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# Neuroimmune crosstalk in the cornea

Wu, Mengliang; Hill, Lisa J.; Downie, Laura E.; Chinnery, Holly R.

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

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# 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	РК	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 1. Introduction

2 The cornea is one of the most densely innervated tissues in the human body, with a nerve fiber density of approximately 46 mm/mm<sup>2</sup> at the basal epithelium layer near the corneal 3 4 apex (Marfurt et al., 2010). Corneal nerves detect endogenous and exogenous signals to 5 maintain the homeostasis of the ocular surface by regulating tear production, modulating 6 blink reflexes, and releasing numerous neurotropic substances (Beuerman and 7 Schimmelpfennig, 1980; Nishida, 2005). The corneal sensory nerves are supplied by the 8 ophthalmic branch of the trigeminal nerve. In the epithelium, they stratify to form the so-9 called sub-basal nerve plexus (SBNP), also termed the intraepithelial corneal basal nerves 10 (Stepp et al., 2020), at the level of the basal epithelium. The superficial nerve terminals 11 (SNT) arising from the SBNP terminate as free nerve endings within the apical layers of the 12 epithelium (Figure 1).

14 Although the cornea lacks blood vessels and is regarded as an immune privileged tissue, there

15 are distinct populations of resident immune cells distributed throughout the corneal

16 epithelium and stroma, including dendritic cells, macrophages, mast cells and innate

17 lymphoid cells (Liu and Li, 2021). In addition to these resident immune cells, inflammatory

- 18 cells such as neutrophils and adaptive T cells can infiltrate into the cornea to participate in the
- 19 pathology of many corneal diseases (Bron et al., 2017; Yu et al., 2022).
- 20

21 Neuroimmune interactions, relating to the physical and functional interplay between nerves 22 and immune cells, have been investigated in many tissues, including the respiratory 23 epithelium (Veres et al., 2009), meninges (Schain et al., 2018), skin (Kashem et al., 2015) 24 and cornea (Hamrah et al., 2016; Jiao et al., 2020a). Many molecular components have been 25 co-identified in the immune and nervous systems. For example, receptors for 26 neurotransmitters that are found on nerves, such as acetylcholine receptors, are also expressed 27 on DCs, macrophages and T cells, suggesting a role for neural modulation of immune 28 responses (Kawashima et al., 2015; Wang et al., 2003). Recent studies have shown that 'anti-29 inflammatory' M2 macrophages, DCs and adaptive immune cells (T cells and B cells) play a 30 pivotal role in the mechanisms involved with peripheral nerve degeneration and regeneration 31 (Chen et al., 2015). With wider use of *in vivo* confocal microscopy (IVCM) in clinical and 32 research settings, and the development of transgenic animal models, considerable attention 33 has been given to understanding the interaction between corneal nerves and immune cells 34 (Bitirgen et al., 2018a; Choi et al., 2017; Gao et al., 2016a).

35

36 Neurogenic inflammation in the cornea provides evidence for corneal neuroimmune 37 crosstalk. Corneal nerve damage induces immune responses to promote the clearance of 38 disrupted axons, and release of neurotrophic factors that trigger inflammation (Lasagni Vitar 39 et al., 2022). The inflammatory response, including activation of immune cells and released 40 cytokines, can further induce nerve damage, to propogate the corneal neuroinflammation 41 (Launay et al., 2016). The reciprocal relationship between immune cells and sensory nerves 42 complicates the picture of neuroimmune interactions in the cornea. Many studies have 43 demonstrated a negative association between corneal innervation and activated immune cells 44 (D'Onofrio et al., 2021; Xu et al., 2021). However, there are also redress regulations in the 45 course of neurogenic inflammation and neuroinflammation, i.e., some immune cell

46 phenotypes, cytokines and neurotrophic factors may contribute to protecting corneal

47 innervation (Gao et al., 2016a; Liu et al., 2018). Understanding the mechanisms underlying

48 corneal neuroimmune crosstalk is important for developing new therapeutic strategies for

49 corneal healing and nerve regeneration.

50

51 This review summarizes and compares findings from studies that have investigated 52 neuroimmune crosstalk (i.e., the reciprocal interaction between immune cells and sensory 53 nerves) in the cornea, with the goal of reframing current dogma that corneal immune cells are 54 negatively associated with corneal nerve integrity. Clinical trial investigations of emerging 55 therapies, and novel treatment approaches currently in pre-clinical development, for corneal 56 nerve regeneration associated with immunomodulation, are also described.

57

58 2. Assessing corneal nerves and immune cells

# 59 2.1. In vivo confocal microscopy

60 In vivo confocal microscopy (IVCM), a non-invasive real-time imaging technique, has 61 advanced the evaluation of the corneal architecture in humans (De Silva et al., 2017; Niederer 62 and McGhee, 2010). IVCM provides high-resolution, en face images to enable qualitative 63 and quantitative analyses of corneal structures, including nerves, epithelial cells and immune 64 cells (Figure 2). Corneal SBNP characteristics have been clinically investigated in health and 65 disease using IVCM (Chinnery et al., 2021a; Cruzat et al., 2017; Malik et al., 2003; 66 Petropoulos et al., 2020). Moreover, IVCM has been useful to identify and assess corneal 67 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 68 69 diseases. Studies have reported corneal immune cell changes using IVCM in conditions such 70 as dry eye disease (Aggarwal et al., 2020), infectious keratitis (Kwon et al., 2018) and 71 pterygium (Wang et al., 2010), as well as systemic neurodegenerative diseases that are not 72 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 73 are also an increasing number of studies reporting negative correlations between corneal 74 75 SBNP density and intraepithelial immune cell density (Cavalcanti et al., 2018; Cruzat et al., 76 2011), suggesting that corneal immune cells may be involved with, or respond to, corneal 77 neuropathy.

79 Although IVCM is a valuable clinical tool for assessing corneal sensory nerves and immune 80 cells, it has some limitations. The visibility of corneal features within the image field can be 81 affected by various factors including image quality, imaging depth and post-capture image 82 enhancements, potentially leading to inconsistencies in the delineation of the sub-basal nerve 83 plexus and subsequent nerve parameter measurements (Patel and McGhee, 2013). In view of 84 these considerations, a tool was developed to assess the methodological quality of clinical 85 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 86 87 studies, to minimise potential biases when quantifying corneal parameters, such as nerve and 88 immune cell densities.

89

78

90 A practical consideration with IVCM is that it can only be used to visualize nerves 91 comprising the SBNP, but rarely the SNT; this latter nerve plexus plays an important role in 92 corneal sensory function, particularly as related to the sensitization of nociceptors. In 93 addition, although the dendriform cells observed using IVCM at the basal epithelium are 94 believed to be  $CD11c^+$  dendritic cells (DCs), based on *ex vivo* immunostaining data of human 95 corneal flatmounts (Yamagami et al., 2005; Zhivov et al., 2005), their specific surface 96 markers cannot be verified in vivo. Currently, phenotypic classification of in vivo corneal 97 epithelial immune cells relies mostly on morphological characteristics, with immune cells 98 without dendrites considered 'immature' DCs, and those with elongated cell processes 99 referred to as 'mature' DCs (Chinnery et al., 2021b). Evidence for the existence of 100 "immature" DCs without dendrites in the healthy corneal epithelium of rodents is lacking; 101 this could be explained by inter-species differences, or, more controversially, may indicate 102 that DCs without dendrites in human corneas represent a different cell population altogether 103 (Loi et al., 2022). For this reason, when referring to immune cells in the human corneal 104 epithelium more broadly, we will refer to them as "immune cells", except when describing 105 findings from clinical studies where the authors have used the term "dendritic cells" (or "DCs"). 106

107

## 108 2.2. Animal models

109 Animal models offer the opportunity to garner more detailed knowledge about the

110 morphology, distribution and phenotype of corneal nerve structures and immune cell

111 populations, owing to the capacity to perform *ex vivo* immunostaining and high-resolution

112 confocal microscopy. This is especially critical when evaluating the apically-projecting SNT

113 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.

116

Transgenic mouse models that allow for direct visualization of peripheral nerves have been 117 118 reviewed recently (Yamakawa et al., 2020). The thy1-YFP transgenic mouse, a 119 neurofluorescent murine model whereby the *thy1* gene promoter expresses yellow fluorescent 120 protein, can be used to readily visualize corneal sensory nerves in vivo, although only 121 approximately 46% of corneal nerves express the reporter protein (Yu and Rosenblatt, 2007) 122 (Figure 3A). The transgenic Gaba<sub>B1</sub>-GFP reporter mouse, expressing fluorescent G-protein 123 coupled receptors for the neurotransmitter  $\gamma$ -aminobutyric acid, has been validated for corneal nerve visualization by labeling about 90% of TuJ1<sup>+</sup> (class III beta-tubulin) corneal nerve 124 125 fibers (Hanack et al., 2015). The TRPM8-eYFP transgenic mouse that expresses fluorescent 126 Transient receptor potential melastatin 8 (TRPM8) also provides a means for observing 127 corneal cold thermoreceptor fibers (Parra et al., 2010).

128

129 In terms of transgenic models used to visualize other corneal features, such as immune cells,

130 corneal DCs can be readily identified in CD11c reporter mice using intravital microscopy

131 (Lee et al., 2010; Seyed-Razavi et al., 2019); when crossed with thy1-YFP mice, nerves, DCs

132 and their physical interactions can be examined using intravital multiphoton microscopy (Fig

133 3B) (Jamali et al., 2020b). Transgenic reporter Cx3cr1 GFP 'knock-in' mice can also be used

to visualize resident corneal epithelial Cx3cr1<sup>+</sup> DCs (Chinnery et al., 2008; Chinnery et al.,

135 2015; Chinnery et al., 2007). Heterozygous  $Cx3cr1^{GFP/+}$  mice have a targeted replacement of

136 the CX3CR1 gene by GFP, enabling visualization of Cx3cr1<sup>+</sup> cells, which are resident to the

137 mouse corneal stroma and epithelium (Fig 3C). Cx3cr1<sup>GFP/GFP</sup> (homozygous) mice also

138 express GFP, but are functionally Cx3cr1-deficient. Interrogation of the function of Cx3cr1

139 signaling in the mouse cornea revealed that Cx3cr1-homozygous mice, lack resident

140 epithelial DCs in the healthy cornea (Fig 3D-F) (Chinnery 2007). In these mice, stromal

141 macrophages appear phenotypically normal, but there is a striking loss of intraepithelial DCs

142 (Fig 3E and F). Other transgenic mouse models, such as CD11c and CD207-diptheria toxin

143 mice, in which corneal DCs can be pharmacologically depleted either systemically or via

subconjunctival injection, also provide evidence for the possible neuroprotective function of

145 DCs during corneal homeostasis and wound healing (Choi et al., 2017; Gao et al., 2016a; Gao

- 146 et al., 2011).
- 147

# 148 3. Corneal innervation

149 3.1. Corneal innervation anatomy and function

150 The outermost layer of the cornea consists of a stratified squamous, non-keratinized 151 epithelium, innervated by a rich sensory nerve supply (Figure 1). Corneal nerves originate 152 from the ophthalmic division of the trigeminal ganglion (cranial nerve V), travelling 153 suprachoroidally and ultimately branching to form the limbal plexus where they penetrate the 154 corneoscleral limbus (Al-Aqaba et al., 2010; Marfurt et al., 1989). The limbal plexus 155 branches and enters the cornea to form the stromal nerve trunks before turning anteriorly 156 towards the ocular surface (Muller et al., 2003). Nerve branches arising from the anterior 157 stromal plexus divide further and run parallel between the basal epithelium and Bowman's 158 layer, forming the so-called SBNP. The plexus of SBNP axons typically forms a distinctive 159 whorl-like pattern approximately 2.5 mm infero-nasal to the corneal apex (Marfurt et al., 160 2010) (Figure 4A). Several nerve branches of the SBNP turn upward, penetrating vertically 161 through the epithelium, and terminating just beneath the epithelial surface as the SNT (Muller 162 et al., 1996) (Figure 4B). Using block-face scanning electron microscopy, the SBNP has 163 recently been shown to have physical and functional interactions with the basal corneal 164 epithelial cells (Courson et al., 2019; Parlanti et al., 2020). The SNT create large nociceptive 165 fields, with an estimated density of about 605 nociceptive terminals per square millimeter in 166 the central cornea of humans that can rapidly respond to mechanical, thermal and chemical 167 stimuli (Marfurt et al., 2010).

168

169 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

171 µm), whereas the slow-conducting C fibers are unmyelinated and thinner in diameter (0.2 to

172 1.5 µm) (Al-Aqaba et al., 2019). Approximately 70% of corneal sensory fibers are of the 173 polymodal subtype, most of which are unmyelinated C type nerves. Polymodal nociceptors 174 convey sharp and sustained pain through slow-conducting C fiber activation in response to 175 mechanical, heat and chemical stimuli, with a sustained discharge of nerve impulses (Downie 176 et al., 2021). Polymodal nociceptors can also be activated by endogenous stimuli and 177 inflammatory mediators (Alamri et al., 2015; MacIver and Tanelian, 1993). In addition to the 178 sensation of pain, polymodal nociceptors contribute to reflex tear secretion caused by corneal 179 stimulation (Acosta et al., 2004). The transient receptor potential vanilloid receptor 1 180 (TRPV1) plays a significant role in nociceptive transduction in polymodal receptors. It has 181 been shown that over 90% of TRPV1<sup>+</sup> corneal afferent neurons are likely to be polymodal 182 nociceptors in guinea pigs (Alamri et al., 2015), and TRPV1 is also expressed in 183 intraepithelial nerve terminals in the mouse corneal epithelium (Jiao et al., 2021). Transient 184 receptor potential ankyrin 1 (TRPA1) and acid-sensing ion channels are also involved in pain 185 sensations transmitted by polymodal nociceptors (Bandell et al., 2004; Callejo et al., 2015). 186 The activities of TRPV1 and TRPA1 are modulated by inflammation, which is proposed to 187 underpin ocular discomfort in inflammatory conditions, such as allergic keratoconjunctivitis 188 (Acosta et al., 2013).

189

190 Another 20% of corneal sensory fibers have mechanoreceptors that are sensitive to 191 mechanical contact and generate one, or at most a few, nerve impulse(s) to convey acute 192 sensations (Belmonte et al., 2004); these mechanoreceptors are all thinly myelinated Aδ type 193 nerves. In contrast to polymodal nociceptors, mechano-nociceptors are responsible for 194 immediate and sharp sensations of pain induced by insults, including touching or scratching 195 the corneal surface (Belmonte et al., 2017). Though TRPV1 is absent from A $\delta$ 196 mechanoreceptors (Murata and Masuko, 2006), another mechanosensitive ion channel 197 (PIEZO2) may contribute to the sharp sensation of pain, which has been identified in corneal 198 afferent neurons in the trigeminal ganglion (Alamri et al., 2015). Future studies are required 199 to confirm its expression in the intraepithelial sensory nerves of the cornea.

200

The remaining 10% of corneal nerve fibers are  $A\delta$  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,

- 206 inflammation can modulate the activity of corneal cold thermoreceptor by inhibiting impulses
- 207 in response to cooling (Acosta et al., 2013). The transient receptor potential melastatin 8
- 208 (TRPM8) cation channel plays an important role in corneal cold sensation (Parra et al., 2010).
- 209 Through the response to cooling and elevated osmolarity, TRPM8-dependent impulses are
- 210 responsible for regulating basal tear secretion (Parra et al., 2010).
- 211

212 Corneal nerves are not only involved in regulating tear production and blinking reflexes to 213 maintain a lubricated and intact corneal epithelium, but also release numerous neurotropic 214 substances, such as substance P (SP) and calcitonin gene-related peptide (CGRP), to 215 modulate wound repair and assist in the maintenance of ocular surface health (Beuerman and 216 Schimmelpfennig, 1980; Nishida, 2005). In mice, an age-related decline in corneal sensory 217 nerve density has been reported (De Silva et al., 2019; Stepp et al., 2018), and reduced SP 218 levels have also been described in the cornea and tear film of both aged mice and humans 219 (Marco et al., 2018). SP released from corneal C fibers has an important role in maintaining 220 corneal nerve structure and sensitivity (Marco et al., 2018), as well as promoting corneal 221 wound healing in synergism with insulin-like growth factor (IGF) (Nagano et al., 2003; 222 Nishida, 2005). A clinical trial demonstrated that eye drops containing SP and IGF-derived 223 peptides induce a rapid re-epithelialisation of persistent epithelial defects in neurotrophic 224 keratopathy (Yamada et al., 2008). The SP receptor is not only expressed on corneal sensory 225 nerves but also on corneal immune cells, suggesting these neurotropic substances have dual 226 neuro- and immune-mediated actions in the cornea. Although SP plays an important role in 227 corneal wound healing by binding to its receptor in epithelial cells and attenuating the 228 cytokine-chemokine network (Yanai et al., 2020), it may adversely affect ocular 229 inflammation in some conditions (Suvas, 2017). In animal models of bacterial keratitis 230 (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 231 232 disease outcomes (Hazlett et al., 2007). Excessive release of SP may further promote 233 leukocyte infiltration and delay the apoptosis of neutrophils, leading to neurogenic 234 inflammation (Lasagni Vitar et al., 2022; Zhou et al., 2008). In addition, SP is also reported 235 to promote inflammatory corneal neovascularisation, with increased infiltration of CD45<sup>+</sup>

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

238

239 On the basis of an abundance of CGRP<sup>+</sup> nerves supplying the cornea, CGRP may also 240 participate in corneal wound healing (He and Bazan, 2016). Regeneration of CGRP<sup>+</sup> nerves 241 after corneal injury accelerated corneal epithelial healing (Cortina et al., 2012), and CGRP 242 has been shown to enhance epithelial wound healing in vitro (Mikulec and Tanelian, 1996). 243 Corneal sensory nerves release CGRP after electrical stimulation and heat or chemical 244 irritants but not cold stimuli, indicating the release of CGRP may requires a vigorous 245 stimulation of polymodal nerve endings (Belmonte et al., 2003). Similar to neuropeptide SP, 246 the release of CGRP is believed to be pro-inflammatory leading to neurogenic inflammation 247 (Belmonte et al., 2004; Lasagni Vitar et al., 2022). However, CGRP can also contribute to the 248 resolution of corneal inflammation, as evidenced by the release of CGRP by macrophages 249 and transformation of macrophages towards the anti-inflammatory phenotype in a mouse 250 model of P. aeruginosa keratitis (Yuan et al., 2022).

251

252 The role of corneal sensory nerves in both maintaining epithelial maintenance and promoting 253 wound healing is also closely linked with corneal epithelial stem cell function. In a mouse 254 model of corneal nerve depletion induced by ophthalmic nerve electrocoagulation, limbal 255 stem cell density was 75% lower than in control eyes, leading to impaired corneal epithelial 256 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 257 258 observation of limbal stem cell deficiency in eyes with neurotrophic keratopathy, where 259 patients are prone to developing persistent epithelial defects and non-healing ulcers (Delic et 260 al., 2022; Dua et al., 2018). A recent study confirmed the dependence of corneal stem cells on 261 corneal innervation, and demonstrated the critical role of sensory nerve TRPV4 in 262 maintaining stemness of limbal basal cells (Okada et al., 2019). Impaired epithelial healing 263 caused by sensory nerve damage can be rescued by TRPV4 gene introduction, which is 264 accompanied by the recovery of stem cell function (Okada et al., 2019). Other neurotrophic 265 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 266 267 maintaining corneal epithelial stem cells (Qi et al., 2007).

268

269 In addition to its well described sensory nerve supply, the cornea is also considered to receive 270 innervation from the autonomic nervous system; this topic has been covered recently in a 271 review by Vitar et al (2022). The mammalian cornea receives sympathetic nerve fibers that 272 predominantly originate from superior cervical ganglion (Marfurt and Ellis, 1993). This 273 sympathic innervation constitutes approximately 15% of the total corneal innervation in 274 rabbits and cats (Morgan et al., 1987; Tervo and Palkama, 1978), but its contribution is 275 considered relatively limited in humans (McDougal and Gamlin, 2015; Toivanen et al., 276 1987). Sympathetic nerve fibers were reported to be predominantly located in the limbal 277 stroma (Ehinger, 1966; Marfurt and Ellis, 1993), but a recent study has shown that around 278 10% of the central intraepithelial nerves in rats express sympathetic nerve markers (He et al., 279 2021). Parasympathetic fibers are also presented in the corneal stroma of rats and cats 280 (Marfurt et al., 1998; Morgan et al., 1987), but these findings have not been confirmed in 281 humans. Though the function of autonomic nerves in the cornea is not well understood, they 282 may contribute to ocular surface health, corneal wound healing responses and/or neurogenic 283 inflammation (Jones and Marfurt, 1996; Yun et al., 2016). The autonomic innervation in the 284 cornea also releases neuropeptides including neuropeptide Y (NPY), acetylcholine and 285 vasoactive intestinal polypeptide (VIP), which may participate in wound healing and 286 angiogenesis (Ekstrand et al., 2003). VIP has also been shown to regulate inflammatory 287 responses and nerve regeneration in the cornea (Zhang et al., 2020b). Animal studies have 288 shown that sympathetic nerve activation inhibits corneal re-epithelialization and promotes 289 neutrophil infiltration after injury, while parasympathetic nerve activation has the opposite 290 effect (Xue et al., 2018). Sympathetic nerves may replace corneal sensory innervation in 291 reactivated herpes stromal keratitis, which is dependent on CD4<sup>+</sup> T cell activation (Yun et al., 292 2022).

293

# 294 *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.

300

# 301 *3.2.1. Dry eye disease*

302 Dry eye disease (DED) is one of the most common disorders affecting the ocular surface. The 303 latest international Dry Eye WorkShop II (DEWS II) report from the Tear Film and Ocular 304 surface Society acknowledged that corneal neurosensory abnormalities have an etiological 305 role in DED (Craig et al., 2017). Using IVCM, some studies have reported reduced SBNP 306 density in patients with DED (Cox et al., 2021; Giannaccare et al., 2019; Labbe et al., 2013), 307 while others have reported that corneal nerve density remains unaffected (Hosal et al., 2005; 308 Zhang et al., 2005). This discrepancy in the literature might, at least in part, be explained by 309 the studies assessing patients with different stages, subtypes and/or severities of DED. In 310 addition, variations in IVCM imaging methods, including the device used, number of images 311 assessed, the method of image selection, and the location and depth of the corneal imaging, 312 may contribute to reported differences in corneal nerve parameters in different studies (De 313 Silva et al., 2017). For example, laser-scanning IVCM enables higher resolution images of 314 intraepithelial nerves than the slit-scanning device, which may result in differential sensitivity 315 to detecting corneal nerves in vivo using the two methods (Cruzat et al., 2017).

316

317 Tear hyperosmolarity and inflammation are considered key elements of the pathophysiology 318 of DED (Bron et al., 2017). Tear hyperosmolarity can trigger innate immune and adaptive 319 (CD4<sup>+</sup> T cell) responses in the cornea (Stern et al., 2013), which involves neuropeptide 320 release from damaged nerves (Figure 5). In desiccating stress-induced models of DED in 321 mice, inhibiting neurokinin-1 receptor (NK1R), the principle SP receptor, reduced DED 322 severity by suppressing T helper cell mediated responses (Taketani et al., 2020; Yu et al., 323 2020). In vitro, substance P can also modulate T cell activation, selectively inducing Th17 324 cell formation, or switching, from memory CD4<sup>+</sup> T cells to amplify inflammation (Cunin et 325 al., 2011). Dendritic cells (DCs) play an important role in the activation of CD4<sup>+</sup> T cells 326 (Schaumburg et al., 2011), and many studies report an increased density of DCs in patients 327 with DED (Xu et al., 2021). In mice, hyperosmolarity can induce morphological and 328 phenotypic maturation of corneal intraepithelial DCs within hours after topical exposure 329 (Senthil et al., 2021). Together, these studies provide support for mechanistic links between 330 nerve stimulation and recruitment and activation of corneal DCs, and also the 331 immunomodulatory roles of neuropeptides in the cornea during DED.

332

333 Neuroinflammation in DED not only occurs in the cornea but can also involve the trigeminal 334 ganglion and the trigeminal brainstem sensory complex. In a DED mouse model induced by 335 unilateral excision of the extraorbital lacrimal gland and Harderian gland, reduced corneal 336 intraepithelial nerves, decreased corneal mechanical hypersensitivity and increased 337 spontaneous electrical activity of the ciliary nerve were observed (Fakih et al., 2019). 338 Notably, increased pro-inflammatory markers were detected in the ipsilateral trigeminal 339 ganglion and the trigeminal brainstem sensory complex (Fakih et al., 2019). Persistent ocular 340 dryness and inflammation triggered corneal nociceptor activity that led to the development of 341 corneal hypersensitivity and peripheral sensitization (Fakih et al., 2019). Similar results were 342 observed in a topical benzalkonium chloride (BAK) model of ocular surface inflammation 343 (Launay et al., 2016). The spread of inflammation from the cornea to the peripheral nervous 344 system in experimental DED, and BAK-induced inflammation, lends further support to 345 neuroimmune crosstalk in the cornea.

346

# 347 *3.2.2. Neurotrophic keratopathy*

348 Neurotrophic keratopathy (also known as neurotrophic keratitis) is defined as "a disease 349 related to alterations in corneal nerves leading to impairment in sensory and trophic function 350 with consequent breakdown of the corneal epithelium, affecting health and integrity of the 351 tear film, epithelium and stroma" (Dua et al., 2018). Dua et al. recently reviewed 352 neurotrophic keratopathy, and suggested a clinically relevant classification with three stages 353 that relate to disease severity and prognosis: 1) Mild: epithelial changes only, without 354 epithelial defect; 2) Moderate: epithelial defect without stromal defect; and 3) Severe: stromal 355 involvement from corneal ulcer to lysis to perforation, with corneal hypo-356 aesthesia/anaesthesia (Dua et al., 2018). Many ocular and systemic diseases that involve 357 corneal nerve impairment can induce neurotrophic keratopathy (Dua et al., 2018). One of the 358 well-accepted theories is that impaired corneal nerves and reduced corneal sensation can 359 result in diminished blink reflexes, reduced tear secretion and a deficit in neurotrophic 360 support (Belmonte and Gallar, 2011; Wilson and Ambrosio, 2001). For example, corneal 361 nerve injury is associated with reduced levels of the neurochemicals SP and acetylcholine; 362 factors that have been shown to promote epithelial wound healing (Semeraro et al., 2014). 363 Animal and clinical studies have demonstrated therapeutic effects with SP and IGF in

- neurotrophic keratopathy (Nagano et al., 2003; Yamada et al., 2008). Therapeutic
- 365 interventions using neurotrophins such as nerve growth factor (NGF) have shown great
- 366 promise in treating neurotrophic keratopathy (Sacchetti and Lambiase, 2017), with recently
- 367 conducted randomized, double-masked, vehicle-controlled trials confirming the efficacy of
- 368 recombinant human NGF for treating neurotrophic keratopathy (Bonini et al., 2018;
- 369 Pflugfelder et al., 2020).
- 370

371 Chronic inflammation triggered by corneal nerve disruption is also involved in the 372 development of neurotrophic keratopathy. Disrupted tear secretion can induce tear 373 hyperosmolarity and increased release of pro-inflammatory factors and neuropeptides, 374 leading to neurogenic inflammation and progression of the disease (Baudouin et al., 2013; 375 Dua et al., 2018). Actived collagenolytic enzymes in corneal inflammation can initiate 376 stromal collagen melting, leading to the disease progressing to a moderate or severe stage 377 (Fini et al., 1992). Studies have shown alterations to immune cell activity and cytokine 378 release in diabetes-induced neurotrophic keratopathy (Lagali et al., 2018; Leppin et al., 379 2014).

380

# 381 *3.2.3. Iatrogenic causes*

382 Refractive surgery leads to corneal nerve degeneration as a result of transecting the sub-383 epithelial or anterior stromal nerves during the procedure. Several studies in animal models 384 have explored the morphological and functional alterations to corneal nerves after 385 experimental photorefractive keratectomy (PRK) (Bech et al., 2018; Medeiros et al., 2018). 386 The regeneration of the SBNP starts shortly after PRK and reaches half the baseline density 387 by day 15 in mice (Bech et al., 2018). In rabbits, nerve fibers sprout during the first month 388 following PRK, however even after six months neither the SBNP nor the stromal nerves are 389 fully recovered (Medeiros et al., 2018). In humans, 70%-80% of the SBNP remains absent at 390 one month after PRK surgery and its regeneration takes much longer, with corneal nerve 391 density only reaching half the pre-operative (baseline) levels after one year (Erie et al., 2005).

392

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).
- 397 Importantly, it is well established that post-LASIK patients are prone to developing dry eye
- 398 signs, including tear instability, decreased tear production and reduced corneal sensitivity (De
- 399 Paiva et al., 2006); these clinical features have been linked to the damaged epithelial sensory
- 400 nerves (Battat et al., 2001).
- 401

402 Laser epithelial keratomileusis (LASEK), which combines elements of LASIK and PRK, is 403 milder in terms of its impact on SBNP density and corneal sensitivity compared to the 404 LASIK at three and six months post-surgery; however, data at longer follow-up periods are 405 not available (Lee et al., 2006). Recent advances in surgical procedures, such as small 406 incision lenticule extraction (SMILE), where the refractive lenticule is extracted through a 407 small incision without the creation of a corneal flap (Sekundo et al., 2011), aim to achieve 408 more rapid post-operative corneal re-innervation (Denoyer et al., 2015). A recent study has 409 reported that eyes subjected to SMILE procedures had significantly higher corneal nerve fiber 410 density and nerve fiber length compared to those that had undergone LASIK at 4.1 years (Liu 411 et al., 2020b); the individuals recruited in this study were of a relatively young age (mean of 412 25 years at the time of surgery). The extent of corneal nerve regeneration after SMILE was 413 age-dependent, with younger patients having more rapid nerve than older individuals (Li et 414 al., 2021a).

415

416 Cataract surgery is another common iatrogenic cause of corneal nerve damage that occurs 417 due to full thickness sensory nerve transection in the operated eye. Reduced corneal nerve 418 fiber number and density, and increased nerve beading, have been described from analyses 419 using corneal IVCM during the first three months after cataract surgery (De Cilla et al., 2014; 420 Misra et al., 2015). A recent study demonstrated that corneal SBNP parameters, including 421 nerve fiber density, fiber length and nerve total branch density, were decreased in both eyes 422 in patients one month after unilateral cataract surgery, suggesting a contralateral eye effect on 423 corneal nerve degeneration (Giannaccare et al., 2020). However, after the unilateral cataract 424 surgery, the density of corneal DCs was described to not differ in either eye relative to 425 preoperative levels (Giannaccare et al., 2020).

426

427 Penetrating keratoplasty (PK) is a surgical procedure that involves full thickness resection of 428 the cornea, which includes severing the corneal nerve network. Corneal nerve fibers 429 regenerate much slower after penetrating keratoplasty compared to cataract or refractive 430 surgery, likely due to the widespread damage to axons during the procedure or the different 431 corneal nerve conditions before the surgeries. At 12 months after PK, no SBNP fibers were 432 detected by IVCM in a group of 20 patients (Darwish et al., 2007). In most patients, 433 regeneration of the corneal SBNP and stromal nerves is observed to take one to 14 years after 434 surgery, but no correlation was observed between the SBNP and stromal nerve trunk density 435 (Al-Aqaba et al., 2012b). This finding suggests that the regenerated SBNP likely penetrated 436 the donor graft directly from the host, and stromal nerves did not contribute to the epithelial 437 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 438 439 decades (Rao et al., 1985; Richter et al., 1996). In deep anterior lamellar keratoplasty 440 (DALK), a partial graft procedure, regeneration of the SBNP and the recovery of corneal 441 sensation are similar to PK (Lin et al., 2014; Zhang et al., 2013). Descemet's membrane 442 endothelial keratoplasty (DMEK) and Descemet-stripping endothelial keratoplasty (DSEK), 443 which are less invasive surgeries, cause acute reductions in SBNP density and corneal 444 sensation, which are predominantly due to the corneal incision and descemetorhexis during 445 surgery; however, most corneal nerve parameters return to baseline within four to 10 months 446 post-operatively (Ahuja et al., 2012; Bucher et al., 2014).

447

448 Corneal collagen cross-linking (CXL) is a widely used treatment, intended to attenuate the 449 clinical progression of keratoconus. The original protocol for the procedure involves removal 450 of the corneal epithelium (giving it the name 'epithelium-off' CXL), thus leading to the 451 destruction of the SBNP (Al-Aqaba et al., 2012a). Transepithelial CXL, where the corneal 452 epithelium is retained *in situ* (so-called 'epithelium-on' CXL), results in significantly less 453 damage to the SBNP (Al-Aqaba et al., 2012a; Caporossi et al., 2012). Physical removal of the 454 epithelium is accepted as the main cause of corneal nerve damage from CXL procedures. 455 Corneal SBNP density returns to pre-operative (baseline) levels at 12 months after 456 epithelium-off CXL (Jordan et al., 2014), while complete regeneration of the SBNP after 457 epithelium-on CXL was observed within six months (Bouheraoua et al., 2014). Corneal 458 sensitivity also showed a faster recovery with the epithelium-on CXL method, compared to 459 epithelium-off CXL (Spadea et al., 2015). Given the lower degree of corneal nerve damage

460 following the 'epithelium on' procedure, it is interesting to note that a recent systematic

461 review reported that there was inadequate evidence that transepithelial CXL is more

462 efficacious than epithelium-off CXL, in terms of disease progression, visual acuity and

463 adverse events (Ng et al., 2021).

464

465 *3.2.4. Corneal infection* 

466 Ocular herpes simplex virus (HSV) infection is often characterized by mild manifestations 467 such as blepharoconjunctivitis during primary infection, followed by long periods of latency 468 in the trigeminal ganglion. Upon reactivation of the latent virus, recurrent infections can 469 present as more severe corneal epithelial dendritic keratitis or a geographical corneal lesion 470 (Lobo et al., 2019). Due to its neurotropic classification, HSV-1 pathophysiology is a topic of 471 great interest in the ophthalmology, immunology and neuroimmunology fields. A decrease in 472 corneal sensory nerve density and sensitivity has been described in a mouse model during 473 primary corneal HSV infection (Chucair-Elliott et al., 2015). The virus can track in a 474 retrograde direction, through the corneal nerve axons into the trigeminal ganglion, leading to 475 long-term latency. Consistent with the concept of a connection between the two eyes by a 476 retrograde neural and/or immune pathway (Lee et al., 2019), Hamrah et al. reported that in 477 patients with unilateral HSV keratitis, both the infected and uninfected contralateral corneas 478 showed similar alterations to corneal nerves, including decreased SBNP density and reduced 479 corneal sensation (Hamrah et al., 2010). In addition, the nerve damage was evident in both 480 the acute and chronic stages of HSV infection, suggesting that the infection can rapidly 481 induce corneal neuropathy. A recent study revealed that the corneal SBNP in the HSV-482 affected eye regenerated slowly during the virus latency phase, however corneal nerve 483 density did not reach the level of the healthy control group even after a follow-up period of 484 29-46 months; in addition, the nerve regeneration did not result in an improvement in corneal 485 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 486 487 both eyes despite an often unilateral presentation.

488

489 Corneal nerve damage also occurs in patients with non-viral infectious keratitis including

490 bacterial, fungal and *Acanthamoeba* keratitis (Cruzat et al., 2011; Muller et al., 2015). Fungal

491 and *Acanthamoeba* keratitis cause a profound decrease in corneal nerve parameters compared

492 to epithelial herpetic keratitis (Kurbanyan et al., 2012), and the degree of corneal neuropathy 493 is reportedly more severe than for bacterial keratitis (Cruzat et al., 2011). Following 494 antimicrobial treatment, corneal nerve regeneration occurred after the resolution of infection, 495 but did not reach baseline levels even at six months after cessation of treatment (Muller et al., 496 2015). Interestingly, a reduced density of corneal nerves and the presence of subclinical 497 corneal immune cell infiltrates was observed in the contralateral (unaffected) eyes in patients 498 with unilateral microbial keratitis. The observed contralateral eye effect, reminiscent of a 499 sympathetic immune response, might be one explanation for the nerve damage in the 500 unaffected eye (Cruzat et al., 2015). In a study of tear cytokines in patients with unilateral 501 bacterial keratitis, interleukin (IL)-1β, IL-6 and IL-8 were elevated only in affected eyes 502 while IL-10 and IL-17a were elevated only in unaffected contralateral eyes (Yamaguchi et 503 al., 2014). The cytokine response in affected eyes might be pathogen-specific, whereas the 504 unaffected eyes may be exhibiting a prophylactic host defense response by producing IL-505 17A.

506

507 The inflammation in the unaffected eyes might occur through a brainstem pathway (Launay 508 et al., 2016). In a non-infectious benzalkonium chloride-induced unilateral corneal injury 509 mouse model, the corneal nerve damage elicited neuron activation and inflammation in both 510 the ipsilateral and contralateral sensory trigeminal complex in the brainstem, characterized by 511 activated microglial cells and a release of pro-inflammatory mediators (Launay et al., 2016). 512 A recent study also reported increased corneal nociceptor activity, in both the ipsilateral and 513 contralateral eyes, in animals with mild unilateral corneal inflammation induced by UV 514 exposure, as well as increased cold thermoreceptor activity bilaterally in a chronic tear 515 deficiency model (Luna et al., 2021). The activation of projecting neurons and altered corneal 516 sensory nerve activity may contribute to ocular pain and inflammation in the contralateral eye 517 (Guzman et al., 2018). These contralateral eye studies provide strong evidence of the 518 existence of functional neuroimmune interactions in the cornea that involve higher order 519 structures, including the trigeminal ganglion and brainstem.

520

## 521 *3.2.5. Glaucoma*

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

524 ocular surface, mostly due to the presense of preservative agents in eye drop solutions. While 525 most studies ascribe the ocular surface changes to the use of eye drops (Martone et al., 2009; 526 Ranno et al., 2011), evidence of corneal nerve changes in patients with untreated glaucoma 527 also exists (Jing et al., 2021). Structural alterations to corneal nerves, including lower nerve 528 fiber length and branch number, have been reported in patients with untreated normal-tension 529 glaucoma (Jing et al., 2021). However, patients with untreated primary open-angle glaucoma 530 (POAG) share similar corneal SBNP features to healthy controls, suggesting that normal-531 tension glaucoma might be a primary neurodegenerative disease that is distinct from POAG 532 (Jing et al., 2021).

533

534 The presence of corneal nerve damage in individuals with medically managed glaucoma 535 provides evidence that topical, intraocular pressure-lowering medications can negatively 536 effect corneal nerves (Villani et al., 2016). The commonly used preservative, benzalkonium 537 chloride, induces the nerve damage due to its known neurotoxic effects (Sarkar et al., 2012). 538 A recent study revealed that multi-therapy glaucoma patients had worse corneal nerve fiber 539 parameters compared to those on unpreserved mono-therapy treatment regimens (Agnifili et 540 al., 2022). Reduced corneal nerve fiber length was also observed in patients using preserved 541 glaucoma mono-therapy compared to a healthy control group (Agnifili et al., 2022). 542 Fogagnolo et al. (2015) investigated the effect of benzalkonium chloride exposure in a 543 clinical trial and found that latanoprost (preserved prostaglandin analog), but not tafluprost 544 (unpreserved prostaglandin analog), induced corneal SBNP changes, including altered nerve 545 branching and nerve beading at 12-months of follow-up. Thus, it is becoming clear that 546 preservative-containing topical treatments used to manage glaucoma can disrupt corneal 547 nerve homeostasis. Furthermore, long-term use of such therapies increases the density of 548 putative dendritic cells in the corneal epithelium (Marsovszky et al., 2014; Zhivov et al., 549 2010). However, whether the increase in corneal immune cells is associated with the 550 neurotoxicity, or whether it is a direct response to the preservative agent(s), is unclear.

551

# 552 3.3. Changes to corneal sensory innervation in systemic conditions

553 As a densely innervated tissue, the cornea is vulnerable to damage from primary systemic

554 diseases and chemotherapy-induced disorders that involve peripheral neurodegeneration

555 (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
- 561 immune cells, enabling early detection, as well as assessment of the progression, of
- 562 peripheral neuropathy and response to treatments (Ferrari et al., 2013; Wang et al., 2015).
- 563

# 564 3.3.1. Diabetes mellitus

Diabetes mellitus is known to affect multiple ocular tissues, including the cornea; progressive 565 566 damage to corneal epithelial cells and sensory nerves defines a condition known as diabetic 567 keratopathy (Bikbova et al., 2018). A number of clinical studies have shown that both type 1 568 and type 2 diabetes can negatively impact corneal innervation, with anatomical changes that 569 include both decreased nerve fiber length and nerve fiber density (Jiang et al., 2016), and 570 increased corneal nerve tortuosity (Ishibashi et al., 2012). Functional impairment has also 571 been reported, including reduced corneal sensitivity to mechanical stimuli (Lv et al., 2014). 572 Notably, decreases in corneal sensitivity are not congruent with the early morphological 573 changes seen in the corneal sub-basal nerves (Messmer et al., 2010; Rosenberg et al., 2000), 574 indicating IVCM may be able to detect corneal neuropathy at an early stage, before the onset 575 of corneal sensory dysfunction.

576

577 The precise role of corneal immune cells in the early stages of diabetes-associated corneal 578 neuropathy is unclear, with contrasting findings from mouse and human studies. Some animal 579 and human studies suggest that diabetic corneal neuropathy (Leppin et al., 2014; Tavakoli et 580 al., 2011) is associated with a higher density of presumed dendritic cells in the corneal 581 epithelium. Lagali et al. (2018) reported that the proportion of so-called 'mature' dendritic 582 cells was increased in individuals with impaired glucose tolerance and peaked within the first 583 10 years of a type 2 diabetes diagnosis. Others report that experimental diabetes in mice 584 reduces corneal dendritic cell density, and that this underpins delayed corneal epithelial 585 wound healing responses after injury (Gao et al., 2016b). It is possible that the contributions 586 of corneal immune cells to diabetic neuropathy relates to the type, stage and/or duration of 587 disease. Alternatively, the recruitment and/or activation of corneal intraepithelial immune

588 cells may be a consequence of systemic inflammation due to chronic metabolic disturbance.

- 589 In this respect, evidence exists from mouse high fat diet models, considered a model of pre-
- 590 type 2 diabetes, whereby higher corneal immune cell density is observed in the early phases
- 591 of the dietary intervention (Jiao et al., 2020b), with corneal immune cell changes apparent
- 592 weeks prior to the onset of nerve pathology (Hargrave et al., 2020).
- 593

# 594 *3.3.2. Central nervous system diseases*

595 Corneal sensory nerve alterations have also been described in central nervous system (CNS) 596 degenerative diseases. In a clinical study of individuals with moderate to severe Parkinson's 597 disease, the density of the corneal SBNP was significantly lower in this population compared 598 to healthy controls, despite no measurable difference in corneal sensitivity (Misra et al., 599 2017). Interestingly, a positive correlation was also observed between corneal SBNP density 600 and Addenbrooke's Cognitive Examination-Revised scores, where higher scores are

601 indicative of better cognitive function (Misra et al., 2017). Similar results of impaired corneal

602 nerves and an association with cognitive function have been reported in individuals with mild

- 603 cognitive impairment or dementia (Ponirakis et al., 2019).
- 604

605 Similar to these observations in humans, a number of animal studies have recently reported 606 corneal nerve degeneration in mouse models of dementia. In transgenic mice overexpressing 607 human non-mutated tau, corneal nerve degeneration occurred prior to cognitive deficits and 608 peripheral neuropathy (Marquez et al., 2021). A recent study from our laboratory identified 609 corneal nerve changes in a mouse model with CNS tauopathy, which was preceded by 610 morphological alterations to the resident corneal epithelial dendritic cells at earlier stages of 611 the disease (Jiao et al., 2020a). In a similar study involving P301L mice, which overexpress 612 human tau protein, corneal nerve loss was greater in eight month-old mice compared to 613 controls, and impaired corneal DC responses after corneal injury were observed in three 614 month old P301L mice compared to controls (Li et al., 2021b). These alterations to corneal 615 neuroimmune parameters in transgenic mice during the early stages of CNS degenerative 616 disease are supported by human data, with altered corneal immune cell morphology described 617 in patients with mild cognitive impairment, in the absence of significant corneal nerve 618 abnormalities (Dehghani et al., 2020). Thus. Given the close relationship between corneal 619 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.

622

623 Severe acute respiratory syndrome coronavirus 2 is a respiratory virus that causes coronavirus 624 disease (COVID-19). Despite initially being considered a pulmonary disease, evidence of 625 neurological manifestations affecting both the central and peripheral nervous systems have 626 been reported (Severo Bem Junior et al., 2020; Wan et al., 2021a). Corneal IVCM imaging 627 has revealed that four weeks after acute COVID-19 infection, patients with ongoing 628 neurological symptoms have a lower corneal nerve fiber density and nerve fiber length, and a 629 higher density of corneal DCs, compared to healthy controls (Bitirgen et al., 2021). A 630 negative correlation between the severity of longCOVID and the extent of corneal nerve fiber 631 loss has also been described (Bitirgen et al., 2021). Barros et al. (2022) reported similar 632 findings among individuals who had overcome COVID-19; these patients showed lower 633 corneal nerve fiber density and nerve fiber length compared to age-matched controls, 634 accompanied by a higher density of immune cells in the central cornea. Overall, the 635 percentage of patients with DCs in the central cornea was higher in COVID-19 patients 636 (diagnosed > 6 months prior) with lower OSDI scores (<5 out of 100) (Barros et al., 2022). In 637 what is a rapidly evolving clinical field, it is possible that IVCM imaging of corneal 638 neuroimmune parameters will be useful as a surrogate marker of peripheral neuropathy, and 639 also as a non-invasive means of assessing immune cell activation in peripheral tissues 640 following COVID-19 disease, particularly in patients experiencing persistence of symptoms 641 known as 'long COVID'.

642

#### 643 3.3.3. Autoimmune diseases

644 Various autoimmune diseases have been demonstrated to have a negative effect on corneal 645 nerve features. In patients with rheumatoid arthritis, IVCM imaging showed more bead-like 646 formations along corneal nerve axons, as well as more hyperreflective (presumably activated) 647 keratocytes in the stroma (Villani et al., 2008). Likewise, decreased corneal nerve fiber 648 density and increased tortuosity were observed in individuals with Grave's orbitopathy, while 649 those with active disease showed more hyperreflective keratocytes compared to those that 650 had an inactive phase of disease (Villani et al., 2010). It is unclear if the corneal nerve 651 impairments occur as a primary component of those autoimmune disorders, or if they are

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

654

## 655 *3.3.4. Other systemic diseases*

656 A range of diseases that historically have been considered to not lead to corneal sequelae 657 have been shown to manifest sub-clinical changes using IVCM imaging. For example, lower 658 corneal nerve density and DED symptoms have been described in individuals with migraine 659 (Kinard et al., 2015). People with migraine also experience more severe DED symptoms and 660 ocular pain compared to those without migraine, suggesting DED symptoms in individuals 661 with migraine may be driven by corneal nerve dysfunction (Farhangi et al., 2020). Damaged 662 corneal nerve structure has also been described in individuals with fibromyalgia, including 663 lower SBNP density and reduced stromal nerve thickness (Erkan Turan et al., 2018; Ramirez 664 et al., 2015). In patients with human immunodeficiency virus (HIV) infection, corneal nerve 665 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 666 667 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 668 669 neuropathy were older than those without sensory neuropathy, and age is an important factor 670 in corneal nerve parameters (De Silva et al., 2019; Roszkowska et al., 2021).

671

#### 672 *4. Corneal immune cells*

Historically, the cornea has been regarded as an immune privileged tissue due, in part, to the 673 674 absence of bone marrow-derived cells, except for intraepithelial DCs at the limbus (Gillette et 675 al., 1982; Streilein et al., 1979). Subsequent studies have revealed that corneal immune 676 privilege involves several mechanisms, including anatomical barriers, immunoregulatory 677 processes and an immunosuppressive microenvironment (Hori et al., 2019). Further, 678 advances in imaging techniques and the development of transgenic mice have contributed to 679 an understanding that there are, in fact, distinct populations of resident immune cells 680 distributed throughout the corneal epithelium and stroma (Figure 6).

## 682 *4.1. Dendritic cells (DCs)*

683 Some studies have historically classified corneal immune cells with long dendrites as 684 'Langerhans cells' (Zhivov et al., 2005), which were generally assumed akin to epidermal DC 685 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 686 687 by the expression of CD103 (Bursch et al., 2007). Similar to the skin, Langerin is expressed 688 in human corneal DCs (Mayer et al., 2007), but it remains unclear whether they are related to 689 the Langerhans cells or dermal Langerin<sup>+</sup> DCs. There is evidence that the murine corneal 690 epithelium is endowed with a resident population of Langerhans cells, while there is also a 691 minor population of Langerin<sup>+</sup> DCs in the stroma that are not Langerhans cells (Hattori et al., 692 2011). In this review, when commenting on mouse studies, we will refer to corneal

- 693 intraepithelial, dendriform  $CD45^+$   $CD11c^+$  cells as DCs.
- 694

695 Dendritic cells belong to a group of antigen-presenting cells (APCs) that typically express 696 Class II major histocompatibility complex (MHC II) antigens. MHC II is required for the 697 presentation of small, endocytosed peptides on the surface of APCs, in the context of co-698 stimulatory molecules CD80 and CD86. Presentation of antigenic peptides by APCs to naïve 699 T-lymphocytes largely underpins the mechanism of adaptive immunity. The previously 700 accepted dogma was that APCs were exclusively present in the peripheral cornea, but absent 701 from the central cornea. Hamrah et al. (2002) earlier described that CD11c<sup>+</sup> DCs were 702 present in both the central and peripheral corneal epithelium of BALB/c mice, with a 703 centripetally decreasing density and that the DCs in the central and paracentral corneal 704 epithelium of BALB/c mice were MHC II<sup>-</sup>, reflecting an immature status (Hamrah et al., 705 2003a; Hamrah et al., 2002).

706

- 707 In the presence of inflammation, resident corneal DCs can undergo maturation by
- vpregulating 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<sup>+</sup> 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
- 713 MHC-II positive in the steady state. Using transgenic Cx3cr1<sup>gfp/gfp</sup> knockin mice (Fig 3D&F),

which are deficient in Cx3cr1 receptor signaling, it was demonstrated that the presence of

- 715 resident MHC II<sup>+</sup> 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

717 Cx3cr1 signaling was also validated using Cx3cr1 knockout mice (Chinnery et al., 2007).

- 718 Cx3cr1 has also been shown to regulate the presence and sampling behaviour of resident
- 719 intraepithelial immune cells to other mucosal sites, including the gut (Niess et al., 2005) and
- 720 olfactory epithelium (Vukovic et al., 2010).
- 721

722 Similar to the reported presence of intraepithelial CD11c<sup>+</sup> MHC II<sup>+</sup> APCs in healthy mouse 723 corneas (Knickelbein et al., 2014), an ex vivo analysis of human corneal tissue showed 724 CD45<sup>+</sup> intraepithelial DCs that co-expressed CD11c<sup>+</sup> and HLA-DR<sup>+</sup>, with a centripetally 725 decreasing density (Yamagami et al., 2005). Similar expression patterns of human corneal 726 CD11c<sup>+</sup> 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 727 728 systems to further investigate the phenotypes of corneal epithelial DCs and their roles in the 729 process of corneal disease and wound healing, including nerve regeneration.

730

731 While macrophages are the predominant immune cell subset in the anterior corneal stroma 732 (Brissette-Storkus et al., 2002; Chinnery et al., 2008; Liu et al., 2017), a minor population of 733 DC subsets, including CD11c<sup>+</sup> CD11b<sup>+</sup> DCs, also reside in this corneal layer (Hamrah et al., 734 2003c). Morphologically, stromal CD11c<sup>+</sup> DCs are indistinguishable from macrophages, 735 however they do appear to be preferentially associated with sites of nerve branch points, 736 where the nerves traverse the basement membrane of the epithelium (Gao et al., 2016a). 737 Similarly, macrophages in the anterior stroma have been observed to extend cellular 738 processes into the basal epithelium, where they appear to interact with the sub-basal nerve 739 plexus (Supplementary Figure 1 and Supplementary Video 1; Chinnery et al., 2017a). A 740 sparse population of plasmacytoid DCs have also been described in the anterior corneal 741 stroma, and are thought to produce interferon-gamma and regulate T cell immunity (Ochando 742 et al., 2006; Reizis et al., 2011; Sosnova et al., 2005). Plasmacytoid DCs contribute to 743 immune responses against corneal HSV-1 infection by promoting survival of regulatory T 744 cells, indicating a critical antiviral role for these cells in the cornea (Jamali et al., 2020a).

## 746 *4.2. Macrophages*

747 Macrophages also reside in the healthy cornea. Although it is well-accepted that tissue 748 macrophages originate from the bone marrow, resident macrophages can also derive from the 749 yolk sac (Gomez Perdiguero et al., 2015). Macrophages participate in innate immune 750 responses by phagocytosing debris and secreting a variety of inflammatory cytokines to assist 751 with tissue remodeling and inflammation (Gordon and Martinez-Pomares, 2017). Following 752 nerve injury, macrophages are recruited to the injury site, contributing to the removal of 753 axonal and myelin debris, which is the source of inhibitory regeneration signals (Chen et al., 754 2015). In the mouse corneal stroma, CD45<sup>+</sup> macrophages are distributed throughout the 755 anterior and posterior stroma (Figure 6), expressing typical markers including CD11b, Iba1 756 and F4/80 (Chinnery et al., 2017a). Approximately 30% of corneal macrophages express 757 MHC II molecules, which enable them to act as APCs in the cornea (Chinnery et al., 2008; 758 Sosnova et al., 2005). In the human cornea, it has been shown that CD45<sup>+</sup>CD11b<sup>+</sup>CD11c<sup>-</sup> 759 resident macrophages are localized in the posterior corneal stroma (Mayer et al., 2007).

760

761 Macrophages are usually classified into pro-inflammatory phenotype (M1) and anti-762 inflammatory phenotype (M2) according to their distinct cytokine expression patterns and 763 functions (Brown et al., 2012; Gordon and Taylor, 2005). In brief, M1 macrophages are 764 activated by lipopolysaccharide, tumor necrosis factor- $\alpha$  and interferon- $\gamma$ , to participate in 765 pro-inflammatory responses including anti-tumor immunity. M2 macrophages can be 766 activated by specific cytokines, including IL-4, IL-10 and IL-13, to promote tissue repair and 767 wound healing. Another marker, C-C chemokine receptor type 2 (CCR2), has been recently 768 used to classify corneal macrophages in mice. Local (subconjunctival) depletion of either 769 CCR2<sup>+</sup> or CCR2<sup>-</sup> macrophages delayed corneal wound healing after epithelial debridement, 770 with corneal CCR2<sup>+</sup> macrophages sharing similar gene expression and function to M1 771 macrophages and CCR2<sup>-</sup> corneal macrophages resembling M2 macrophages (Liu et al., 772 2017). Based on their phenotype and gene expression, CCR2<sup>-</sup> corneal macrophages likely 773 originate from the yolk sac, similar to CCR2<sup>-</sup> macrophages and yolk sac-derived microglia 774 (Liu et al., 2017). Using single-cell profiling and cell fate mapping of ocular immune cells, 775 Wieghofer et. al., demonstrated that despite most of the corneal macrophages exhibiting a 776 molecular signature similar to bone marrow-derived monocytes, including continuous 777 replenishment from the circulation during adulthood, there is also a contribution of 778 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 respectto their phenotype, origin and contribution to wound healing.
- 781

#### 782 4.3. Contribution of infiltrating immune cells to corneal nerve regeneration

783 In addition to resident DCs and macrophages, infiltrating immune cells that are recruited 784 following corneal injury have an important role in corneal inflammatory responses. 785 Neutrophils are the dominant infiltrating cells in the early stage of inflammation, 786 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 787 788 local production of chemokines, such as the subset of CXC chemokines (Carlson et al., 2006; 789 Lin et al., 2007). Animal studies have shown that neutrophils infiltrate the injured cornea in 790 two phases; an initial phase within 12 hours and a second peak of neutrophil migration at 24 791 to 48 hours that involves a small heat shock protein released by activated resident 792 macrophages (Li et al., 2006; Oh et al., 2012). The recruited corneal neutrophils contribute to 793 corneal re-epithelialization and angiogenesis (Gong and Koh, 2010; Li et al., 2006). Further, 794 neutrophil accumulation is beneficial to corneal nerve regeneration after epithelial injury. 795 Systemic depletion of neutrophils in wild-type mice resulted in a significant reduction in 796 corneal nerve density, which was associated with reduced expression of vascular endothelial 797 growth factor (VEGF) (Li et al., 2011a). This result was supported by evidence of impaired 798 corneal nerve regeneration after injury in wild-type mice treated with anti-VEGF antibody (Li 799 et al., 2011a). In addition to VEGF, other factors or cytokines may also participate in corneal 800 nerve regeneration during the acute, neutrophil-intense phase of an inflammatory response. In 801 neutropenic wild-type mice that have reduced neutrophil recruitment and delayed wound 802 healing after corneal injury, topical application of recombinant IL-20 promoted corneal 803 wound healing and nerve regeneration (Zhang et al., 2017). It is clear that neutrophils play 804 key roles in corneal wound healing, and that an optimal balance of inflammatory cell 805 infiltrates is required to achieve ideal rates of re-epithelialization and accompanying sensory 806 nerve recovery.

807

808 In addition to resident corneal DCs and macrophages, a small population of  $\gamma\delta$  T cells are 809 resident to the limbal epithelium of the steady state cornea; these cells comprise 90% of the T 810 cell population at the limbus in mice (Li et al., 2007). Corneal  $\gamma\delta$  T cells have a dual effect on

811 ocular immunity. In one respect,  $\gamma\delta$  T cells play a vital role in maintaining ocular immune tolerance. Studies have shown that either TCR $\delta^{-/-}$  mice or mice treated with anti- $\gamma\delta$  TCR 812 813 antibody fail to develop Anterior Chamber-Associated Immune Deviation (ACAID) (Skelsey 814 et al., 2001; Xu and Kapp, 2001), suggesting that this subset of T cells contributes to the 815 induction of ACAID to soluble antigen. Moreover, mice treated with anti- $\gamma\delta$  TCR antibody 816 are at a higher risk of corneal allograft rejection due to the failure to develop ocular immune 817 privilege (Skelsey et al., 2001). However, other studies have indicated that  $\gamma\delta$  T cells promote 818 inflammation by inducing neutrophil infiltration (Li et al., 2007), which is critical for corneal 819 wound healing. Another study has shown that IL-22 is a key factor mediating wound healing, and that topical treatment with IL-22 in TCR $\delta^{-/-}$  mice promotes epithelial wound closure (Li 820 821 et al., 2011b). The γδ T cell-dependent inflammatory cascade involving VEGF and IL-17 822 after corneal abrasion was found to be beneficial for promoting corneal nerve regeneration, 823 providing evidence for corneal neuroimmune crosstalk following sterile injury (Li et al., 824 2011a).

825

## 826 5. Corneal neuroimmune interactions

827 Interactions between components of the nervous and immune systems have been investigated 828 in many tissues, including the gut (Yoo and Mazmanian, 2017), lung and skin (Blake et al., 829 2019), and more recently in the cornea. The peripheral nervous system can respond rapidly to 830 irritants and nociceptive stimuli, modulating local immune responses and, reciprocally, 831 immune cells can affect neuronal function by releasing neurotransmitters and cytokines 832 (Kawashima et al., 2015; Rosas-Ballina et al., 2011). Since the cornea is exposed directly to 833 the external environment, its neural and immune systems are at the front line of responses to 834 exogenous stimuli. Using advanced, post-acquisition image processing programs, high-835 resolution confocal microscopy images can be analyzed to quantify the degree of physical 836 overlap between fluorophore channels (Figure 7). These approaches are based on the 837 percentage overlap of user-generated rendered surfaces that localize to the structures of 838 interest (Figure 7B-D). This quantitative approach is useful for measuring close 839 neuroimmune interactions between corneal nerves and immune cells in 3D tissue 840 preparations, however such findings should be interpreted with some caution, as unlike ultra-841 high resolution techniques such as serial block face scanning electron microsopy (Parlanti et 842 al., 2020), confocal resolution limits do not allow the true visualization of cell contacts. 843 Elucidating neuroimmune crosstalk in the cornea is important, and helpful, to better

- understand the physiology and pathophysiology of corneal diseases, and potentially can be
- 845 used to develop novel therapies aimed at promoting corneal nerve regeneration.
- 846

# 847 5.1. Interactions between corneal nerves and intraepithelial immune cells

848 Over the past decade there has been increasing evidence for reciprocal spatiotemporal and 849 biochemical communication between corneal nerves and immune cells. In the healthy human 850 cornea, most of the intraepithelial immune cells are localized to the level of the basal 851 epithelial cells and SBNP (Zhivov et al., 2005). A recent study reported that the density of 852 resident immune cells was positively correlated with corneal nerve branch density and total 853 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 854 855 that involve corneal neuropathy have described concurrent alterations to intraepithelial 856 immune cells and corneal sensory nerves. Corneal immune cell density has also been 857 proposed as a potential marker of neuroinflammation in small fiber neuropathy (Kamel et al., 858 2019). Further to this, the degree of interaction between corneal epithelial immune cells and 859 nerve axons, as determined visually using IVCM images, has provided evidence of at least 860 spatial interaction in human corneas. By quantifying the number of corneal immune cells 861 with at least one or more cellular processes in close contact with nerve fibres (Figure 7B), the 862 interaction between these two constituents of the corneal epithelium can be measured and 863 compared across populations with various diseases. Additional stratification of neuroimmune 864 contacts can be performed by assessing the number of immune cells 'without dendrites' and 865 cells 'with dendrities' that appear to cross or make contact with nerve fibres (Figure 7J) 866 (Stettner et al., 2016). In a study of chronic inflammatory demyelinating 867 polyradiculoneuropathy (CIDP), the number of DCs in close proximity to corneal nerve 868 fibres was reported to be higher in patients with painful inflammatory neuropathy compared 869 to both healthy controls and patients with painless neuropathy (Figure 7J) (Stettner et al., 2016). 870

871

# 872 5.1.1. Evidence for neuroimmune crosstalk in corneal homeostasis

873 In steady-state conditions of the ocular surface, corneal nociceptors can detect changes in

- 874 temperature, osmolarity and other elements of tear composition, to initiate the release of
- 875 various neuromediators to maintain corneal homeostasis. Tear neuromediators including SP,

876 CGRP and VIP are present in healthy individuals (Yoon et al., 2022). SP has been reported to

877 regulate reflex tear production and the expression of tight junction proteins in the corneal

878 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,

883 which regulates functional neuroimmune crosstalk.

884

885 Additional supportive evidence on the importance of neuroimmune crosstalk during ocular 886 homeostasis stems from studies on ocular immune privilege. Ocular immune privilege is a 887 phenomenon whereby the cornea and anterior chamber are able to limit local immune 888 responses to preserve corneal integrity and function. One of the critical mechanisms is that 889 the naïve T cell sensitization and effector T cell activation in the local lymphoid compartment 890 is suppressed by regulatory T cells (Treg), leading to ocular surface immune quiescence 891 (Amouzegar et al., 2016). In the aqueous humor, neuropeptides such as CGRP and VIP 892 inhibit the activation of Th1 cells and macrophages to maintain a relative immunosuppressive 893 microenvironment (Taylor, 2007). Corneal nerve integrity also plays an important role in 894 ocular immune privilege. Corneal nerve injury can induce ocular surface CD11c<sup>+</sup> cells 895 converting into contrasuppressor cells that inhibit Treg cells, leading to the loss of immune 896 privilege (Neelam et al., 2018).

897

898 The expression of transient receptor potential (TRP) channels also serves as another form of 899 functional connection between sensory nerves and immune cells. TRPV1, TRPV2 and 900 TRPM8 are expressed on both corneal sensory nerves and tissue-resident immune cells 901 express TRPV1 (Jiao et al., 2021; Khalil et al., 2016; Link et al., 2010). In the CNS, TRPV1 902 is localized to the mitochondria of microglia, and promotes microglial migration (Miyake et 903 al., 2015) and regulates glutamatergic signaling in neurons (Marrone et al., 2017). In the 904 normal cornea, stromal macrophage expression of TRPV1 is evident, and is localized to 905 CD68<sup>+</sup> lysosomes. After sterile injury, the proportion of TRPV1<sup>+</sup> macrophages is lower, 906 suggesting a potential role for TRPV1 by resident macrophages in maintaining tissue 907 homeostasis (Jiao et al., 2021). It is possible that corneal stromal macrophages, through

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

911

# 912 5.1.2. Evidence for neuroimmune crosstalk in diabetes mellitus

913 Peripheral neuropathy is a common co-morbidity of patients living with diabetes. In 914 individuals with type 2 diabetes, corneal nerve fiber density and branch density are lower, 915 while the density of corneal DCs has been reported to be higher, relative to healthy control 916 participants (D'Onofrio et al., 2021; Tavakoli et al., 2011). Lagali et al. (2018) reported a 917 higher density of so-called 'mature' epithelial DCs (i.e., DCs with long dendritic processes) 918 in individuals with type 2 diabetes, compared to healthy controls. Interestingly, the corneal 919 DCs in patients with type 2 diabetes were more likely to be arranged in a cluster at the level 920 of the SBNP, and were positively associated with tumor necrosis factor receptor super family 921 member 9 protein levels in the plasma. In a pediatric population of individuals with type 1 922 diabetes, early corneal nerve loss and a higher DC density were reported, compared to age-923 matched healthy controls (Ferdousi et al., 2019). While several clinical studies have reported 924 concomitant changes to sensory nerves and immune cell populations in corneas of individuals 925 with local or systemic pathologies, direct evidence for functional neuroimmune crosstalk, and 926 its role in corneal homeostasis and pathology, mostly derives from animal studies involving 927 transgenic and knock-out mouse models.

928

929 The synergistic interactions between corneal nerves and immune cells during epithelial 930 wound healing in diabetic corneas has been reviewed recently (Yu et al., 2022). Animal 931 models of type 1 and type 2 diabetes are commonly used to examine mechanisms of corneal 932 neuropathy; several studies have demonstrated corneal nerve loss in mice and rats with 933 established diabetes (Coppey et al., 2020; Leppin et al., 2014), and dietary-induced metabolic 934 dysfunction that precedes type 2 diabetes (Alamri et al., 2019; Jiao et al., 2020b). In mice 935 with type 1 diabetes, induced pharmacologically using streptozotocin (STZ), an increase in 936 corneal DC density was associated with a lower corneal nerve fiber density (Leppin et al., 937 2014). In this study, close physical associations between mature corneal DCs and the SBNP 938 were described using immunostained corneal flatmounts and confocal microscopy, 939 suggesting a potential interaction between DC activation and corneal nerve fiber damage. In a

940 high-fat, diet-induced metabolic disturbance mouse model, our laboratory also observed a 941 lower SBNP density and a higher density of intraepithelial DCs in the central cornea (Jiao et 942 al., 2020b). In contrast, the sum length of the apically located SNT was similar in animals 943 who consumed either a high-fat or standard diet (Jiao et al., 2020b). Given that most of the 944 DCs are located in the basal corneal epithelium (Zhivov et al., 2005), these results provide 945 evidence for neuroimmune interactions between epithelial DCs and sub-basal nerves, in the 946 context of metabolic disturbance. Further analyses in this study identified that the percentage 947 of mature DCs (MHC-II<sup>+</sup>) that were in close proximity to nerve axons in the SBNP was 948 higher in the high-fat diet mice, relative to controls (Jiao et al., 2020b).

949

950 Although it is unclear whether resident corneal intraepithelial immune cells are 951 neuroprotective or neurotoxic, or both depending on the condition, some clues have been 952 provided in recent years. Gao et al. (2016b) reported that in STZ-induced diabetic mice with 953 a corneal epithelial debridement wound, the number of resident and infiltrating DCs was 954 lower and sensory nerve regeneration was delayed compared to animals without diabetes. 955 This study also found that neutralization of ciliary neurotrophic factor (CNTF) delayed 956 corneal sensory nerve regeneration after injury in the normal cornea. CNTF belongs to the 957 neurotrophic factor family, and most of the CNTF in the cornea is produced by DCs (Gao et 958 al., 2016b), suggesting that activated DCs may directly mediate nerve regeneration through 959 production of neurotrophic factors, such as CNTF. When DCs were locally depleted, 960 exogenous CNTF promoted reinnervation of the wounded cornea. In addition, blocking the 961 CNTF-specific receptor induced corneal sensory nerve degeneration (Gao et al., 2016b). 962 These findings provide evidence that DCs provide neurotrophic support to sensory nerves in 963 the mouse corneal epithelium, and align closely with evidence of a neurotrophic role for 964 intraepidermal Langerhans cells in cutaneous sensory nerve density and function (Doss and 965 Smith, 2014).

966

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-

971 DC overlap has its challenges, as the complexity of both the cell processes and the abundance

972 of nerve axons can prove difficult when making binary decisions around cells that are, or are 973 not, in contact with nerves. Using image analysis software to quantify the colocalization of 974 rendered surfaces of fluorophore channels, we measured the degree of DC-nerve surface 975 overlap in the normal mouse cornea, and in corneas following topical exposure to the TRPV1 976 nerve antagonist, capsaicin, or the inflammatory cytokine TNF- $\alpha$  (Figure 7E-H). First, there 977 was no regional difference in the degree of interaction between corneal DCs and nerves in the 978 central versus the peripheral cornea. However, within 10 minutes of exposure to topically 979 applied TNF- $\alpha$ , a known activator of corneal DCs, a higher degree of neuroimmune contact 980 was observed, compared to topical application of capsaicin or vehicle controls. These data 981 demonstrate the interaction between corneal DCs is dynamic, rapid and sensitive to changes 982 in the inflammatory milieu of the ocular surface. Whether such physical neuroimmune 983 interactions can be regulated by neuropeptide release, or if these dynamic interactions occur 984 in human corneas, is so far unexplored.

985

986 Another study from our laboratory considered the relationship between corneal nerves and 987 DCs in the context of local nerve trauma caused by sterile epithelial abrasion in mice. We 988 observed that the degree of physical 'contacts', as measured by surface overlaps of rendered 989 images of DCs and transient receptor potential vanilloid receptor 1 (TRPV1)-expressing 990 corneal nerves, was higher in the injured epithelium compared to intact, uninjured controls 991 (Figure 7I) (Jiao et al., 2021). Regarding the potential function of nerve-associated DCs, 992 Parlanti *et al.* (2020) revealed that intraepithelial CD45<sup>+</sup> immune cells (presumed DCs) 993 phagocytose and degrade axonal debris in a mouse model of corneal trephine wounds; this 994 finding was evidenced by the presence of electron dense material, identified as lysosomes 995 using scanning electron microscopy. Clearance of axonal debris after injury has been 996 identified as an important processes in axonal regeneration in the peripheral nervous system 997 (Dubovy, 2011), thus it is possible that corneal epithelial DCs participate similarly after 998 corneal nerve injury.

999

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).

1011

# 1012 5.1.4. Evidence for neuroimmune crosstalk in dry eye disease

1013 Interactions between corneal sensory nerves and DCs are also evident in pathological 1014 conditions that affect the ocular surface, including DED. Decreased corneal SBNP features, 1015 including nerve fiber length, fiber density, fiber area and total branch density, have been 1016 observed using IVCM in patients with DED, along with an increased density of presumed 1017 DCs relative to healthy controls (Shetty et al., 2016). Notably, central corneal DC density in 1018 patients with DED was positively correlated with ocular discomfort and SBNP features 1019 including nerve fiber width, branch points and total fiber area, suggesting that increased 1020 inflammatory cells and neural changes are associated with ocular discomfort experienced by 1021 the patients with DED (Shetty et al., 2016). Tepelus et al. (2017a) also reported increased 1022 corneal DC density and decreased nerve fiber density in patients with Sjögren's or non-1023 Sjögren's DED compared with age-matched control subjects. However, these authors 1024 observed a negative correlation between corneal DC and nerve densities; it should be noted 1025 that this correlation analysis included all study participants, including healthy controls. The 1026 different findings in these two studies may be explained by the subtypes of DED studied 1027 and/or the methods used for the IVCM image analyses. Patients with evaporative DED were 1028 recruited in Shetty et. al.'s study with corneal nerve features analyzed by automatic 1029 ACCMetrics software. In Tepelus et al.'s study, over half of the participants were diagnosed 1030 with dry eye due to Sjögren's syndrome, which is typically of the aqueous-deficient subtype, 1031 and a semi-automated tracing program (NeuronJ) was used to quantify the corneal nerves.

1032

1033 In a mouse study, a decrease in the corneal SBNP density and an increase in DC density was

1034 reported after exposure to environmental dry eye stress for three days (Simsek et al., 2018).

1035 Using intravital multiphoton microscopy, Jamali et al. (2020b) investigated the functional

1036 significance of physical connections between corneal nerves and DCs in a mouse model of

1037 DED. In this study, corneal DCs were found to be less frequently in contact with central and 1038 peripheral corneal nerves during DED, compared with control corneas. Using time-lapse 1039 intravital microscopy, this study also showed the DCs that were in contact with nerves 1040 presented shorter track lengths, track speeds and displacement compared to those not in 1041 neuronal contact, suggesting that corneal nerve interactions may directly regulate the 1042 morphology and spatiotemporal dynamics of corneal DCs Jamali et al. (2020b). Moreover, 1043 corneal DCs may indirectly protect sensory nerves in DED by inhibiting ocular inflammation 1044 and regulating the expression of neurotrophic factors and neurotransmitters (Choi et al., 1045 2017). Increased inflammatory cell infiltration and decreased expression of calcitonin gene-1046 related peptide (CGRP), nerve growth factor (NGF) and SP were observed in DC-depleted 1047 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 1048 1049 important for sensory nerve maintenance and corneal inflammatory regulation in DED.

1050

# 1051 5.1.5. Evidence for neuroimmune crosstalk in corneal infection

1052 Pathological alterations to corneal immune cells and sensory nerves are well documented in 1053 infectious keratitis, in particular HSV-1 and herpes zoster virus (HZV) infection. In patients 1054 with unilateral herpes zoster ophthalmicus (HZO), a bilateral increase in corneal DC density 1055 and decrease in SBNP length have been reported (Cavalcanti et al., 2018). Corneal 1056 denervation in the contralateral (uninfected) eye provides evidence for ocular inflammation-1057 associated nerve degeneration (Cavalcanti et al., 2018). Although the mechanism underlying 1058 sensory nerve degeneration with corneal HSV/HZV infection has not been fully elucidated, 1059 the theory that viral replication is the only cause of nerve damage is debatable (Yun et al., 1060 2014). Due to its reliance on nerves, virus-induced neuronal death acts against the virus' 1061 survival. Although inflammation plays a pivotal role in the tissue response to pathogens, 1062 over-reactive and/or unresolved inflammatory responses can be harmful to local cells and 1063 tissues. It has been proposed that inflammation from the viral infection may lead to corneal 1064 nerve damage after HSV infection (Yun et al., 2014). In a mouse model of acute HSV 1065 infection, IL-6, produced during the early phase of the immune response, contributed to 1066 corneal denervation, and neutralization of IL-6 partially preserved corneal nerve structure 1067 (Chucair-Elliott et al., 2016). In a rabbit model of corneal HSV infection, treatment with 1068 topical pigment epithelial-derived factor (PEDF) plus docosahexaenoic acid (DHA) promoted 1069 corneal nerve regeneration and improved resolution of the inflammatory response (He et al.,
1070 2017b). However, in a mouse model of HSV keratitis, despite the increased DC density and 1071 corneal nerve damage after infection, local depletion of corneal DCs resulted in more severe 1072 loss of corneal nerves (Hu et al., 2015). Gao et al. (2016a) reported similar results in sterile 1073 injury model, where local depletion of corneal DCs resulted in a loss of nerve density and 1074 delayed nerve regeneration. Likewise, our decorin-intervention study revealed that topical 1075 decorin imparted a therapeutic benefit on corneal nerve regeneration in wild-type mice 1076 following sterile injury but not in Cx3cr1<sup>gfp/gfp</sup> mice that lack resident and infiltrating corneal 1077 intraepithelial DCs (Wu et al., 2020). Taken together, although alleviating inflammation 1078 appears beneficial to corneal nerves, depletion of local corneal DCs may lead to a series of 1079 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 1080 1081 crosstalk between DCs and corneal nerves.

1082

1083 Divergent findings in the literature regarding the potential contribution of corneal DCs to 1084 generating and resolving inflammatory responses may be explained by these immune cells 1085 having a dual role in the pathophysiology of corneal infection. First, consistent with their 1086 traditional function as APCs, corneal DCs initiate adaptive T-cell immune responses that in 1087 turn can protect corneal nerves by inhibiting virus replication (He et al., 2017a; St Leger and 1088 Hendricks, 2011). However, despite evidence that DCs have neuroprotective functions in the 1089 cornea, over-reactive DCs and inflammation may delay corneal neuroregeneration (Wan et 1090 al., 2022). Recent research from our laboratory has shown that CNS tauopathy is associated 1091 with the activation of corneal DCs, which precedes the gradual loss of neighboring corneal 1092 sensory nerves (Jiao et al., 2020). Another study also demonstrated that mice with unilateral 1093 trephine-injured corneas had contralateral corneal nerve degeneration and DC infiltration 1094 (Lee et al., 2019). Moreover, an increased number of mature DCs and effector lymphocytes 1095 were observed in the draining lymph nodes, suggesting that immune cells might migrate 1096 through the blood circulation to both corneas, causing further damage to the sensory nerves 1097 (Lee et al., 2019).

1098

# 1099 5.1.6. Effects of corneal sensory nerve activation on immune cells

1100 Given the intimate physical interactions between immune cells and sensory nerves in the

1101 corneal epithelium, it is not surprising that immune cell homeostasis and inflammatory

1102 responses can be modulated by sensory nerve activation. Ablation of TRPV1-expressing 1103 corneal sensory nerves increases corneal susceptibility to bacteria by modulating local innate 1104 immunity, including reducing neutrophil bactericidal activity (Lin et al., 2021) and reducing 1105 the capacity of corneal DCs to counter bacterial adhesion (Wan et al., 2021b). Sensory 1106 neurons release mediators, including SP and CGRP, which directly attract and activate 1107 immune cells (Ansel et al., 1993; Ding et al., 2008). Previous studies have reviewed the 1108 important role of neuropeptides (e.g., SP, CGRP and VIP) in neuroinflammation at the ocular 1109 surface (Bignami et al., 2016; Mantelli et al., 2010). There is evidence that following the 1110 stimulation of polymodal nociceptors, the corneal sensory nerve endings release CGRP 1111 (Belmonte et al., 2003), which can influence the development of local inflammation (Yin et 1112 al., 2019). Increased levels of SP and VIP in the tears have also been described in association 1113 with inflammatory responses in allergic conjunctivitis (Fujishima et al., 1997; Motterle et al., 1114 2006). Patients who experience contact lens discomfort also have elevated tear film levels of 1115 the pro-inflammatory cytokine IL-17A, relative to those who have asymptomatic contact lens 1116 wear (Downie et al., 2018; Gad et al., 2019). A recent study reported that unilateral eye 1117 injuries in mice can lead to mucosal immune responses in the contralateral eye, which is 1118 associated with TRPV1 activation in the injured eye and the release of SP in the opposite eye 1119 (Guzman et al., 2018).

1120

1121 Neuropeptides can not only be released by sensory neurons, but also by immune cells 1122 including DCs, macrophages, neutrophils and T cells (Suvas, 2017). These neuropeptides, 1123 including SP and CGRP, can be detected in the tears of healthy humans, suggesting their role 1124 in ocular surface physiology (Yoon et al., 2022). After corneal nerve damage, the release of 1125 neuropeptides increases rapidly followed by the activation and infiltration of immune cells 1126 (Barbariga et al., 2018). The role of neuropeptides in corneal neurogenic inflammation is 1127 evidenced by immune cells largely expressing neuropeptide receptors (Morelli et al., 2020). 1128 For example, in a mouse model of corneal alkali burn, there is a rapid increase of SP and its 1129 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 1130 1131 amplify inflammation, including immune cell infiltration and cytokine release (Cunin et al., 1132 2011). These inflammatory influxes result in a positive feedback loop that perpetuates the 1133 inflammation and can lead to ocular surface diseases, such as DED and corneal 1134 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).

1136 The involvement of SP in inflammatory corneal neovascularization has been identified in

1137 both humans and animal models (Barbariga et al., 2018).

1138

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.

1145

#### 1146 5.2. Interactions between corneal nerves and macrophages

1147 In addition to the role of corneal DCs in mediating neuroimmune interactions in the 1148 peripheral nervous system (Feng et al., 2009), macrophages are also critically involved in 1149 nerve regeneration. Macrophages have both pro-inflammatory and anti-inflammatory 1150 phenotypes. There is evidence that macrophages contribute to Wallerian degeneration, a 1151 process whereby distal portions of injured peripheral nerves progressively degenerate, with 1152 the breakdown of axons and myelin (Chen et al., 2015; Namikawa et al., 2006). Following 1153 peripheral nerve injury, macrophages are recruited by cytokines and chemokines that are 1154 released by Schwann cells. Once at the site of nerve injury, macrophages participate in 1155 clearing myelin and axonal debris, enabling axonal regeneration to occur (Chen et al., 2007; 1156 Dubovy, 2011; Gaudet et al., 2011).

1157

1158 However, the mechanism in the corneal epithelium might differ, since most sensory nerves in 1159 the corneal epithelium are unmyelinated and there are no Schwann cells surrounding 1160 intraepithelial nerve axons. Instead, corneal epithelial cells are believed to function as 1161 Schwann cells, including modulating macrophage recruitment by secreting cytokines and 1162 regulatory factors, such as matrix metalloproteinase-9 (Pal-Ghosh et al., 2011; Stepp et al., 1163 2017). In the normal murine cornea, an intimate physical connection has been described 1164 between corneal macrophages and peripheral stromal nerve trunks (Seved-Razavi et al., 1165 2014). These peripherally located, nerve-associated macrophages were found to rapidly 1166 respond to the damage of terminal axons in the central corneal epithelium, which was partly

1167 mediated by Cx3cr1 signaling. Our recent study revealed that stromal macrophages express 1168 TRPV1 channels that are localized to CD68<sup>+</sup> lysosomes (Jiao et al., 2021). We observed a 1169 lower proportion of TRPV1<sup>+</sup> macrophages in the injured cornea relative to homeostatic 1170 conditions, suggesting that the expression of TRPV1 in resident macrophages is implicated in 1171 corneal homeostasis (Jiao et al., 2021). Functionally, the consequences of TRPV1 1172 expression by corneal stromal macrophages, and whether they contribute directly to nerve 1173 homeostasis, is unclear. In other systems, TRPV1 expression by CD68<sup>+</sup> macrophages has 1174 been reported in the synovium of healthy individuals and osteoarthritis patients (Lv et al., 1175 2021). In rats, activation of TRPV1 via intra-articular injections of TRPV1 agonists lowered 1176 the proportion of M1 (inflammatory) macrophages in the synovium, and improved 1177 osteoarthritis severity (Lv et al., 2021). These findings suggest that targeting TRPV1 1178 expression may be a useful avenue for manipulating the profile of inflammatory macrophages 1179 in tissues, which may be beneficial for promoting corneal nerve regeneration.

1180

1181 He et al. (2017c) reported that in diabetic mice with wounded corneas, topical treatment with 1182 PEDF<sup>+</sup>DHA promoted sensory nerve regeneration and increased the recruitment of M2 1183 macrophages. As previously discussed, M1 macrophages are broadly considered pro-1184 inflammatory, whereas M2 macrophages are immunosuppressive cells that contribute to the 1185 repair of tissues and axons after injury (Chen and Bonaldo, 2013; Italiani and Boraschi, 1186 2014). In a diabetic mouse model of corneal injury, topical treatment with netrin-1 (an axon 1187 guidance factor) enhanced corneal nerve fiber regeneration, as well as M2 macrophage 1188 transition (Zhang et al., 2018b). Similar results were obtained in another study from the same 1189 laboratory, in which topical application of resolvin-D1 promoted corneal nerve regeneration 1190 and enhanced M2 macrophage activation (Zhang et al., 2018c). The crosstalk between 1191 corneal nerves and macrophages might be beneficial to resolving inflammation. In vitro 1192 studies have shown that a synergistic effect between macrophages and sensory neuron-1193 induced neuropeptide CGRP release, which was also observed in mice with P. aeruginosa 1194 keratitis (Yuan et al., 2022). The released CGRP regulated the transformation of M1 1195 macrophages to the M2 subtype, promoting the resolution of corneal inflammation (Yuan et 1196 al., 2022). Moreover, there is evidence that restoration of the distribution of corneal CCR2<sup>-</sup> 1197 macrophages (similar to M2 macrophages) promotes corneal nerve regeneration through 1198 increased secretion of neurotrophins, including brain-derived neurotrophic factor (BDNF), 1199 NGF, neurotrophin (NTF)-3 and NTF-5 (Liu et al., 2018). The CCR2<sup>-</sup> macrophage subset

- 1200 preferentially express the  $\alpha$ -7 nicotinic acetylcholine receptor ( $\alpha$ 7nAChR). The activation of
- 1201  $\alpha$ 7nAChR further enhances the expression of anti-inflammatory genes in the CCR2<sup>-</sup>
- 1202 macrophage subset, which is necessary for corneal wound healing (Xue et al., 2018).
- 1203

1204 In our recent intervention study that investigated topical decorin for treating traumatic central 1205 corneal nerve injury in mice, the density of corneal stromal macrophages was lower, and 1206 nerve regeneration was higher, in decorin-treated corneas compared to controls (Wu et al., 1207 2020). This finding is not contradictory to previous studies, since the macrophages recruited 1208 to the site of the injured nerves are expected to be a mix of phenotypes, and the total number 1209 of macrophages is known to decrease with the resolution of inflammation (Lee et al., 2018). 1210 For example, in the acute phase after partial sciatic nerve ligation, the M1 phenotype 1211 dominates the macrophage population by day three post-injury. However, at day 14, the 1212 percentage of M2 macrophages increases, with both subpopulations of macrophages equally 1213 represented (Lee et al., 2018). A recent study using Macrophage Fas-Induced Apoptosis 1214 (MAFIA) transgenic mice, in which macrophages can be depleted by intraperitoneal injection 1215 of the dimerizer drug AP20187, corneal nerve structure and function were preserved after 1216 corneal HSV infection (Chucair-Elliott et al., 2017). Therefore, inhibiting the pro-1217 inflammatory activity of M1 macrophages might be another means for promoting corneal 1218 nerve regeneration during the early phase after injury.

1219

### 1220 5.3. Interactions between corneal nerves and other infiltrating immune cells

1221 Aside from DCs and macrophages, other immune cells such as neutrophils and T cells, that 1222 are considered to typically not be resident to the cornea, are recruited to sites of nerve injury, 1223 contributing to the inflammatory process (Liou et al., 2011; Moalem and Tracey, 2006). In a 1224 corneal epithelial abrasion mouse model, the depletion of natural killer cells delayed corneal 1225 nerve regeneration and increased the infiltration of neutrophils (Liu et al., 2012). Exaggerated 1226 inflammatory responses may damage the corneal sensory nerves subsequent to cytokine 1227 secretion. Corneal infiltration of inflammatory cells, and a higher expression of IL-17 and 1228 IFN-y mRNA, occur in association with corneal nerve degeneration in mice with 1229 experimental DED (Choi et al., 2017). Elevated tear IFN- $\gamma$  is also a feature of evaporative 1230 DED in humans (Jackson et al., 2016).

1232 Tian et al. (2018) reported that after topical application of PEDF in mice with corneal HSV 1233 infection, the attenuation of neutrophil infiltration and reduced expression of IL-6, IL-1β and 1234 TNF- $\alpha$  were all accompanied by reduced corneal nerve degeneration. These inflammatory 1235 cytokines can modulate ion channels that concentrate in the nerve terminals (McMahon and 1236 Koltzenburg, 1990), including reversing the operation of the sodium-calcium exchanger and 1237 increasing calcium influx, which can otherwise promote axonal degeneration (Persson et al., 1238 2013). In addition, our laboratory's latest study demonstrated that the neuroregenerative 1239 effect of topical decorin on the injured mouse cornea might be associated with fewer 1240 infiltrating neutrophils in the early phase post-injury (i.e., within 24 hours). Moreover, the decorin-induced neutrophil inhibition was absent in Cx3cr1<sup>gfp/gfp</sup> mice that lack corneal 1241 1242 epithelial DCs, indicating a potential interaction between DCs and neutrophils with topical 1243 decorin treatment (unpublished data). Another recent study suggests that following excimer 1244 laser annular keratectomy in thy1-YFP mice, YFP<sup>+</sup> bone marrow cells infiltrating the cornea are myeloid-derived suppressor cells (MDSCs), expressing cell surface markers CD11b<sup>+</sup>Gr1<sup>+</sup> 1245 1246 (Sarkar et al., 2013). An in vitro study also showed that MDSCs promoted the neurite growth 1247 of a co-cultured trigeminal ganglion by secreting NGF (Sarkar et al., 2013). Thus, the 1248 attenuation of corneal neutrophil and NGF-secreting myeloid cells are possible targets for the 1249 development of therapies aimed at improving nerve regeneration after injury (Sarkar et al., 1250 2013).

1251

1252 CD4<sup>+</sup> T cells also play a critical role in corneal nerve pathology. There is evidence that CD4<sup>+</sup> T cells are required in HSV infection-induced neurogenic inflammation in the cornea. CD4-1253 1254 depleted mice showed faster resolution of corneal inflammation and recovery of the blink 1255 reflex at 20 to 70 day post-infection with HSV, which was not observed in wide type mice 1256 (Yun et al., 2014). The regeneration of the corneal nerve plexus and terminals was also 1257 observed in CD4-depleted mice, indicating CD4<sup>+</sup> T cells may negatively affect corneal re-1258 innervation via neurogenic inflammation (Yun et al., 2014). CD4<sup>+</sup> T cells also repress 1259 sensory nerve growth in HSV-infected corneas via the production of VEGF (Yun et al., 1260 2020). These findings confirm that neurogenic inflammation can inhibit nerve regeneration, or induce nerve degeneration, leading to a positive feedback and worsened corneal 1261 1262 neuropathy.

1263

#### 1264 5.4. Corneal neuroimmune interactions in ocular pain

1265 Most corneal sensory nerves have polymodal nociceptors; their activation can lead to 1266 sensations of discomfort and pain (Patapoutian et al., 2009). Pain can be classified into three 1267 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 1268 1269 inflammation; and 3) neurogenic pain, induced by nerve injury (Patapoutian et al., 2009). 1270 Resident and infiltrating immune cells can be activated in both tissue injury-related 1271 inflammation and neurogenic inflammation, and contribute to pain responses by releasing 1272 pro-inflammatory cytokines. Local inflammation is a key component of many ocular 1273 diseases, characterized by activated immune cells and increased release of pro-inflammatory 1274 cytokines and neurotrophins. Many studies have demonstrated an upregulation in 1275 inflammatory factors including IL-1, IL-3, IL-6, TNF-α and MMP-9 in DED (Bron et al., 1276 2017). These mediators can sensitize corneal nociceptors and lower their threshold for 1277 activation, leading to the inflammation-related pain (Belmonte et al., 2017). Nociceptors may 1278 then respond to normally innocuous stimuli (allodynia) in the absence of a noxious stimulus, 1279 or show exaggerated responses to a noxious stimulus (hyperalgesia) (Patapoutian et al., 1280 2009).

1281

1282 Furthermore, inflammatory cytokines such as TNF- $\alpha$  and IL-1 $\beta$  can directly stimulate 1283 nociceptors and increase the membrane excitability of corneal nerves via activation of p38 1284 mitogen-associated protein kinase (MAPK) (Binshtok et al., 2008; Wei et al., 2021). The 1285 action potential firing further leads to an increased release of pro-inflammatory neuropeptides 1286 including SP and CGRP, which is associated with a downregulation of TRPV1 receptor 1287 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., 1288 2009), contributing to spontaneous neuropathic pain and a release of pro-inflammatory 1289 1290 neuropeptides (Baron, 2006). The pro-inflammatory neuropeptides and cytokines released by 1291 activated nociceptors and immune cells result in a positive feedback loop, enhancing 1292 spontaneous and stimulus-evoked nerve impulse activity in polymodal nociceptors. 1293

#### 1294 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).

1302

1303 The immune system is generally affected by aging (Sadighi Akha, 2018). Age-related 1304 inflammation, also known as inflammaging, plays a critical role in neurodegenerative 1305 diseases, including Alzheimer's disease and Parkinson's disease (Scheiblich et al., 2020). 1306 Aging can also affect immune cell phenotypes and their distribution at the ocular surface 1307 (Golebiowski et al., 2020; McClellan et al., 2014). In mice, a higher proportion of CD11b<sup>+</sup> 1308 CD11c<sup>+</sup> DCs was observed in aged mouse corneas compared to in younger eyes (Jung et al., 1309 2021). Among the DC subtypes, CD11b- CD11c+ DCs induce cytotoxic CD8<sup>+</sup> T cell and Th1 1310 responses, while CD11b<sup>+</sup> CD11c<sup>+</sup> DCs initial Th17 and Treg responses (Sun et al., 2020). CD11b<sup>+</sup> CD11c<sup>+</sup> DCs contribute to the development of DED (Lee et al., 2012; Maruoka et 1311 1312 al., 2018), thus their increased presence in aging may contribute to the pathophysiology of 1313 age-related DED and sensory nerve degeneration.

1314

1315 IVCM is also beginning to shed light on the effects of aging on human corneal immune cell 1316 density and distribution. A higher ratio of central to mid-peripheral corneal intraepithelial DC 1317 density was found in healthy humans of older age (Golebiowski et al., 2020). This age-related 1318 change in corneal intraepithelial DC distribution might be associated with corneal nerve 1319 alterations in the whorl region, which is also affected by aging (Badian et al., 2021). Healthy 1320 volunteers aged over 50 years showed counterclockwise or non-rotatory corneal nerve whorl 1321 patterns, accompanied by more dot-like features in IVCM images that are presumed to be 1322 bulbous thickenings of the nerve fibers or possibly immune cells (Badian et al., 2021). The 1323 relationship between inflammaging and corneal denervation in the cornea, and whether 1324 corneal neuroimmune interactions are impacted by aging, requires further investigation.

1325

#### 1326 5.6. Autonomic nerves in corneal neuroimmune interactions

1327 Despite only forming a minor contribution to corneal innervation, autonomic nerves also 1328 participate in corneal neuroimmune interactions. After the loss of corneal sensory nerves, 1329 sympathetic nerves invade into the stroma, forming large sprouts. The sympathetic nerve 1330 fibers that hyperinnervate the cornea may promote HSV-associated inflammation by 1331 releasing catecholamines (Yun et al., 2016). Depletion of CD4<sup>+</sup> T cells results in a lower 1332 sympathetic innervation of the corneal stroma, indicating a central role for CD4<sup>+</sup> T cells in 1333 the interactions between sympathetic nerves and inflammatory cells (Yun et al., 2022). In a 1334 mouse model of corneal abrasion, activation of sympathetic nerves after injury inhibited 1335 corneal re-epithelialization and promoted neutrophil infiltration and cytokine release, which 1336 was associated with the polarization of corneal macrophages (Xue et al., 2018). The pro-1337 inflammatory effect of sympathetic nerves has also been reported in a corneal alkali burn 1338 model, whereby the acute stimulation of corneal sensory nerves induced sympathetic 1339 activation and noradrenaline release, which led to further corneal inflammation and 1340 opacification (Lasagni Vitar et al., 2021).

1341

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<sup>+</sup> or CCR2<sup>-</sup> macrophages after corneal injury (Xue et al., 2018).

1349

#### 1350 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<sup>+</sup>), activation of CD4<sup>+</sup> T cells and the release of pro-inflammatory cytokines (e.g., IL-

1358 1 $\beta$ , Il-6, TNF- $\alpha$ ). However, there are also redress regulations existing in this picture of 1359 reciprocal neuroimmune interactions. In some conditions, activated epithelial DCs and other 1360 myeloid cells may have a neuroprotective effect on sensory nerves. These cells may directly 1361 phagocytose and degrade axonal debris, or produce neuroprotective factors such as CNTF 1362 and NGF. Moreover, anti-inflammatory macrophages ("M2" or CCR2<sup>-</sup>) also promote corneal innervation by inhibiting further inflammation. Inhibiting the process of neuroinflammation 1363 1364 and/or augmenting neuroprotective mechanisms provides potential therapeutic strategies for 1365 corneal neuropathy, but future studies are warranted.

1366

1367 It can be difficult to distinguish between neurogenic inflammation and tissue inflammation in 1368 some corneal conditions, such as non-viral infection and diabetic keratopathy. Some 1369 inflammatory components in neuroimmune interactions may have different roles in each 1370 context (Figure 9). For example, during corneal wound healing, activated DCs can be 1371 beneficial to nerve regeneration. A possible explanation is that in this scenario when the 1372 corneal epithelium is lost and the tissue suffers a reduction in its DC population, recovery of 1373 the epithelium and DC populations are foundational to the corneal nerve regeneration 1374 process. In contrast, in inflammation-dominated conditions, such as diabetic keratopathy and 1375 DED, DCs, as member of the innate immune system, likely produce pro-inflammatory 1376 mediators that may contribute to corneal denervation. This concept of DCs having pro-1377 inflammatory and neurotoxic roles is consistent with a large body of evidence of elevated 1378 corneal DC numbers, concomitant with fewer corneal nerves, in a range of corneal 1379 inflammatory conditions (Cavalcanti et al., 2018; D'Onofrio et al., 2021; Shetty et al., 2016). 1380 In the more complicated context of infection, DCs may present dual effects depending on the 1381 course of the disease. DC-induced adaptive T-cell immune responses after infection are 1382 beneficial for inhibiting virus replication and clearing bacteria (Frank et al., 2012), whereas 1383 chronic inflammation can lead to further nerve degeneration. Similarly, nerve-derived SP can 1384 promote epithelial wound healing after injury, providing an anatomical foundation for nerve 1385 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., 1386 2022). Therefore, understanding the prodominat neuroimmune interactions in different 1387 1388 corneal conditions and during steady-state is important for further translational studies.

- 1390 Although our understanding of corneal neuroimmune interactions has increased in recent
- 1391 years (Tables 1&2), many questions remain. In particular, there is a need for further
- 1392 knowledge regarding how nerve-derived factors affect immune cell recruitment and function,
- and how macrophage polarization in the cornea shapes nerve regeneration after injury.
- 1394 Further investigations into the dynamic alterations of identified immune cell phenotypes in
- 1395 the cornea, as well as mechanistic studies of specific therapies, are necessary to gain further
- 1396 insights into the functional significance of corneal neuroimmune interactions.

					Relationship	
			Changes to corneal	Changes to	between	
First author (year)	Animal model	Intervention	nerves after	immune cells	corneal	
			intervention	after intervention	nerves and	
					immune cells	
Namavari (2012)		Tanical Comerchanin 7a	Increased corneal nerve	Increased immune	ND	
(Namavari et al., 2012)	Mouse model of corneal nerve-transecting lameliar hap surgery	Topical Semaphorin 7a	length	cell influx (CD45+)	NR	
$1 \frac{1}{10} \frac{2012}{100} \frac{1}{100} $	Mouse model of corneal epithelial abrasion	Depletion of NK coll	Delayed corneal nerve	Increased		
Liu (2012) (Liu et al., 2012)		Depletion of NK cell	regeneration	neutrophil influx		
					Colocalization	
Leppin (2014) (Leppin et	Mouse model of diabetes mellitus	ΝΔ	Decreased corneal	Increased corneal	of nerve	
al., 2014)			nerve density	DC density	fibres and	
					DCs	
$H_{\rm H}$ (2015) (Hu of al. 2015)	Mouse model of HSV keratitic	Local doplation of DC	Decreased corneal	Depletion of	NR	
Thu (2013) (Thu et al., 2013)		Local depietion of DC	nerve density	corneal DCs		
Gao (2016) (Gao et al.,	Mouse model of dishetes mollitus		Decreased corneal	Decrease corneal	ND	
2016b)	Mouse model of diabetes mellitus		nerve density	DC density	INIT	
Gao (2016) (Gao et al.,	Mouse model of corneal injury and local deplotion of DCs	Topical CNTF	Increased corneal nerve	Depletion of	NR	
2016b)	Modse model of comeaningly and local depletion of Des		density	corneal DCs		
Gao (2016) (Gao et al	Mouse model of corneal epithelial debridement	Local depletion of DC	Decreased corneal nerve density	Depletion of	DC dendrites cross nerve	
2016a)						
20108)				comea Des	endings	
				Decreased		
Chucair-Elliott (2016)			Retained corneal nerve	macrophage		
(Chucair-Elliott et al.,	Mouse model of HSV keratitis	Topical dexamethasone	density, increased	density	NR	
2016)			corneal sensitivity	Decreased CD8+ T		
				cell density		
				Depletion of		
Choi (2017) (Choi et al., 2017)			Decreased corneal nerve density	corneal DCs,		
	Mouse model of dry eye disease	Local depletion of DC		increased		
				infiltration of	NR	
				inflammatory cells		
				(CD4+ CD19+		
				CD45+ CD11b+)		

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
He (2017) (He et al., 2017c)	Mouse model of diabetes mellitus and corneal injury	Topical PEDF+DHA	Increased corneal nerve density	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. <i>,</i> 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.

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)		Decrease nerve fiber density, branch			
(Tavakoli et al.,	Diabetes mellitus	density and length; increased nerve	Increased DC density	NR	
2011)		tortuosity			
Cruzat (2011)		Decreased nerve density total	Increased DC density	Negative correlation between nerve	
(Cruzat et al.,	Infectious keratitis	number of nerves and branching	Increased size and dendrites of DC	density and DC density	
2011)					
Villani (2013)	Siögren's syndrome dry	Decreased corneal nerve fiber			
(Villani et al.,	eve and MGD	density, increased bead density and	Increased DC density	NR	
2013)	-,	tortuosity			
Cruzat (2015)	Unilateral infectious	Decreased nerve density, total	Increased DC density in both eyes	Negative correlation between nerve	
(Cruzat et al.,	keratitis	number of nerves and number of		density and DC density	
2015)		branches in both eyes			
Stettner (2016)	Chronic inflammatory	Decreased nerve fiber density, nerve branch density and length	Increased density of DCs that		
(Stettner et al.,	demyelinating		contact nerve fibers, increased	NR	
2016)	polyneuropathy		density of nondendritic cells		
Shetty (2016)		Decreased nerve fiber density. fiber		Positive correlation between DC	
(Shetty et al.,	Dry eye disease	length and nerve branch density	Increased DC density	density and corneal nerve fiber density	
2016)					
Choi (2017) (Choi	Dry eye disease	Decreased nerve density, increased	Increased DC density, process and	Positive correlation between DC	
et al., 2017)	1 - 1	nerve tortuosity and beading	area	process length and nerve density	
Tepelus (2017)	Mucous Membrane	Decreased nerve density, increased			
(Tepelus et al.,	Pemphigoid	nerve tortuosity	Increased DC density	NR	
2017b)		,			
Tepelus (2017)	Non-Sjögren's and	Decreased nerve fiber density.	Increased DC density	Negative correlation between nerve	
(Tepelus et al.,	Sjögren's syndrome dry	increased nerve tortuosity		density and DC density	
2017a)	еуе	,			

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
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 reepithelialization 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<sup>+</sup> cells suppressed corneal nerve regeneration in HSV-1 infected mice, and depletion of the CD4<sup>+</sup> 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 scarderived 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- $\beta$ 1), 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 nonselectively 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 reinnervation. 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 singlearm, 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  $\mu$ m) 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<sup>+</sup> DCs and thy1+ neurons. Scale bars =  $100 \,\mu$ m. (D) Cx3cr1<sup>GFP/+</sup> mice label Cx3cr1<sup>+</sup> cells that are resident to corneal stroma and epithelium. MHC-II<sup>+</sup> Cx3cr1<sup>+</sup> cells localized in the epithelium are DCs. (E) Cx3cr1<sup>GFP/GFP</sup> mice label Cx3cr1<sup>+</sup> 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 =  $50 \mu m$ .

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<sup>+</sup>CD11c<sup>+</sup> dendritic cells localized at the epithelial layer in an intact cornea, showing their typical dendriform shape. (B1-B4) Resident CD45<sup>+</sup>Iba1<sup>+</sup> macrophages in the stroma in an intact cornea. (C1-C4) Infiltrating NIMP<sup>+</sup> Cx3cr1<sup>-</sup> neutrophils in the stroma in a sterile injured cornea, characterized with a round cell shape and multi-lobed nucleus. Cx3cr1<sup>+</sup> 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<sup>+</sup> DCs and PGP9.5<sup>+</sup> 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<sup>+</sup>BIII<sup>+</sup> 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 ( $\beta$ III+) 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<sup>+</sup> sensory nerves (A) and CD45<sup>+</sup> 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<sup>+</sup> 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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