Anatomy and Pathology

A Novel Association Between Resident Tissue Macrophages and Nerves in the Peripheral Stroma of the Murine Cornea

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PURPOSE. To characterize the interactions between resident macrophage populations and nerves in naïve and injured corneas of the mouse eye.

METHODS. Corneas from wild-type (WT) *C57BL/6J*, *BALB/cJ*, and transgenic Cx₃cr1-eGFP mice were subjected to a 1-mm central epithelial debridement injury. The eyes were fixed and immunostained as flat mounts with a range of antibodies to identify macrophages, neurons, and Schwann cells. Interactions between nerves and immune cells were analyzed and quantitated using three-dimensional reconstructions of confocal microscopy images. Naïve eyes acted as controls.

RESULTS. A distinctive association between resident immune cells and corneal nerves was noted in the peripheral or perilimbal stromal nerve trunks. These epineurial cells were mostly Cx_3cr1^+ $Iba-1^+$ major histocompatibility complex (MHC) class II$^+$ $F4/80^+$ $CD11b^+$ macrophages. The number of nerve-associated macrophages was greater in WT BALB/c mice than in C57BL/6J mice. There were no qualitative or quantitative differences in the circumferential distribution of nerve-associated macrophages in the cornea. Sterile corneal epithelial debridement led to a dissociation of macrophages from peripheral nerve trunks as early as 2 hours postinjury, with numbers returning to baseline after 72 hours. This dissociation was Cx₃cr1 dependent.

CONCLUSIONS. This study is the first to highlight a direct physical association between nerves and resident immune cells in the murine cornea. Furthermore, we reveal that this association in normal eyes is responsive to central corneal epithelial injury and is partly mediated by Cx₃cr1 signaling. This association may serve as an indicator of malfunctioning neuroimmune communication in disease states such as neurotrophic keratitis and peripheral neuropathy.

Keywords: cornea, nerve associated macrophages, dendritic cells, Cx3cr1 deficiency, sterile injury

The cornea, like other tissues that have epithelial interfaces with the external environment such as the skin, lungs, and digestive tract, is highly innervated by sensory nerves.[1] The cornea is the most densely innervated tissue in the body and is supplied by the ophthalmic division of the trigeminal nerve.[2,3] Stimulation of these sensory fibers results in pain and activation of the ocular surface defense mechanisms including tear production, blinking, and other defensive reflex actions.[4]

Large nerve fiber bundles enter the corneal stroma peripherally in a radial pattern and travel in a centripetal direction parallel to the corneal surface and spiral toward the center.[3,5] Entering the corneal limbus predominantly in the mid and deep stroma, nerve bundles traverse the limbal zone where they lose their perineurial and myelin sheaths.[3] Continuing within the collagenous stroma surrounded only by Schwann cells, stromal nerve fibers continue to divide into smaller branches and eventually become more superficial and form a basal epithelial nerve plexus.

The density, number, degree of branching, and tortuosity of corneal nerves are of clinical importance, as wound healing and ultimately corneal integrity rely upon a competent corneal nerve supply.[6] Dysfunctional corneal innervation has been implicated in neuropathological changes in the eye arising from surgery,[7]

diabetic neuropathy,[8] and dry eye syndrome.[9] A recent study reported a correlation between dendritic cell (DC) numbers and decreased subbasal corneal nerve density following corneal infection,[10] suggesting a possible interaction between the immune and nervous systems in pathogenesis of the disease.

Heterogeneous populations of macrophages and DCs present in the healthy mammalian corneal epithelium and stroma are a component of immune defenses at the ocular surface, exhibiting a progression in phenotype from an antigen-presenting cell (APC) function at the exposed corneal surface to that of an innate immune barrier function deeper in the stroma.[11–13] In the murine cornea, the majority of intraepithelial CD11c$^+$ DCs and a subpopulation of stromal macrophages express major histocompatibility complex (MHC) class II,[12,14] whereas the majority of stromal macrophages express F4/80[15] and the chemokine receptor Cx₃cr1.[14]

The nervous system exerts an important level of control of immune cell activity. Peripheral neuropeptides, released by sensory nerves, are able to influence the activity of macrophages and DCs by modulating their chemotaxis, maturation, phagocytosis, and T-cell stimulatory capacity.[16–19] The shared expression of neuropeptides and relevant neuropeptide receptors by nerves and resident or infiltrating immune cells

concordantly illustrates a degree of communication between the nervous system and the immune system.[20]

Cx₃cr1-deficient mice have been reported to have a normal response to acute noxious stimuli, bacterial challenge, and immune cell migration.[21,22] However, recent studies have highlighted that fractalkine/Cx₃cr1 signaling plays a role during neural injury, as Cx₃cr1-deficient mice had deficits in inflammatory and neuropathic nociceptive responses.[23]

During peripheral nervous system (PNS) injury, macrophages associated with large nerve bundles play a role in clearance of myelin debris[24,25] and provide necessary mitogenic factors and cytokines important in the subsequent injury response.[26-28] Functional interactions between sensory nerves and inflammatory cells are likely facilitated by a degree of physical proximity to promote efficiency in signal transmission between the immune cells and nerves. Indeed, close neuro-immune associations have been described in the skin, gut, and other tissues.[19,29-32] For example, mast cells, eosinophils, and plasma cells have been described in close proximity to nerve fibers in the gut mucosa[29] and dura mater.[31] In the skin, up to 80% of epidermal Langerhans cells are reported to be in contact with nerves.[19] Due to varying degrees of homology between the cornea and the tissue microenvironments of mucosal and cutaneous surfaces, together with its developmental origins similar to the dura mater, direct neuroimmune interactions would not be unexpected.

In the present study we sought to establish the degree and distribution of neuroimmune interaction in the naïve cornea. We examined differences that may arise due to genetic backgrounds; we also investigated whether deficiency in Cx₃cr1, the chemokine receptor for fractalkine/neurotactin (Cx₃cl1) and an important molecule in the injury of the nervous system,[33] had any effect on neuroimmune interactions in the mouse cornea.

METHODS

Animals

Wild-type (WT) and transgenic (Tg) $Cx_3cr1^{GFP/+}$ and $Cx_3cr1^{GFP/GFP}$ mice on either a $BALB/cJ$ or $C57BL/6J$ background, together with $CD11c^{eYFP}$, were used in the present study. Mice were housed at the Monash Animal Research Platform (MARP) under specific pathogen-free conditions and treated in accordance with Monash University animal welfare guidelines and complied with ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. $Cx_3cr1^{GFP/GFP}$ mice are Cx₃cr1 deficient, with both alleles of the Cx_3cr1 gene replaced by the green fluorescence protein (GFP) reporter gene, whereas $Cx_3cr1^{GFP/+}$ animals retain a functional allele.[22] Cx_3cr1^{eGFP} Tg mice from were originally obtained from Stefan Jung and coauthors,[22] but have been bred and housed internally for several years.

Sterile Corneal Injury

Mice were deeply anesthetized using an intraperitoneal injection of ketamine (80 mg/kg) and xylazine (10 mg/kg). Following central corneal debridement with an Algerbrush II (Alger Equipment Co., Inc., Lago Vista, TX),[34] 2 μL sterile PBS was applied to the eyes, and eyelids were closed with tape. Eyes were then enucleated 2, 24, and 72 hours posttreatment and fixed in 4% paraformaldehyde (PFA).

Flat Mount Immunostaining

Naïve eyes were collected, fixed, and stored in 4% PFA overnight. Dura mater, ear skin, and cremaster muscle were dissected from animals fixed by cardiac perfusion. Tissues were further dissected into smaller pieces and washed once in PBS. Corneas were cut into pie-shaped wedges by radial incisions as previously described.[14] For circumferential distribution analysis, the orientation of the cornea was marked by removing a small wedge from the temporal cornea. Tissue flat mounts were incubated in 20 mM EDTA for 60 minutes at 37°C, then placed into blocking buffer (PBS + 3% bovine serum albumin + 0.3% Triton X-100). Flat mounts were then incubated overnight in blocking buffer containing primary antibodies, including rat anti-MHC class II monoclonal Ab (clone M5/114, 1:200 dilution; BD Pharmingen, San Jose, CA), rabbit anti-Iba-1 (1:300 dilution; Wako, Osaka, Japan), rat anti-F4/80 (1:300 dilution; Serotec, Raleigh, NC), rat anti-CD11b (1:200 dilution; BD Pharmingen), rat anti-CD68 (1:200 dilution; Serotec), rabbit anti-βIII tubulin (1:500 dilution; Promega, Madison, WI), rat anti-Substance P (1:100 dilution, clone NC 1; Chemicon, Temecula, CA) and guinea pig anti-calcitonin gene-related peptide (CGRP) (1:100; Acris, Hiddenhausen, Germany) followed by their corresponding secondary (2-hour incubation) and tertiary antibodies (45-minute incubation). Secondary antibodies included goat anti-rabbit 647 (1:300; Invitrogen, Eugene, OR), donkey anti-guinea pig Cy3 (1:300; Jackson ImmunoResearch, West Chester, PA), and biotinylated goat anti-rat Ab (1:300; GE Healthcare, Piscataway, NJ). Streptavidin Cy3 was used as a tertiary fluorochrome (1:300; Jackson ImmunoResearch). To identify Schwann cells, corneas were incubated with the lectin stain Alexa Fluor 488-conjugated wheat germ agglutinin[35] (WGA, 1:4 dilution for 10 minutes; Invitrogen). To visualize nuclei, all tissues were incubated with Hoechst for 10 minutes at room temperature.

Confocal Microscopy and Image Analysis

Tissue flat mounts were examined by confocal microscopy (Leica TCS SP5-II inverted confocal), and Z-stack series were generated using 1-μm increments. Final compilation and analysis of images were performed using Imaris software (7.1.1; Bitplane, Zurich, Switzerland), with the Z-profile of each image used as a reference to confirm physical contact between nerves and immune cells. The number of direct neuroimmune interactions was determined by counting the number of macrophages in direct contact with the perilimbal nerve trunks. Nerve trunks were classified as radially oriented nerves stemming from the perilimbal ring. The total number of macrophages was counted 300 μm along a nerve trunk and represented as cells per 100-μm length (/100 μm).

Statistical Analysis

Results are presented as mean ± standard error of the mean (SEM). Statistical significance was determined for each experiment by t-test or one-way analysis of variance (GraphPad Prism software, La Jolla, CA). Differences between experimental and control groups were considered significant at $P < 0.05$.

RESULTS

Interactions Between Resident Immune Cells and Corneal Nerves in Normal Murine Eyes

There was no apparent relationship between corneal nerves and immune cells in the central corneal stroma (CC) (Fig. 1A). Resident MHC class II⁺ cells were found to be closely associated with a proportion of βIII-tubulin⁺ corneal nerve fibers throughout the stroma of the paracentral cornea (PCC) and the majority of large nerve trunks of the peripheral cornea
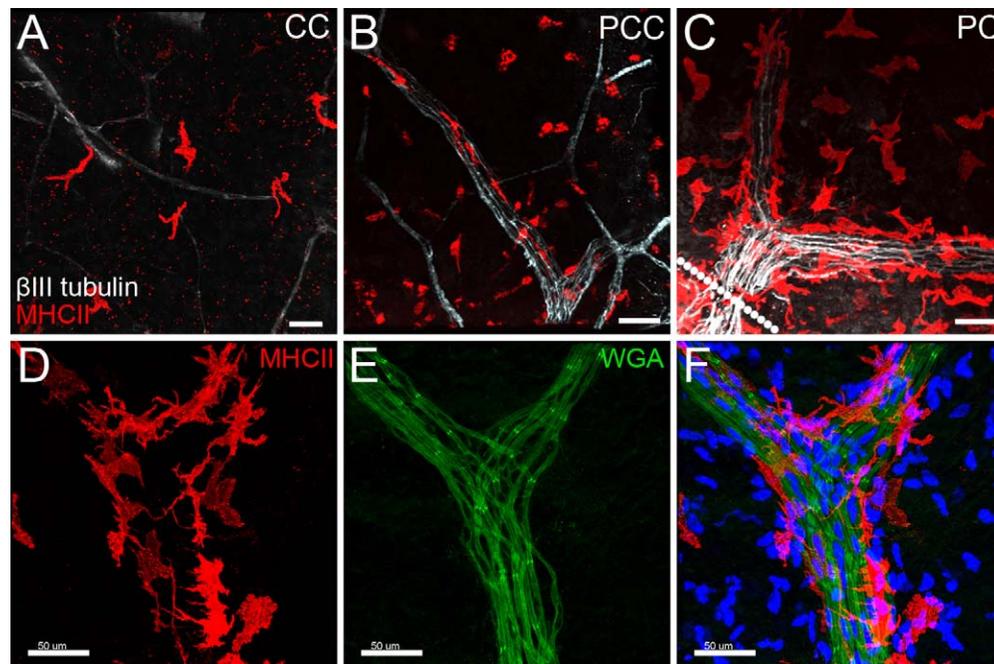
FIGURE 1. (**A–C**) Confocal microscopic analysis of naïve ocular flat mounts illustrating the range of interaction between MHC class II$^+$ (*red channel*) immune cells and β-III tubulin$^+$ nerve fibers (*white channel*), from minimal interaction in the central cornea (CC) and some interaction in the paracentral cornea (PCC) to the greatest association in the peripheral corneal stroma (PC, *dashed line* indicating the limbal border). (**D–F**) MHC class II$^+$ (*red*) resident immune cells intimately associated with WGA-lectin$^+$ (*green channel*) Schwann cells that surround corneal nerves (see Supplementary Video S1). *Scale bars*: 50 μm.

(PC) (Figs. 1B, 1C). Major histocompatibility complex class II$^+$ cells in the stroma were intimately associated with WGA$^+$ Schwann cells of peripheral nerve trunks (Figs. 1D–F, Supplementary Video S1). The large corneal nerve trunks that were surrounded by resident myeloid-derived immune cells also expressed neuropeptides Substance P (SP) and CGRP (Figs. 2A, 2B).

As macrophages and DCs can both express MHC class II and both populations are known to exist in the mouse cornea,[11,12,14,36] we sought to perform further phenotypic characterization of the MHC class II$^+$ cells that we had found associated with the peripheral nerve trunks. Previous studies have shown that the *CD11c^{eYFP}* Tg mouse model[37] is valuable for discriminating tissue resident MHC class II$^+$ cell subsets as CD11c$^+$ DCs or CD11c$^-$ macrophages.[12,38] Using *CD11c^{eYFP}* Tg mice, analysis of stained corneal flat mounts revealed few interactions between CD11c$^+$ DCs and peripheral stromal nerve trunks (Fig. 2C). By contrast, the majority of cells surrounding peripheral nerve trunks were positive for markers associated with macrophages including Iba-1, F4/80, CD11b, and CD68 (Figs. 3A–L).

In order to determine whether these nerve-associated macrophages (NAMs) were unique to the cornea, flat mounts of iris, cremaster muscle, dura mater, and dermis from naïve mice were stained and analyzed in a similar manner. Resident immune cells were also associated with thick βIII-tubulin$^+$ peripheral nerves in these tissues (Supplementary Fig. S1, arrows). The colocalization and extent of the association, however, were not as distinctive as noted in corneal nerve trunks.

Differences Between Mouse Strains and in the Circumferential Distribution of Nerve-Associated Macrophages

Wild-type BALB/cJ mice had a significantly greater number of NAMs, 2.3 ± 0.2 cells/100 μm (mean ± SEM), compared to C57BL/6J mice, 1.5 ± 0.1 cells/100 μm (Fig. 4A). Previous studies have documented a strain-related difference in the anatomical/topographical circumferential distribution of lymphatics and immune cells in the periphery of mammalian corneas.[39,40] Quantitative analysis revealed no significant differences in the circumferential distribution of NAMs in the cornea of BALB/c ($n = 6$) or C57BL/6 mice ($n = 8$) (Fig. 4B).

The Effect of Sterile Injury on Nerve-Associated Macrophages in WT and Cx$_3$cr1-Deficient Mice

We next considered whether NAMs were likely to be responsive to changes in corneal integrity or involved in wound healing. We initially postulated that the numbers may increase following injury. On the contrary, as early as 2 hours following sterile injury to central corneal epithelium, the numbers of NAMs in the peripheral cornea of WT BALB/cJ naïve mice were found to significantly decrease compared to those of naïve controls (1.3 ± 0.1 cells/100 μm; $n = 12$). This marked reduction in the density of NAMs was maintained at 24 hours postinjury (1.2 ± 0.3 cells/100 μm; $n = 6$), but returned to baseline after 72 hours (2.1 ± 0.1 cells/100 μm; $n = 5$) (Fig. 5A).

In light of the role of Cx$_3$cl1 (fractalkine)- and Cx$_3$cr1-bearing cells during neural injury,[23,33] we sought to determine the potential role for Cx$_3$cr1 signaling in regulating the density of NAMs in normal naïve corneas and following sterile injury to the corneal epithelium. To this end we compared the number of MHC II$^+$ cells surrounding peripheral nerve trunks in naïve WT, *Cx$_3$cr1$^{GFP/+}$*, and *Cx$_3$cr1$^{GFP/GFP}$* mice.

No significant difference was noted in the number of NAMs in naïve peripheral corneas of BALB/c or C57BL/6 Cx$_3$cr1 mice (data not shown). However, unlike the diminution in NAMs observed in WT mice, sterile injury of the central corneal epithelium did not influence the density of NAMs in Cx$_3$cr1
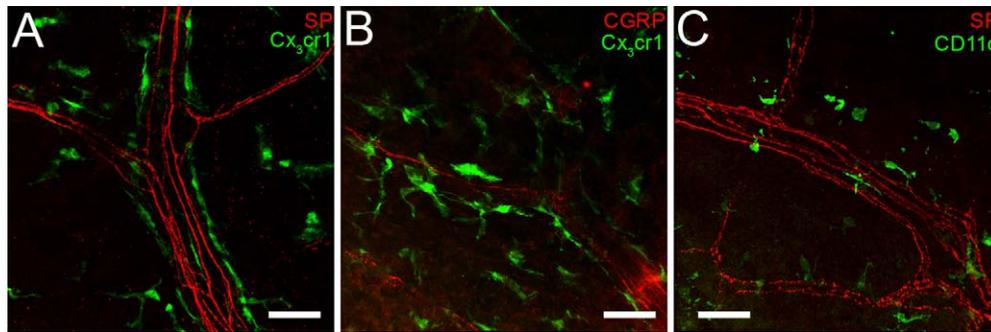
FIGURE 2. Neuropeptide expression highlighted that the majority of peripheral nerve trunks strongly expressed SP (**A**), whereas CGRP was only faintly visible (**B**). (**C**) Representative image of *CD11c^eYFP* cornea highlighting rare interaction between CD11c⁺ DCs and peripheral corneal nerve trunks. *Scale bars*: 50 μm.
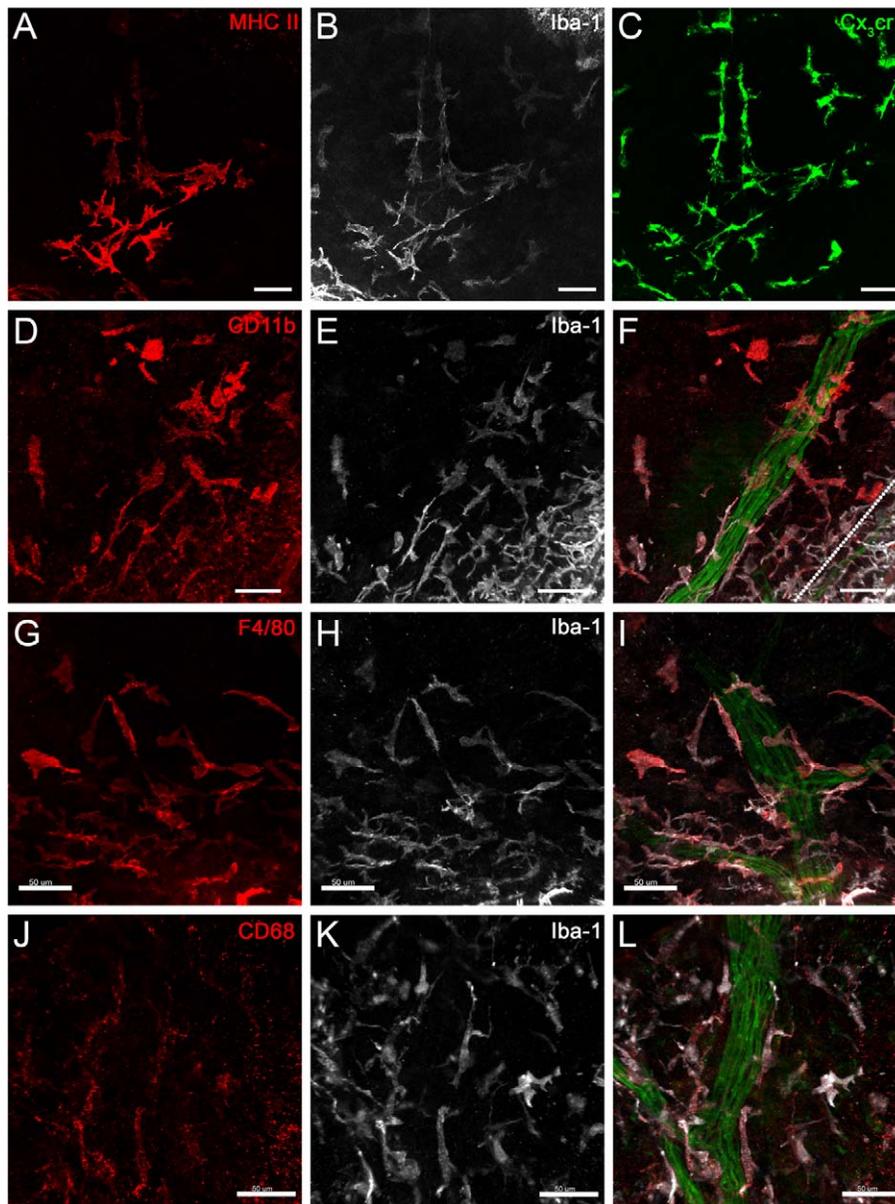

FIGURE 3. Neuroimmune interactions in the peripheral cornea occur between MHC class II⁺ (*red channel*), and Iba-1⁺ (*white channel*) Cx₃cr1⁺ (*green channel*) myeloid-derived immune cells (**A–C**). Further immunophenotyping highlights that Iba-1⁺ (*white channel*) cells intimately associating with WGA-lectin⁺ (*green channel in composite*) peripheral nerve trunks were CD11b⁺ (**D–F**), F4/80⁺ (**G-I**), and CD68⁺ (**J–L**). *Scale bars*: 50 μm.
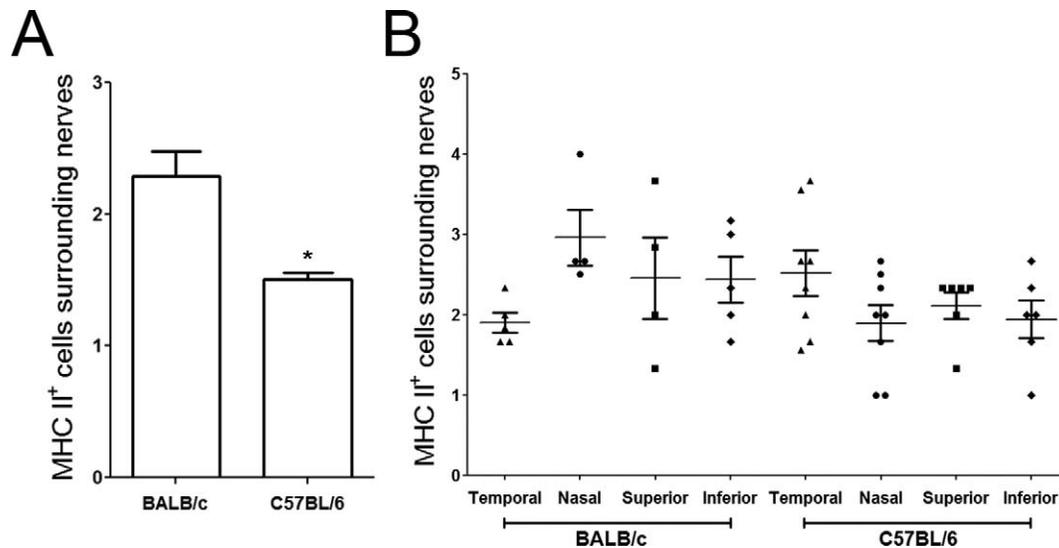
FIGURE 4. (A) Quantitative analysis of nerve-associated macrophages along peripheral nerve trunks in BALB/c and C57BL/6J mouse strains ($n = 5$–11). (B) No significant difference was noted in the circumferential distribution of nerve-associated macrophages in either strain. *t-test, $P = 0.015$.

heterozygous or homozygous mice (Fig. 5B). That is, the response of NAM seen following injury was Cx3cr1 dependent.

DISCUSSION

Despite recent interest in corneal innervation in relation to local and systemic diseases such as dry eye disease,[9,41] infectious keratitis,[42] and diabetes,[43] and the raised awareness of interactions between the immune and nervous systems in other organs such as the skin and lung,[19,32] there is a paucity of evidence relating to the interplay of nerves and resident immune cells in the cornea. Noninvasive analysis of corneal nerves in patients with normal, inflamed, or infected corneas using in vivo confocal microscopy (IVCM) has been used since the mid-1990s to correlate changes in the density and morphology of corneal nerves in a range of pathologies or systemic diseases related to the cornea.[8,10,44] Studies utilizing IVCM or ex vivo immunostaining of corneas have reported dendritic-shaped cells in close proximity to intraepithelial nerve terminals.[2,10] The technical limitation of IVCM restricts analysis to the central corneal region, usually in an area 400 μm by 400 μm,[45] and of course does not provide definitive proof of cell phenotype.

The present study demonstrates close associations between nerve trunks in the stromal layers of the peripheral murine cornea and resident macrophages. While rare interactions were noted in the central and paracentral corneal stroma, as well as the subbasal plexus layer between CD11c^{+} intraepithelial DCs and CD11c^{−} Iba-1^{+} macrophages in the anterior corneal stroma (data not shown), the most distinct association was between large peripheral nerve trunks and resident stromal macrophages. Peripheral nerve trunks enter the mouse cornea predominantly in the deep stroma[46] where the local immune cells are more likely of a macrophage phenotype.[12,14]

To our knowledge, the closest comparable previous observation of resident immune cells associating with peripheral nerves is the descriptions of MHC-positive ramified macrophages and F4/80-positive cells irregularly scattered throughout the sciatic nerve in the normal rat[28] and mouse,[47] respectively. Although resident MHC class II^{+} cells in the present study were occasionally noted surrounding large βIII-tubulin[+] peripheral nerve fibers in the skin, dura, iris, and connective tissue of the cremaster muscle, the association was not as distinctive as that observed in the peripheral nerve trunks of the cornea. We postulate that the lack of a myelin sheath around nerves in the cornea may facilitate a closer interaction between immune cells and nerves than in other tissues.

We next compared the density of NAMs between BALB/c and C57BL/6 mice, as previous studies have reported differences between these genetic backgrounds that may influence the nature and degree of their innate immune responsiveness, and a greater density of immune cells and lymphatics has been described in C57BL/6 corneas.[39] Macrophages from C57BL/6 mice have been reported to exhibit enhanced innate immune response, including greater production of nitric oxide[48] and inflammatory cytokine release[49] following stimulation with IFN-γ and lipopolysaccharide (LPS), when compared to those from BALB/c mice. Our data revealed a slight but statistically significant higher number of NAMs in BALB/c corneas compared to C57BL/6J. How this relates to the above functional innate responsiveness is presently unknown.

Corneal nerves have been shown to enter the human cornea in an evenly distributed circumferential pattern,[45,50] contrary to the previously held view that they predominantly enter the cornea at the nasal and temporal positions.[51] The nasotemporal axis of the human eye is more exposed to the external environment and more susceptible to damage arising after exposure to UV light and irritation from dry and dusty environments, which have been shown to play an etiological role in pterygium and pinguecula.[52] Recent investigations of the difference in the anatomical/topographical circumferential distribution of blood and lymphatic vessels in naïve and inflamed corneas have reported a greater frequency of vessels in the nasal quadrant of BALB/c and C57BL/6 mice.[40] We found no significant differences in the circumferential distribution or number of peripheral nerve trunks and NAMs in either BALB/c or C57BL/6 mice throughout the peripheral stroma.

Nerve–macrophage interactions have been well documented following peripheral nerve injury,[47,53,54] with macrophages responsible for the phagocytosis of cellular debris,[24,25] production of mitogenic factors that act on Schwann cells[26] and fibroblasts,[55] and inflammatory cytokines including interleukin-1 (IL-1), IL-12, and tumor necrosis factor-α (TNF-α).[56] The injury of the central 1-mm corneal epithelium in the present model produced a diminution in NAM density as early
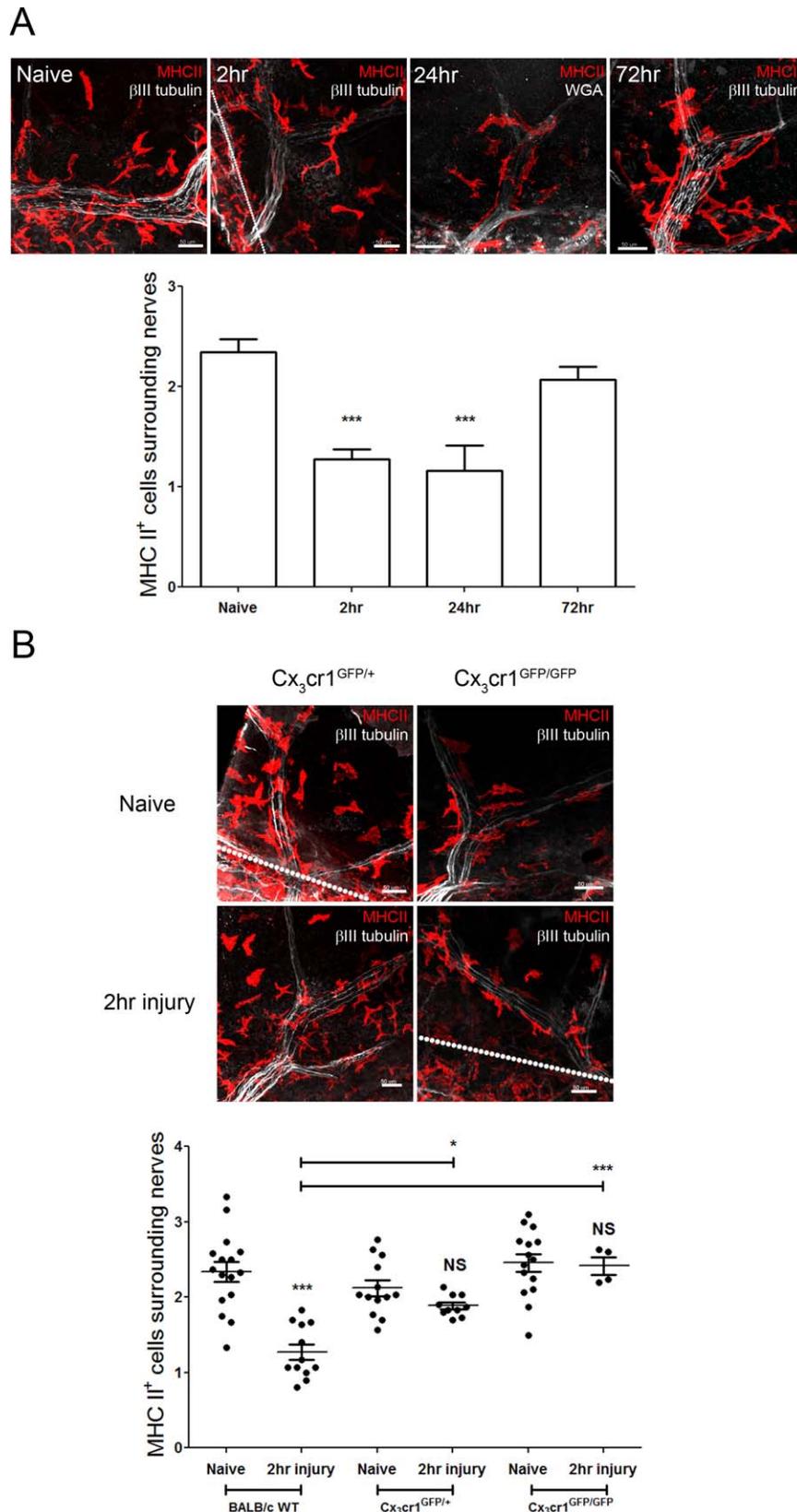
FIGURE 5. Quantitative analysis of nerve-associated macrophages in WT and Cx_3cr1-deficient BALB/c mice after sterile injury. (**A**) A significant decrease was noted in the number of MHC class II[+] NAMs of WT BALB/c corneas 2 and 24 hours after sterile injury. Numbers returned to baseline 72 hours after sterile injury ($n = 5$–16). (**B**) Partial and total loss of fractalkine receptor highlighted that the decrease in NAM density is Cx_3cr1 dependent. One-way analysis of variance, NS, not significant, *$P < 0.05$, ***$P < 0.001$. *Dashed lines* indicate the limbal border. *Scale bars*: 50 μm.

as 2 hours following the treatment, which lasted up to 24 hours. This suggests that NAMs disassociate from nerves in response to the damage of terminal axons in the central corneal epithelium. This may represent an early warning system of breaches to corneal integrity, and may be a previously unsuspected component of the ocular surface defensive mechanisms. Further immunophenotypic characterization of these cells is required to determine whether NAMs returning to the nerves after 72 hours are different from those surrounding the nerve bundles under normal conditions. Namely, they are an M1 (proinflammatory) or M2 (immune-modulating) phenotype.

In light of previous reports of the role of Cx_3cl1 and Cx_3cr1 signaling during neural injury,[23,33] we investigated whether a deficiency in Cx_3cr1 affected the number of NAMs in the naïve corneas of either BALB/c or C57BL/6 mice. Interestingly, the reduction in NAM density observed in naïve corneas following sterile corneal injury did not occur in either Cx_3cr1 heterozygous or homozygous mice. These findings support previous reports that the intermediate phenotype resulting from a loss of one allele is significant enough to alter fractalkine receptor function in injury/disease states,[57,58] and suggests that functional fractalkine signaling is important for the rapid disassociation of macrophages from corneal nerves following injury.

The present study provides a basis for investigations into the potential interplay between peripheral nerves and resident immune cells following injury in the cornea and other tissues. The ease of visual examination of the corneal microenvironment makes this an excellent experimental template for general investigations of sterile inflammation, as well as functional relationships between nerves and immune cells. These data may serve as an indicator of malfunctioning neuroimmune communication in disease states or in neurotrophic keratitis and peripheral neuropathy.

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