to resident corneal macrophages (Wieghofer et al., 2021). Thus, it is

becoming clear that stromal macrophages in the mouse cornea are heterogenous with respect to their phenotype, origin and contribution to wound healing.

4.3. Contribution of infiltrating immune cells to corneal nerve regeneration

In addition to resident DCs and macrophages, infiltrating immune cells that are recruited following corneal injury have an important role in corneal inflammatory responses. Neutrophils are the dominant infiltrating cells in the early stage of inflammation, extravasating from the limbal vessels and migrating through the corneal stroma to the site of damage (Hanlon et al., 2014; Scapini et al., 2000). The migration of neutrophils relies on the local production of chemokines, such as the subset of CXC chemokines (Carlson et al., 2006; Lin et al., 2007). Animal studies have shown that neutrophils infiltrate the injured cornea in two phases; an initial phase within 12 hours and a second peak of neutrophil migration at 24 to 48 hours that involves a small heat shock protein released by activated resident macrophages (Li et al., 2006; Oh et al., 2012). The recruited corneal neutrophils contribute to corneal re-epithelialization and angiogenesis (Gong and Koh, 2010; Li et al., 2006). Further, neutrophil accumulation is beneficial to corneal nerve regeneration after epithelial injury. Systemic depletion of neutrophils in wild-type mice resulted in a significant reduction in corneal nerve density, which was associated with reduced expression of vascular endothelial growth factor (VEGF) (Li et al., 2011a). This result was supported by evidence of impaired corneal nerve regeneration after injury in wild-type mice treated with anti-VEGF antibody (Li et al., 2011a). In addition to VEGF, other factors or cytokines may also participate in corneal nerve regeneration during the acute, neutrophil-intense phase of an inflammatory response. In neutropenic wild-type mice that have reduced neutrophil recruitment and delayed wound healing after corneal injury, topical application of recombinant IL-20 promoted corneal wound healing and nerve regeneration (Zhang et al., 2017). It is clear that neutrophils play key roles in corneal wound healing, and that an optimal balance of inflammatory cell infiltrates is required to achieve ideal rates of re-epithelialization and accompanying sensory nerve recovery.

In addition to resident corneal DCs and macrophages, a small population of γδ T cells are resident to the limbal epithelium of the steady state cornea; these cells comprise 90% of the T cell population at the limbus in mice (Li et al., 2007). Corneal γδ T cells have a dual effect on

ocular immunity. In one respect, γδ T cells play a vital role in maintaining ocular immune tolerance. Studies have shown that either TCRδ$^{-/-}$ mice or mice treated with anti-γδ TCR antibody fail to develop Anterior Chamber-Associated Immune Deviation (ACAID) (Skelsey et al., 2001; Xu and Kapp, 2001), suggesting that this subset of T cells contributes to the induction of ACAID to soluble antigen. Moreover, mice treated with anti-γδ TCR antibody are at a higher risk of corneal allograft rejection due to the failure to develop ocular immune privilege (Skelsey et al., 2001). However, other studies have indicated that γδ T cells promote inflammation by inducing neutrophil infiltration (Li et al., 2007), which is critical for corneal wound healing. Another study has shown that IL-22 is a key factor mediating wound healing, and that topical treatment with IL-22 in TCRδ$^{-/-}$ mice promotes epithelial wound closure (Li et al., 2011b). The γδ T cell-dependent inflammatory cascade involving VEGF and IL-17 after corneal abrasion was found to be beneficial for promoting corneal nerve regeneration, providing evidence for corneal neuroimmune crosstalk following sterile injury (Li et al., 2011a).

5. Corneal neuroimmune interactions

Interactions between components of the nervous and immune systems have been investigated in many tissues, including the gut (Yoo and Mazmanian, 2017), lung and skin (Blake et al., 2019), and more recently in the cornea. The peripheral nervous system can respond rapidly to irritants and nociceptive stimuli, modulating local immune responses and, reciprocally, immune cells can affect neuronal function by releasing neurotransmitters and cytokines (Kawashima et al., 2015; Rosas-Ballina et al., 2011). Since the cornea is exposed directly to the external environment, its neural and immune systems are at the front line of responses to exogenous stimuli. Using advanced, post-acquisition image processing programs, high-resolution confocal microscopy images can be analyzed to quantify the degree of physical overlap between fluorophore channels (Figure 7). These approaches are based on the percentage overlap of user-generated rendered surfaces that localize to the structures of interest (Figure 7B-D). This quantitative approach is useful for measuring close neuroimmune interactions between corneal nerves and immune cells in 3D tissue preparations, however such findings should be interpreted with some caution, as unlike ultra-high resolution techniques such as serial block face scanning electron microsopy (Parlanti et al., 2020), confocal resolution limits do not allow the true visualization of cell contacts. Elucidating neuroimmune crosstalk in the cornea is important, and helpful, to better

understand the physiology and pathophysiology of corneal diseases, and potentially can be used to develop novel therapies aimed at promoting corneal nerve regeneration.

5.1. Interactions between corneal nerves and intraepithelial immune cells

Over the past decade there has been increasing evidence for reciprocal spatiotemporal and biochemical communication between corneal nerves and immune cells. In the healthy human cornea, most of the intraepithelial immune cells are localized to the level of the basal epithelial cells and SBNP (Zhivov et al., 2005). A recent study reported that the density of resident immune cells was positively correlated with corneal nerve branch density and total branch density in the normal human cornea (Colorado et al., 2019), suggesting the presence of corneal immune cells in healthy corneas may be benefical to nerve health. Many diseases that involve corneal neuropathy have described concurrent alterations to intraepithelial immune cells and corneal sensory nerves. Corneal immune cell density has also been proposed as a potential marker of neuroinflammation in small fiber neuropathy (Kamel et al., 2019). Further to this, the degree of interaction between corneal epithelial immune cells and nerve axons, as determined visually using IVCM images, has provided evidence of at least spatial interaction in human corneas. By quantifying the number of corneal immune cells with at least one or more cellular processes in close contact with nerve fibres (Figure 7B), the interaction between these two constituents of the corneal epithelium can be measured and compared across populations with various diseases. Additional stratification of neuroimmune contacts can be performed by assessing the number of immune cells ‘without dendrites’ and cells ‘with dendrities’ that appear to cross or make contact with nerve fibres (Figure 7J) (Stettner et al., 2016). In a study of chronic inflammatory demyelinating polyradiculoneuropathy (CIDP), the number of DCs in close proximity to corneal nerve fibres was reported to be higher in patients with painful inflammatory neuropathy compared to both healthy controls and patients with painless neuropathy (Figure 7J) (Stettner et al., 2016).

5.1.1. Evidence for neuroimmune crosstalk in corneal homeostasis

In steady-state conditions of the ocular surface, corneal nociceptors can detect changes in temperature, osmolarity and other elements of tear composition, to initiate the release of various neuromediators to maintain corneal homeostasis. Tear neuromediators including SP,

CGRP and VIP are present in healthy individuals (Yoon et al., 2022). SP has been reported to regulate reflex tear production and the expression of tight junction proteins in the corneal epithelium, suggesting a role in maintaining the barrier function of the ocular surface (Ko et al., 2009; Kovacs et al., 2005). Moreover, both human and mouse DCs express SP receptors, including NK1R and NK2R (Marriott and Bost, 2001), with evidence that NK1R signaling promotes the survival of DCs (Janelsins et al., 2009). Therefore, it is reasonable to speculate that SP contributes to maintaining resident DC populations in the densely innervated cornea, which regulates functional neuroimmune crosstalk.

Additional supportive evidence on the importance of neuroimmune crosstalk during ocular homeostasis stems from studies on ocular immune privilege. Ocular immune privilege is a phenomenon whereby the cornea and anterior chamber are able to limit local immune responses to preserve corneal integrity and function. One of the critical mechanisms is that the naïve T cell sensitization and effector T cell activation in the local lymphoid compartment is suppressed by regulatory T cells (Treg), leading to ocular surface immune quiescence (Amouzegar et al., 2016). In the aqueous humor, neuropeptides such as CGRP and VIP inhibit the activation of Th1 cells and macrophages to maintain a relative immunosuppressive microenvironment (Taylor, 2007). Corneal nerve integrity also plays an important role in ocular immune privilege. Corneal nerve injury can induce ocular surface $CD11c^+$ cells converting into contrasuppressor cells that inhibit Treg cells, leading to the loss of immune privilege (Neelam et al., 2018).

The expression of transient receptor potential (TRP) channels also serves as another form of functional connection between sensory nerves and immune cells. TRPV1, TRPV2 and TRPM8 are expressed on both corneal sensory nerves and tissue-resident immune cells express TRPV1 (Jiao et al., 2021; Khalil et al., 2016; Link et al., 2010). In the CNS, TRPV1 is localized to the mitochondria of microglia, and promotes microglial migration (Miyake et al., 2015) and regulates glutamatergic signaling in neurons (Marrone et al., 2017). In the normal cornea, stromal macrophage expression of TRPV1 is evident, and is localized to $CD68^+$ lysosomes. After sterile injury, the proportion of $TRPV1^+$ macrophages is lower, suggesting a potential role for TRPV1 by resident macrophages in maintaining tissue homeostasis (Jiao et al., 2021). It is possible that corneal stromal macrophages, through

expression of TRPV1, modulate excitatory neurotransmission between corneal nerves and stromal cells, including keratocytes that express the glutamate receptor NMDAR1 (Sloniecka et al., 2015).

5.1.2. Evidence for neuroimmune crosstalk in diabetes mellitus

Peripheral neuropathy is a common co-morbidity of patients living with diabetes. In individuals with type 2 diabetes, corneal nerve fiber density and branch density are lower, while the density of corneal DCs has been reported to be higher, relative to healthy control participants (D'Onofrio et al., 2021; Tavakoli et al., 2011). Lagali *et al.* (2018) reported a higher density of so-called 'mature' epithelial DCs (i.e., DCs with long dendritic processes) in individuals with type 2 diabetes, compared to healthy controls. Interestingly, the corneal DCs in patients with type 2 diabetes were more likely to be arranged in a cluster at the level of the SBNP, and were positively associated with tumor necrosis factor receptor super family member 9 protein levels in the plasma. In a pediatric population of individuals with type 1 diabetes, early corneal nerve loss and a higher DC density were reported, compared to age-matched healthy controls (Ferdousi et al., 2019). While several clinical studies have reported concomitant changes to sensory nerves and immune cell populations in corneas of individuals with local or systemic pathologies, direct evidence for functional neuroimmune crosstalk, and its role in corneal homeostasis and pathology, mostly derives from animal studies involving transgenic and knock-out mouse models.

The synergistic interactions between corneal nerves and immune cells during epithelial wound healing in diabetic corneas has been reviewed recently (Yu et al., 2022). Animal models of type 1 and type 2 diabetes are commonly used to examine mechanisms of corneal neuropathy; several studies have demonstrated corneal nerve loss in mice and rats with established diabetes (Coppey et al., 2020; Leppin et al., 2014), and dietary-induced metabolic dysfunction that precedes type 2 diabetes (Alamri et al., 2019; Jiao et al., 2020b). In mice with type 1 diabetes, induced pharmacologically using streptozotocin (STZ), an increase in corneal DC density was associated with a lower corneal nerve fiber density (Leppin et al., 2014). In this study, close physical associations between mature corneal DCs and the SBNP were described using immunostained corneal flatmounts and confocal microscopy, suggesting a potential interaction between DC activation and corneal nerve fiber damage. In a

high-fat, diet-induced metabolic disturbance mouse model, our laboratory also observed a lower SBNP density and a higher density of intraepithelial DCs in the central cornea (Jiao et al., 2020b). In contrast, the sum length of the apically located SNT was similar in animals who consumed either a high-fat or standard diet (Jiao et al., 2020b). Given that most of the DCs are located in the basal corneal epithelium (Zhivov et al., 2005), these results provide evidence for neuroimmune interactions between epithelial DCs and sub-basal nerves, in the context of metabolic disturbance. Further analyses in this study identified that the percentage of mature DCs (MHC-II^{+}) that were in close proximity to nerve axons in the SBNP was higher in the high-fat diet mice, relative to controls (Jiao et al., 2020b).

Although it is unclear whether resident corneal intraepithelial immune cells are neuroprotective or neurotoxic, or both depending on the condition, some clues have been provided in recent years. Gao *et al.* (2016b) reported that in STZ-induced diabetic mice with a corneal epithelial debridement wound, the number of resident and infiltrating DCs was lower and sensory nerve regeneration was delayed compared to animals without diabetes. This study also found that neutralization of ciliary neurotrophic factor (CNTF) delayed corneal sensory nerve regeneration after injury in the normal cornea. CNTF belongs to the neurotrophic factor family, and most of the CNTF in the cornea is produced by DCs (Gao et al., 2016b), suggesting that activated DCs may directly mediate nerve regeneration through production of neurotrophic factors, such as CNTF. When DCs were locally depleted, exogenous CNTF promoted reinnervation of the wounded cornea. In addition, blocking the CNTF-specific receptor induced corneal sensory nerve degeneration (Gao et al., 2016b). These findings provide evidence that DCs provide neurotrophic support to sensory nerves in the mouse corneal epithelium, and align closely with evidence of a neurotrophic role for intraepidermal Langerhans cells in cutaneous sensory nerve density and function (Doss and Smith, 2014).

5.1.3. Evidence for neuroimmune crosstalk in acute corneal inflammation and sterile injury

In addition to DCs and sensory nerves localizing to the corneal epithelium, DC processes appear to interact closely with sensory nerve axons and terminals (Gao et al., 2016a; Jamali et al., 2020b; Jiao et al., 2021; Jiao et al., 2020b; Leppin et al., 2014). Quantification of nerve-DC overlap has its challenges, as the complexity of both the cell processes and the abundance

of nerve axons can prove difficult when making binary decisions around cells that are, or are not, in contact with nerves. Using image analysis software to quantify the colocalization of rendered surfaces of fluorophore channels, we measured the degree of DC-nerve surface overlap in the normal mouse cornea, and in corneas following topical exposure to the TRPV1 nerve antagonist, capsaicin, or the inflammatory cytokine TNF-α (Figure 7E-H). First, there was no regional difference in the degree of interaction between corneal DCs and nerves in the central versus the peripheral cornea. However, within 10 minutes of exposure to topically applied TNF-α, a known activator of corneal DCs, a higher degree of neuroimmune contact was observed, compared to topical application of capsaicin or vehicle controls. These data demonstrate the interaction between corneal DCs is dynamic, rapid and sensitive to changes in the inflammatory milieu of the ocular surface. Whether such physical neuroimmune interactions can be regulated by neuropeptide release, or if these dynamic interactions occur in human corneas, is so far unexplored.

Another study from our laboratory considered the relationship between corneal nerves and DCs in the context of local nerve trauma caused by sterile epithelial abrasion in mice. We observed that the degree of physical 'contacts', as measured by surface overlaps of rendered images of DCs and transient receptor potential vanilloid receptor 1 (TRPV1)-expressing corneal nerves, was higher in the injured epithelium compared to intact, uninjured controls (Figure 7I) (Jiao et al., 2021). Regarding the potential function of nerve-associated DCs, Parlanti *et al.* (2020) revealed that intraepithelial $CD45^+$ immune cells (presumed DCs) phagocytose and degrade axonal debris in a mouse model of corneal trephine wounds; this finding was evidenced by the presence of electron dense material, identified as lysosomes using scanning electron microscopy. Clearance of axonal debris after injury has been identified as an important processes in axonal regeneration in the peripheral nervous system (Dubovy, 2011), thus it is possible that corneal epithelial DCs participate similarly after corneal nerve injury.

In addition to providing neurotrophic support to corneal nerves in systemic conditions that are known to cause peripheral neuropathy, DCs also contribute to corneal nerve recovery following local, sterile trauma. A recent study in our laboratory demonstrated that topical application of decorin, a small leucine-rich proteoglycan, was associated with the recruitment of corneal intraepithelial DCs at an early stage after sterile epithelial injury (i.e., six hours),

and enhanced sensory nerve regeneration after one week of treatment (Wu et al., 2020). We attribute this effect to decorin being able to modulate the expression of cytokines and/or neurotrophins by the DCs. This DC-dependent neuroregenerative effect of decorin was supported by findings in Cx3cr1$^{gfp/gfp}$ mice, which lack resident and infiltrating corneal intraepithelial DCs (Wu et al., 2020). In these mice, decorin did not impart a therapeutic benefit on corneal nerve regeneration following sterile injury (Figure 8).

5.1.4. Evidence for neuroimmune crosstalk in dry eye disease

Interactions between corneal sensory nerves and DCs are also evident in pathological conditions that affect the ocular surface, including DED. Decreased corneal SBNP features, including nerve fiber length, fiber density, fiber area and total branch density, have been observed using IVCM in patients with DED, along with an increased density of presumed DCs relative to healthy controls (Shetty et al., 2016). Notably, central corneal DC density in patients with DED was positively correlated with ocular discomfort and SBNP features including nerve fiber width, branch points and total fiber area, suggesting that increased inflammatory cells and neural changes are associated with ocular discomfort experienced by the patients with DED (Shetty et al., 2016). Tepelus *et al.* (2017a) also reported increased corneal DC density and decreased nerve fiber density in patients with Sjögren's or non-Sjögren's DED compared with age-matched control subjects. However, these authors observed a negative correlation between corneal DC and nerve densities; it should be noted that this correlation analysis included all study participants, including healthy controls. The different findings in these two studies may be explained by the subtypes of DED studied and/or the methods used for the IVCM image analyses. Patients with evaporative DED were recruited in Shetty *et. al.*'s study with corneal nerve features analyzed by automatic ACCMetrics software. In Tepelus *et al.*'s study, over half of the participants were diagnosed with dry eye due to Sjögren's syndrome, which is typically of the aqueous-deficient subtype, and a semi-automated tracing program (NeuronJ) was used to quantify the corneal nerves.

In a mouse study, a decrease in the corneal SBNP density and an increase in DC density was reported after exposure to environmental dry eye stress for three days (Simsek et al., 2018). Using intravital multiphoton microscopy, Jamali *et al.* (2020b) investigated the functional significance of physical connections between corneal nerves and DCs in a mouse model of

DED. In this study, corneal DCs were found to be less frequently in contact with central and peripheral corneal nerves during DED, compared with control corneas. Using time-lapse intravital microscopy, this study also showed the DCs that were in contact with nerves presented shorter track lengths, track speeds and displacement compared to those not in neuronal contact, suggesting that corneal nerve interactions may directly regulate the morphology and spatiotemporal dynamics of corneal DCs Jamali *et al.* (2020b). Moreover, corneal DCs may indirectly protect sensory nerves in DED by inhibiting ocular inflammation and regulating the expression of neurotrophic factors and neurotransmitters (Choi et al., 2017). Increased inflammatory cell infiltration and decreased expression of calcitonin gene-related peptide (CGRP), nerve growth factor (NGF) and SP were observed in DC-depleted mice with experimental dry eye, and these changes were accompanied by a loss of paracentral corneal nerves (Choi et al., 2017). Thus, corneal DC-nerve interactions are important for sensory nerve maintenance and corneal inflammatory regulation in DED.

5.1.5. Evidence for neuroimmune crosstalk in corneal infection

Pathological alterations to corneal immune cells and sensory nerves are well documented in infectious keratitis, in particular HSV-1 and herpes zoster virus (HZV) infection. In patients with unilateral herpes zoster ophthalmicus (HZO), a bilateral increase in corneal DC density and decrease in SBNP length have been reported (Cavalcanti et al., 2018). Corneal denervation in the contralateral (uninfected) eye provides evidence for ocular inflammation-associated nerve degeneration (Cavalcanti et al., 2018). Although the mechanism underlying sensory nerve degeneration with corneal HSV/HZV infection has not been fully elucidated, the theory that viral replication is the only cause of nerve damage is debatable (Yun et al., 2014). Due to its reliance on nerves, virus-induced neuronal death acts against the virus' survival. Although inflammation plays a pivotal role in the tissue response to pathogens, over-reactive and/or unresolved inflammatory responses can be harmful to local cells and tissues. It has been proposed that inflammation from the viral infection may lead to corneal nerve damage after HSV infection (Yun et al., 2014). In a mouse model of acute HSV infection, IL-6, produced during the early phase of the immune response, contributed to corneal denervation, and neutralization of IL-6 partially preserved corneal nerve structure (Chucair-Elliott et al., 2016). In a rabbit model of corneal HSV infection, treatment with topical pigment epithelial-derived factor (PEDF) plus docosahexaenoic acid (DHA) promoted corneal nerve regeneration and improved resolution of the inflammatory response (He et al.,

2017b). However, in a mouse model of HSV keratitis, despite the increased DC density and corneal nerve damage after infection, local depletion of corneal DCs resulted in more severe loss of corneal nerves (Hu et al., 2015). Gao *et al.* (2016a) reported similar results in sterile injury model, where local depletion of corneal DCs resulted in a loss of nerve density and delayed nerve regeneration. Likewise, our decorin-intervention study revealed that topical decorin imparted a therapeutic benefit on corneal nerve regeneration in wild-type mice following sterile injury but not in $Cx3cr1^{gfp/gfp}$ mice that lack resident and infiltrating corneal intraepithelial DCs (Wu et al., 2020). Taken together, although alleviating inflammation appears beneficial to corneal nerves, depletion of local corneal DCs may lead to a series of changes in the local immune network, resulting in delayed nerve regeneration. Elucidating the roles of DCs in corneal immune responses is necessary to more clearly define the crosstalk between DCs and corneal nerves.

Divergent findings in the literature regarding the potential contribution of corneal DCs to generating and resolving inflammatory responses may be explained by these immune cells having a dual role in the pathophysiology of corneal infection. First, consistent with their traditional function as APCs, corneal DCs initiate adaptive T-cell immune responses that in turn can protect corneal nerves by inhibiting virus replication (He et al., 2017a; St Leger and Hendricks, 2011). However, despite evidence that DCs have neuroprotective functions in the cornea, over-reactive DCs and inflammation may delay corneal neuroregeneration (Wan et al., 2022). Recent research from our laboratory has shown that CNS tauopathy is associated with the activation of corneal DCs, which precedes the gradual loss of neighboring corneal sensory nerves (Jiao et al., 2020). Another study also demonstrated that mice with unilateral trephine-injured corneas had contralateral corneal nerve degeneration and DC infiltration (Lee et al., 2019). Moreover, an increased number of mature DCs and effector lymphocytes were observed in the draining lymph nodes, suggesting that immune cells might migrate through the blood circulation to both corneas, causing further damage to the sensory nerves (Lee et al., 2019).

5.1.6. Effects of corneal sensory nerve activation on immune cells

Given the intimate physical interactions between immune cells and sensory nerves in the corneal epithelium, it is not surprising that immune cell homeostasis and inflammatory

responses can be modulated by sensory nerve activation. Ablation of TRPV1-expressing corneal sensory nerves increases corneal susceptibility to bacteria by modulating local innate immunity, including reducing neutrophil bactericidal activity (Lin et al., 2021) and reducing the capacity of corneal DCs to counter bacterial adhesion (Wan et al., 2021b). Sensory neurons release mediators, including SP and CGRP, which directly attract and activate immune cells (Ansel et al., 1993; Ding et al., 2008). Previous studies have reviewed the important role of neuropeptides (e.g., SP, CGRP and VIP) in neuroinflammation at the ocular surface (Bignami et al., 2016; Mantelli et al., 2010). There is evidence that following the stimulation of polymodal nociceptors, the corneal sensory nerve endings release CGRP (Belmonte et al., 2003), which can influence the development of local inflammation (Yin et al., 2019). Increased levels of SP and VIP in the tears have also been described in association with inflammatory responses in allergic conjunctivitis (Fujishima et al., 1997; Motterle et al., 2006). Patients who experience contact lens discomfort also have elevated tear film levels of the pro-inflammatory cytokine IL-17A, relative to those who have asymptomatic contact lens wear (Downie et al., 2018; Gad et al., 2019). A recent study reported that unilateral eye injuries in mice can lead to mucosal immune responses in the contralateral eye, which is associated with TRPV1 activation in the injured eye and the release of SP in the opposite eye (Guzman et al., 2018).

Neuropeptides can not only be released by sensory neurons, but also by immune cells including DCs, macrophages, neutrophils and T cells (Suvas, 2017). These neuropeptides, including SP and CGRP, can be detected in the tears of healthy humans, suggesting their role in ocular surface physiology (Yoon et al., 2022). After corneal nerve damage, the release of neuropeptides increases rapidly followed by the activation and infiltration of immune cells (Barbariga et al., 2018). The role of neuropeptides in corneal neurogenic inflammation is evidenced by immune cells largely expressing neuropeptide receptors (Morelli et al., 2020). For example, in a mouse model of corneal alkali burn, there is a rapid increase of SP and its receptor NK1R in the cornea, coinciding with an increased density of infiltrating leukocytes (Bignami et al., 2014). In addition, SP can selectively induce T helper cell activation to amplify inflammation, including immune cell infiltration and cytokine release (Cunin et al., 2011). These inflammatory influxes result in a positive feedback loop that perpetuates the inflammation and can lead to ocular surface diseases, such as DED and corneal neovascularization. Sensory nerve-derived SP binds to NK1R on vascular endothelial cells

and promotes vascular endothelial cell proliferation and tube formation (Liu et al., 2020a). The involvement of SP in inflammatory corneal neovascularization has been identified in both humans and animal models (Barbariga et al., 2018).

Based on the above consideration of the evidence, it is reasonable to hypothesize that the role of DCs in modulating corneal reinnervation may depend on the stage of the inflammatory response. At some stages, activated DCs may be important for promoting corneal nerve regeneration by releasing neurotrophic factors. In conditions of chronic neuropathy, corneal nerve damage may cause excessive DC activation, which could be regulated by either direct neuroimmune contact, or indirectly by neurotransmitters.

5.2. Interactions between corneal nerves and macrophages

In addition to the role of corneal DCs in mediating neuroimmune interactions in the peripheral nervous system (Feng et al., 2009), macrophages are also critically involved in nerve regeneration. Macrophages have both pro-inflammatory and anti-inflammatory phenotypes. There is evidence that macrophages contribute to Wallerian degeneration, a process whereby distal portions of injured peripheral nerves progressively degenerate, with the breakdown of axons and myelin (Chen et al., 2015; Namikawa et al., 2006). Following peripheral nerve injury, macrophages are recruited by cytokines and chemokines that are released by Schwann cells. Once at the site of nerve injury, macrophages participate in clearing myelin and axonal debris, enabling axonal regeneration to occur (Chen et al., 2007; Dubovy, 2011; Gaudet et al., 2011).

However, the mechanism in the corneal epithelium might differ, since most sensory nerves in the corneal epithelium are unmyelinated and there are no Schwann cells surrounding intraepithelial nerve axons. Instead, corneal epithelial cells are believed to function as Schwann cells, including modulating macrophage recruitment by secreting cytokines and regulatory factors, such as matrix metalloproteinase-9 (Pal-Ghosh et al., 2011; Stepp et al., 2017). In the normal murine cornea, an intimate physical connection has been described between corneal macrophages and peripheral stromal nerve trunks (Seyed-Razavi et al., 2014). These peripherally located, nerve-associated macrophages were found to rapidly respond to the damage of terminal axons in the central corneal epithelium, which was partly

mediated by Cx3cr1 signaling. Our recent study revealed that stromal macrophages express TRPV1 channels that are localized to $CD68^+$ lysosomes (Jiao et al., 2021). We observed a lower proportion of $TRPV1^+$ macrophages in the injured cornea relative to homeostatic conditions, suggesting that the expression of TRPV1 in resident macrophages is implicated in corneal homeostasis (Jiao et al., 2021). Functionally, the consequences of TRPV1 expresssion by corneal stromal macrophages, and whether they contribute directly to nerve homeostasis, is unclear. In other systems, TRPV1 expression by $CD68^+$ macrophages has been reported in the synovium of healthy individuals and osteoarthritis patients (Lv et al., 2021). In rats, activation of TRPV1 via intra-articular injections of TRPV1 agonists lowered the proportion of M1 (inflammatory) macrophages in the synovium, and improved osteoarthritis severity (Lv et al., 2021). These findings suggest that targeting TRPV1 expression may be a useful avenue for manipulating the profile of inflammatory macrophages in tissues, which may be beneficial for promoting corneal nerve regeneration.

He et al. (2017c) reported that in diabetic mice with wounded corneas, topical treatment with $PEDF^+$DHA promoted sensory nerve regeneration and increased the recruitment of M2 macrophages. As previously discussed, M1 macrophages are broadly considered pro-inflammatory, whereas M2 macrophages are immunosuppressive cells that contribute to the repair of tissues and axons after injury (Chen and Bonaldo, 2013; Italiani and Boraschi, 2014). In a diabetic mouse model of corneal injury, topical treatment with netrin-1 (an axon guidance factor) enhanced corneal nerve fiber regeneration, as well as M2 macrophage transition (Zhang et al., 2018b). Similar results were obtained in another study from the same laboratory, in which topical application of resolvin-D1 promoted corneal nerve regeneration and enhanced M2 macrophage activation (Zhang et al., 2018c). The crosstalk between corneal nerves and macrophages might be beneficial to resolving inflammation. *In vitro* studies have shown that a synergistic effect between macrophages and sensory neuron-induced neuropeptide CGRP release, which was also observed in mice with *P. aeruginosa* keratitis (Yuan et al., 2022). The released CGRP regulated the transformation of M1 macrophages to the M2 subtype, promoting the resolution of corneal inflammation (Yuan et al., 2022). Moreover, there is evidence that restoration of the distribution of corneal $CCR2^-$ macrophages (similar to M2 macrophages) promotes corneal nerve regeneration through increased secretion of neurotrophins, including brain-derived neurotrophic factor (BDNF), NGF, neurotrophin (NTF)-3 and NTF-5 (Liu et al., 2018). The $CCR2^-$ macrophage subset

preferentially express the α-7 nicotinic acetylcholine receptor (α7nAChR). The activation of α7nAChR further enhances the expression of anti-inflammatory genes in the $CCR2^-$ macrophage subset, which is necessary for corneal wound healing (Xue et al., 2018).

In our recent intervention study that investigated topical decorin for treating traumatic central corneal nerve injury in mice, the density of corneal stromal macrophages was lower, and nerve regeneration was higher, in decorin-treated corneas compared to controls (Wu et al., 2020). This finding is not contradictory to previous studies, since the macrophages recruited to the site of the injured nerves are expected to be a mix of phenotypes, and the total number of macrophages is known to decrease with the resolution of inflammation (Lee et al., 2018). For example, in the acute phase after partial sciatic nerve ligation, the M1 phenotype dominates the macrophage population by day three post-injury. However, at day 14, the percentage of M2 macrophages increases, with both subpopulations of macrophages equally represented (Lee et al., 2018). A recent study using Macrophage Fas-Induced Apoptosis (MAFIA) transgenic mice, in which macrophages can be depleted by intraperitoneal injection of the dimerizer drug AP20187, corneal nerve structure and function were preserved after corneal HSV infection (Chucair-Elliott et al., 2017). Therefore, inhibiting the pro-inflammatory activity of M1 macrophages might be another means for promoting corneal nerve regeneration during the early phase after injury.

5.3. Interactions between corneal nerves and other infiltrating immune cells

Aside from DCs and macrophages, other immune cells such as neutrophils and T cells, that are considered to typically not be resident to the cornea, are recruited to sites of nerve injury, contributing to the inflammatory process (Liou et al., 2011; Moalem and Tracey, 2006). In a corneal epithelial abrasion mouse model, the depletion of natural killer cells delayed corneal nerve regeneration and increased the infiltration of neutrophils (Liu et al., 2012). Exaggerated inflammatory responses may damage the corneal sensory nerves subsequent to cytokine secretion. Corneal infiltration of inflammatory cells, and a higher expression of IL-17 and IFN-γ mRNA, occur in association with corneal nerve degeneration in mice with experimental DED (Choi et al., 2017). Elevated tear IFN-γ is also a feature of evaporative DED in humans (Jackson et al., 2016).

Tian *et al.* (2018) reported that after topical application of PEDF in mice with corneal HSV infection, the attenuation of neutrophil infiltration and reduced expression of IL-6, IL-1β and TNF-α were all accompanied by reduced corneal nerve degeneration. These inflammatory cytokines can modulate ion channels that concentrate in the nerve terminals (McMahon and Koltzenburg, 1990), including reversing the operation of the sodium-calcium exchanger and increasing calcium influx, which can otherwise promote axonal degeneration (Persson et al., 2013). In addition, our laboratory's latest study demonstrated that the neuroregenerative effect of topical decorin on the injured mouse cornea might be associated with fewer infiltrating neutrophils in the early phase post-injury (i.e., within 24 hours). Moreover, the decorin-induced neutrophil inhibition was absent in $Cx3cr1^{gfp/gfp}$ mice that lack corneal epithelial DCs, indicating a potential interaction between DCs and neutrophils with topical decorin treatment (unpublished data). Another recent study suggests that following excimer laser annular keratectomy in thy1-YFP mice, YFP^+ bone marrow cells infiltrating the cornea are myeloid-derived suppressor cells (MDSCs), expressing cell surface markers $CD11b^+Gr1^+$ (Sarkar et al., 2013). An *in vitro* study also showed that MDSCs promoted the neurite growth of a co-cultured trigeminal ganglion by secreting NGF (Sarkar et al., 2013). Thus, the attenuation of corneal neutrophil and NGF-secreting myeloid cells are possible targets for the development of therapies aimed at improving nerve regeneration after injury (Sarkar et al., 2013).

$CD4^+$ T cells also play a critical role in corneal nerve pathology. There is evidence that $CD4^+$ T cells are required in HSV infection-induced neurogenic inflammation in the cornea. CD4-depleted mice showed faster resolution of corneal inflammation and recovery of the blink reflex at 20 to 70 day post-infection with HSV, which was not observed in wide type mice (Yun et al., 2014). The regeneration of the corneal nerve plexus and terminals was also observed in CD4-depleted mice, indicating $CD4^+$ T cells may negatively affect corneal re-innervation via neurogenic inflammation (Yun et al., 2014). $CD4^+$ T cells also repress sensory nerve growth in HSV-infected corneas via the production of VEGF (Yun et al., 2020). These findings confirm that neurogenic inflammation can inhibit nerve regeneration, or induce nerve degeneration, leading to a positive feedback and worsened corneal neuropathy.

5.4. Corneal neuroimmune interactions in ocular pain

Most corneal sensory nerves have polymodal nociceptors; their activation can lead to sensations of discomfort and pain (Patapoutian et al., 2009). Pain can be classified into three subtypes based on the anticipated underlying mechanisms: 1) nociceptive pain, stimulated by noxious stimuli; 2) inflammatory pain, due to hypersensitivity and low thresholds caused by inflammation; and 3) neurogenic pain, induced by nerve injury (Patapoutian et al., 2009). Resident and infiltrating immune cells can be activated in both tissue injury-related inflammation and neurogenic inflammation, and contribute to pain responses by releasing pro-inflammatory cytokines. Local inflammation is a key component of many ocular diseases, characterized by activated immune cells and increased release of pro-inflammatory cytokines and neurotrophins. Many studies have demonstrated an upregulation in inflammatory factors including IL-1, IL-3, IL-6, TNF-α and MMP-9 in DED (Bron et al., 2017). These mediators can sensitize corneal nociceptors and lower their threshold for activation, leading to the inflammation-related pain (Belmonte et al., 2017). Nociceptors may then respond to normally innocuous stimuli (allodynia) in the absence of a noxious stimulus, or show exaggerated responses to a noxious stimulus (hyperalgesia) (Patapoutian et al., 2009).

Furthermore, inflammatory cytokines such as TNF-α and IL-1β can directly stimulate nociceptors and increase the membrane excitability of corneal nerves via activation of p38 mitogen-associated protein kinase (MAPK) (Binshtok et al., 2008; Wei et al., 2021). The action potential firing further leads to an increased release of pro-inflammatory neuropeptides including SP and CGRP, which is associated with a downregulation of TRPV1 receptor expression (Yang et al., 2010). In addition, corneal sensory nerve damage can alter the expression of transduction channels, including TRPV1, TRPA1 and TRPM8 (Staaf et al., 2009), contributing to spontaneous neuropathic pain and a release of pro-inflammatory neuropeptides (Baron, 2006). The pro-inflammatory neuropeptides and cytokines released by activated nociceptors and immune cells result in a positive feedback loop, enhancing spontaneous and stimulus-evoked nerve impulse activity in polymodal nociceptors.

5.5. Role of aging in corneal neuroimmune interactions

Many studies have investigated the effects of aging on corneal innervation in rodents and humans. Reduced SBNP density and corneal sensation were observed in aged mice (22 months) relative to younger animals (2 months) (De Silva et al., 2019; Stepp et al., 2018). In rat corneas, the density of sensory nerves was similar between 10 and 56 week old animals, but significantly lower by 120 weeks (He et al., 2021). Similarly in humans, increasing age has been associated with a decline in corneal nerve fiber density and length, as well as lower concentrations of the neuropeptides SP and CGRP in tears (Tummanapalli et al., 2020).

The immune system is generally affected by aging (Sadighi Akha, 2018). Age-related inflammation, also known as inflammaging, plays a critical role in neurodegenerative diseases, including Alzheimer's disease and Parkinson's disease (Scheiblich et al., 2020). Aging can also affect immune cell phenotypes and their distribution at the ocular surface (Golebiowski et al., 2020; McClellan et al., 2014). In mice, a higher proportion of $CD11b^+$ $CD11c^+$ DCs was observed in aged mouse corneas compared to in younger eyes (Jung et al., 2021). Among the DC subtypes, CD11b- CD11c+ DCs induce cytotoxic $CD8^+$ T cell and Th1 responses, while $CD11b^+$ $CD11c^+$ DCs initial Th17 and Treg responses (Sun et al., 2020). $CD11b^+$ $CD11c^+$ DCs contribute to the development of DED (Lee et al., 2012; Maruoka et al., 2018), thus their increased presence in aging may contribute to the pathophysiology of age-related DED and sensory nerve degeneration.

IVCM is also beginning to shed light on the effects of aging on human corneal immune cell density and distribution. A higher ratio of central to mid-peripheral corneal intraepithelial DC density was found in healthy humans of older age (Golebiowski et al., 2020). This age-related change in corneal intraepithelial DC distribution might be associated with corneal nerve alterations in the whorl region, which is also affected by aging (Badian et al., 2021). Healthy volunteers aged over 50 years showed counterclockwise or non-rotatory corneal nerve whorl patterns, accompanied by more dot-like features in IVCM images that are presumed to be bulbous thickenings of the nerve fibers or possibly immune cells (Badian et al., 2021). The relationship between inflammaging and corneal denervation in the cornea, and whether corneal neuroimmune interactions are impacted by aging, requires further investigation.

5.6. Autonomic nerves in corneal neuroimmune interactions

Despite only forming a minor contribution to corneal innervation, autonomic nerves also participate in corneal neuroimmune interactions. After the loss of corneal sensory nerves, sympathetic nerves invade into the stroma, forming large sprouts. The sympathetic nerve fibers that hyperinnervate the cornea may promote HSV-associated inflammation by releasing catecholamines (Yun et al., 2016). Depletion of $CD4^+$ T cells results in a lower sympathetic innervation of the corneal stroma, indicating a central role for $CD4^+$ T cells in the interactions between sympathetic nerves and inflammatory cells (Yun et al., 2022). In a mouse model of corneal abrasion, activation of sympathetic nerves after injury inhibited corneal re-epithelialization and promoted neutrophil infiltration and cytokine release, which was associated with the polarization of corneal macrophages (Xue et al., 2018). The pro-inflammatory effect of sympathetic nerves has also been reported in a corneal alkali burn model, whereby the acute stimulation of corneal sensory nerves induced sympathetic activation and noradrenaline release, which led to further corneal inflammation and opacification (Lasagni Vitar et al., 2021).

The parasympathetic system also participates in corneal neuroimmune crosstalk, but likely as a mechanism to balance the pro-inflammatory effects induced by sympathetic nerves. After corneal injury, sympathetic nerve activation promotes inflammatory responses, while the parasympathetic signaling inhibits neutrophil influx and the release of pro-inflammatory cytokines to promote corneal epithelial regeneration (Xue et al., 2018). A possible mechanism is that sympathetic and parasympathetic neurons respectively stimulate $CCR2^+$ or $CCR2^-$ macrophages after corneal injury (Xue et al., 2018).

5.7. Summary of corneal neuroimmune interactions

The current evidence suggests a reciprocal interaction between corneal immune cells and sensory nerves (Figure 9). After corneal nerve injury, disrupted axons induce inflammatory responses directly or by triggering the release of numerous neuromediators, leading to the neurogenic inflammation. Both neurogenic inflammation and tissue inflammation can further induce corneal nerve damage. Corneal inflammation is characterized by the infiltration of neutrophils, transformation of macrophages into pro-inflammatory phenotypes ("M1" or $CCR2^+$), activation of $CD4^+$ T cells and the release of pro-inflammatory cytokines (e.g., IL-

1β, Il-6, TNF-α). However, there are also redress regulations existing in this picture of reciprocal neuroimmune interactions. In some conditions, activated epithelial DCs and other myeloid cells may have a neuroprotective effect on sensory nerves. These cells may directly phagocytose and degrade axonal debris, or produce neuroprotective factors such as CNTF and NGF. Moreover, anti-inflammatory macrophages ("M2" or $CCR2^-$) also promote corneal innervation by inhibiting further inflammation. Inhibiting the process of neuroinflammation and/or augmenting neuroprotective mechanisms provides potential therapeutic strategies for corneal neuropathy, but future studies are warranted.

It can be difficult to distinguish between neurogenic inflammation and tissue inflammation in some corneal conditions, such as non-viral infection and diabetic keratopathy. Some inflammatory components in neuroimmune interactions may have different roles in each context (Figure 9). For example, during corneal wound healing, activated DCs can be beneficial to nerve regeneration. A possible explanation is that in this scenario when the corneal epithelium is lost and the tissue suffers a reduction in its DC population, recovery of the epithelium and DC populations are foundational to the corneal nerve regeneration process. In contrast, in inflammation-dominated conditions, such as diabetic keratopathy and DED, DCs, as member of the innate immune system, likely produce pro-inflammatory mediators that may contribute to corneal denervation. This concept of DCs having pro-inflammatory and neurotoxic roles is consistent with a large body of evidence of elevated corneal DC numbers, concomitant with fewer corneal nerves, in a range of corneal inflammatory conditions (Cavalcanti et al., 2018; D'Onofrio et al., 2021; Shetty et al., 2016). In the more complicated context of infection, DCs may present dual effects depending on the course of the disease. DC-induced adaptive T-cell immune responses after infection are beneficial for inhibiting virus replication and clearing bacteria (Frank et al., 2012), whereas chronic inflammation can lead to further nerve degeneration. Similarly, nerve-derived SP can promote epithelial wound healing after injury, providing an anatomical foundation for nerve regeneration (Yanai et al., 2020). However, in inflammation-dominated corneal conditions, excessive SP may induce T help cell activation to amplify inflammation (Lasagni Vitar et al., 2022). Therefore, understanding the prodominat neuroimmune interactions in different corneal conditions and during steady-state is important for further translational studies.

Although our understanding of corneal neuroimmune interactions has increased in recent years (Tables 1&2), many questions remain. In particular, there is a need for further knowledge regarding how nerve-derived factors affect immune cell recruitment and function, and how macrophage polarization in the cornea shapes nerve regeneration after injury. Further investigations into the dynamic alterations of identified immune cell phenotypes in the cornea, as well as mechanistic studies of specific therapies, are necessary to gain further insights into the functional significance of corneal neuroimmune interactions.

Table 1. Key animal studies on corneal nerve and immune cell changes and their interactions (in the order of the publication year).

First author (year)	Animal model	Intervention	Changes to corneal nerves after intervention	Changes to immune cells after intervention	Relationship between corneal nerves and immune cells
Namavari (2012) (Namavari et al., 2012)	Mouse model of corneal nerve-transecting lamellar flap surgery	Topical Semaphorin 7a	Increased corneal nerve length	Increased immune cell influx (CD45+)	NR
Liu (2012) (Liu et al., 2012)	Mouse model of corneal epithelial abrasion	Depletion of NK cell	Delayed corneal nerve regeneration	Increased neutrophil influx	NR
Leppin (2014) (Leppin et al., 2014)	Mouse model of diabetes mellitus	NA	Decreased corneal nerve density	Increased corneal DC density	Colocalization of nerve fibres and DCs
Hu (2015) (Hu et al., 2015)	Mouse model of HSV keratitis	Local depletion of DC	Decreased corneal nerve density	Depletion of corneal DCs	NR
Gao (2016) (Gao et al., 2016b)	Mouse model of diabetes mellitus	NA	Decreased corneal nerve density	Decrease corneal DC density	NR
Gao (2016) (Gao et al., 2016b)	Mouse model of corneal injury and local depletion of DCs	Topical CNTF	Increased corneal nerve density	Depletion of corneal DCs	NR
Gao (2016) (Gao et al., 2016a)	Mouse model of corneal epithelial debridement	Local depletion of DC	Decreased corneal nerve density	Depletion of corneal DCs	DC dendrites cross nerve endings
Chucair-Elliott (2016) (Chucair-Elliott et al., 2016)	Mouse model of HSV keratitis	Topical dexamethasone	Retained corneal nerve density, increased corneal sensitivity	Decreased macrophage density Decreased CD8+ T cell density	NR
Choi (2017) (Choi et al., 2017)	Mouse model of dry eye disease	Local depletion of DC	Decreased corneal nerve density	Depletion of corneal DCs, increased infiltration of inflammatory cells (CD4+ CD19+ CD45+ CD11b+)	NR

First author (year)	Animal model	Intervention	Changes to corneal nerves after intervention	Changes to immune cells after intervention	Relationship between corneal nerves and immune cells
He (2017) (He et al., 2017c)	Mouse model of diabetes mellitus and corneal injury	Topical PEDF+DHA	Increased corneal nerve density	Increased M2 macrophage density, decreased neutrophil density	NR
He (2017) (He et al., 2017b)	Rabbit model of HSV keratitis	Topical PEDF+DHA	Increased corneal nerve density	Increased neutrophil and macrophage density at 7 days, decreased neutrophil and macrophage density at 14 days	NR
Chucair-Elliott (2017) (Chucair-Elliott et al., 2017)	MAFIA transgenic mouse model and HSV keratitis	AP20187 (to systemically deplete macrophages)	Increased corneal nerve density	Depletion of macrophages	NR
Zhang (2018) (Zhang et al., 2018c)	Mouse model of diabetes mellitus and corneal epithelial injury	Topical resolvin D1	Increased corneal nerve density	Increased M2 macrophage density	NR
Zhang (2018) (Zhang et al., 2018b)	Mouse model of diabetes mellitus and corneal epithelial injury	Netrin-1	Increased corneal nerve density	Decreased M1 macrophage density, increased M2 macrophage density, decreased neutrophil infiltration	NR
Simsek (2018) (Simsek et al., 2018)	Mouse model of dry eye	NA	Decreased corneal nerve density	Increased DC density	NR
Tian (2018) (Tian et al., 2018)	Mouse model of HSV keratitis	PEDF	Increased corneal nerve density	Decreased neutrophil density	NR

First author (year)	Animal model	Intervention	Changes to corneal nerves after intervention	Changes to immune cells after intervention	Relationship between corneal nerves and immune cells
Harris (2018) (Harris et al., 2018)	Mouse model of radiation keratopathy	NA	Decreased corneal nerve density	Increased density of CD45+ immune cells, increased density of MHC II+ cells	Negative correlation between nerve density and CD45+ cell density
Liu (2018) (Liu et al., 2018)	Mouse model of antibiotic-induced dysbiosis of gut microbiota and corneal epithelial abrasion	Fecal transplant/probiotic treatment	Increased corneal nerve density	Increased density of CCR2- macrophages	NR
Liu (2018) (Liu et al., 2018)	Mouse model of corneal epithelial abrasion	Local depletion of CCR2- macrophages	Decreased corneal nerve density	Depletion of CCR2- macrophages	NR
Lee (2019) (Lee et al., 2019)	Mouse model of unilateral corneal injury	NA	Decreased corneal nerve density in both eyes	Increased density of DCs and mature DCs (CD86+ or MHC II+) in both eyes	NR
Jiao (2020) (Jiao et al., 2020a)	Mouse model of CNS tauopathy (transgenic rTg4510)	NA	Decreased density of corneal nerve axons, increased percentage of beaded nerve axons	Lower density of DCs Higher proportion of CD86+ DCs	NR
Wu (2020) (Wu et al., 2020)	Mouse model of corneal epithelial abrasion	Topical decorin	Increased corneal nerve length	Increased DC density, decreased macrophage density	NR
Jiao (2020) (Jiao et al., 2020b)	Mouse model of high-fat diet (pre-diabetes)	NA	Decreased corneal nerve density	Increased DC density	Increased percentage of nerve-associated MHC-II+ cells

First author (year)	Animal model	Intervention	Changes to corneal nerves after intervention	Changes to immune cells after intervention	Relationship between corneal nerves and immune cells
Jiao (2021) (Jiao et al., 2021)	Mouse model of corneal epithelial abrasion	NA	Decreased TRPV1+ corneal nerve density	Increase percentage of TRPV1+ nerve associated DC	Physical contacts between DC and nerve axons
Wang 2021 (Wang et al., 2021)	Mouse model of Type 1 diabetes with corneal epithelial abrasion	Topical calcitriol	Increased corneal nerve density	Lower density of neutrophils, increased proportion of M2 macrophages	NR

Abbreviations: CCR2, C-C chemokine receptor type 2; CNTF, ciliary neurotrophic factor; CNS, central nervous system; DC, dendritic cell; DHA, docosahexaenoic acid; HSV, herpes simplex virus; MAFIA, Macrophage Fas-Induced Apoptosis; NA, not applicable; NK, natural killer; NR, not reported; PEDF, pigment epithelial-derived factor.

Table 2. Key human studies on corneal nerve and immune cell changes and their interactions (in the order of the publication year).

First author (year)	Disease/condition	Changes to corneal nerves compared to control group	Changes to corneal immune cells compared to control group, as described by the study authors	Relationship between corneal nerves and immune cells
Tavakoli (2011) (Tavakoli et al., 2011)	Diabetes mellitus	Decrease nerve fiber density, branch density and length; increased nerve tortuosity	Increased DC density	NR
Cruzat (2011) (Cruzat et al., 2011)	Infectious keratitis	Decreased nerve density, total number of nerves and branching	Increased DC density Increased size and dendrites of DC	Negative correlation between nerve density and DC density
Villani (2013) (Villani et al., 2013)	Sjögren's syndrome dry eye and MGD	Decreased corneal nerve fiber density, increased bead density and tortuosity	Increased DC density	NR
Cruzat (2015) (Cruzat et al., 2015)	Unilateral infectious keratitis	Decreased nerve density, total number of nerves and number of branches in both eyes	Increased DC density in both eyes	Negative correlation between nerve density and DC density
Stettner (2016) (Stettner et al., 2016)	Chronic inflammatory demyelinating polyneuropathy	Decreased nerve fiber density, nerve branch density and length	Increased density of DCs that contact nerve fibers, increased density of nondendritic cells	NR
Shetty (2016) (Shetty et al., 2016)	Dry eye disease	Decreased nerve fiber density, fiber length and nerve branch density	Increased DC density	Positive correlation between DC density and corneal nerve fiber density
Choi (2017) (Choi et al., 2017)	Dry eye disease	Decreased nerve density, increased nerve tortuosity and beading	Increased DC density, process and area	Positive correlation between DC process length and nerve density
Tepelus (2017) (Tepelus et al., 2017b)	Mucous Membrane Pemphigoid	Decreased nerve density, increased nerve tortuosity	Increased DC density	NR
Tepelus (2017) (Tepelus et al., 2017a)	Non-Sjögren's and Sjögren's syndrome dry eye	Decreased nerve fiber density, increased nerve tortuosity	Increased DC density	Negative correlation between nerve density and DC density

First author (year)	Disease/condition	Changes to corneal nerves compared to control group	Changes to corneal immune cells compared to control group, as described by the study authors	Relationship between corneal nerves and immune cells
Bitirgen 2017 (Bitirgen et al., 2017)	Multiple sclerosis	Decreased nerve fiber density, fiber length and nerve branch density	Increased DC density	NR
Cavalcanti 2018 (Cavalcanti et al., 2018)	Unilateral HZO	Decreased total nerve length, total nerve number and nerve branch number in both eyes	Increased DC density in both eyes, increased DC size and dendrite number in affected eyes	Negative correlation between total nerve length and DC density
Bitirgen 2018 (Bitirgen et al., 2018a)	Behcet's disease	Decreased nerve fiber density and length	Increased DC density	NR
Bitirgen 2018 (Bitirgen et al., 2018b)	Fabry disease	Decreased nerve fiber density and nerve fiber length, decreased corneal sensitivity	increased DC density	NR
Aggarwal 2018 (Aggarwal et al., 2018)	Fuchs' endothelial corneal dystrophy	Decreased total nerve length and number	Increased DC density	NR
Colorado 2019 (Colorado et al., 2019)	Healthy human	NA	NA	Positive correlation between corneal nerve branch density and resident DC density
Dehghani 2020 (Dehghani et al., 2020)	Mild cognitive impairment	Decreased fiber length, fiber density and branch density, but not significant	Similar DC density and higher DC field area and perimeter	NR
Klitsch 2020 (Klitsch et al., 2020)	Fibromyalgia syndrome	Decreased nerve fiber density and length	No difference in DC density, decreased density of DCs that contact nerve fibers	NR
D'Onofrio 2021 (D'Onofrio et al., 2021)	type 1 diabetes mellitus, type 2 diabetes mellitus, and latent autoimmune diabetes of adults	Decreased nerve fiber density and length	Increased DC density	Negative correlation between nerve density and DC density in type 1 diabetes mellitus

First author (year)	Disease/condition	Changes to corneal nerves compared to control group	Changes to corneal immune cells compared to control group, as described by the study authors	Relationship between corneal nerves and immune cells
Bitirgen 2021 (Bitirgen et al., 2021)	Long COVID	Decreased nerve fiber density and length	Increased DC density	NR

Abbreviations: CL, contact lens; DC, dendritic cell; HZO, Herpes Zoster Ophthalmicus; NA, not applicable; NR, not reported; MGD, meibomian gland dysfunction

6. Recent advances in promoting corneal nerve regeneration

Multiple ocular and systemic diseases can lead to corneal nerve degeneration. Severe cases of corneal nerve dysfunction can result in neurotrophic keratopathy, characterized by tear film abnormalities, corneal epithelial destruction and ulceration, and potentially corneal perforation. Despite the lack of epidemiological evidence regarding the prevalence of neurotrophic keratopathy, it is estimated that the incidence of neurotrophic keratopathy caused by herpetic keratitis and iatrogenic nerve damage is 1.22/10000 cases and 0.02/10000 cases respectively (Dua et al., 2018). Current clinical treatments for neurotrophic keratopathy primarily aim to promote corneal epithelial healing, but generally have limited capacity to encourage nerve regeneration or to restore corneal sensation (Pflugfelder et al., 2020). Although epithelial healing can provide anatomical structural support for corneal reinnervation and promote the secretion of neurotrophic mediators that enhance neurite survival (You et al., 2000), new treatments that are able to effectively promote corneal nerve regeneration and corneal sensitivity recovery are under investigation.

6.1. Neurotrophic/growth factors

Neurotrophic factors are a group of regulatory molecules that promote the survival of neurons and guide the growth of axons in the central and peripheral nervous systems. In skin, neurotrophic factors play important roles in cutaneous nerve regeneration as well as re-epithelialization and wound contraction (Ashrafi et al., 2016). Although several neurotrophic therapies have been shown to have positive experimental effects on corneal epithelial healing, very few demonstrate the ability to promote corneal reinnervation and restore corneal sensitivity in humans. Among them, NGF seems the most promising therapy for corneal reinnervation.

NGF, and its receptors Trk A (high affinity) and p75NTR (low affinity), are expressed in both healthy and injured corneas (Chung et al., 2013; Esquenazi et al., 2005; Qi et al., 2007; You et al., 2000). Lambiase *et al.* (1998) first reported outcomes from the application of topical NGF in patients with neurotrophic corneal ulcers. In this uncontrolled study, the authors described improved corneal healing in all 12 patient cases, and recovery of corneal sensitivity in most cases within 10 days to six weeks of treatment. Thereafter, a similar study with a larger sample population confirmed these results, however this study also lacked a

control group (Bonini et al., 2000). Topical recombinant human NGF (rhNGF) has been evaluated in two randomized, double-masked, vehicle-controlled trials in patients with neurotrophic keratitis (Bonini et al., 2018; Pflugfelder et al., 2020). The first study, a Phase 2 trial (NCT01756456), recruited 156 adult patients with moderate or severe neurotrophic keratitis, who were randomly treated with one of vehicle, 10 ug/ml, or 20 ug/ml rhNGF. Relative to vehicle, the rhNGF-treated groups showed a significantly higher proportion of patients who achieved corneal healing (defined as <0.5 mm lesion staining) after four or eight weeks of treatment. Another multi-center, randomized controlled trial (NCT02227147) confirmed the efficacy of rhNGF for treating neurotrophic keratitis. Both trials reported no significant safety concerns; most adverse events were ocular, mild, and judged to not be related to the study treatment. However, recent retrospective studies have reported more adverse events including difficulty sleeping and continued corneal thinning (Hatcher et al., 2021), as well as a rapid onset of corneal opacity identified as acute calcific band keratopathy (Qureshi et al., 2022). Neither of the two previous clinical trials showed a significant difference in corneal sensitivity (assessed using the Cochet-Bonnet esthesiometer) between rhNGF treatment and placebo, despite previous evidence that NGF can promote SBNP regeneration in a rabbit model after PRK and improve corneal sensitivity recovery in patients after LASIK (Esquenazi et al., 2005; Joo et al., 2004).

Given that immune cells can secrete growth factors such as NGF that have potential effects on corneal nerves, they may be an important component of the corneal neuroimmune interactions that underlie immune-mediated nerve repair. There is thus scope to target the function of immune system cells to repair injured nerves in the cornea. For example, in mice with excimer laser annular keratectomy, immune cells derived from bone marrow infiltrate the cornea (Sarkar et al., 2013); *in vitro* experiments demonstrate that these cells promote neurite growth by secreting NGF (Sarkar et al., 2013). Another recent study revealed that VEGF produced by $CD4^+$ cells suppressed corneal nerve regeneration in HSV-1 infected mice, and depletion of the $CD4^+$ cells promoted reinnervation of corneal sensory nerves (Yun et al., 2020). These findings provide evidence for targeting cells of the immune as a potential therapeutic approach for corneal nerve regeneration. A similar therapeutic strategy, involving the targeting of immune cells has been described in a preclinical model of optic nerve injury, using the transplantation of activated macrophages to promote optic nerve regeneration (Lazarov-Spiegler et al., 1996).

There is mounting evidence that a variety of other neurotrophic factors, and their receptors, exist in the cornea, mainly in epithelial cells and stromal keratocytes (Chung et al., 2013; You et al., 2000). The topical application of neurotrophic factors, including glial cell-derived neurotrophic factor (GDNF), PEDF and CNTF, can promote corneal nerve regeneration in diabetic mice (Di et al., 2017a; Gao et al., 2016b; Tian et al., 2018). A novel neurotrophic factor, mesencephalic astrocyte-derived neurotrophic factor (MANF), is highly expressed in the healthy mouse cornea, but not in diabetic mouse corneas during homeostasis and corneal injury. Application of recombinant human MANF was reported to accelerate corneal reinnervation and improve corneal sensitivity in normal and diabetic mouse corneas after injury (Wang et al., 2020). Other growth factors are under investigation, including VEGF, which has been shown to improve corneal nerve regeneration in normal and diabetic mice after corneal injury (Brash et al., 2019; Di et al., 2017b). Subcutaneous injection of insulin-like growth factor-1 (IGF-1) in diabetic rats has also been found to preserve corneal nerve innervation (Aghanoori et al., 2019).

Semaphorins are a class of membrane and secreted proteins that are identified as repulsive guidance molecules for axonal growth cones (Luo et al., 1993; Pasterkamp, 2012). In a murine dry eye model, topical application of selective Semaphorin 3A (Sema3A) inhibitor preserved corneal SBNP density and corneal nerve sensitivity, as well as the expression of TRPV1 (Yamazaki et al., 2017). The neuroregenerative effect of Sema3A inhibition was also demonstrated in a mouse corneal transplantation model (Omoto et al., 2012). However, in contrast, a study by Zhang *et al.* (2018a) reported that Sema3A promoted corneal nerve regeneration after epithelial debridement. These conflicting results might be explained by differences in the administration of the various agents. Specifically, Zhang *et al.* used a slow release delivery method, achieved by inserting pellets containing Sema3A into an intrastromal micropocket. The Sema3A inhibitor in the other two studies (Omoto et al., 2012; Yamazaki et al., 2017) was applied topically or by subconjunctival injection. Further studies are needed to elucidate whether or not Sema3A is neuroprotective in the context of corneal injury.

6.2. Anti-fibrotics

The corneal stromal microenvironment is considered to influence corneal nerve regeneration, due to the expression of neurotrophins and cytokines, as well as the development of fibrosis after injury (Chaudhary et al., 2012; Hamrah et al., 2003b). The scarring response has an inhibitory effect on nerve axon growth, which is associated with accumulation of scar-derived axon growth inhibitory ligands (Davies et al., 1999; Kawano et al., 2012). A recent study reported that activation of myofibroblasts in the corneal stroma inhibited nerve regeneration in a cat model of photorefractive keratectomy (PRK) (Jeon et al., 2018). The myofibroblasts showed diverse effects on axon growth, secreting transforming growth factor beta 1 (TGF-β1), which inhibits neurite outgrowth via collapsin response mediating protein 2 (CRMP2) signalling (Jeon et al., 2018). Topical application of the anti-fibrosis agent mitomycin C (MMC) after PRK has been shown to accelerate sensory nerve regeneration and decrease myofibroblast differentiation (Hindman et al., 2019; Jeon et al., 2018). However, Medeiros *et al.* (2018) reported corneal nerve toxicity from MMC application after PRK, consistent with the neurotoxic effects of this agent (Sui et al., 2014). MMC acts non-selectively against cell proliferation and differentiation, leading to a potential risk of various corneal complications, including delayed epithelial healing and endothelial decompensation (Arranz-Marquez et al., 2019). Anti-fibrotics with more specific targets and less adverse effects might be considered as potential therapeutic strategies for corneal nerve regeneration, however further research is required to expand our understanding of the interactions between corneal fibrosis and nerve regeneration.

6.3. Autologous serum and platelet-rich plasma eye drops

Autologous serum and platelet-rich plasma eye drops provide a cocktail of neurotrophins, neuropeptides and growth factors that may be beneficial for supporting corneal re-innervation. Several studies have shown that autologous serum tears (20%), dosed eight times daily for one to eight months, can improve corneal SBNP regeneration in patients with corneal neuropathy (Aggarwal et al., 2019; Aggarwal et al., 2015). Sjögren syndrome-related DED patients treated with autologous serum tears (five times daily for one year) showed reduced number of sub-basal nerve branches and a reduced degree of beading compared to those treated with artificial tears (Semeraro et al., 2016). However, no changes to corneal intraepithelial DC density were observed after treatment with autologous serum tears

(Aggarwal et al., 2015). In addition, application of human platelet lysate for 14 consecutive days enhanced corneal nerve regeneration in wounded rat corneas, and human platelet lysate promoted neuronal growth in an *in vitro* study (Huang et al., 2021). A randomized controlled trial evaluating the efficacy and safety of cord blood eye drops in patients with neurotrophic keratitis is ongoing (NCT03084861).

6.4. Omega-3 fatty acid supplementation

Long-chain omega-3 fatty acids, in particular eicosapentaenoic acid (EPA) and DHA, have anti-inflammatory and neuroprotective properties (Calder, 2017; Downie et al., 2018; Robson et al., 2010). There is pre-clinical evidence that topical application of resolvin-D1, a metabolite of DHA, can promote corneal nerve regeneration and restore corneal sensitivity in diabetic mice with corneal epithelial abrasion (Zhang et al., 2018c). Several studies have shown the DHA enhances the neuroprotective efficacy of other neurotrophic factors, including NGF and PEDF, for murine corneal reinnervation (Esquenazi et al., 2005; He et al., 2017b).

Menhaden oil, a natural source of omega-3 polyunsaturated fatty acids, can preserve corneal nerve fibers in chronic obese and type 2 diabetic rats (Coppey et al., 2020). A randomized controlled trial from our laboratory reported improvements in corneal SBNP parameters in DED patients, relative to placebo, after a moderate daily dose of oral omega-3 fatty acid supplementation for 90 days (Chinnery et al., 2017b; Deinema et al., 2017). Another single-arm, open label trial reported that 12-months of supplementation with a moderate-dose of oral omega-3 fatty acids led to increased corneal nerve fiber length in patients with type 1 diabetes (Lewis et al., 2017). Overall, participants tolerated the omega-3 fatty acid supplementation well, with no significant adverse events. Considering the totality of the available evidence, a recent systematic review and meta-analysis by our group concluded that there was low certainty evidence for a neuroprotective effect on peripheral nerves with systemic omega-3 fatty acid supplementation, and that further research was required to clarify the role of this intervention as a potential therapeutic for peripheral nerve disease (Zhang et al., 2020a). Participants' baseline omega-3 fatty acid dietary intake is an important potential modifier of the therapeutic efficacy of omega-3 fatty acid interventions, but has not been routinely measured in clinical trials of this intervention (Downie et al., 2019; Zhang and

Downie, 2019). Addressing these evidence gaps and lending further support to a role for oral omega-3 supplementation in treating corneal neuropathy, a recent randomized, double-masked, placebo-controlled trial completed by our team evaluated the effects of six months of oral omega-3 fatty acid supplementation on corneal nerve parameters, with adjustment for the baseline systemic Omega-3 index; this study found a significant corneal neuroregenerative effect with the omega-3 intervention in patients with type 1 diabetes (Britten-Jones et al., 2021). The observed neuroregenerative effect of oral omega-3 fatty acid supplementation might be, at least in part, associated with an anti-inflammatory effect. Consistently, treatment with topical DHA, neuroprotectin D1 and resolvins in preclinical studies have also demonstrated improvements in corneal nerve density in concert with an attenuation of local inflammation (Cortina et al., 2013; He et al., 2017b; Zhang et al., 2018c).

7. Future directions and perspectives

Neuroimmune crosstalk in the cornea has attracted much attention in recent years. Despite considerable progress towards understanding the intricate interplay between corneal immune cells and sensory nerves, a range of complex questions and challenges remain. For example, how does the presence and activation of corneal DCs and the physical interactions between immune cells and sensory nerves affect corneal nerve health? Although the majority of clinical studies report a negative association between the number of corneal epithelial immune cells and nerve density, usually in the context of disease, animal studies using corneal DC depletion techniques clearly show that the absence of intraepithelial DCs is detrimental to corneal nerve health. It is important to note too, that the vast majority of clinical studies using IVCM images to measure corneal immune cells tend to broadly classify all intraepithelial immune cells located alongside the sub-basal nerve plexus as ‘dendritic cells’. In some cases, there is progress towards the classification of corneal intraepithelial immune cells based on morphology, and even a shift in the terminology from Langerhans cells or dendritic cells to ‘immune cells’, which is appropriate for now. It is possible that, with time, the immunological identity of these morphological subtypes will be further refined, in which case a re-assessment of studies examining corneal neuroimmunology may be required to improve our understanding of the relationship between corneal nerves and specific cellular populations. Future research, taking advantage of the local depletion or functional inhibition of specific immune cell subsets, available with the use of animal models, is required to grow this field of research.

Another question that remains is whether the number of epithelial immune cells, and/or the ratio of morphological subtypes (namely ‘mature’ immune cells with dendrites, and so-called ‘immature’ cells without dendrites) in an individual’s cornea is associated with later nerve damage and/or recovery. This information could provide useful predictions about the expected degree of nerve damage, and the relative rate of neuroregeneration, in patients undergoing surgical procedures, such as cross-linking and refractive surgery, that are well known to cause long-standing corneal nerve damage. Perhaps more boldly, the neuroregenerative capacity of corneal epithelial DCs, at least in animal models for now, may represent a novel therapeutic strategy for cell-based delivery of biological factors, including CNTF, that may promote local nerve regeneration *in vivo*.

Another underexplored are of corneal neuroimmunology relates to the macrophage subpopulations in the corneal stroma, and whether they contribute differentially to intraepithelial sensory nerve degeneration and repair. Although not directly located in the epithelium, nerve-associated macrophages are positioned along the nerve trunks in the stroma, and appear to rapidly dissociate within hours of epithelial nerve injury (Seyed-Razavi et al., 2014). Later, macrophages likely contribute to overall resolution of inflammation after injury, which may indirectly influence nerve recovery. Thus it is possible that macrophages may represent another cell target for improving corneal neuroregeneration after injury.

Finally, after unilateral corneal injury, what is/are the dominant factor/s that underpin(s) contralateral ocular inflammation and corneal nerve degeneration? Given the growing number of studies reporting contralateral eye effects on corneal nerves and immune cells, this is an important question that could provide more clues into the functional significance of corneal neuroimmune interactions.

With the increased use of IVCM in research and clinical settings, as well as more clinical trials exploring new therapies for corneal neuropathy, it is highly likely that new insights into corneal neuroimmune interactions will be revealed. In addition, continued improvements in techniques for immune cell identification and characterisation *in vivo*, new imaging technologies for visualizing corneal sensory nerve terminals, and robust techniques for

evaluating corneal nerve function will likely be the foundation for gaining a deeper understanding of neuroimmune interactions and developing novel therapeutics for treating corneal neuropathy.

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Figure captions

Figure 1. Schematic illustration of corneal anatomy. SBNP, sub-basal nerve plexus; SNT, superficial nerve terminals. Note: diagram not to scale.

Figure 2. In vivo confocal microscope images of corneal nerves and immune cells in a healthy individual. (A) Normal appearance of the sub-basal nerve plexus that forms a whorl-like pattern in the paracentral cornea. (B) Sub-basal nerve plexus and immune cells in the healthy central cornea. Magnified images on the right panel show so-called 'immature' dendritic cells (DCs) without dendrites (red rectangle) and 'mature' DCs with dendrites (yellow rectangle, based on the classification by Lagali et al., 2018). Note that the 'mature' DC process appears to contact the nerve axon. Scale bar (100 µm) applies to images A and B. Adapted and modified from Chinnery et al., 2021.

Figure 3. Transgenic mouse models for visualization of corneal nerves and immune cells. (A) Thy1-YFP transgenic mice visualize corneal sensory nerves in vivo. Adapted and modified from Namavari et al., 2011. (B-C) CD11c-YFP × thy1-YFP mice label both $CD11c^{+}$ DCs and thy1+ neurons. Scale bars = 100 µm. (D) $Cx3cr1^{GFP/+}$ mice label $Cx3cr1^{+}$ cells that are resident to corneal stroma and epithelium. MHC-II^{+} $Cx3cr1^{+}$ cells localized in the epithelium are DCs. (E) $Cx3cr1^{GFP/GFP}$ mice label $Cx3cr1^{+}$ cells but there is a lack of epithelial DCs due to Cx3cr1-deficiency. (F-G) Wild-type and Cx3cr1-deficient mice show a complete loss of intraepithelial DCs (magenta) in Cx3cr1-deficient mice, but normal presence of stromal macrophages (cyan) (G). Panel B and C adapted and modified from Jamali et al., 2020b. Panel D from Chinnery et.al., 2017a.

Figure 4. Corneal intraepithelial nerves in a C57BL/6J mouse. (A) Sub-basal nerve plexus (SBNP) nerves forming a whorl-like pattern in the inferonasal, paracentral cornea. (B) Superficial nerve terminals (SNT) arising from the SBNP and extending towards the epithelial surface. (C) Merged image of the two layers with false-colored projections. Magenta: SBNP, cyan: SNT. Scale bars = 50 µm.

Figure 5. Schematic illustration of neural arc of inflammation in dry eye disease. Abbreviations: TRPV1, transient receptor potential vanilloid 1; TRPM8, transient receptor potential melastatin 8; SP, substance P.

Figure 6. Corneal immune cells in mice during homeostasis and inflammation. (A1-A4) Resident $CD45^+CD11c^+$ dendritic cells localized at the epithelial layer in an intact cornea, showing their typical dendriform shape. (B1-B4) Resident $CD45^+Iba1^+$ macrophages in the stroma in an intact cornea. (C1-C4) Infiltrating $NIMP^+$ $Cx3cr1^-$ neutrophils in the stroma in a sterile injured cornea, characterized with a round cell shape and multi-lobed nucleus. $Cx3cr1^+$ cells in C2 are macrophages. Scale bar (50 μm) in C4 applies to all images.

Figure 7. Corneal neuroimmune interactions based on physical proximity between epithelial DCs and nerves. (A-D) $CD45^+$ DCs and $PGP9.5^+$ sensory nerves in the epithelium of a normal mouse cornea. Surface rendering of CD45 and PGP9.5 channels in Imaris reveal the voxels that represent surface overlap (D). No difference in the degree of surface overlap ('neuroimmune interaction') in the central and peripheral cornea (E). After short (i.e. 10 minute) exposure to topical agonists in vivo, the degree of neuroimmune interaction was greater after exposure to TNF-a (F-H). The degree of surface overlap between $TRPV1^+BIII^+$ corneal nerves with corneal DCs was higher after sterile corneal injury (I). In humans, IVCM imaging enables visualization and quantitative analysis of 'non-dendritic' cells (NCF) and 'dendritic cells' (DCF) in contact with nerve fibres (J; NCF). The number of DCs in contact with nerve fibres is higher in patients with inflammatory neuropathy. Panel I adapted from Jiao et al., 2021; panel J adapted from Stettner et al., 2016.

Figure 8. Pre-clinical evidence for functional neuroimmune interactions in corneal sterile injury, in wild-type mice (with DCs, arrowheads; A) and $Cx3cr1^{gfp/gfp}$ (without DCs; B, C). (D-H) Improved corneal nerve (βIII+) regeneration following sterile injury was observed after topical decorin application in wild type mice, but not in $Cx3cr1^{gfp/gfp}$ mice. Scale bar in A&B = 100 μm, scale bar in D-G =50 μm.

Figure 9. Schematic illustration of proposed corneal neuroimmune interactions. Abbreviations: BDNF, brain-derived neurotrophic factor; CNTF, ciliary neurotrophic factor; NGF, nerve growth factor; SBNP, sub-basal nerve plexus; SNT, superficial nerve terminals.

Supplementary information:

Supp Figure 1: *En face* flatmount of PGP9.5^{+} sensory nerves (A) and CD45^{+} stromal macrophages (B) in the healthy central cornea of a mouse. A color-coded depth projection showing the presence of smaller cellular processes (arrows) from CD45^{+} stromal cells which are visible in the sub-basal nerve plexus.

Supplementary Video 1: 3D rendered reconstruction of stromal macrophages (purple) located in the anterior stroma of the central mouse cornea. Cellular processes from the stromal macrophages appear to interact with the sub-basal nerve axons which are located in the basal layer of the epithelium.

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