MACROPHAGES IN CORNEAL EPITHELIAL WOUND HEALING

by

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DISSERTATION

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DEDICATION

This dissertation is dedicated to my loving mother, Zhaofang Wang, and my father, Zongkuan Zhang. I studied abroad for years and was not able to share time with you. When you met difficulties, I wasn't beside you and I was not able to help. I owe you too much for your unconditional love, unwavering support and unlimited sacrifice. Thank you both for everything you've done for me.

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Abstract

Purpose: After corneal epithelial injury, the ensuing inflammatory response is necessary for efficient wound healing. While beneficial healing effects are attributed to recruited neutrophils and platelets, little is known regarding the relative distribution of macrophage phenotypes within the cornea and whether macrophages contribute to the inflammatory cascade that is so important for corneal wound healing. The objectives of this research were: (1) To characterize macrophages in normal and wounded mouse corneas; (2) To determine if macrophage-derived IL-20 plays a beneficial role in corneal wound healing; (3) To determine if oncomodulin, a potent stimulus for nerve regeneration known to be present in macrophages and neutrophils, plays a beneficial role in corneal wound healing.

Methods: In all wounded corneas, a 2mm diameter central epithelial region was mechanically debrided with a gulf-club spud. (1) Fluorescently tagged antibodies raised against mouse macrophage markers (F4/80, CD115, CX3CR1 and CD206) together with known M1 (CD80, CD86) and M2 (CD301) pro- and anti-inflammatory markers, respectively, were used to identify and localize macrophages within corneal wholemounts of wildtype mice before and after injury (24, 48, 72h and 7days). To enumerate different macrophage phenotypes within the cornea, whole mounts were sub-divided into five regions: (a) limbus (L), (b) paralimbus (PL), (c) parawound (PW), (d) wound (W) and (e) wound center (WC). Within each region, a single camera field (40X, 150 x 150 μm) was recorded and cells staining positively for macrophage markers were counted. (2) Injured female wildtype C57BL/6 mouse corneas were topically treated every 4h up to 24h with 10μl of neutralizing IL-20 antibody (200μg/ml) or control antibody (non-immune isotype

matched IgG). To examine the effects of recombinant IL-20 (rIl-20) on epithelial wound healing, wildtype mice, neutrophil-depleted wildtype mice (anti-Ly6G antibody pre-treatment) , and mutant mice known to have reduced neutrophil infiltration (γδ T cell deficient mice (TCR-/-) and CD11a deficient mice (CD11a-/-)) received 10μL of rIL-20 dissolved in phosphate buffered saline (200ng/mL) every 4h up to 24h, while appropriate control mice received buffer only. The rate of corneal wound closure, the numbers of dividing basal epithelial cells, infiltrating neutrophils, and platelets, and the density of epithelial nerves were evaluated. Some corneas were prepared for immunofluorescence microscopy to localize IL-20 and its receptor, IL-20R1. (3) Some wildtype mice receiving corneal epithelial abrasions were topically treated with oncomodulin-specific blocking peptide P1 (100ng/5ul) or control peptide P3 (100ng/5ul), respectively. The process was repeated every 6 hours for 24 hours. The rate of corneal wound closure, epithelial nerve density, and number of dividing epithelial cells were evaluated to assess the functional contribution of oncomodulin on epithelial wound healing. Some dissected mouse corneas were immunostained with anti-oncomodulin antibody together with CD301 (M2 macrophage marker), Ly6G (neutrophil marker), and FITC-avidin (mast cell marker) to assess the cellular localization of oncomodulin. Non-immune isotype matched antibody served as a control for non-specific staining.

Results: (1) CD301+ CD80- cells were present at the limbus and showed a strong association with limbal vessels. CD301+ cells were negative for CX3CR1 but positive for all other macrophage markers examined (F4/80, CD115, and CD206), suggesting these cells are CD301+ M2 macrophages. Within the uninjured mouse cornea, macrophages failed to stain with antibodies against M1 markers (CD86 and CD80). However, CD80+

cells were observed at the limbus and additional staining with FITC-avidin established their identity as mast cells. Prior to injury, CD301+ macrophages were found at the L and PL. 24h after wounding, CD301+ macrophage numbers declined by 50% at the L ($P \leq 0.05$) and were elevated at the PL, PW, W and WC ($P \leq 0.05$). By 48h and 72h post-injury, CD301+ macrophage numbers returned to baseline at the L while their numbers remained elevated at the PL, PW, W and WC. Seven days after wounding, CD301+ macrophage numbers showed a second decline to 67% of baseline at the L while their numbers in the PL and PW returned to baseline values and remained elevated at the W and WC. Mast cell numbers at the limbus remained unchanged after wounding. (2) Corneal epithelium and keratocytes showed positive staining for IL-20. CD301+ macrophages and CD11C+ dendritic cells were negative for IL-20. Immunostaining confirmed IL-20R1 was expressed on wildtype corneal epithelial cells prior to injury. Topical application of neutralizing IL-20 antibody markedly delayed epithelial wound closure between 8 and 24h post-injury ($P \leq 0.05$) and, unlike control mice, the wound remained open at 24h. In addition, fewer dividing epithelial cells were detected across the cornea ($P \leq 0.05$) and epithelial nerve recovery was also markedly depressed by anti-IL-20 treatment, while neutrophil infiltration increased ($P \leq 0.05$). Topical administration of rIL-20 accelerated wound closure, increased epithelial cell division and enhanced nerve recovery; similar findings were also observed in injured neutropenic, TCR-/- and CD11a-/- mice. The already low neutrophil infiltration in TCR-/- mice was inhibited further by rIL-20 treatment ($P \leq 0.05$). In wildtype mice, topical application of rIL-20 decreased neutrophil infiltration and platelet accumulation while the overall rate of wound closure was not affected. Further investigation revealed these mice had 39% more

dividing epithelial cells at the PL and a higher nerve density at the WC at 24h after rIL-20 treatment. (3) Oncomodulin staining was predominantly found in mast cell granules, while weaker staining was observed in the infiltrating neutrophils. Corneal CD301+ macrophages showed no evidence of oncomodulin staining. During the first 24h after corneal abrasion, topical application of the oncomodulin receptor blocking peptide P1 did not affect the epithelial wound closure rate. However, nerve regeneration was significantly depressed at the PL and W ($P \leq 0.05$) when compares to control peptide P3 treated corneas.

Conclusions: In summary, macrophages with an anti-inflammatory phenotype (M2, CD301+) were detected at the limbus and within the corneal stroma. Pro-inflammatory macrophages (M1) were not detected, even after corneal epithelial abrasion. CD301+ macrophage infiltration into the wounded cornea is biphasic and the corneal epithelium and keratocytes appear to be the source of IL-20, a cytokine that is necessary for efficient corneal epithelial wound closure, cell division and nerve recovery. The fact that IL-20 also inhibits neutrophil and platelet recruitment identifies IL-20 as a possible beneficial therapeutic agent capable of sustaining wound healing while limiting neutrophil and platelet infiltration. Oncomodulin, a potent stimulus for nerve regeneration, was detected in the cornea but not in macrophages. Oncomodulin staining was observed in mast cells and infiltrating neutrophils (after corneal injury) and oncomodulin appears to contribute to nerve regeneration as blocking the binding of oncomodulin to its receptor using the P1 peptide delayed nerve regeneration during the first 24h after abrasion. Mast cells, unlike neutrophils, are resident cells at the limbus and the observation that oncomodulin staining

is found in mast cells raises the possibility that these cells play an important role in maintaining the health of epithelial nerves in the uninjured cornea.

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ABBREVIATIONS

IL ---------- Interleukin

L ---------- Limbus

PL ---------- Paralimbus

PW ---------- Parawound

W ---------- Wound

WC ---------- Wound center

µm ---------- Micrometer

µg ---------- Microgram

ml ---------- Milliliter

mg ---------- Milligram

ng ---------- Nanogram

µl ---------- Microliter

IgG ---------- Immunoglobulin G

WT ---------- Wildtype

CD ---------- Cluster of differentiation

rIL-20 ---------- Recombinant IL-20

TCR ---------- T cell receptor

IL-20R1 ---------- IL-20 receptor 1

FITC ---------- Fluorescein isothiocyanate

E ---------- Embryonic (day)

P ---------- Postnatal (day)

H---------- Hour(s)

ALL ---------- Anterior limiting laminar

PLL---------- Posterior limiting laminar

GAG---------- Glycosaminoglycan

APCs---------- Antigen Presenting Cells

MHC ---------- Major histocompatibility complex

NK cell---------- Natural killer cell

VEGF---------- Vascular endothelial growth factor

NGF---------- Nerve growth factor

PDGF---------- Platelet-derived growth factor

TGF-β---------- Transforming growth factor -β

EGF---------- Epidermal growth factor

IGF-1---------- Insulin-like growth factor-1

ICAM-1---------- Intercellular adhesion molecule-1

TACs ---------- Transient amplifying cells

TNF-α ---------- Tumor necrosis factor-alpha

FGF---------- Fibroblast growth factor

GFP ---------- Green fluorescent protein

MCP-1---------- Monocyte chemoattractant protein-1

PBS ---------- Phosphate buffered saline

BSA ---------- Bovine serum albumin

FBS ---------- Fetal bovine serum

PE ---------- Phycoerythrin

APC ---------- Allophycocyanin

BDNF---------- Brain-derived neutrophic factor

GDNF---------- Glial cell line-derived neutrophic factor

SEM---------- Standard error of mean

ANOVA---------- Analysis of variance

ARVO---------- The Association for Research in Vision and Ophthalmology

Chapter 1- Introduction

After corneal epithelial injury, the ensuing inflammatory response is necessary for efficient wound healing. While beneficial healing effects are attributed to recruited neutrophils and platelets, little is known regarding the relative distribution of macrophage phenotypes within the cornea and whether macrophages contribute to the inflammatory cascade that is so important for corneal wound healing. The purpose of this thesis is to characterize the macrophage distribution within the mouse cornea, determine how this distribution changes in response to epithelial abrasion, and to determine if two potential macrophage-derived products (IL-20 and Oncomodulin) regulate inflammation and corneal wound healing. Before I describe the experiments, it is necessary to set the stage and provide the reader with an overview of corneal development, anatomy, injury and inflammation, wound healing, and macrophage biology. Using this background information, I will state the working hypothesis, provide the rationale behind the hypothesis, and outline the three Specific Aims of this thesis.

1.1 Cornea

The cornea is an optically transparent outmost layer and anterior window for the eye which serves not only as a refractive medium, but also as a protective barrier against particles that may harm the eye. Maintaining corneal transparency is critical to the preservation of clear vision. Worldwide, approximately 17 million people have had corneal surgery and 1.5 million people undergo refractive surgery in the United States each year (Cortina et al., 2010). These surgeries involve injury to the corneal epithelium and its associated nerves. Our laboratory has been investigating the role of inflammation

in corneal wound healing that accompanies epithelial injury for many years and found inflammation is beneficial to wound healing and promotes efficient epithelial wound closure and nerve regeneration. Before expanding on this concept and getting to the subject of this dissertation, it will be useful to provide an overview of the cornea.

1.1.1 Corneal Development

During corneal development, the different cell types within the cornea have distinct embryologic origins. The corneal epithelium is derived from lens surface ectoderm, while the other layers of the cornea are produced from a different population of cells sitting in the Neural Crest (Pei and Rhodin, 1970).

In human, the development of the cornea starts as early as five weeks after fertilization. Between the fifth and sixth week, the inductive signal from the lens reaches the surface ectoderm, stimulating basal cuboidal epithelial cells in the surface ectoderm to increase in number. These basal cuboidal epithelial cells, together with the periderm (a superficial layer of the surface ectoderm) form the corneal epithelium (O'Rahilly, 1975; Pei and Rhodin, 1970). Around the eighth week, after the epithelial cells secrete collagen to form the primary stroma, mesenchymal cells from the neural crest migrate across the primary stroma and form a continuous layer where the migrating cells change shape to form a cuboidal epithelial layer called the corneal endothelium. Other neural crest cells migrate into the space between the corneal epithelium and endothelium and differentiate into keratoblasts, which finally become keratocytes. The keratocytes increase in number and synthesize high levels of collagen and keratan sulfate proteoglycans to form the mature stroma. The basement membrane of the anterior epithelium can be recognized after corneal epithelium formation at the fifth week during corneal development. Then the

anterior limiting lamina, which develops from the superficial mesenchymal cells of the stroma, is seen as extremely fine fibrils becoming a distinct layer at 19 weeks (Sevel and Isaacs, 1988). By the end of the eighth week of gestation, a low density layer appears anterior to posterior endothelium, which is known as the posterior limiting lamina or Descemet's membrane (O'Rahilly, 1975; Wulle and Lerche, 1969).

The mouse serves as a commonly used animal model to study corneal pathobiology. Mouse corneal studies benefit from a wide range of available mouse-specific reagents (e.g., antibodies and recombinant proteins) and mice with specific targeted mutations. However, the mouse cornea has some structural differences that need to be kept in mind when extrapolating findings to human corneal pathobiology. For this reason, it is worth reviewing mouse corneal development and anatomy to provide a basis for making comparisons with the human cornea.

In mouse, the development of the cornea starts by embryonic day (E) 12.5. By E 12.5, only one to two cell layers of surface ectoderm are seen in the corneal epithelium. In the following two days, periocular mesenchymal cells migrate to the gap between the corneal epithelium and anterior lens to form the presumptive corneal stroma and endothelium by differentiating into keratocytes and corneal endothelial cells, respectively (Haustein, 1983; Pei and Rhodin, 1970). During the next days E 14.5-15.5, almost all layers of the mouse cornea have been defined. The surface ectoderm which covers anterior mesenchyme becomes the corneal epithelium, posterior mesenchymal cells become the corneal endothelium, while keratocytes continue to increase in number and synthesize the stromal matrix utill E16.5; by E17.5, collagen lamellae are evident (Cvekl and Tamm, 2004; Gould et al., 2004; Haustein, 1983; Pei and Rhodin, 1970). At birth,

Descemet's membrane is still not visible by light microscopy but can be observed by electron microscopy (Kidson et al., 1999; Smith et al., 2001). By postnatal day (P) 6-8, Descemet's membrane is clearly detected. In mouse, unlike human, the anterior limiting lamina is not present (Hazlett, 1993; Rehbinder, 1978).

1.1.2 Corneal Anatomy

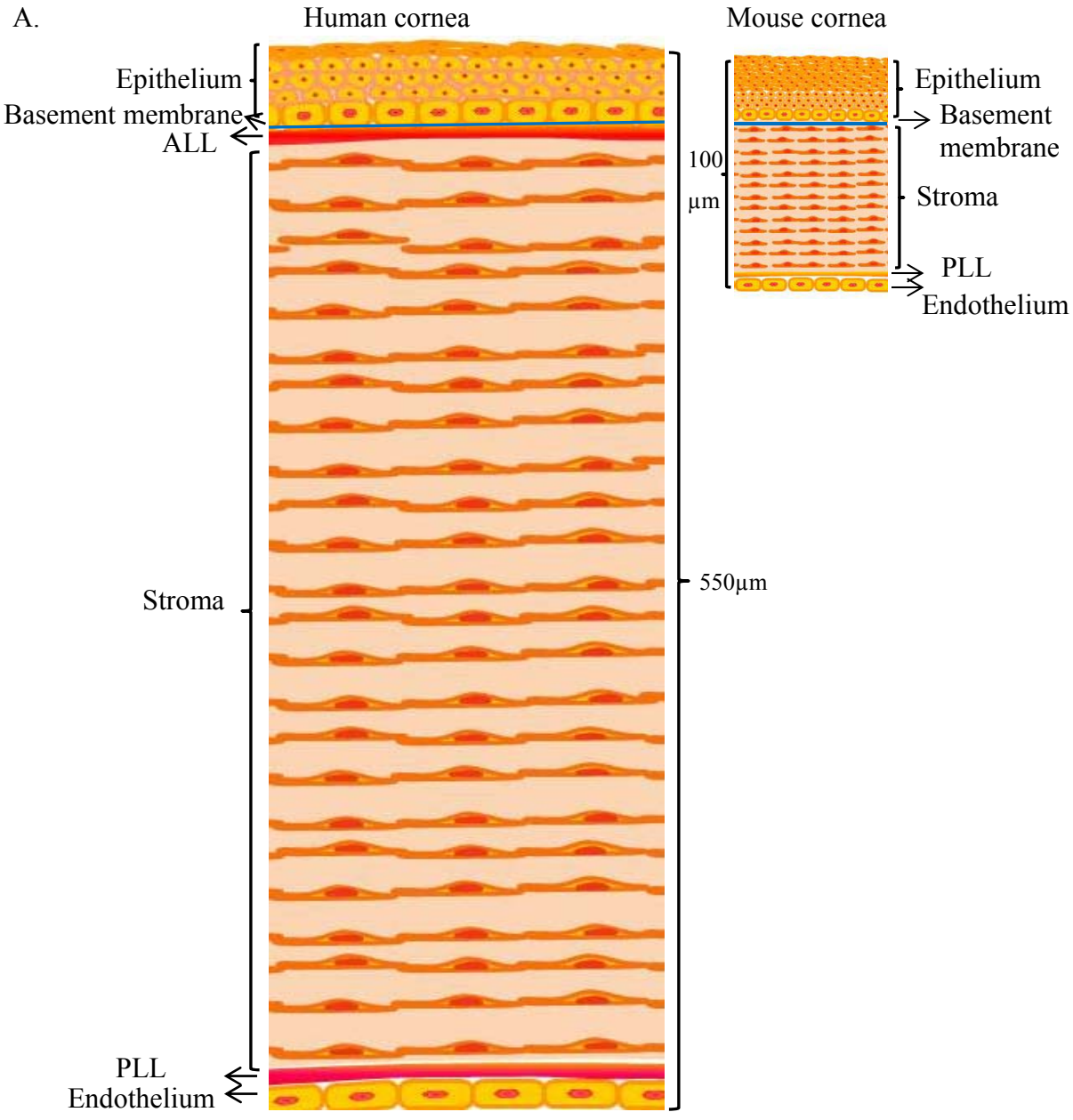
The transparent cornea is continuous with the opaque sclera and the semi-transparent conjunctiva. The limbus is the transition zone between the cornea and sclera which is highly vascularized and contains a reservoir of stem cells located in Palisades of Vogt (Goldberg and Bron, 1982; Van Buskirk, 1989). The Palisades of Vogt is present in humans, but absent in mice (Mort et al., 2012; Notara et al., 2011). The anterior corneal surface is covered by the tear film, which protects cornea form dehydration and helps to form a smooth epithelial surface. The posterior corneal surface is bathed by aqueous humor in the anterior chamber. In humans, the cornea is composed of 5 distinct layers: epithelium, anterior limiting lamina (Bowman's layer), stroma, posterior limting lamina (Descemet's membrane), endothelium (Figure 1) (as reviewed by (Secker and Daniels, 2008)). The mouse cornea is somewhat different in that it only has 4 layers because the anterior limiting lamina is not formed (Hazlett, 1993; Rehbinder, 1978).

Recently, Dua and colleagues reported the existence of a 6th layer in the human cornea. This "pre-Descemet's posterior stroma layer" has been named as "Dua's layer" (Dua et al., 2013). However, the evidence supporting the existence of this 6th layer has come under criticism. In the clinic, the "big bubble" technique is used in deep anterior lamellar keratoplasty to separate Descemet's membrane from the stroma in keratoconus patients. But the gross organization of the stromal lamellae has undergone considerable

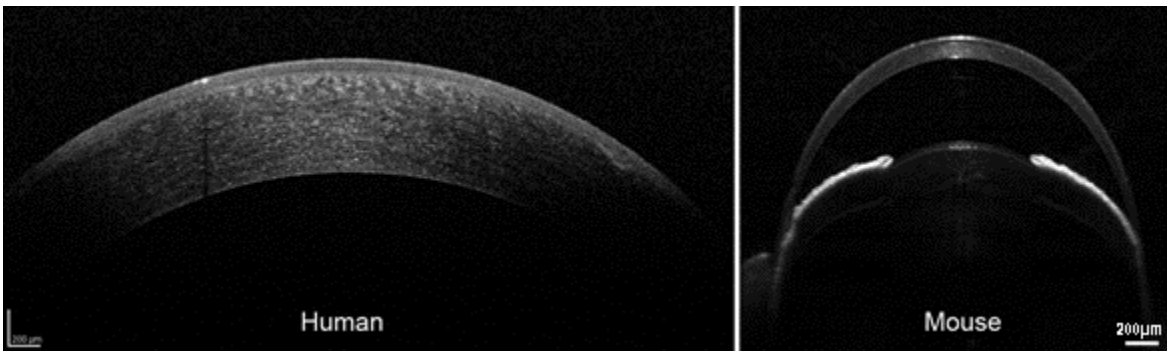
change as result of keratoconus (Meek et al., 2005). For this reason, caution must be exercised when interpreting observations in the keratoconic cornea and extrapolating them to the normal human cornea. Furthermore, bubble measurements are influenced by the location of the injection site, which may be difficult to replicate from cornea to cornea. The immunostained and electron microscope images provided by Dua are difficult to interpret since the posterior lamellae were cut at an oblique angle. Clearly, additional studies in normal human cornea are needed to verify the existence of this 6th layer.

Figure 1 – Human and mouse central corneal anatomy.

Five layers are present in the human cornea (A, left panel) while in the mouse cornea (A, right panel), four layers are present and the anterior limiting laminar (ALL) is absent. PLL refers to the posterior limiting laminar. Figure A is drawn to emphasize layer differences in the human and mouse cornea (not drawn to scale). The OCT image (B) shows the true difference in thickness between the human and mouse cornea. (Courtesy of Samuel Hanlon).



B.



Epithelium

The human cornea is covered by a 5-7 multilayered epithelium which contains regularly arranged, nonkeratinized squamous epithelial cells (Kenyon, 1979). The more superficial two to three layers of epithelial cells are flattened, possess tight junctions (*zonulae occludens*) that seal off the intercellular space and, form a permeability barrier for the cornea (Ban et al., 2003). Superficial cells have abundant microvilli and microplicae and this arrangement is thought to increase the surface area which enhances metabolite transportation and tear film adhesion (Dohlman, 1971; Nichols et al., 1983). The innermost basal cell layer consists of cuboidal cells which are attached to the basement membrane by hemidesmosomes. Basal cells are mitotically and metabolically active and it is within this layer that epithelial cell proliferation occurs (reviewed by (Dua et al., 1994)). Between the overlying squamous superficial epithelial cells and basal cells, are wing cells which are named because of their wing-like elongated cytoplasmic processes.

Since the superficial corneal epithelial cells are constantly shed into the tear pool by blinking, they must be continually replaced in order to maintain corneal homeostasis. In humans, stem cells reside in the Palisades of Vogt at the corneoscleral limbus (Davanger and Evensen, 1971; Dua and Azuara-Blanco, 2000; Sundmacher and Reinhard, 1998) and resultant daughter cells move centrally to replenish the loss of epithelial cells from the corneal surface. The most popular concept is the X,Y,Z hypothesis in which X represents the proliferation of basal epithelial cells, Y represents the centripetal migration of cells, and Z represents the cell loss from the epithelial surface. To maintain the equilibrium of the corneal epithelium, it requires that X+Y=Z (Thoft and Friend, 1983).

Normally, complete turnover of human corneal epithelial cells is about 1 week (Hanna et al., 1961). In mouse, corneal epithelial turnover time is approximately 1-2 weeks (Douvaras et al., 2013; Hanna and O'Brien, 1960).

Anterior Limiting Lamina (Bowman's Layer)

In the human cornea, the anterior limiting lamina (ALL) lies just beneath the epithelium and is about 8-12μm thick and contains non-bundled randomly woven 20-25nm diameter thick type I collagen fibers (Komai and Ushiki, 1991; Polack, 1961). Removal of the ALL (which also removes the epithelial basement membrane) delays subbasal nerve regeneration after epithelial abrasion, suggesting a possible role of the ALL in efficient anterior stromal wound healing and corneal epithelial innervation (Lagali et al., 2009). Removing the ALL also results in increased keratocyte density and activity (Lagali et al., 2009; Roszkowska et al., 2011), which appears under confocal imaging as an aggregation of keratocytes into regions that scatter light (Lagali et al., 2009). This observation suggests the ALL may serve as a barrier for keratocyte interactions with epithelial cells. However, in many healthy mammals, such as in rat, mouse and most carnivores, the ALL is absent, leaving only the basement membrane separating the epithelium from stroma (Smith et al., 2001). Hence, the ALL appears to be unnecessary in these animals for normal corneal function yet in humans, the congenital absence of the ALL is a pathologic feature of certain diseases such as sclerocornea, Peter's anomaly and osteogenesis imperfecta type II (Hayashi et al., 2002). In summary, more research is needed to understand the function of the ALL and why it is dispensable in so many mammalian species.

Stroma

The human corneal stroma is a 500μm thick highly organized transparent tissue, and accounts for most of the corneal thickness. The optical transparency property of the stroma is thought to be due to the uniform size and regular organization of collagen fibrils, which minimizes light scatter (Clark, 2001; Hart and Farrell, 1969; Maurice, 1957), making the stroma transparent to visible light at wavelengths greater than 400nm (Freegard, 1997). The collagen fibrils in the stroma are organized into thin flattened sheets, called lamellae. Transmission electron microscope studies suggest the mean number of lamellae across the entire thickness of the human corneal stroma is 242 (Bergmanson et al., 2005). The human corneal stroma is largely comprised of three distinct collagens. Collagen fibrils (Type I collagen) are the main lamellar component with additional contributions from type V and VI collagen (Marshall et al., 1991; Newsome et al., 1982). It has been reported that collagen fibril diameter may be regulated by type V collagen (Birk et al., 1986). In addition to the three major collagen types, other minor collagen types are known to be present in the cornea (e.g., Types III, IV, VII, VIII, XII, XIII, XVII, XVIII, and XX) (reviewed by(Ihanamaki et al., 2004)). Between the collagen fibrils, the interfibrillar space is occupied by glycosaminoglycans (GAGs) that are attached to core proteins to form two types of proteoglycans (keratan sulphate and dermatan sulphate). Proteoglycans are negatively charged and through repulsive forces and the ability to attract sodium ions, they imbide water which exerts a swelling pressure that keeps individual collagen fibrils from aggregating with one another; aggregation would lead to light scatter and loss of corneal transparency (Hedblom, 1961).

Keratocytes are the major cell type in the corneal stroma and they are located between the collagen lamellae in the interlamellar space. Connected to one another by gap junctions (Hasty and Hay, 1977), they form a cellular network and are responsible for synthesis and maintenance of collagen fibrils and proteoglycans. Keratocytes also contain "corneal crystallins" such as aldehyde dehydrogenase 3 and transketolase which are believed to maintain cellular transparency and optimize refraction within the stroma (Jester, 2008). Furthermore, following corneal injury, the keratocyte network may function as a "cellular highway" for neutrophil stromal migration (Gagen et al., 2010; Poole et al., 2003). As well, because of their remarkable phagocytic abilities, keratocytes may serve an important role in the normal turnover of the corneal stroma extracellular matrix during injury or infection (Lande et al., 1981).

Posterior limting lamina (Descemet's membrane)

The posterior limiting lamina (PLL) is a 8-12μm thick acellular layer beneath the stroma which is an extension of the endothelial basement membrane (Day, 1950; Wislocki, 1952). It's well accepted that the PLL is generated by endothelial cells and can be regenerated after injury (Cogan and Kuwabara, 1971; Jakus, 1956). Type IV collagen is the basement membrane-specific collagen that is found in the PLL (Newsome et al., 1981; Sundarraj and Willson, 1982). Proteins such as laminin and fibronectin are also found in this layer (Millin et al., 1986; Newsome et al., 1981). The PLL is an unusually thick basement membrane (thickens with age) which functions in support, filtration and compartmentalization of the cornea (Kenney et al., 1979). The most common disease that is associated with a PLL abnormality is Fuch's corneal dystrophy, which is characterized

by extracellular collagenous deposits on the posterior aspect of the PLL that results in a thickened PLL and a dysfunctional endothelium with premature corneal endothelial cell degeneration and apoptosis that leads to progressive stromal edema and blindness (Borderie et al., 2000; Klintworth, 2009; Szentmary et al., 2005).

Endothelium

The corneal endothelium is the innermost single layer and consists of interdigitating hexagonal cells that line the posterior limiting lamina. This endothelial layer is continuous with the endothelium of the trabecular meshwork and forms a critical interface between the stroma and anterior chamber. The endothelial cells communicate with one another via gap junctions while incomplete tight junctions (macula occludens) comprise a leaky barrier. Hydrophilic GAGs in the stroma are negatively charged and attract sodium ions and water. This "negative swelling pressure" is responsible for fluid leaking into the stroma and the leak rate is controlled by the endothelial tight junctions. To counteract the leak, the endothelium works as a fluid pump and drives excess fluid from the stroma into the anterior chamber. This Pump-Leak hypothesis is thought to be the mechanism which allows the corneal stroma to maintain an ideal state of hydration (76%) that preserves corneal transparency and prevents corneal edema. Due to the leaky property of the endothelial barrier, nutrients can also flow from aqueous humour into cornea (Edelhauser, 2006; Riley, 1985; Srinivas, 2010). Corneal endothelial cell density decreases with age. For humans, the corneal endothelial cell density is 2577±134 cells/mm^2 in aged eyes, which is significant lower than the 3233±113 cells/mm^2 in young eyes (Sanchis-Gimeno et al., 2005). The highest endothelial cell density occurs just after

birth and in infancy at around 4200 cells/mm^2 (Muller et al., 2000). Cell density decreases rapidly before age 18 and then slows to a loss rate of about 0.6% per year (Bourne and McLaren, 2004). Since human corneal endothelial cells are not able to regenerate, they enlarge and change shape to accommodate for loss. However, if the corneal endothelial cell density drops below 1000 cells/mm^2, corneal stromal edema and bullous keratopathy will occur (Wilson and Roper-Hall, 1982). Since the hydration status of the stroma is directly related to the pump-leak rate of aqueous humor across the endothelium, wounding or damage to the corneal endothelium impairs the fluid pump which makes it difficult to reverse the inward fluid transport and the cornea becomes edematous and visual acuity is lost (Yee et al., 1985).

1.1.3 Antigen Presenting Cells (APCs) in the Cornea

Besides the cells mentioned above, the cornea has bone marrow-derived cells which function as antigen presenting cells (APCs). Two functional APC groups are identified as "professional" and "nonprofessional" APCs. Professional APCs have high expression of major histocompatibility complex (MHC) class II antigen hence high T cell stimulatory capacity. Conversely, nonprofessional APCs express low levels of MHC class II antigen thus low T cell stimulatory capacity (Banchereau et al., 2000). Although the central corneal bone marrow-derived cells such as dendritic cells are MHC class II negative (Hamrah et al., 2003b), nearly half of peripheral cornea and limbal dendritic cells do express MHC class II (Hamrah et al., 2003a) and, during inflammation, MHC class II antigen expression is markedly upregulated in the center of the cornea (Hamrah et al., 2003a; Hamrah et al., 2002).

Damage to the ocular surface provides an opportunity for invading microrganisms to gain access to the corneal stroma. While the initial injury triggers an innate immune response (i.e., acute inflammation), resident APCs are able to sample the inflamed tissue, detect and ingest foreign organisms (bacteria, fungi, and viruses) or their breakdown products, leave the cornea and migrate to the lymph nodes where they can initiate an immune response by presenting antigen to T-cells within the lymph nodes. In evidence is the observation of corneal resident bone marrow-derived cells expressing green fluorescent protein migrating into lymph nodes during inflammation, consistent with the hypothesized function of these resident cells as APCs (Liu et al., 2002).

Langerhans cells belong to the dendritic family and function as APCs at the ocular surface. In the past, Langerhans cells were thought to be localized to the conjunctiva and corneal limbus (Gillette et al., 1982; Rodrigues et al., 1981). Although Langerhans cells are normally not detected in the central cornea, after injury or infection, large numbers are seen infiltrating all regions of the corneal epithelium (Lewkowicz-Moss et al., 1987; Miller et al., 1993). However, a significant number of MHC class II negative Langerhans cells are found in the central normal uninflamed corneal epithelium. These Langerhans cells express activation markers (MHC class II, CD80 and CD86 costimulatory markers) during inflammation and activated Langerhans cells are thought to be important for a wide range of immune-inflammatory responses such as graft rejection and herpetic keratitis(Hamrah et al., 2002).

Dendritic cells are a heterogeneous group of leukocytes derived from a myeloid lineage, also working as APCs in the cornea. A large number of immature dendritic cells occupy the central cornea while more mature dendritic cells are found in the peripheral

cornea (Hamrah et al., 2003a; Hamrah et al., 2003b). Immature dendritic cells (MHC class II low or negative) show a high capacity for antigen capature but a low ability to stimulate T cells. Conversely, mature dendritic cells (MHC class II positive) are poor in antigen capture but have an enhanced ability for T cell stimulation (Banchereau et al., 2000; Banchereau and Steinman, 1998). Dendritic cells are able to infiltrate into the wounded cornea and play important multifaceted roles during inflammation. Depletion of dendritic cells delays corneal wound closure and reduces inflammatory genes IL-1β, CXCL10, and TSLP expression in corneal epithelium (Gao et al., 2011; Ma et al., 2009). Dendritic cell infiltration is regulated by ICAM-1, IFN-ɣ and natural killer (NK) cells. ICAM-1-/- mice show markedly reduced dendritic cell recovery after corneal epithelial wounding. Topical application of anti-IFN-ɣ to the wounded cornea results in significant lowered dendritic cell recovery. Depletion of NK cells results in reduced dendritic cell recovery during wound healing, Mice with low dendritic cell numbers show delayed epithelial wound healing compare to control mice, which suggests dendritic cells are necessary for efficient corneal epithelial wound healing(Gao et al., 2013).

1.1.4 Corneal Nerve

The cornea is one of the most densely innervated tissues in the body that is supplied by sensory and sympathetic nerves. Most of the sensory nerves are derived from the ophthalmic branch of the trigeminal nerve while some inferior nerves may originate from the maxillary branch of the trigeminal nerve (Ruskell, 1974). The sympathetic nerves branch from the superior cervical ganglion (Marfurt and Ellis, 1993) and their density is significantly lower than the sensory nerves in the cornea (Marfurt et al., 1989; Tervo and Palkama, 1978; Toivanen et al., 1987). The nerves enter the corneal stroma form the

periphery and extend as bundles running parallel to the cornea surface. After entering the stroma, the nerves rapidly lose their myelin sheath which is thought to help in the maintenance of corneal transparency. In humans, thick peripheral nerve trunks derived from the trigeminal nerve make their way through the stroma toward the center and give off perpendicular branches that are oriented toward the corneal surface. After penetrating the anterior limiting lamina, the perpendicular nerve fibers form a subepithelial plexus which runs parallel to the corneal surface. Axons from the sub-epithelial plexus penetrate the epithelial basal lamina and give rise to the sub-basal nerve plexus. Branches from the sub-basal nerve plexus then run vertically into the epithelium extending and branching between epithelial cells and eventually terminating at the superficial epithelial cells (Muller et al., 2003).

The corneal sensory nerves produce the sensation of pain in response to irritation and this induces reflex tearing to protect the cornea. In addition to serving a sensory role, corneal epithelial nerves play a critical role in the maintenance of corneal epithelial integrity and, following epithelial injury, they are able to promote wound healing through the release of substance P to stimulate corneal epithelial cell growth and migration (Nagano et al., 2003; Nakamura et al., 2003; Nakamura et al., 1997; Reid et al., 1993). Corneal sensory nerves express a variety of neuropeptides including calcitonin gene-related peptide (Jones and Marfurt, 1991; Marfurt and Echtenkamp, 1995), pituitary adenylate cyclase-activating peptide (Elsas et al., 1996; Wang et al., 1995) and galanin (Grimes et al., 1994), which are thought to enhance epithelial cell migration, adhesion and/or proliferation (Baker et al., 1993; Marfurt et al., 2001; Wang et al., 1995).

The sympathetic nerves produce various neurochemicals like norepinephirine, serotonin and neuropeptide Y which provide a neurotrophic effect to support epithelial integrity. For example, several studies show norepinephirine and neuropeptide Y promote corneal epithelial cell proliferation and migration (Jones and Marfurt, 1996; Murphy et al., 1998; Shigeri and Fujimoto, 1993), while topical application of serotonin on wounded corneas promotes epithelial proliferation (Kwitko et al., 1992; Osborne and Tobin, 1987).

Damage to the corneal epithelium will also result in damage to the epithelial nerves. Hence, following injury, regeneration of both the epithelium and its associated nerves is vital to the restoration of corneal integrity and function. In the clinic, patients can present with accidental corneal injuries, corneal disease (e.g., keratitis), or surgical trauma in which corneal denervation is evident. This is often associated with increased epithelial permeability, decreased epithelial proliferation, and poor wound healing (Araki et al., 1994; Baker et al., 1993).

In humans and mice, corneal nerve density changes with age. Nerve density increases with age during the embryonic period in mice (McKenna and Lwigale, 2011), continues to increase after birth and then decreases markedly by an average of 53.9% after 8 weeks of age (Wang et al., 2012). In humans, nerve density increases after birth and then declines with age with a mean decline of 0.25% to 0.3% per year (Grupcheva et al., 2002; Parissi et al., 2013).

We focus on corneal epithelial wound healing study in mice, so it's important to elaborate the progress of mouse corneal nerve regeneration. During corneal development, the corneal nerve terminal density reaches the highest at 24 days after birth (9.9±1.4 mm/mm^2), while the maximum corneal nerve terminal number peaks at 60 days after birth

(32.6±6.8 mm) (Wang et al., 2012). However, after corneal epithelial wounding, even though corneal epithelium fully closes around 24 hours, corneal nerve density does not reach its baseline. After corneal epithelial abrasion, two waves of neutrophil infiltration peak at 12 to 18 hours and 30 to 36 hours (Li et al., 2006b), with corresponding accumulations of platelets. These leukocytes release VEGF which promotes corneal nerve regeneration (Li et al., 2011a). Depletion of neutrophils reduces VEGF production by 70% and results in more than 80% reduction in corneal nerve regeneration, while platelet depletion leads to 62% reduction in epithelial branches and 57% reduction in subbasal nerve regeneration (Li et al., 2011a). Nerve growth factor (NGF) shows neuroprotective function in both central and peripheral neuron maintenance (Sofroniew et al., 2001). Murine and human corneal epithelial cells, keratocytes, and endothelial cells secrete NGF which contributes to corneal epithelial wound closure (Lambiase et al., 2000) and nerve regeneration (Esquenazi et al., 2005). Corneal epithelial wounding increases NGF production which contributes to wound healing while topical application of anti-NGF significantly delays wound healing (Lambiase et al., 2000). Oncomodulin is another growth factor expressed by macrophages and neutrophils that supports central and periphery nerve regeneration both in vitro and in vivo (Kurimoto et al., 2013; Yin et al., 2009; Yin et al., 2006). Oncomodulin stimulates axon regeneration in vitro (Yin et al., 2003) while decreased oncomodulin production or antibody blockade of oncomodulin function inhibit axon regeneration in vivo (Kurimoto et al., 2013).

1.2 Corneal Inflammation and Epithelial Wound Healing

1.2.1 Corneal Inflammation

Corneal inflammation is a complex cascade involving the infiltration of various inflammatory cells into the wounded cornea.

After a simple, non-pentrating (i.e., basal lamina is not penetrated) epithelial abrasion in the mouse, neutrophils are the first type of inflammatory cell to migrate out from limbal vessels into the anterior avascular corneal stroma (Petrescu et al., 2007; Wilson et al., 2001). Neutrophil transmigration out of limbal vessels is facilitated by specific adhesion molecules like leukocyte-specific β-2 integrin CD18 (Li et al., 2006b). Absence of CD18 or specific CD18 family members like CD11a/CD18-LFA-1 or CD11b/CD18-Mac-1, delays neutrophil emigration and results in delayed wound healing (Li et al., 2006a, b). Neutrophil emigration occurs in two waves and the first wave peaks at 12 to 18 hours while the second wave peaks at 30 to 36 hours. Neutrophil infiltration into the wounded corneal stroma is essential for efficient re-epithelialization as evidenced by experiments with neutropenic mice and mice with targeted deletions in CD18 and P-selectin which show diminished and/or delayed neutrophil recrutiment. Epithelial wound healing is restored in CD18- and P-selectin-deficient mice after adoptive transfer of wildtype neutrophils or wildtype platelets, respectively (Li et al., 2006b; Li et al., 2006c). Infiltrating neutrophils and platelets carry vascular endothelial growth factor (VEGF), a trophic factor that is prestored in neutrophil granules and platelet α-granules and is known to promote neurite out growth (Gong and Koh, 2010; Liu et al., 2010; Sondell et al., 1999; Taichman et al., 1997). VEGF levels within the cornea rise and fall during the course of the inflammatory response in a pattern that mimics the rise and fall of neutrophil

infiltration. Corneal nerve regeneration is impaired by neutrophil depletion prior to corneal wounding or by administration of anti-VEGF antibody (Li et al., 2011a).

Various studies in other tissues suggest neutrophil recruitment is associated with platelet recruitment during an inflammatory response (Nishijima et al., 2001; Nishijima et al., 2004). With respect to corneal wounding, Li and colleagues found platelets are able to extravasate from limbal vessels but unable to move into the injured corneal stroma (Li et al., 2011b). Platelet and neutrophil extravasation appears to be co-dependent as depletion of either one will significantly affect the accumulation of the other (Cooper et al., 2004; Li et al., 2006c; Nishijima et al., 2004). Platelets contain many growth factors including platelet-derived growth factor (PDGF), transforming growth factor -β (TGF-β), VEGF, epidermal growth factor (EGF), and insulin-like growth factor-1 (IGF-1) (Eppley et al., 2004; Heldin et al., 1981). PDGF enhances corneal epithelial cell migration *in vitro* (Imanishi et al., 2000) and promotes corneal epithelial wound healing *in vivo* (Tuominen et al., 2001). The binding of EGF to the corneal epithelial cell EGF receptor promotes cell proliferation both *in vitro* and *in vivo* (Watanabe et al., 1993; Wilson et al., 1992). TGF-β antagonizes the action of EGF and may function as a negative modulator of corneal epithelial cell proliferation (Honma et al., 1997). IGF-1 promotes epithelial cell migration and proliferation (Lee et al., 2006; Yanai et al., 2006). Combined administration of IGF-1 with substance-P significantly enhances corneal epithelial wound closure both *in vitro* and *in vivo* (Nakamura et al., 1997; Nishida et al., 1996).

Another important inflammatory cell that contributes to corneal inflammation and wound healing is the γδ T cell. In the mouse, the γδ T cells are a population of GL3 positive T cells (Goodman and Lefrancois, 1989) residing in the limbal epithelium and,

after corneal injury, are necessary for neutrophil infiltration, platelet localization at the limbus and NK cell accumulation. This cellular cascade positively influences epithelial and nerve recovery (Li et al., 2011a; Li et al., 2007). Intercellular adhension molecule-1 (ICAM-1) is a five domain transmembrane glycoprotein that serves as a ligand for CD11a/CD18 and CD11b/CD18 (Brake et al., 2006; Huang and Springer, 1995; Ley, 1996; Roebuck and Finnegan, 1999). Byeseda et al. showed γδ T cell migration into the wounded cornea requires ICAM-1 (Byeseda et al., 2009). Mice deficient in the γδ T cell receptor show reduced platelet localization at the limbus, reduced neutrophil infiltration into stroma, and delayed epithelial wound healing (i.e., fewer dividing epithelial cells and delayed wound closure) (Li et al., 2007). A critical mechanism by which γδ T cells regulate the inflammatory response is through the release of IL-22 which stimulates the epithelium to secrete a CXCL1 chemokine which in turn promotes neutrophil recruitment, thereby positively influencing wound closure (Li et al., 2011b).

After epithelial abrasion, NK cells migrate into the corneal stroma, peak at 24 to 30 hours after injury and appear to limit neutrophil infiltration into the injured cornea. Depletion of NK cells or antibody blockade of NK cell receptor NKG2D significantly increases neutrophil infiltration and results in delayed epithelial wound closure and nerve regeneration. However, neutrophil depletion does not influence NK cell accumulation. Adoptive transfer of NK cells into NK-depleted wildtype mice reduced neutrophil accumulation and restored epithelial cell division and nerve recovery. NK cells likely limit neutrophil infiltration by killing neutrophils expressing the NKG2D ligand. Thus NK cells appear to prevent excessive neutrophil infiltration while maintaining the beneficial effects that neutrophils bring to the injured cornea ((Liu et al., 2012).

1.2.2 Corneal Epithelial Wound Healing

The process of corneal epithelial wound healing can be divided into three different phases: cell migration, cell proliferation and cell adhesion (Dua et al., 1994).

Cell migration

The epithelial cell migration period is composed of two phases: the latent phase and the linear healing phase. In the latent phase, the epithelial wound may become slightly larger in 4 to 6 hours after epithelial injury due to sloughing of necrotic cells and the rearrangement of epithelial cells at the wound edge. The latent phase is named for the time interval between wounding and the onset of cell migration. This phase also includes desquamation of superficial cells, loss of the columnar shape of basal cells, and breakdown of hemidesmosomes between basal cells and the basement membrane which results in epithelial thinning at the wound edge. The epithelium develops a provisional attachment complex (focal contacts) that allows for cell movement (Crosson et al., 1986; Dua et al., 1994). In the linear healing phase, approximately 8 to 30 hours after wouding, the epithelial cells flatten, spread, and move as an intact sheet across the wounded area until the injured area is fully covered. The provisional focal contacts are selectively degraded by plasmin in such a way as to allow cell release from the basement membrane, pseudopod extension and formation of new focal contacts. The process repeats until migrating cells cover the wound. The average cell migration speed is constant (64±2μm/hour) and independent of wound size. The cell migration process is not dependent on cell proliferation (Crosson et al., 1986).

Cell proliferation

In the cell proliferation period, limbal basal epithelial cells serve as a reservoir of stem cells for corneal epithelial cell replacement and regeneration (Hanna and O'Brien, 1960). These cells have great potential for cell division and show increased proliferation after corneal injury (Cotsarelis et al., 1989). Besides renewing the stem cell population, stem cell mitosis gives rise to rapidly dividing transient amplifying cells (TACs) which have a limited capacity to divide. The TACs (basal cells) differentiate into post-mitotic cells and terminally differentiated cells which correspond to epithelial suprabasal cells (Schermer et al., 1986; Tseng, 1989).

Cell adhension

The cell adhesion period requires the newly regenerated corneal epithelium to firmly attach to basement membrane. After wounding, migrating cells show increased filipodial extensions rich in fimbrin and α-actin. Cell surface receptors interact with extracellular protein deposits containing fibronectin and fibrin which stimulate epithelial cells to release plasminogen activator which can convert plasminogen to plasmin, lysing temporary cell adhesions which facilitates cell migration and wound closure (reviewed by (Dua et al., 1994)). Hemidesmosomes reform to strengthen the attachment of the corneal epithelium to the basement membrane. If the basement membrane is intact, hemidesmosomes develop within a week. Otherwise, the time required for resynthesis of the basement membrane can delay firm adhesion by 6 weeks (Khodadoust et al., 1968).

1.3 Corneal Macrophages

Macrophages are known to reside within both human (Mayer et al., 2007; Yamagami et al., 2006) and mouse cornea (Brissette-Storkus et al., 2002; Hamrah et al., 2003a; Nakamura et al., 2005; Sosnova et al., 2005). After corneal epithelial injury, the ensuing inflammatory response is necessary for efficient wound healing. While beneficial healing effects are clearly attributed to recruited neutrophils and platelets for epithelial wound closure and nerve recovery (Li et al., 2011a; Li et al., 2006b; Li et al., 2006c), the contribution of macrophages to corneal wound healing has not been fully investigated.

Macrophages are large mononuclear phagocytic cells first described by Elie Metchnikoff in the 1880s. In 1908, Elie Metchnikoff was awarded the Nobel Prize for his hard work and insight into cellular phagocytosis (Kaufmann, 2008). Macrophages are generated from a common myeloid progenitor that is shared with neutrophils in the bone marrow and are released into the circulation as monocytes. Monocytes represent 10% of leukocytes in human blood and 4% of leukocytes in mouse blood (Auffray et al., 2009). After spending a few days in the circulation, monocytes migrate from the bloodstream into tissues throughout the body where they differentiate into resident macrophages. Half of the monocytes are stored in clusters in the cords of the subcapsular red pulp in the spleen, where they serve as a storage reservoir for immature monocytes. During inflammation or injury, splenic monocytes become activated and are released into the circulation as evidenced by the appearance of increased blood monocytes and a decrease in splenic monocytes. After recruitment to a site of injury or infection, monocytes differentiate into macrophages and participate in inflammation and wound healing (Swirski et al., 2009).

The morphology of macrophages varies among different tissues making it difficult to identify macrophages only by their appearance. In different tissues, macrophages carry specific names as summarized Table 1 (Hashimoto et al., 2011; Weidenbusch and Anders, 2012).

Table 1. Macrophages in different body tissues.

Tissue Name	Macrophage Name
Bone	Osteoclast
Central nervous system	Microglia
Connective tissue	Histiocyte
Spleen	Red pulp macrophage; Marginal zone macrophage; Marginal zone metallophilic macrophage; White pulp macrophage.
Chirion villi of the placenta	Hofbauer cell
Lymph node	Subcapsular sinus macrophage; Medullar macrophage; CD11chiCD169+ macrophage; Tingible body macrophage.
Thymus	Subcapsular macrophage; Cortex macrophage; Cortico-medullary macrophage; Medulla macrophage.
Liver	Kupffer cell
Lung	Alveolar macrophage; Interstitial macrophage.
Stomach, ileum, colon	Intestinal macrophage
Peritoneal cavitiy	Peritoneal macrophage
Gut	Lamina propria macrophage; Serosal macrophage.
Skin	Epidermal macrophage; Dermal macrophage
Kidney	Mesangial cell

Macrophages are classified by Metchnikoff as phagocytes which is a combination of the Greek words ‘‘phago’’ (devour) and ‘‘cytes’’ (cells) and phagocytes are well known to play an important role in host resistance to bacterial infections. The committed bone marrow macrophage precursors along with circulating blood monocytes and tissue macrophages comprise a family of cells that is defined as the mononuclear phagocyte system (Hume, 2006). Macrophages also work as antigen presenting cells in the body which is important both in innate immune defense as well as in the regulation of adaptive immunity. In mice, direct injection of macrophages isolated from bone marrow shows these cells are able to migrate into lymph nodes and activate CD8 T cell proliferation (Pozzi et al., 2005). After treating macrophages with IFNɣ to induce MHC class II expression and antigen recognition, the injected primed macrophages can be recognized by T cells and they elicit a Th1-polarized primary immune responses in mice (Desmedt et al., 1998). T lymphocytes expressing the surface molecule CD4 are known as helper T cells, which can be divided into Th1 and Th2 subtypes (Mosmann and Coffman, 1989). Th1 cells are important in cell mediated immunity and release Th-1 cytokines IL-2, IFN-ɣ for example (Grewe et al., 1998), which produce pro-inflammatory responses. Th2 cells are important regulators of the humoral immune response and release Th-2 cytokines like IL-4, IL-5, IL-10 (Grewe et al., 1998), to trigger anti-inflammatory responses. However, Th2 responses counteract Th1 responses to maintain a balance within the body since an excessive pro-inflammatory response will lead to tissue damage (Berger, 2000).

Besides their phagocytic and antigen presenting functions, macrophages are known to play a central role during all phases of wound healing through the release of inflammatory cytokines and growth factors (Guo and Dipietro, 2010; Kharraz et al.,

2013). In most tissues, immediately after injury, the innate immune system is activated and inflammatory cells are recruited from the circulation to the wound area. Circulating monocytes also enter the wound area and differentiate into mature macrophages that contribute to wound healing (Ross and Odland, 1968) and, along with local resident macrophages, are activated by proinflammatory mediators (Zhang and Mosser, 2008). Macrophages are able to phagocytose apoptotic cells and protect tissue from exposure to the toxic contents that are released by these apoptotic cells which may cause secondary necrosis during normal tissue repair (Erwig and Henson, 2008; Fadok, 1999; Khanna et al., 2010). Macrophages can assist in the removal of excessive neutrophils (Eming et al., 2007; Savill et al., 1989; Silva, 2011) in the wound area and this serves to limit neutrophil-mediated tissue damage caused by neutrophils releasing proteolytic enzymes like elastase and cathepsin G which may destroy normal tissue and negatively influence wound repair (Briggaman et al., 1984; Dovi et al., 2003; Dovi et al., 2004).

Macrophages promote tissue wound healing through the generation of various inflammatory cytokines and growth factors that modulate extracellular matrix content and remodeling. The inflammatory cytokines that are produced by macrophages include IL-1, IL-6 and tumor necrosis factor-alpha (TNF-α) while the growth factors are fibroblast growth factor (FGF), epidermal growth factor (EGF), transforming growth factor-beta (TGF-β) and platelet-derived growth factor (PDGF) (Barrientos et al., 2008; Finnerty et al., 2006; Rappolee et al., 1988). Cytokines IL-1, IL-6 and TNF-α all contribute to inflammation and re-epithelialization in skin (Gallucci et al., 2004; Mast and Schultz, 1996; Tang and Gilchrest, 1996). In skin, EGF promotes keratinocyte proliferation in response to re-epithelialization (Jiang et al., 1993). FGF, TGF-β and PDGF contribute to

inflammation, re-epithelialization, matrix formation and tissue remodeling (Lin et al., 2006; Riedel et al., 2007; Tsunawaki et al., 1988; Uutela et al., 2004). In wildtype mice depleted of macrophages by clodronate liposomes or in mutant mice depleted of CD11c+ cells using diphtheria-toxin injection, defects in re-epithelialization, granulation tissue formation, angiogenesis, wound associated contraction, and cytokine production are observed (Goren et al., 2009; Lucas et al., 2010; Mirza et al., 2009; Ren et al., 2008). In aging and diabetic patients and diabetic mice, macrophage dysfunction impairs resolution of inflammation and wound healing (Khanna et al., 2010; Swift et al., 2001).

1.4 IL-20 and the IL-10 superfamily

In skin, macrophages appear to facilitate wound healing through the release of IL-20, a cytokine which induces keratinocyte proliferation and differentiation (Blumberg et al., 2001; Roupe et al., 2010). IL-20 belongs to the IL-10 cytokine super family which includes IL-10, IL-19, IL-20, IL-22, IL-24 and IL-26 (Blumberg et al., 2001; Pestka et al., 2004). IL-20 contains 176 amino acids and the amino acid sequence is 76% identical in human and mouse (Blumberg et al., 2001). IL-22 is another member of IL-10 family. Previous studies from our laboratory determined that γδ T cells release IL-22 which stimulates the epithelium to produce the CXCL1 chemokine which in turn promotes neutrophil recruitment, thereby positively influencing wound closure. In mutant mice lacking γδ T cells, IL-22 production is markedly reduced and epithelial healing is impaired and addition of rIL-22 restores epithelial wound healing (Li et al., 2011b). Receptors for IL-20 and IL-22 exist as heterodimers and IL-20 binds IL-20R1/IL-20R2 and IL-22R1/IL-20R2 while IL-22 binds IL-22R1/IL-10R2. Hence, the IL-22R1 subunit is

shared by IL-20 and IL-22 (Logsdon et al., 2012). Since the mouse corneal epithelium shows positive staining for IL-22R1 (Li et al., 2011b), the possibility is raised that the binding of macrophage-derived IL-20 to corneal epithelium may influence wound healing.

Among the various neurotrophic factors that macrophages secrete, oncomodulin appears to be the only one that stimulates retinal ganglion cell axon outgrowth (Yin et al., 2003). Oncomodulin was originally thought to be expressed only in fetal and cancerous tissues. However, it is now clear that oncomodulin is part of the parvalbumin family. As such, it is a calcium binding protein now known to be expressed in adult tissues beginning with its discovery in the outer hair cells in the organ of Corti in a variety of adult vertebrates (Sakaguchi et al., 1998). Recently, macrophage- and neutrophil-derived oncomodulin has been reported to support central and peripheral nerve regeneration both in vitro and in vivo (Kurimoto et al., 2013; Yin et al., 2009; Yin et al., 2006). Adding activated macrophages (more oncomodulin production) to cultured medium of retinal ganglion cells stimulates axon regeneration (Yin et al., 2003). While in vivo, reducing oncomodulin levels via neutrophil depletion or blocking oncomodulin function with a peptide antagonist P1 significantly diminished axon regeneration in the rat retina (Kurimoto et al., 2013). Nerve degeneration in the CNS is associated with disruptions in Ca2+ homeostasis. Oncomodulin is a calcium binding protein and mice over-expressing parvalbumin (oncomodlin) exhibit neuroprotection (Dekkers et al., 2004). Hence, in the cornea, oncomodlin may protect injured nerves by seving as a buffer against damaging increases in Ca2+ influx. Since the cornea possesses abundant macrophages before and after injury, whether corneal macrophages release oncomodulin and contribute to corneal epithelial wound healing remains to be known.

In the past, macrophages were thought to be absent from the normal avascular cornea given the high success rate with human corneal transplants attributable to the understanding that the cornea was an immune-privileged site (Niederkorn, 1999). Later, a significant number of macrophages were shown to reside within the corneal stroma. Brissette-Storkus et al. examined BALB/c mouse corneal macrophages and found 200-300 macrophages/ mm^2 throughout the stroma with the highest density of macrophages observed in the anterior and posterior thirds of the stroma (Brissette-Storkus et al., 2002). Hamrah et al. found a population of CD11c-CD11b+ cells in the posterior corneal stroma in BALB/c mice which were thought to be macrophages based on cell morphology. Chinnery et al. calculated the baseline density and distribution of macrophages in the corneal stroma of CX3CR1 GFP heterozygous transgenic mice on a C57BL/6 background. In this mutant mouse, all monocyte derived cells express enhanced green fluorescent protein (GFP). In total, 366 macrophages /mm^2 were found evenly distributed through the corneal stroma but with significantly fewer macrophages being found in the paracentral cornea (Chinnery et al., 2008). Nakamura et al. reported that GFP labeled bone marrow derived cells can migrate into the cornea. The immunohistochemical experiments showed some of the migrating GFP+ cells were CD11b+ which was consistent with a macrophage phenotype (Nakamura et al., 2005).

Even though many lines of evidence support the existence of macrophages in the normal cornea, the number of macrophages and their function during corneal wound healing is still not clear. Hamrah and Nakamura used immunostaining to confirm CD11b+ cells are macrophages, but actually CD11b is not a specific marker for macrophages. CD11b also labels dendritic cells and granulocytes. Though Chinnery and Nakamura used

GFP tagged bone marrow-derived cells to show proof of macrophage existence and migration into corneal stroma, one should keep in mind that GFP is likely to be present in all bone marrow-derived leukocytes since CX3CR1 is expressed in all cells during leukocyte development (Corcione et al., 2009; Imai et al., 1997). Thus the number of calculated macrophages would be exaggerated especially after wounding since large numbers of GFP-expressing neutrophils are likely to enter the injured cornea.

Even though macrophages are known to reside within the mouse cornea, nothing is known regarding the distribution of macrophage phenotypes and how macrophages respond to corneal injury with respect to wound healing. My research centers on the corneal macrophages and my working hypothesis is that efficient corneal epithelial wound healing following epithelial abrasion is dependent on macrophages and their derived products, IL-20 and oncomodulin. The rationale supporting this hypothesis is that IL-20 and oncomodulin are reported to contribute to keratinocyte proliferation/differentiation and retinal ganglion cell axon regeneration, respectively. To test the hypothesis, my research is divided into three specific aims:

Aim 1: To characterize macrophages in normal and wounded mouse corneas.

Aim 2: To determine if macrophage-derived IL-20 plays a beneficial role in corneal wound healing.

Aim 3: To determine if oncomodulin plays a beneficial role in corneal wound healing.

Chapter 2- Aim 1: To characterize macrophages in normal and wounded mouse corneas

2.1 Introduction

Early on, macrophages were recognized as phagocytic cells for pathogen elimination and housekeeping functions. Later, macrophages were shown to function as antigen presenting cells in innate and adaptive immunity. In other tissues, macrophages are broadly divided into pro-inflammatory M1 (classically-activated) and anti-inflammatory M2 (alternatively-activated) phenotypes which exert pro- and anti-inflammatory effects on wound healing, respectively (Gordon, 2003; Martinez et al., 2009). The classical activation of macrophages, named for anti-microbial macrophages, release proinflammatory mediators and respond to tissue injury while other macrophages, named for alternatively-activated macrophages, express functional alternative activation markers and downregulate inflammation and promote tissue repair (reviewed by(Laskin, 2009)). Macrophages exhibit significant functional plasticity that is determined by environmental stimuli and this plasticity exists as a continuum in which M1 and M2 macrophages occupy the two extremes. In this continuum, the M1 extreme represents the pro-inflammatory state while the M2 extreme represents the anti-inflammatory state (Gordon and Taylor, 2005; Mosser and Edwards, 2008). This classification of M1 and M2 macrophages provides an important tool for understanding how macrophage polarization regulates the informatory process.

Macrophages that are stimulated by molecules released from activated T helper 1 type lymphocytes or NK cells, in particular IFN-ɣ, bacterial lipopolysaccharide, and interleukins like IL-12 and IL-18 tend to polarize macrophages to the M1 phenotype. M1

macrophages produce pro-inflammatory cytokines like IL-1β, TNF-α and monocyte chemoattractant protein-1 (MCP-1) which enhance microbicidal capacity, strengthen the cell mediated adaptive immunity and benefit the host (Benoit et al., 2008; Gordon, 2003; Gordon and Taylor, 2005). However, excessive or prolonged M1 polarization can cause tissue injury and host organ failure (Bozza et al., 2007; Lopez-Bojorquez et al., 2004; Mehta et al., 2004). In contrast, M2 macrophages are involved in the resolution of inflammation. Cytokines that are produced by T helper 2 type lymphocytes such as IL-4 and IL-13 stimulate alternative activation of macrophages to M2 phenotype and contribute to immunity against parasites, wound healing and tissue remodeling (Gordon, 2003; Martinez et al., 2009). M2 macrophages are further classified into at least 3 subcategories, M2a, M2b and M2c. Different subtypes express different subsets of M2 marker genes with distinct functions (Mosser and Edwards, 2008). Macrophages that are stimulated by IL-4 and IL-13 differentiate into M2a; Immunoglobulin complexes that combine with a Toll-like receptor ligand stimulate macrophages to turn into M2b; While glucocorticoid hormones, IL-10, and TGF-β are able to polarize macrophage to M2c (Mantovani et al., 2004; Martinez et al., 2008; Mosser and Edwards, 2008).

A significant number of macrophages are known to reside within the mouse cornea (Brissette-Storkus et al., 2002; Hamrah et al., 2003a; Nakamura et al., 2005; Sosnova et al., 2005). Yet little is known regarding the relative distribution of macrophage phenotypes within the cornea and whether macrophages contribute to the inflammatory cascade that is so important for corneal wound healing. Given that leukocyte infiltration (e.g., neutrophils and platelets) can be beneficial to corneal wound healing while dysregulation of leukocyte infiltration impairs the healing process, and that the cornea

contains a resident population of macrophages which may be capable of influencing wound healing, the characterization of the macrophage response in the injured cornea to wound healing is potentially important. The working hypothesis for Aim 1 is that corneal macrophages can be characterized as M1 and M2 phenotypes and their number and distribution within the cornea is expected to change after epithelial abrasion.

2.2 Materials and Methods

Animal model

Studies were carried out at Baylor College of Medicine (Houston, TX) and all mice for use were 8-12 weeks old and were treated following the guidelines in the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. We used female mice in all our experiments because the inflammatory and wound healing pattern is different between male and female mice. We would like to keep consistence with our published data which were collected from female mice (Byeseda et al., 2009; Li et al., 2011b; Liu et al., 2012). Twenty female C57BL/6J wildtype mice were purchased from Jackson Laboratory (Bar Harbor, ME). The central corneal wound was made following an established protocol (Li et al., 2006b; Li et al., 2006c). Briefly, mice were anesthetized by intraperitoneally injecting pentobarbital sodium solution (50mg/kg) and a 2mm diameter central corneal epithelial region was marked by a trephine and mechanically debrided with a golf club spud under a dissecting microscope. This is a wound that does not penetrate the epithelial basement membrane (Figure 2).

Immunohistology

After injury (24, 48, 72h and 7days), wildtype mice were sacrificed. Dissected corneas (including limbus) were fixed for 30 mins in phosphate buffered saline (PBS, pH 7.2) containing 2% paraformaldehyde. Fixed corneas were washed in PBS 3×5 mins each and then permeabilized with 0.1% Triton X-100 for 30 mins. To block nonspecific binding, corneas were incubated with anti-Fc receptor mAb (CD16/32; BD Pharmingen, San Jose, CA) and 2% bovine serum albumin (BSA) at room temperature for 30 minutes. Then corneas were incubated with fluorescent-labeled antibodies raised against mouse macrophage markers. The antibodies used to identify, localize and enumerate macrophages within corneal wholemounts were: phycoerythrin (PE)-conjugated anti-F4/80 (AbD Serotec, Raleigh, NC); Alexa fluor 488-conjugated anti-CD301(AbD Serotec, Raleigh, NC); allophycocyanin (APC)-conjugated anti-CD115 (eBioscience); PE-conjugated anti-CD206 (AbD serotec, Raleigh, NC); purified anti-CX3CR1 (R&D systems); alexa fluor 647-conjugated anti-CD80 (AbD Serotec, Raleigh, NC) and PE-conjugated anti-CD86 (eBioscience, San Diego, CA). Other antibodies used were: PE or APC-conjugated anti-CD31 (BD Pharmingen, San Jose, CA) and FITC-avidin (Invitrogen, Grand Island, NY). Non-immune isotype matched fluorochrome matched antibodies served as controls for non-specific staining. Unwounded corneas were used for comparison. Immunostained corneas were imaged as wholemounts using a 40X oil immersion lens mounted on a DeltaVison Core Spectris microscope system (Applied Precision, Issequah, WA).

Macrophage cell counts

To enumerate the different macrophage phenotypes within the cornea, whole mounts were sub-divided into five regions: (1) limbus (L), (2) paralimbus (PL), (3) parawound (PW), (4) wound (W) and (5) wound center (WC). For L, PL, PW, and W, four camera fields (40X, 150×150μm) were recorded according to the 3, 6, 9, and 12 o'clock pattern diagramed in Figure 3; for WC, four non-overlapping images were also recorded from within the defined region. Cells staining positively for the various macrophage markers were counted and the average number of macrophage phenotypes was determined for each region.

To compare macrophage numbers before and 24h after wounding, four camera fields (40X, 150×150μm) from the limbus were recorded at the 3, 6, 9, and 12 o'clock positions. Cells staining positively for CD301 and/or F4/80 were counted and the average number of CD301+ and/or F4/80+ cells was determined.

Statistical analysis

Data were analyzed using a student's t-test or a two-way analysis of variance (ANOVA) with a Tukey post-test for pair-wise multiple comparisions. Data are expressed as means±SEM and P values ≤ 0.05 were considered statistically significant.

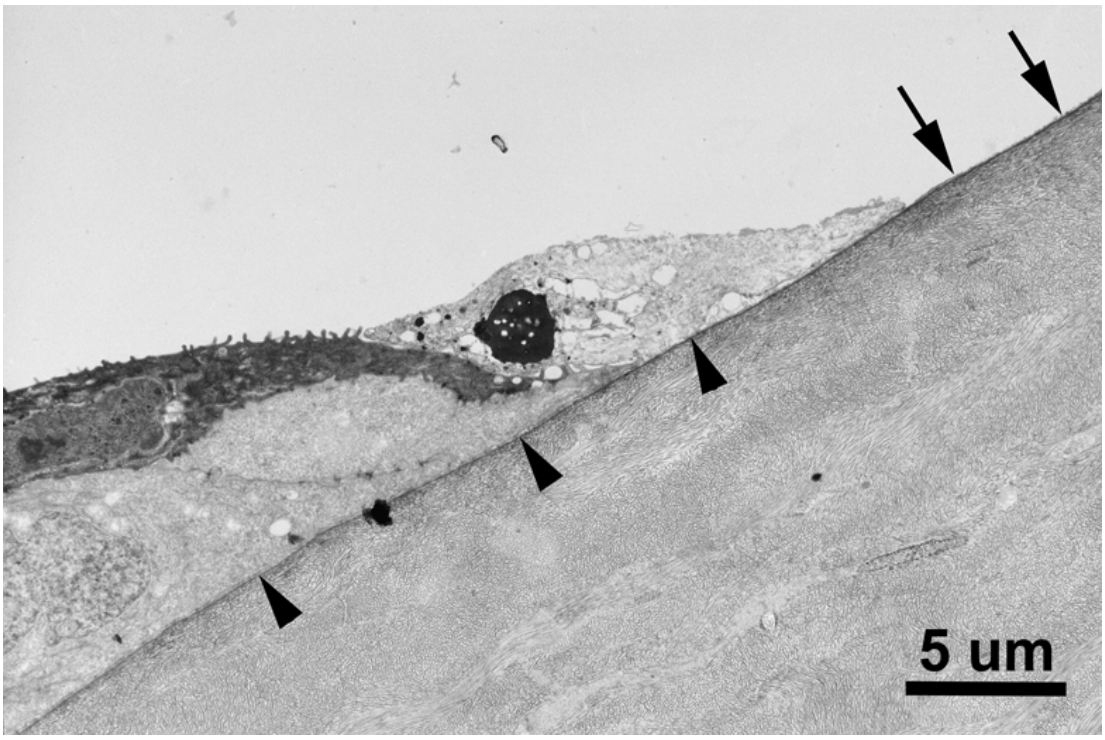


Figure 2 – Scape injury does not penetrate the basement membrane.

Following epithelial abrasion, the basement membrane is clearly present beneath the wounded epithelium (arrow heads) and in the abraded region (arrows).

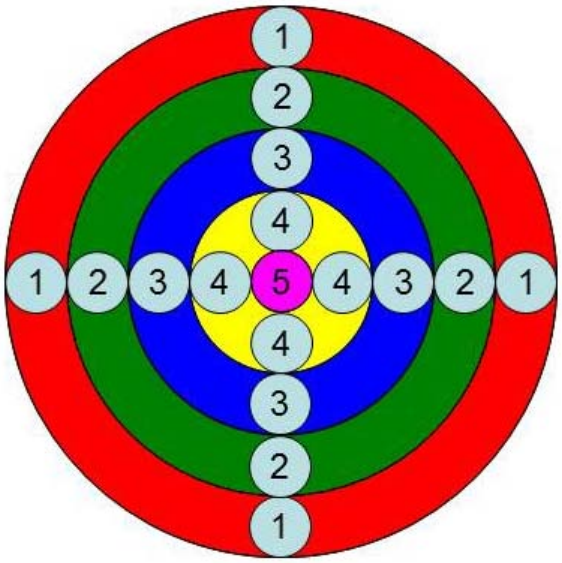


Figure 3 - Corneal schematic for enumerating macrophages.

The corneal wholemount was divided into 5 regions for counting macrophages: (1) limbus, (2) paralimbus, (3) parawound, (4) wound and (5) wound center. Corneal epithelium remained intact in the peripheral regions (1 and 2) while in the central regions (3, 4, and 5), the corneal epithelium was removed by debridment.

2.3 Results

Macrophage characterization in the uninjured cornea

Antibodies directed against markers known to be expressed on macrophages can be used to identify macrophages and evaluate how macrophage phenotypes and numbers are affected during the course of an inflammatory response (Barros et al., 2013). Within the uninjured mouse cornea, staining for CD86 was not detected. However, distinct CD80+ (potentially M1 macrophages) and CD301+ (potentially M2 macrophages) cells were present at the limbus. Further observations showed CD80+ cells are also in the conjunctiva. CD80+ cells were negative for all macrophage markers tested, but positive for CX3CR1. In addition, these cells stained positively with FITC-avidin (Figure 4). The combined positive staining for CD80 and FITC-avidin, coupled with a lack of staining for common macrophage markers, suggests the CD80+ cells are mast cells (Bergstresser et al., 1984; Tharp et al., 1985) and this is consistent with the immunofluorescence images of these cells taken after FITC-avidin staining showing large numbers of granules within these cells and immediately adjacent to these cells in the extracellular space, indicating mast cell degranulation. These mast cells are located adjacent to the limbal vessels (Figure 5).

CD301+ CD80- cells were also present at the limbus and showed a strong association with blood vessels. Further investigation revealed these perivascular CD301+ cells were negative for CX3CR1, CD86, CD80 but positive for all other macrophage markers examined (F4/80, CD115, and CD206) (Figure 6 and Table 2), consistent with an M2 macrophage phenotype. These cells were most abundant around limbal vessels and relatively rare in the paralimbal region of the cornea. In corneal wholemounts, CD301+

M2 macrophages adjacent to the limbal vessels showed an elongated shape while macrophages located more than one macrophage cell diameter away from the vessel showed a more rounded shape (Figure 7). Beyond the limbus, within the avascular corneal stroma, a very small population of F4/80+ cells was identified but because these cells failed to label with any other macrophage markers (Figure 8), their M1/M2 phenotype could not be determined, so they were not studied further.

CD301+ macrophages localize to the wounded cornea

To determine how perivascular CD301+ macrophage numbers are affected during the course of an inflammatory response, corneas that received a central epithelial abrasion were immunostained with anti-CD301 and examined at various time points out to 7 days. Macrophage numbers were counted at the L, PL, PW, W and WC in wholemount unwounded and injured corneas. Prior to injury, CD301+ macrophages were found at the L and PL. 24h after wounding, CD301+ macrophage numbers declined by 50% at the L ($P \leq 0.01$) and were elevated at the PL, PW, W and WC. By 48h and 72h post-injury, CD301+ macrophage numbers returned to baseline at the L while their numbers remained elevated at the PL, PW, W and WC (Figure 9; $P \leq 0.05$). Seven days after wounding, CD301+ macrophage numbers showed a second decline to 67% of baseline at the L ($P \leq 0.01$) while their numbers in the PL and PW returned to baseline values and remained elevated at the W and WC (Figure 9). Moreover, the decline in CD301+ macrophages was mirrored by a decline in F4/80+ macrophage numbers at the limbus 24h after wounding (Figure 10; $P \leq 0.01$). At all post-injury times, CD80+ mast cell numbers remained unchanged and these cells were only detected within the L (Figure 9). CD301+

macrophage numbers were also counted in the anterior and posterior stroma in wholemount unwounded and injured corneas up to 7 days. Before injury, CD301+ macrophages were rare in both anterior and posterior stroma. 24h after wounding, CD301+ macrophages numbers increased in the anterior stroma. By 48h and 72h after injury, CD301+ macrophage numbers significantly increased in the anterior stroma compared to posterior stroma ($P \leq 0.05$). Seven days after wounding, CD301+ macrophage mumbers returned to baseline in both anterior and posterior stroma (Figure 11).

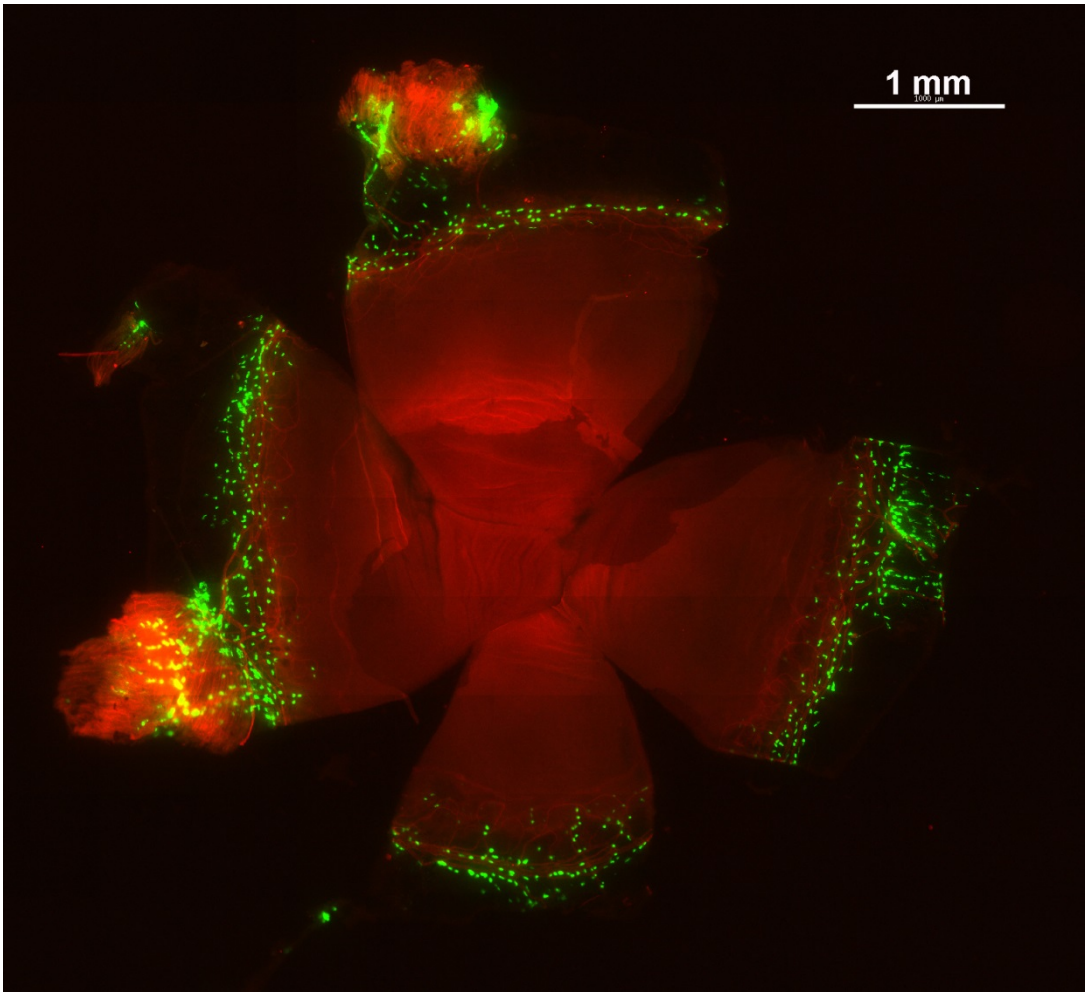


Figure 4 - Mast cell distribution in the cornea.

A low power (20X) montaged image of the cornea where the green stained avidin positive mast cells are located at the limbus and conjunctiva. Mast cells were absent in other regions of the cornea. (Courtesy of Thomas Nettleton)

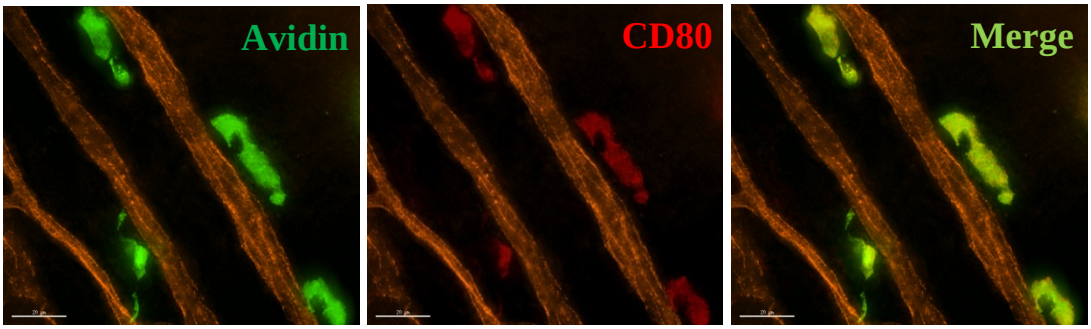


Figure 5 - Mast cells occupied a perivascular position within the limbus.

Green stained avidin+ mast cells were located adjacent to orange stained limbal vessels and large numbers of granules were seen within these cells. Mast cells also stained positively for CD80+ (red). The third image shows the combined positive staining for CD80+ avidin+ mast cells.

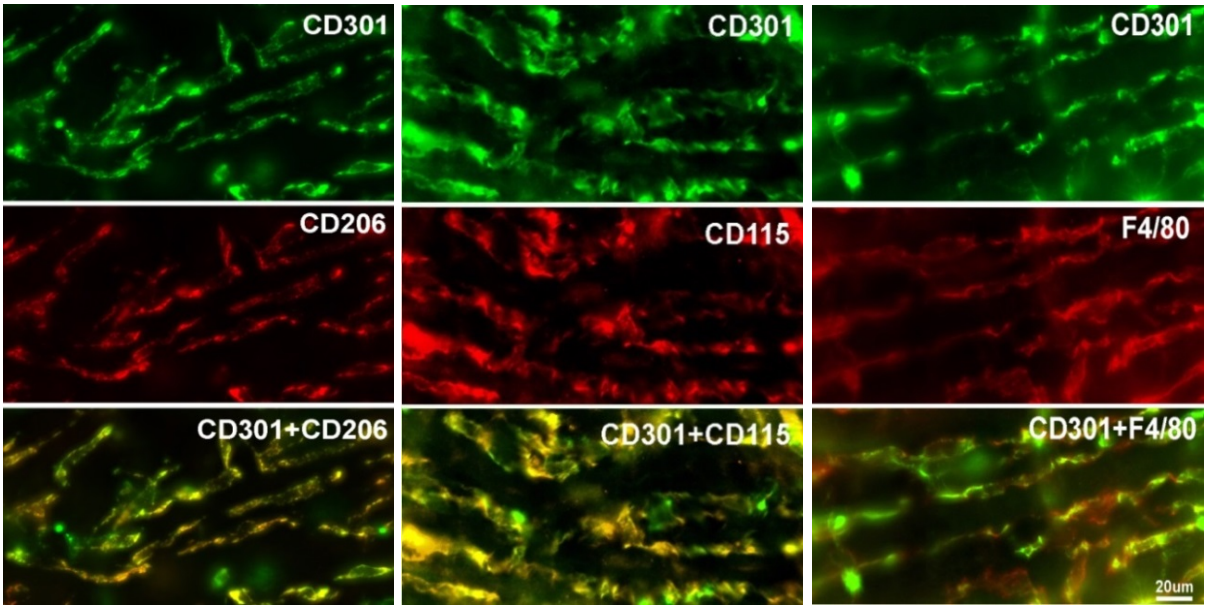


Figure 6 - Macrophage phenotype revealed by immunostaining.

At the limbus, CD301+ macrophages (green) were co-labeled (red) with antibodies directed against CD206, CD115, and F4/80.

Table 2. Macrophage marker expression in CD301+ cells.

CD301+ cells are positive for most macrophage markers except CX3CR1, CD86 and CD80.

	F4/80	CD206	CD115	CX3CR1	CD86	CD80
CD301	+	+	+	-	-	-

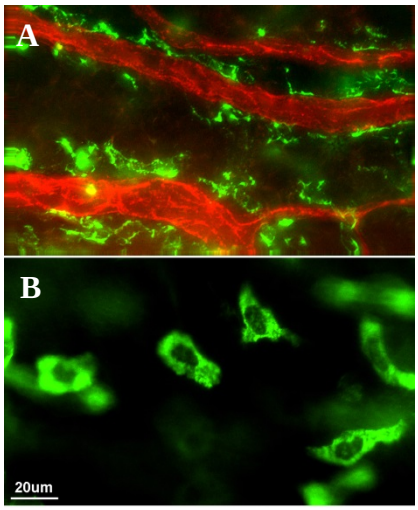


Figure 7 - CD301+ macrophage morphology at the limbus.

Green stained elongated CD301+ macrophages lie next to red stained limbal vessels (A), while CD301+ macrophages that were a short distance (one cell diameter) away from the limbal vessels had a more rounded shape (B).

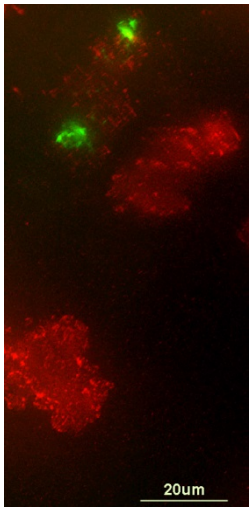
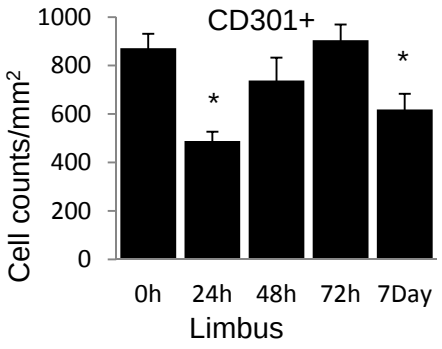
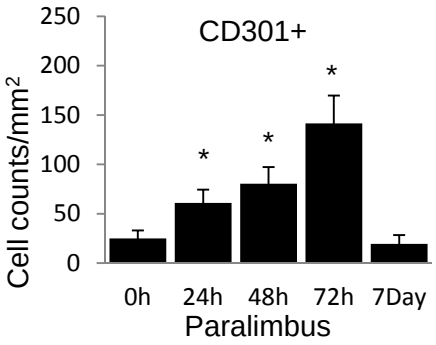
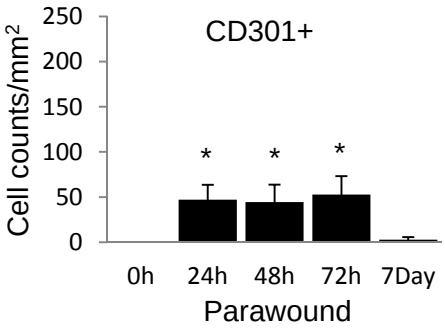


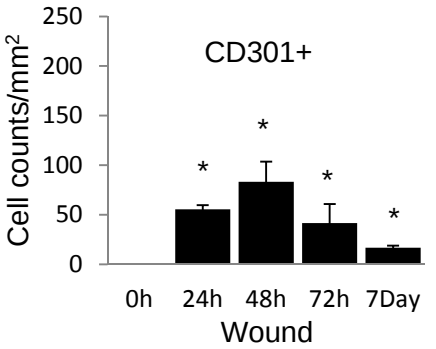
Figure 8 - Macrophage staining in the central corneal stroma.

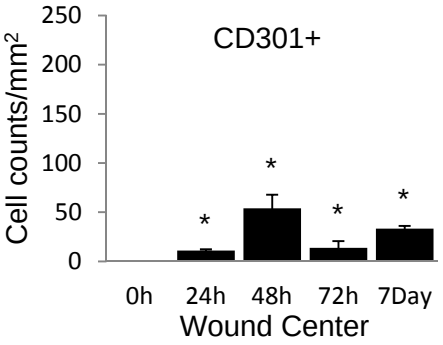
Within the avascular central corneal stroma, all CD301+ cells (green) are F4/80+ (red) but not all F4/80+ cells expressed CD301.


CD301+
Cell counts/mm2
1000
800
600
400
200
0
0h
24h
48h
72h
7Day
Limbus


CD301+
Cell counts/mm2
250
200
150
100
50
0
0h
24h
48h
72h
7Day
Paralimbus


CD301+
Cell counts/mm2
250
200
150
100
50
0
0h
24h
48h
72h
7Day
Parawound


CD301+
Cell counts/mm2
250
200
150
100
50
0
0h
24h
48h
72h
7Day
Wound


CD301+
Cell counts/mm2
250
200
150
100
50
0
0h
24h
48h
72h
7Day
Wound Center

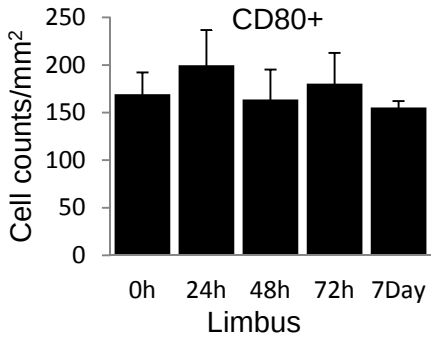

CD80+
Cell counts/mm2
250
200
150
100
50
0
0h
24h
48h
72h
7Day
Limbus

Figure 9 - Macrophage and mast cell distribution before and after corneal injury. CD301+ macrophage and CD80+ mast cell counts at the limbus, paralimbus, parawound, wound and wound center area in corneal wholemounts before and after wounding. At the limbus, CD301+ macrophage numbers showed a drop at 24h and 7 days. Beyond the limbus (i.e. PL, PW, W, and WC), CD301+ macrophages increased in number after wounding when compared to uninjured cornea, while CD80+ mast cell numbers remained unchanged and were restricted to the limbus. * indicates $P \leq 0.05$ compared to control.

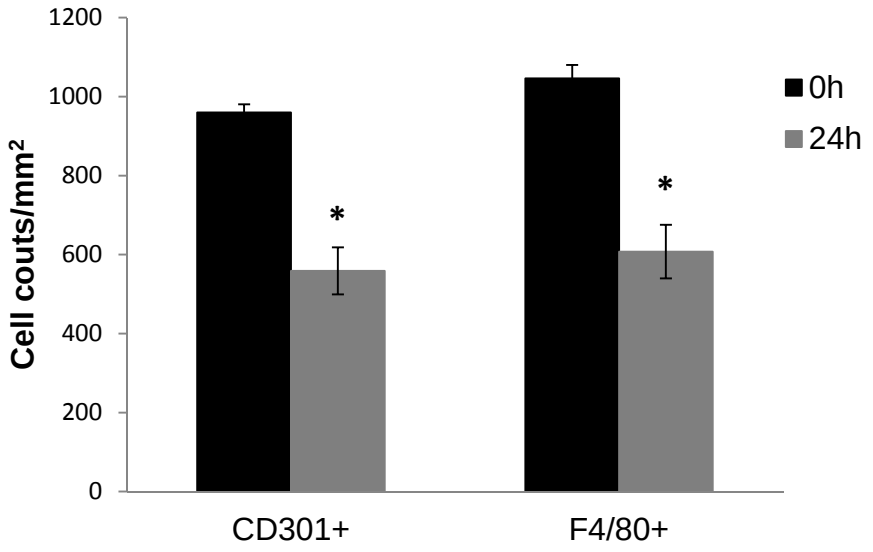


Figure 10 - Macrophage number decreased 24h after wounding at limbus.

CD301+ and/or F4/80+ macrophage counts at limbus in corneal wholemounts before and 24h after wounding. 24h after corneal injury, both CD301+ macrophages and F4/80+ macrophages showed a significant drop about 42% at limbus. * indicates $P \leq 0.01$ compared to macrophage counts in unwounded cornea.

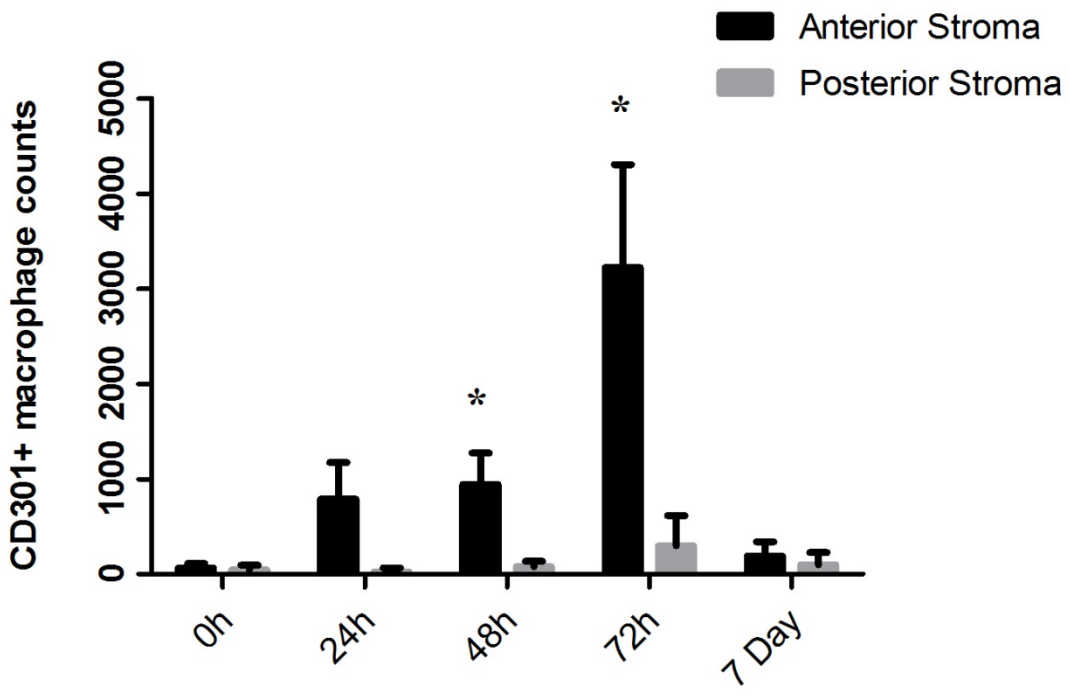


Figure 11 - Infiltrating macrophages localized preferentially to the anterior corneal stroma.

CD301+ macrophage total counts in the anterior and posterior corneal stroma in corneal wholemounts before and after wounding. Anterior CD301+ macrophage numbers increased significantly 48h and 72h after wounding compared to baseline, while 7 days after injury their numbers returned to baseline. Posterior CD301+ macrophage numbers remained low and did not change after injury. * indicates $P \leq 0.05$ compared to anterior macrophage baseline counts.

2.4 Discussion

The purpose of Aim 1 was to characterize macrophages in normal and wounded mouse corneas. In agreement with prior studies (Brissette-Storkus et al., 2002; Hamrah et al., 2003a; Nakamura et al., 2005; Sosnova et al., 2005), our results show a significant number of resident macrophages can be detected within the uninjured mouse cornea. Macrophages can be polarized toward a pro-inflammatory (M1) or an anti-inflammatory (M2) phenotype, but they can also exist somewhere between these two extremes. Antibodies directed against markers known to be expressed on macrophages can be used to characterize the macrophage phenotype and evaluate how this phenotype is affected during the course of an inflammatory response (Barros et al., 2013). Because of the complexity and difficulty of characterizing macrophage phenotypes by only using one marker, it's reasonable to use fluorescently tagged antibodies raised against mouse macrophage markers together with known M1and M2 markers to identify and localize macrophages within corneal wholemounts before and after injury. Monitoring changes in macrophage number and phenotype following corneal abrasion can provide new information that will help understand their role in inflammation and wound healing.

The majority of macrophages are located in the peripheral cornea and the surrounding limbal tissues where the macrophages assume a perivascular position. The identity of these cells as macrophages is confirmed by their positive staining for CD301 and a panel of macrophage markers that include F4/80, CD115, and CD206.

F4/80 is a monoclonal antibody that recognizes a 160-kDa glycoprotein on the surface of most macrophage populations in mice (Austyn and Gordon, 1981) and has been widely used to characterize macrophage populations as a macrophage-specific marker in a

range of immunological studies (Gordon et al., 1992; Hume, 2006; Hume et al., 1984; Perry et al., 1985). CD115, also known as colony stimulating factor 1 receptor, is an integral transmembrane glycoprotein encoded by the *CSF1R* gene (c-fms proto-oncogene) (Sherr et al., 1985) and is a member of the type III protein tyrosine kinase receptor family. It binds with macrophage colony stimulating factor 1 which contributes to the production, differentiation and function of macrophages (Pollard, 2009; Sweet and Hume, 2003). CD206 contains 8 C-type lectin-like domains, encodes the mannose receptor C-type lectin and is a type I transmembrane protein that is expressed abundantly on macrophages and dendritc cells (McGreal et al., 2004; Stahl and Ezekowitz, 1998). Recent studies show CD206 on macrophages mediates the uptake of diverse allergens and acts as a phagocytic receptor for various pathogens (Boskovic et al., 2006; Emara et al., 2011).

CD80 and CD86 are well known markers for M1 macrophages in other tissues. However, our data clearly show CD80+ cells in the cornea are negative for CD86 and F4/80, suggesting they are not macrophages. Indeed, further observation of CD80+ cells stained with FITC-avidin confirmed the identity of these cells as mast cells and not M2 macrophages. FITC-avidin staining is a well-accepted marker for mast cells (Bergstresser et al., 1984; Tharp et al., 1985). The avidin binds to the highly negatively charged heparin sulfate contained within the mast cell granules (Tharp et al., 1985; Zhang et al., 1991). Previous reports show mast cells are widely spread throughout the vascularized conjunctiva and are involved in ocular allergy (Fukuda et al., 2009; Irani and Schwartz, 1994; Miyazaki et al., 2008) and wound healing (reviewed by (Asboe-Hansen, 1968)). While our research found evidence of limbal mast cell degranulation after injury, mast cell

numbers remained constant. The role of limbal mast cells in corneal inflammation and wound healing remains to be determined (see Future Studies, Chapter 5).

While additional F4/80+ cells were detected within the corneal stroma, these resident cells failed to label with other macrophage markers and were not studied further. Instead, we focused on the behavior of CD301+ macrophages because following abrasion injury, their numbers decrease at the limbus (a similar drop in F4/80+ cells is also observed) and increase at all regions across the cornea, consistent with migration toward the wound center rather than a change in macrophage phenotype (i.e., downregulation of CD301 at the limbus and upregulation of CD301 in the cornea).

In the mouse cornea, CD301+ macrophages localize to the limbus and the peripheral cornea. The majority of limbal macrophages occupy a perivascular position, a potentially important anatomical position where they would have the opportunity to interact with emigrating leukocytes (e.g., neutrophils) and platelets following an epithelial abrasion injury and, through the release of inflammatory mediators, influence infiltrating cells within the limbus. After corneal epithelial injury, the reduction in CD301+ macrophage numbers at the limbus is matched by increased numbers throughout the corneal stroma, consistent with cell migration toward the central wounded cornea. The vast majority (91%) of infiltrating stromal CD301+ macrophages are confined to the anterior stroma. While the mechanism for replenishing the limbal macrophage numbers was not investigated, it is clear that limbal CD301+ macrophage numbers return to baseline just prior to a second decrease in their numbers at 7 days post-injury, which seems to support sustained macrophage infiltration toward the central cornea.

Collectively, these data suggest CD301+ macrophage infiltration into the wounded cornea is biphasic.

Chapter 3- Aim 2: To determine if macrophage-derived interleukin-20 (IL-20) plays a beneficial role in corneal wound healing

3.1 Introduction

After corneal epithelial injury, rapid wound healing is essential to restore normal corneal function. Effective corneal epithelial wound healing involves not only epithelial proliferation, but also nerve regeneration. Corneal nerves release neurotrophic factors to help maintain the integrity of the corneal epithelium. Ophthalmic surgeries involving injury to the corneal epithelium show reduced corneal nerve densities even after many years post-surgery (Moilanen et al., 2008; Niederer et al., 2007). Denervation can result in ocular surface problems such as punctate epithelial erosion and rose bengal staining, etc. (Wilson, 2001). Although epithelial nerve regeneration returns to baseline in WT mice 8 weeks after a 2mm diameter abrasion injury (Namavari et al., 2011), the nerve morphology is abnormal.

In addition to phagocytic activities, macrophages also secrete various growth factors and have been linked to central and peripheral axon regeneration and epithelial cell proliferation (Kurimoto et al., 2010; Yin et al., 2009; Yin et al., 2006). While macrophages are known to reside within the mouse cornea (Brissette-Storkus et al., 2002; Hamrah et al., 2003a; Nakamura et al., 2005; Sosnova et al., 2005), their contribution to corneal wound healing has not been studied.

In skin, macrophages contribute to wound healing through the release of IL-20. IL-20 is a member of IL-10 family which induces keratinocyte proliferation and differentiation (Blumberg et al., 2001; Roupe et al., 2010). IL-22, another IL-10 family member, is released by γδ T cells and stimulates the epithelial cells to produce CXCL1

chemokine. CXCL1 is a potent chemokine that promotes neutrophil recruitment. Platelet recruitment is closely tied and dependent on neutrophil recruitment. Both platelets and neutrophils contain vascular endothelial growth factor (VEGF) and increased levels of VEGF in the injured cornea contribute to epithelial wound healing. Indeed, depletion of γδ T cells, and hence, decreased levels of IL-22, impairs epithelial wound healing while application of recombinant IL-22 promotes epithelial recovery (Li et al., 2011b).

IL-20 receptors exist as heterodimers as IL-20R1/IL-20R2 and IL-22R1/IL-20R2. Since the mouse corneal epithelium shows positive staining for IL-22R1 (Li et al., 2011b), and IL-20 and IL-22 share the same receptor subunit, IL-22R1 (Logsdon et al., 2012), the possibility is raised that like IL-22, the binding of IL-20 by the epithelium may influence corneal wound healing.

The aim of this chapter was to determine the cellular sources of IL-20 and whether IL-20 plays a beneficial role in corneal wound healing in mice.

3.2 Materials and Methods

Animal model

Studies were carried out at Baylor College of Medicine (Houston, TX) and all mice for use were 8-12 weeks old and were treated following the guidelines in the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Female wildtype mice, γδ T cell deficient mice (TCR-/-), and CD11a deficient mice (CD11a-/-) were purchased from Jackson Laboratory (Bar Harbor, ME) and were on a C57BL/6 background. Neutropenic mice were prepared by intraperitoneally injecting anti-Ly6G (0.25mg/mouse;BD Pharmingen). The central corneal wound was made following an established protocol (Li et al., 2006b; Li et al., 2006c). Briefly, mice were anesthetized by

intraperitoneally injecting pentobarbital sodium solution (50mg/kg) and a 2mm diameter central corneal epithelial region was marked by a trephine and mechanically debrided with a golf club spud under a dissecting microscope.

Immunohistology

Wildtype mice were sacrificed and dissected corneas (including limbus) were fixed for 30 mins in phosphate buffered saline (PBS, pH 7.2) containing 2% paraformaldehyde. Fixed corneas were washed in PBS 3 times, 5 mins each and then permeabilized with 0.1% Triton X-100 for 30 mins. Corneas were then blocked for 30 min with 1% bovine serum albumin. Dissected corneas were incubated with rabbit anti-IL-20R1 (Millipore, Billerica, MA), rabbit anti-IL-20R2 (Bioss, Wobum, MA) and rat anti-IL-20R2 (Abcam, Cambridge, MA) antibodies. For detection of IL-20, 6 and 18 hour wounded wildtype mice were sacrificed and dissected corneas (including limbus) were incubated in IMDM (Gibco, Grand Island, NY) +20% FBS with 3μM monensin (Biolegend, San Diego, CA) at 37°C for 12 hours. Monensin inhibits Golgi function and increases the intracellular retension of proteins destined for secretion (reviewed by (Tartakoff, 1980)). Then the corneas were fixed, washed, and permeabilized as mentioned above. Corneas were blocked for 30 min with 1% donkey serum. Then the corneas were incubated with goat-anti-mouse IL-20 primary antibody (R&D Systems, Minneapolis, MN) overnight and then incubated with donkey-anti-goat Alexa Fluor 488 secondary antibody (Molecular Probes, Grand Island, NY). Other antibodies used were: PE or APC-conjugated anti-CD31 (BD Pharmingen, San Jose, CA) for detection of blood vessels; PE-conjugated anti-β-III tubulin (R&D Systems, Minneapolis, MN) for detection of nerves, fluorescein

isothiocyanate (FITC)-conjugated monoclonal anti-α-tubulin (Sigma, St. Louis, MO) for detection of mitotic spindles and FITC-conjugated anti-Ly6G (BD Pharmingen, San Jose, CA) for detection of neutrophils. Non-immune isotype matched antibodies served as controls to evaluate non-specific staining. Immunostained corneas were imaged as wholemounts using a 40X oil immersion lens mounted on a DeltaVison Spectris Core microscope (Applied Precision, Issequah, WA).

Local effect of IL-20

To study the local effect of IL-20 on corneal epithelial wound healing, IL-20 function was blocked by topical application of a neutralizing goat-anti-mouse IL-20 antibody (R&D systems). Briefly, 15 wildtype mice received corneal epithelial abrasions and were divided into 3 groups. In the first group, 5 mice received a topical application (10µl) of goat-anti-mouse IL-20 antibody dissolved in PBS (200µg/ml). The process was repeated every 4 hours for 24 hours. In the second group of 5 mice, non-immune isotype matched IgG was substituted for anti-IL-20 while the remaining 5 mice in the third group received PBS only. The rate of corneal wound closure was determined using an established protocol (Li et al., 2006a, b; Li et al., 2006c). Briefly, sodium fluorescein was applied to the wounded cornea every 4 hours beginning at wounding and up to 24 hours. The extent of fluorescein staining demarcates the size of the open wound and images were photographed with a digital camera and analyzed by Optimus 6.2 software (Media Cybernetics). The relationship of wound size at various time points establishes the rate of wound closure after injury. At 24h post-injury, epithelial nerve recovery was analyzed in fixed corneal wholemounts at 4 regions (PL, PW, W and WC). Nerves were imaged after

staining with anti-β-III tublin antibody and serial epithelial images were collected at 0.2μm Z-steps through the entire thickness of the epithelium, deconvolved and saved as a maximum projected image. The projected images were analyzed using a custom nerve tracing program (Matlab) that quantifies nerve density. Dividing basal epithelial cells, where mitotic spindles stained positively with anti-tubulin antibody, were counted from L to WC and compared between anti-IL-20 treated and control groups. Neutrophils were counted and compared at the wound margin area (PW) and comparisons were made between anti-IL-20 treated and control groups.

To further examine the role of IL-20 on corneal epithelial wound healing, wildtype mice with and without neutrophil depletion and mutant mice known to have reduced neutrophil infiltration, TCR-/- and CD11a-/- mice, received central corneal epithelial abrasions. Immediately after wounding, the mice received a topical application (10μL) of mouse recombinant IL-20 (rIL-20; R&D Systems) dissolved in PBS (200ng/mL) every 4h for up to 24h. The appropriate control mice only received the vehicle (PBS; n=5). Corneal wound closure, epithelial nerve density, dividing epithelial cells and neutrophil influx were measured and counted as mentioned above. Platelet numbers were also counted in wounded wildtype mice treated with rIL-20 or PBS, respectively.

IL-20 ELISA Assay

IL-20 was analyzed in corneal extracts collected at 0, 24, 48, 72 hours and 7 days after epithelial injury using an ELISA kit (R&D systems, Minneapolis, MN) and following the manufacturer's instructions. Briefly, 10 corneas from each time point were

dissected (including limbus) and digested in 500μL of radioimmunoprecipitation buffer containing protease inhibitors (Roche). All samples were stored at -80°C until needed.

CXCL1 ELISA Assay

CXCL1 expression from unwounded and wounded corneas was analyzed using an ELISA kit (R&D systems, Minneapolis, MN) following the manufacturer's instructions. Briefly, 6 unwounded corneas and 18 wounded corneas were dissected (including limbus) immediately after wounding. Six unwounded corneas and 6 injured corneas were pooled separately into 1ml of RPMI 1640 medium. Six additional wounded corneas were cultured with rIL-20 (200ng/ml) for 6h while the remaining 6 wounded corneas were cultured with rIL-22 (200ng/ml) for 6h. All culture supernatants (in vitro) were collected and stored at -80°C until needed.

Statistical analysis

Data were analyzed using a two-way analysis of variance (ANOVA) with a Tukey post-test for pair-wise multiple comparisions. Data are expressed as means±SEM and P values ≤ 0.05 were considered statistically significant.

3.3 Results

Localization of IL-20 in mouse cornea

Four of the IL-10 family members, IL-19, IL-20, IL-22 and IL-24 were reported stimulating human keratinocyte proliferation in vitro (Sa et al., 2007). In analyzing the tissue for members of the IL-10 family known to participate in corneal epithelial wound healing, we found γδ T cells were positive for IL-22 (Li et al., 2011b). Studies in normal skin suggest IL-20 is important for keratinocyte proliferation and differentiation (Blumberg et al., 2001). Monocytes are the only leukocytes known to express IL-20 (Wolk et al., 2002). We hypothesized macrophages may express IL-20 in mouse cornea. However, corneal macrophages showed no immunostaining for IL-20 before or after epithelial abrasion. Instead, we found strong IL-20 staining in corneal epithelial cells and keratocytes before and after injury (Figure 12).

Antibody neutralization of IL-20 delays corneal wound healing

After corneal abrasion, topical application of neutralizing goat-anti-mouse IL-20 antibody markedly delayed corneal wound closure as measured by fluorescein dye exclusion when compared to controls which received PBS only or non-immune isotype IgG antibody. In the two control groups, corneal epithelial wounds closed by 24h after injury and there was no statistical difference for wound closure rates between the two groups. However, in the anti-IL-20 treated group, the wound closure rate was significantly delayed between 8 and 24h post injury ($P \leq 0.01$), and the wound remained open at 24h (Figure 13). Fewer dividing corneal epithelial cells were detected across the cornea (L to the WC) following anti-IL-20 antibody treatment (Figure 14).

IL-20 enhances corneal epithelial wound healing in TCR-/- and WT mice

Immunostaining confirmed that like IL-22R1, the IL-20R1 was also expressed on corneal epithelial cells (Figure 15), while IL-20R2 staining was not detected. TCR-/- mice were used to assess the direct effect of IL-20 on epithelial wound closure in the absence of IL-22; TCR-/- mice lack γδ T cells which are the cellular source of IL-22 secretion in the mouse cornea. Topical application of rIL-20 on wounded TCR-/- mouse corneas resulted in accelerated wound closure from 8h up to 24h compared to controls (PBS only) as measured by fluorescein dye exclusion ($P \leq 0.01$) (Fig. 16). As well, the number of dividing epithelial cells increased across the cornea following rIL-20 treatment in TCR-/- mice (Figure 16) when compared to controls ($P \leq 0.05$). Following corneal abrasion, WT mice treated with rIL-20 maintained the same wound closure rate as control WT mice that received topical PBS (Figure 17). At 24h post-injury, these mice also showed 39% more dividing epithelial cells at the PL (Figure 17). Collectively, the data suggest IL-20 potentiates corneal wound healing by enhancing corneal epithelial cell division and the rate of wound closure.

IL-20 promotes corneal nerve regeneration after epithelial wounding

Efficient corneal wound healing also involves epithelial nerve regeneration. Since IL-20 enhanced epithelial wound closure and increased epithelial cell division, we wished to determine if IL-20 facilitates corneal epithelial nerve recovery. After topical application of anti-IL-20, we found epithelial nerve recovery at 24h post-injury was decreased in WT mice compared to control. While no statistical difference was found between the control and anti-IL-20 treated groups at the PL, nerve density was markedly

diminished in the PW, W and WC after anti-IL-20 treatment (Figure 18). In TCR-/- mice, rIL-20 markedly improved corneal nerve recovery at the PW, W and WC when compared to control ($P \leq 0.01$) (Figure 18). WT mice treated with rIL-20 showed a higher nerve density at the WC at 24h post-injury (Figure 18). These findings suggest IL-20 is an important cytokine for corneal nerve recovery after a central epithelial abrasion.

IL-20 expression increased after corneal epithelial abrasion

The ELISA data show, 24h after wounding, IL-20 expression increased from 7.5pg/ml to 18.7pg/ml. No further increase was observed but levels remained elevated out to 7 days post-injury (Figure 19).

IL-20 negatively regulates neutrophil recruitment

IL-20, like IL-22, belongs to the IL-10 superfamily. Previously, we observed that anti-IL-22 treatment decreased neutrophil recruitment in the injured mouse cornea (Li et al., 2011b). Unexpectedly, and in contrast to anti-IL22, anti-IL20 treatment enhanced neutrophil recruitment as demonstrated by increased neutrophil counts at the PL at 24h post-injury (Fig. 20) while WT mice that received topical applications of rIL-20 showed a marked reduction in neutrophil recruitment (Figure 20). Neutrophil numbers were the same (24h) or lower (16h) at the parawound in TCR-/- mice which received rIL-20 compared to TCR-/- mouse corneas which only received PBS (Figure 20).

IL-20 and IL-22 belong to the IL-10 family and there is receptor overlap in the use of the IL-22R1 subunit. Our present data suggest IL-20 inhibits neutrophil recruitment while our previous data showed IL-22 promotes neutrophil recruitment. The ability of IL-

22 to promote neutrophil recruitment is linked to its ability to induce epithelial expression of CXCL1, a potent neutrophil chemoattractant (Li et al., 2011b). Since IL-20 inhibits neutrophil recruitment, we wished to determine what effect it has on CXCL1 production (Figure 21). The supernatant of unwounded and wounded corneas cultured ex vivo were analyzed by ELISA. Without injury, 6h cultured cornea supernant yeilded 606.355 pg/ml CXCL1; with injury alone, the cultured supernant contained 43.4% more CXCL1. As expected, with injury and culture with exogenous rIL-22 ex vivo for 6h, there was a marked increase in CXCL1 (1601.99 pg/ml, which is 2.6-flod more than unwounded corneas). With injury and culture in rIL-20 ex vivo for 6h, CXCL1 production was inhibited (350.618 pg/ml) and significantly lower than that observed in injured or uninjured corneas. Collectively, these observations suggest IL-20 may serve to negatively regulate and limit neutrophil infiltration into the injured cornea.

IL-20 negatively regulates platelet recruitment

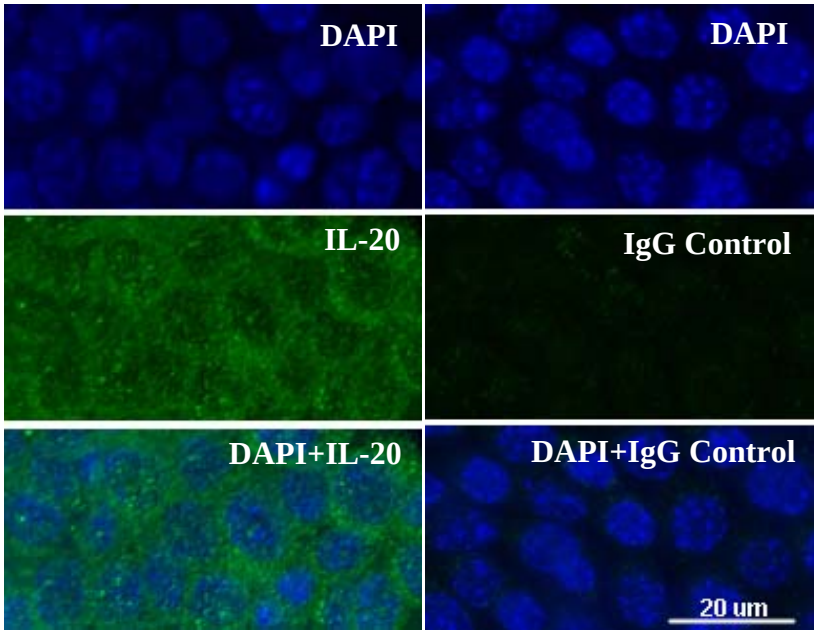
Since platelet recruitment into the cornea is linked to and dependent on neutrophil recruitment (Li et al., 2006c), the ability of IL-20 to inhibit neutrophil recruitment after corneal epithelial injury may have similar effects on platelet recruitment. Topical application of rIL-20 on wounded wildtype mouse corneas inhibited platelet recruitment at 24h post-injury by 70% ($P \leq 0.05$). The inhibition of platelet recruitment involved reductions in both intravascular and extravascular platelets (Figure 22).

IL-20 promotes corneal epithelial wound healing independently of its effects on neutrophil and platelet recruitment

Previously, we reported that diminished or excessive neutrophil recruitment can delay corneal wound closure (Li et al., 2006a). Platelet depletion also results in delayed corneal wound healing. (Li et al., 2006c). In the present study, anti-IL-20 antibody treatment of injured WT corneas increased neutrophil recruitment and this likely contributed to delayed corneal wound healing. In injured TCR-/- mice, neutrophil recruitment is diminished compared to WT mice and, as expected, topical application of rIL-20 did not enhance neutrophil recruitment but it did restore corneal wound healing. To further explore the possibility that IL-20 has direct effects on wound healing, independent of its inhibitory effects on neutrophils, we sought to determine if rIL-20 could restore corneal epithelial recovery in two additional but distinct mouse models known to have markedly reduced neutrophil infiltration after wounding, CD11a-/- mutant mice and WT mice made neutropenic (70% neutrophils were depleted). In wounded CD11a-/- and neutropenic mouse corneas, the rate of wound closure from 16h to 24h ($P \leq 0.05$) was faster than that observed for matched controls which received PBS only (Figure 23). As well, the number of dividing epithelial cells increased significantly across the cornea from PL to W after topical application of rIL-20 in CD11a-/- and neutropenic mice ($P \leq 0.05$) (Figure 24). Similarly, nerve recovery was enhanced in CD11a-/- mice at the PW, W and across the cornea from PL to W in neutropenic mice ($P \leq 0.05$) (Figure 25). The direct healing effect of rIL-20 is also illustrated by the maintenance of normal wound closure rates in WT mice at a time when platelet and neutrophil recruitment is blunted (Figure 22, 23).

Collectively, these data suggest IL-20 has a direct positive influence on corneal wound healing that is distinct from its inhibitory effects on neutrophil and platelet infiltration.

A.



B.

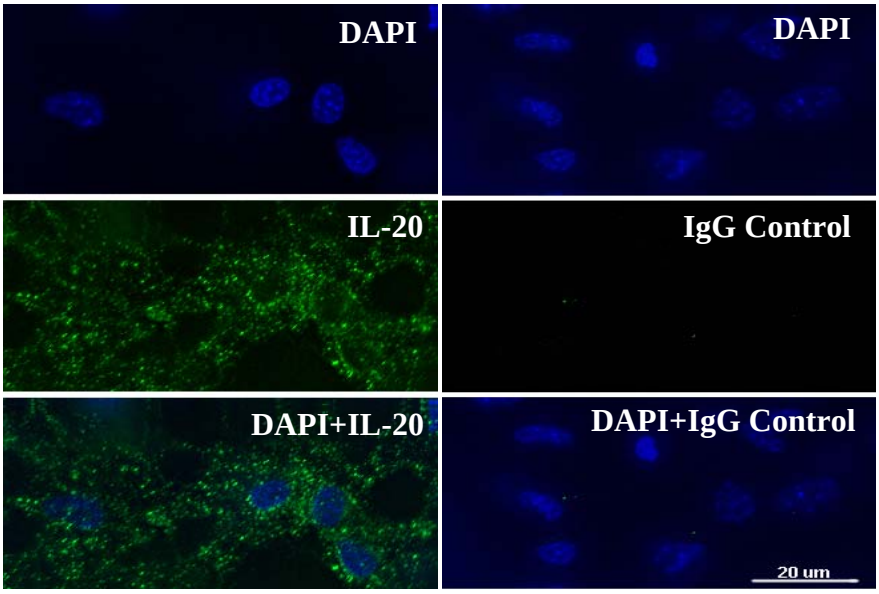
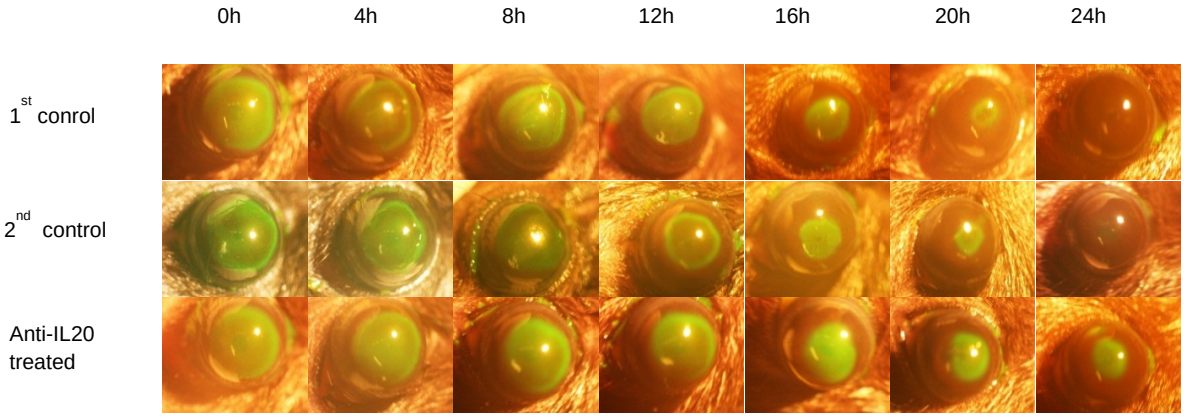


Figure 12 - IL-20 immunostaining in the injured mouse cornea.

At 18h post-injury, IL-20 staining was evident in corneal epithelial cells (A, green) and keratocytes (B, green).


0h
4h
8h
12h
16h
20h
24h
1st conrol
2nd control
Anti-IL20 treated

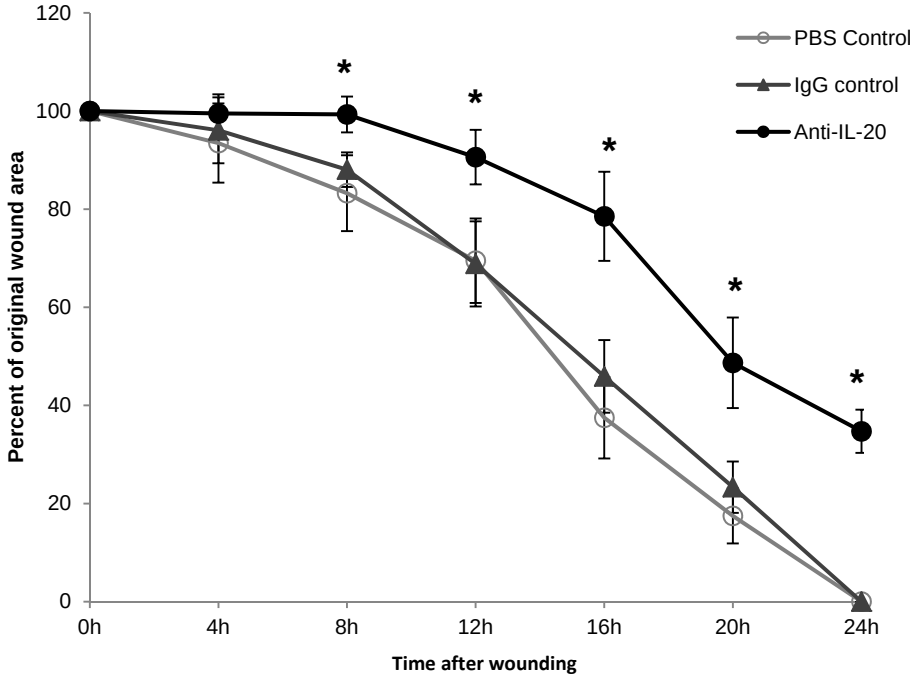

PBS Control
IgG control
Anti-IL-20
Percent of original wound area
Time after wounding
0
20
40
60
80
100
120
0h
4h
8h
12h
16h
20h
24h
*
*
*
*
*

Figure 13 - Effect of anti-IL-20 on epithelial healing.

In the first control group, mice received PBS only; In the second control group, mice received 200ug/ml non-immune isotype IgG dissolved in PBS; In the treated group, mice received 200ug/ml goat-anti-mouse IL-20 antibody dissolved in PBS. Sodium fluorescein staining (upper panel) showed the corneal epithelial wound closed by 24h in the two control groups, while the wound remained open in the anti-IL-20 antibody treated group. The rate of wound closure over the first 24h after injury is shown in the lower panel. * = $P \leq 0.05$ compared to control.

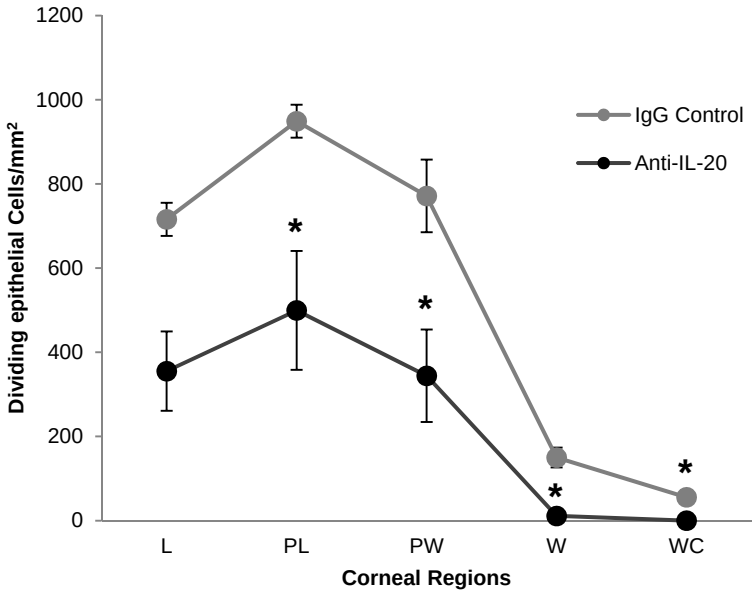


Figure 14 - IL-20 inhibition suppressed epithelial cell division after wounding. Topical application of neutralizing anti-IL-20 antibody to wildtype mice markedly suppressed epithelial cell division across the cornea at 24h post-injury. * = $P \leq 0.05$ compared to control.

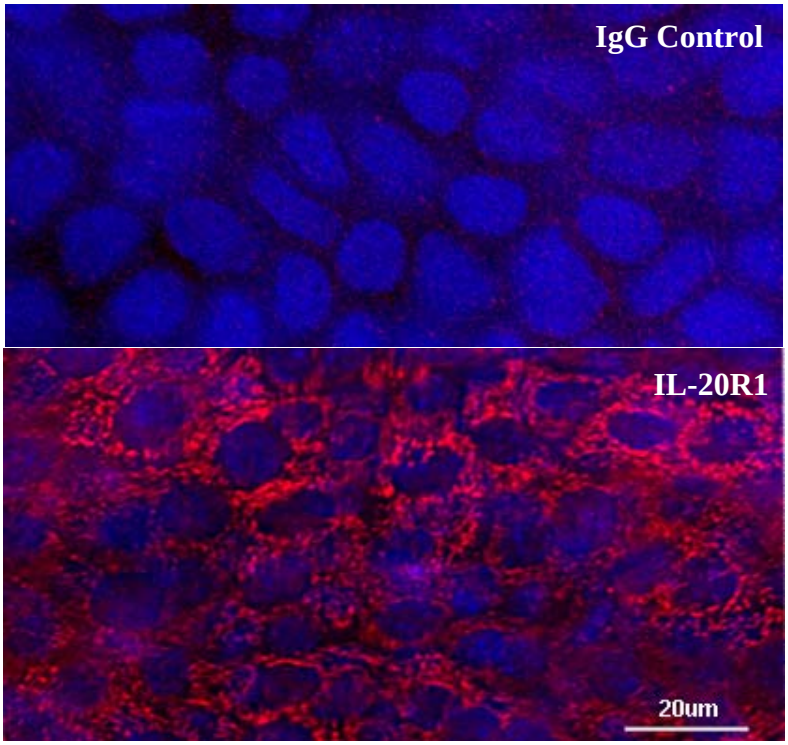
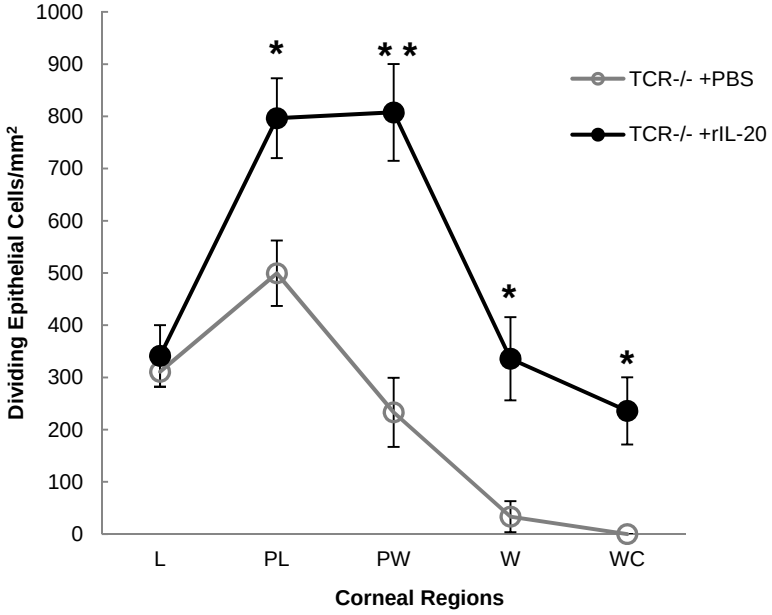


Figure 15 - IL-20R1 staining on corneal epitheial cells.

IL-20R1 (red) staining was observed on paralimbal corneal basal epithelial cells at 24h post-injury. Nuclei are stained with DAPI (blue).


1000
900
800
700
600
500
400
300
200
100
0
Dividing Epithelial Cells/mm²
L
PL
PW
W
WC
Corneal Regions
TCR-/- +PBS
TCR-/- +rIL-20
*
* *
*
*

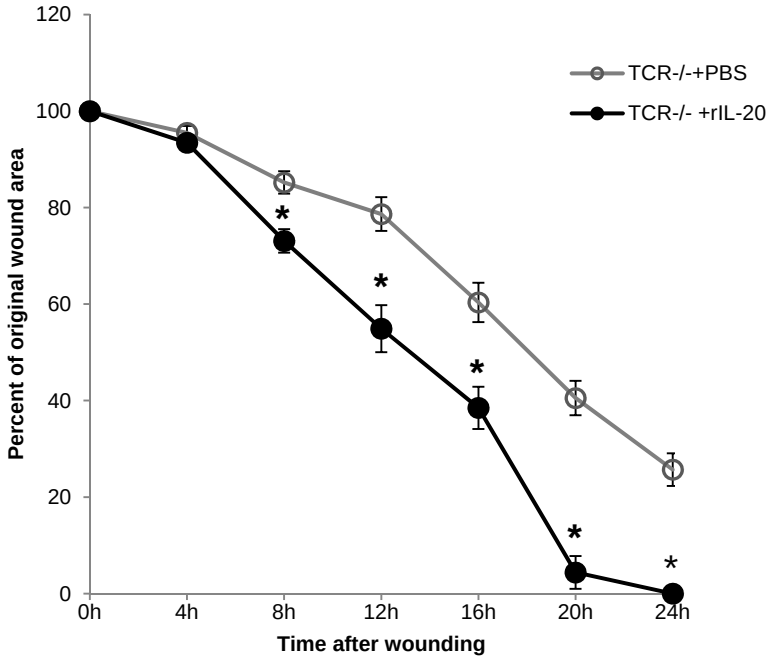

120
100
80
60
40
20
0
Percent of original wound area
0h
4h
8h
12h
16h
20h
24h
Time after wounding
TCR-/-+PBS
TCR-/- +rIL-20
*
*
*
*
*

Figure 16 - IL-20 promoted cell division and wound closure in TCR-/- mice.

Topical application of rIL-20 promoted epithelial cell division across the cornea in TCR-/- mice following corneal epithelial abrasion (24h). rIl-20 accelerated corneal wound closure in TCR-/- mice from 8h to 24h. * = $P \leq 0.05$ compared to control. * * = $P \leq 0.01$ compared to control.

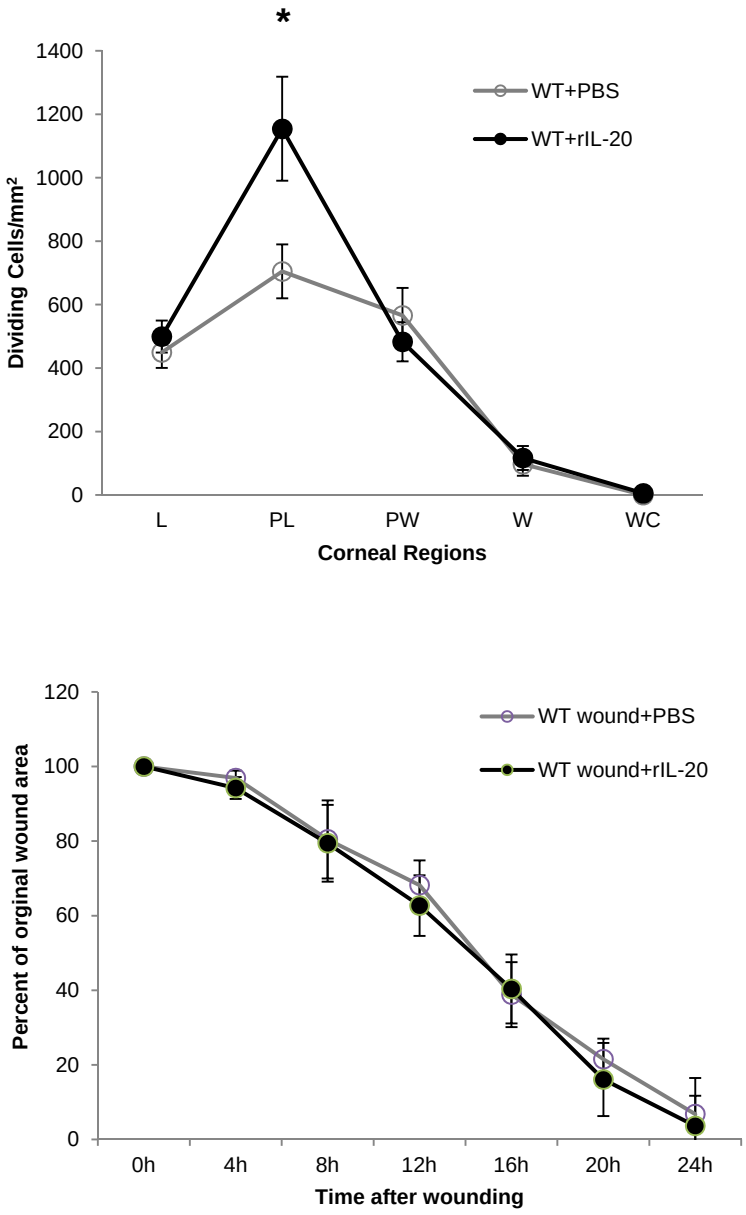
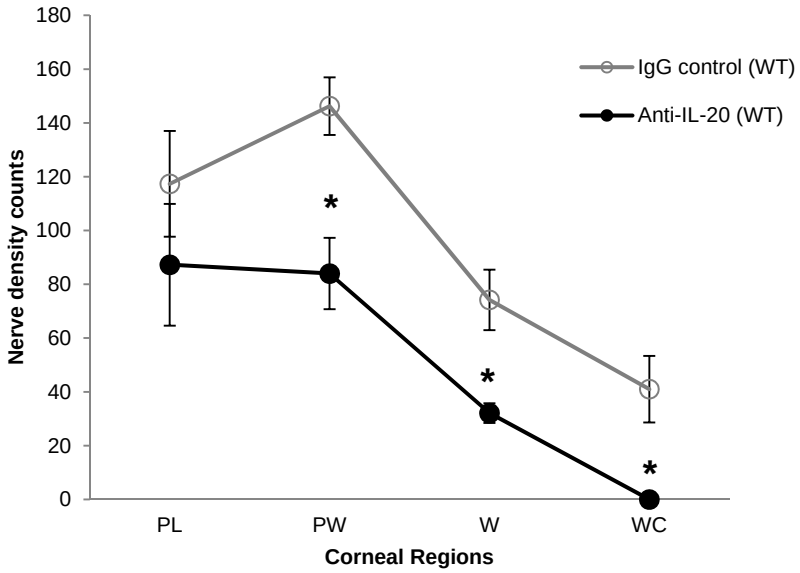
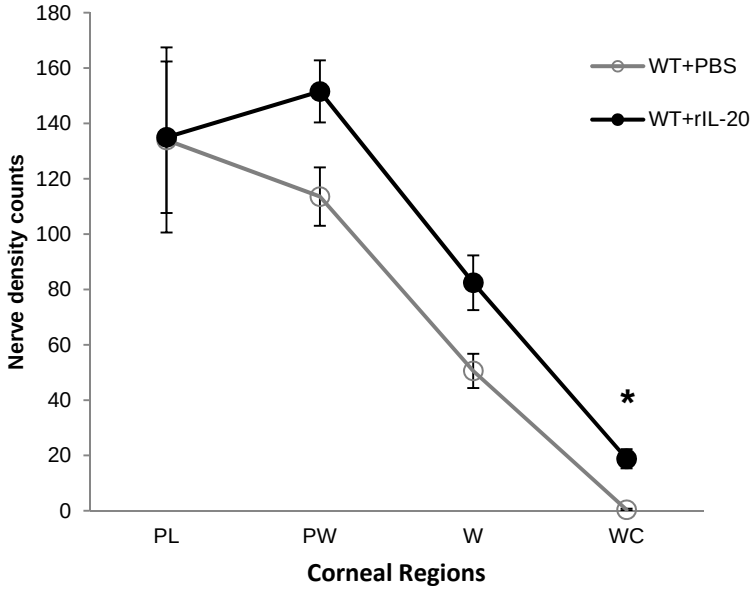


Figure 17 - IL-20 promoted cell division, but not wound closure, in wildtype mice. In WT mice, rIL-20 significantly increased epithelial cell division at PL at 24h after injury. In the presence of rIL-20, the rate of epithelial wound closure was not different from control (PBS) injured mice. * = P≤0.05 compared to control.


180
160
140
120
100
80
60
40
20
0
Nerve density counts
IgG control (WT)
Anti-IL-20 (WT)
*
*
*
PL
PW
W
WC
Corneal Regions


180
160
140
120
100
80
60
40
20
0
Nerve density counts
WT+PBS
WT+rIL-20
*
PL
PW
W
WC
Corneal Regions

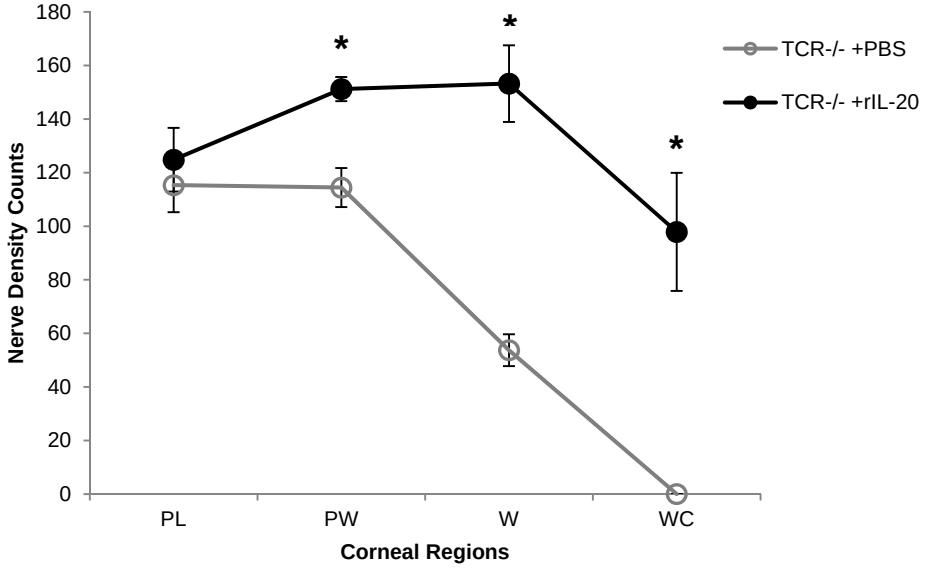


Figure 18 - IL-20 was necessary for nerve regeneration.

Topical application of neutralizing anti-IL-20 antibody markedly suppressed nerve recovery in WT mice from PW to WC, while application of rIL-20 significantly enhanced nerve regeneration in WT mice at WC and in TCR-/- mice across the cornea. * = $P \leq 0.05$ compared to control.

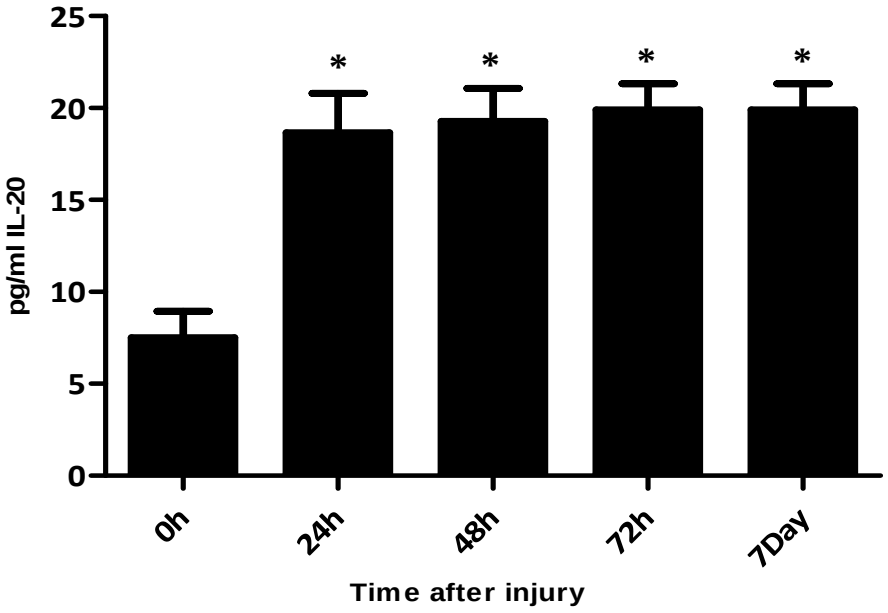
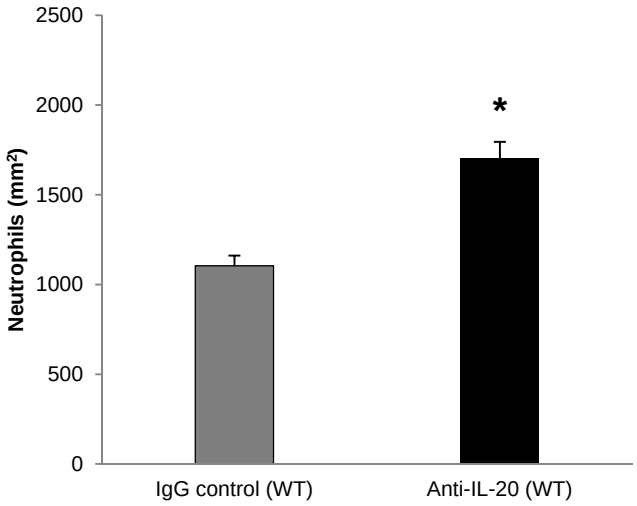
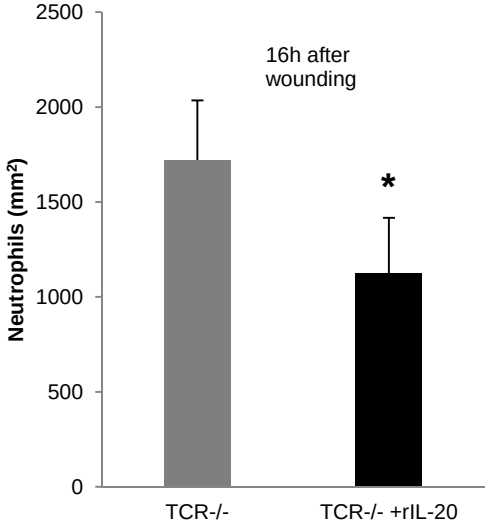
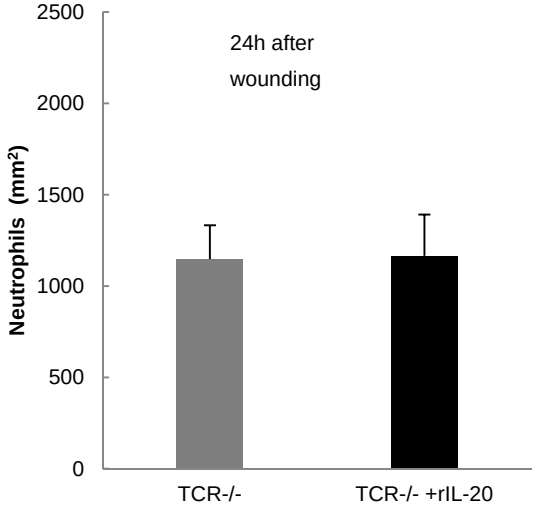


Figure 19 - IL-20 expression increased after corneal epithelial abrasion.

IL-20 production was measured by ELISA from corneal extracts collected at 0, 24, 48, 72 hours and 7 days after epithelial injury (see Methods for details). IL-20 increased sharply at 24h and remained elevated through 7 days.


2500
2000
1500
1000
500
0
Neutrophils (mm²)
*
IgG control (WT)
Anti-IL-20 (WT)


2500
2000
1500
1000
500
0
Neutrophils (mm²)
16h after
wounding
*
TCR-/-
TCR-/- +rIL-20


2500
2000
1500
1000
500
0
Neutrophils (mm²)
24h after
wounding
TCR-/-
TCR-/- +rIL-20

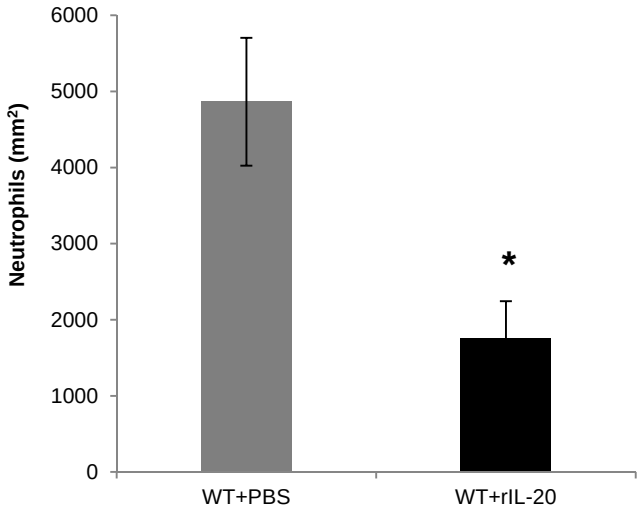


Figure 20 - IL-20 inhibited neutrophil infiltration.

Topical application of neutralizing anti-IL-20 antibody enhanced neutrophil infiltration 24h after injury in WT mice. Compared to control, topical application of rIL-20 to TCR-/- mouse corneas suppressed neutrophil infiltration16h after injury while no differences was found for neutrophil infiltration at 24h. In WT mice, rIL-20 markedly suppressed neutrophil recruitment at 24h after injury. * = $P \leq 0.05$ compared to control.

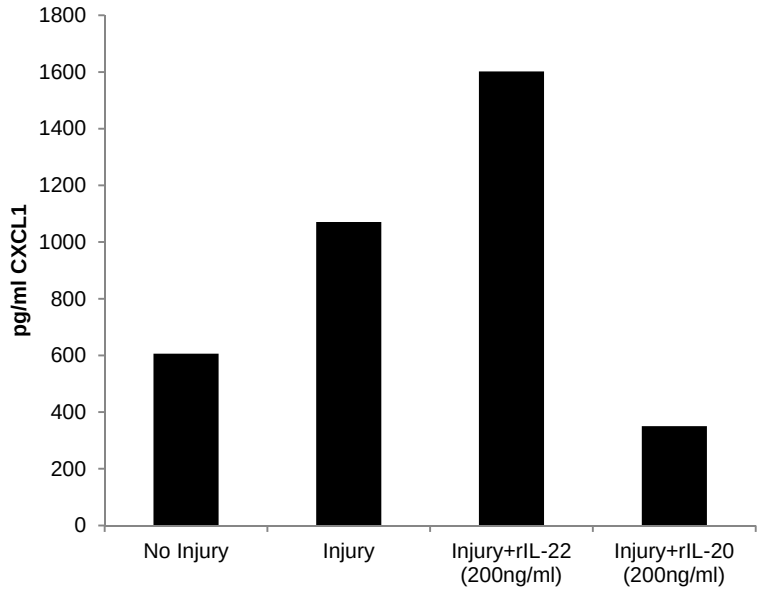


Figure 21 - IL-20 suppressed CXCL1 production in wounded wildtype mouse corneas.

CXCL1 production was measured by ELISA from cultured unwounded and wounded mouse cornea supernatants (see Methods for details). CXCL1 production increased with injury and, as expected, production was further enhanced by the addition of rIL-22. In contrast, the addition of rIL-20 to the injured corneas markedly suppressed CXCL1 production.

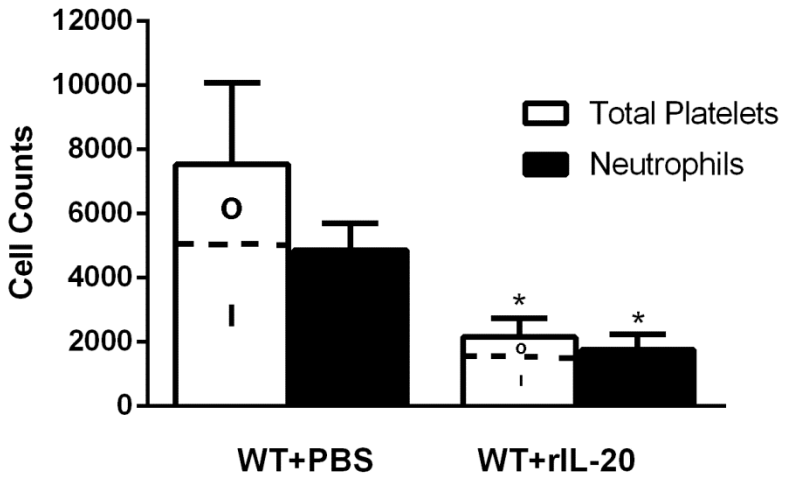


Figure 22 - IL-20 inhibited platelet and neutrophil recruitment in WT mice.

Topical application of rIL-20 significantly inhibited both platelet recruitment at limbus and neutrophil recruitment at paralimbus in wounded wildtype mice at 24h post-injury ($P \leq 0.05$). The inhibition of platelet recruitment involved reductions in both intravascular (I) and extravascular (O) platelets. * = $P \leq 0.05$ compared to control.

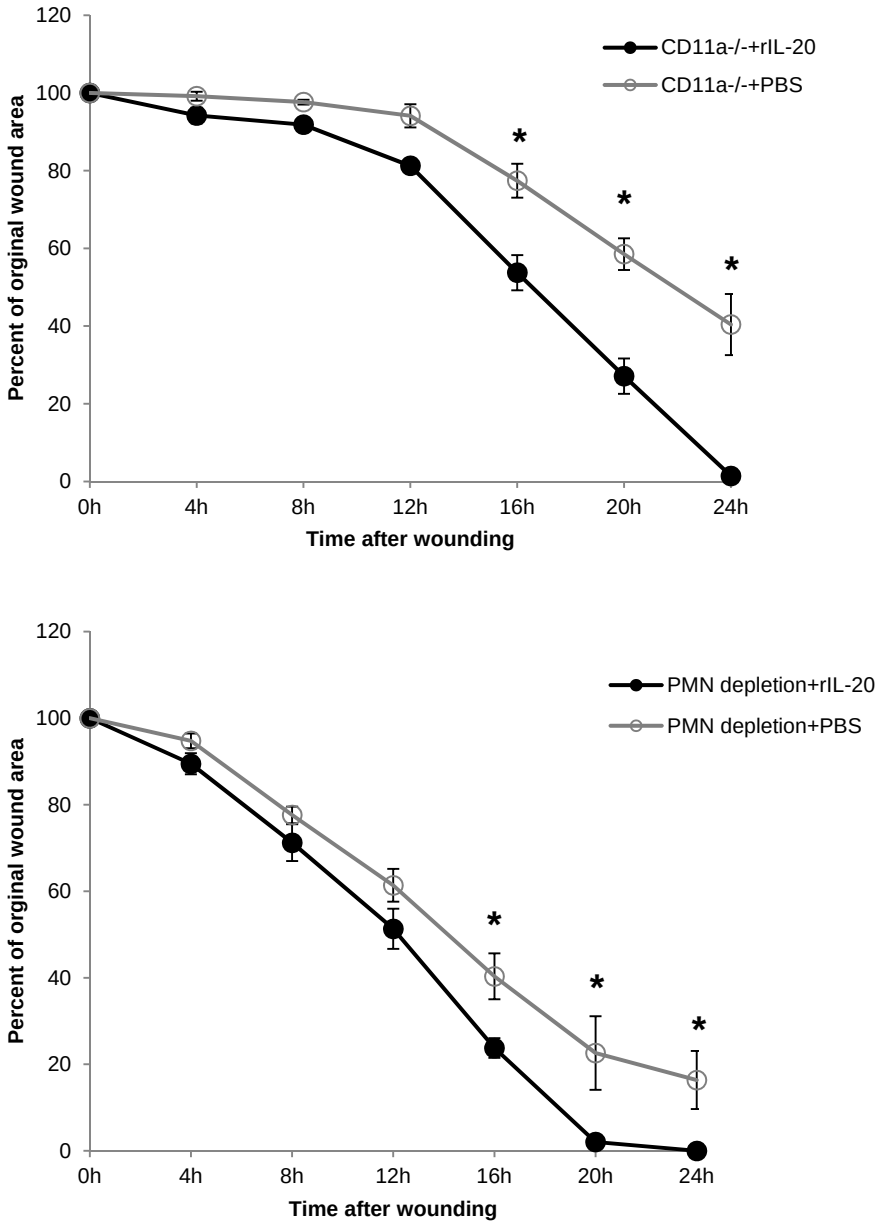


Figure 23 - IL-20 promoted epithelial wound closure in CD11a-/- and neutropenic mice.

After topical application of rIL-20, the rate of wound closure was elevated from16 to 24h in CD11a-/- and neutropenic (neutrophil-depleted) mice. * = P≤0.05 compared to control.

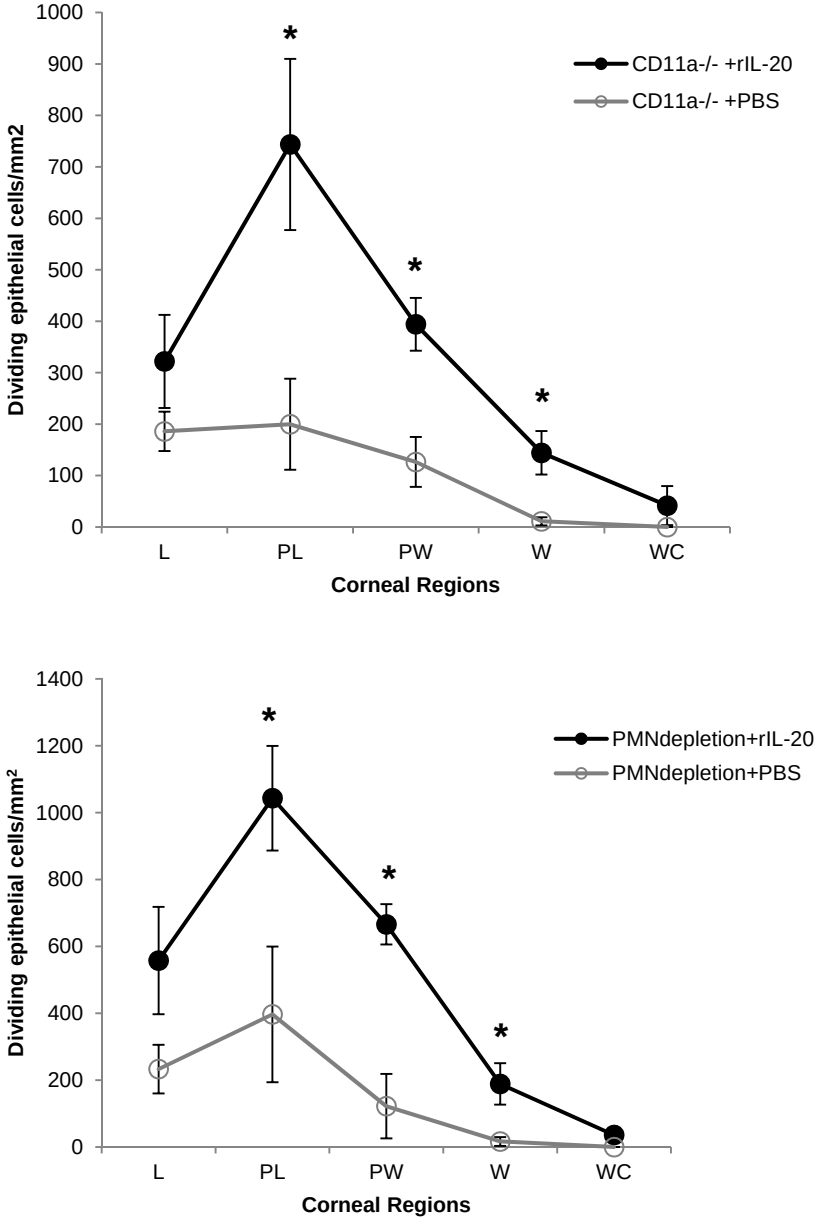
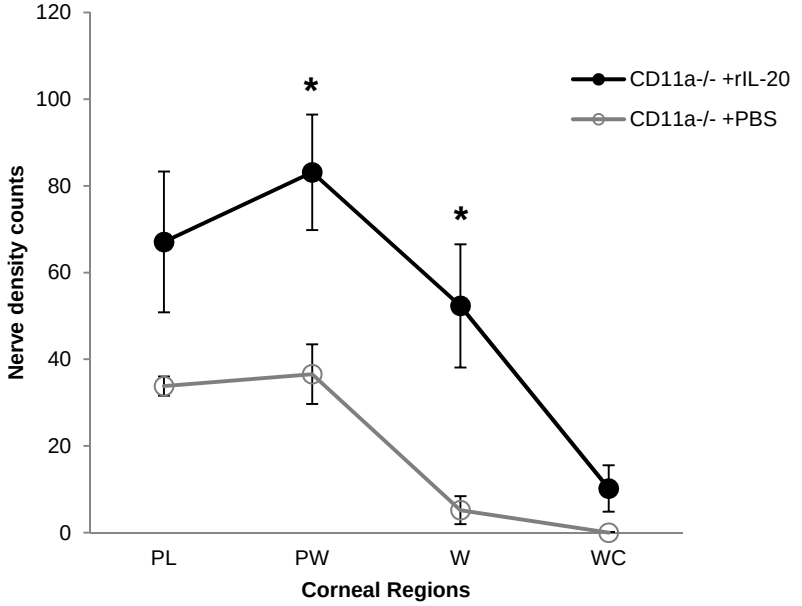


Figure 24 - IL-20 promoted cell division in injured CD11a-/- and neutropenic mouse corneas.

After topical application of rIL-20, corneal epithelial cell division increased significantly from PL to WC in both CD11a-/- and neutropenic (neutrophil-depleted) mice. * = P≤0.05 compared to control.


120
100
80
60
40
20
0
Nerve density counts
*
*
CD11a-/- +rIL-20
CD11a-/- +PBS
PL
PW
W
WC
Corneal Regions

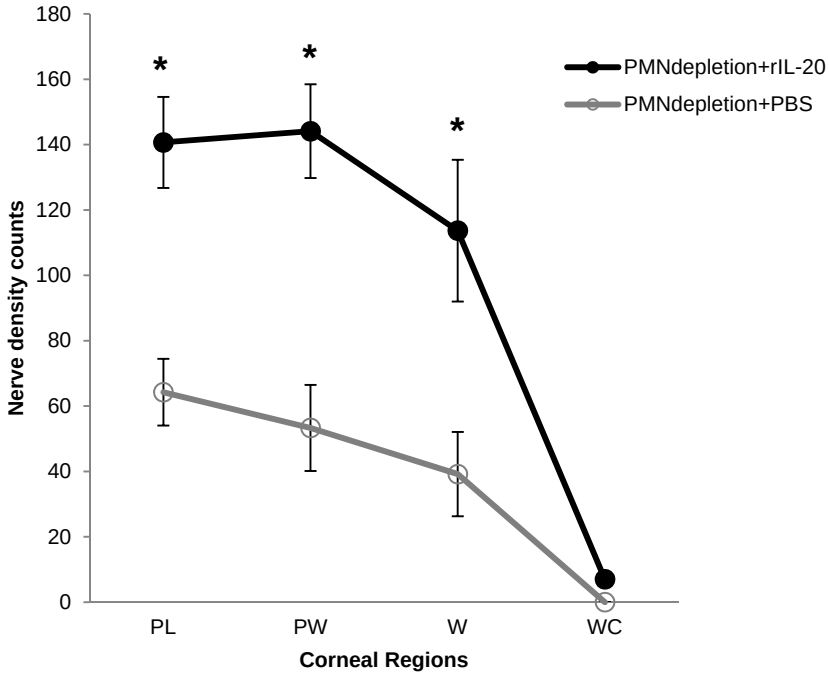

180
160
140
120
100
80
60
40
20
0
Nerve density counts
*
*
*
PMNdepletion+rIL-20
PMNdepletion+PBS
PL
PW
W
WC
Corneal Regions

Figure 25 - IL-20 promoted corneal nerve regeneration in CD11a-/- and neutropenic mice.

After topical application of rIL-20, nerve recovery increased at PL and PW in CD11a-/- mice. Nerve recovery was also enhanced in neutropenic mice across the injured corneas from PL to W. * = $P \leq 0.05$ compared to control.

3.4 Discussion

The purpose of this study was to determine the cellular sources of IL-20 and whether IL-20 plays a beneficial role in corneal wound healing in mice. Immunostaining shows, after injury, corneal epithelial cells and keratocytes are positive for IL-20, a cytokine known to induce keratinocyte proliferation and differentiation (Blumberg et al., 2001). Additional immunostaining shows the corneal epithelium is also positive for the IL-20 receptor subunit IL-20R1, raising the possibility that IL-20 influences corneal wound healing. Indeed, topical application of neutralizing IL-20 antibody markedly delays epithelial wound closure, epithelial cell division, and epithelial nerve recovery while it enhances neutrophil infiltration. Conversely, using three distinct mouse models with known reductions in neutrophil infiltration following corneal abrasion and resultant delays in corneal wound healing (neutropenic wildtype mice, TCR-/- and CD11a-/- mutant mice), topical application of rIL-20 restores wound healing as evidenced by accelerated wound closure, increased epithelial cell division and enhanced nerve recovery. Topical application of rIL-20 on wounded wildtype mouse corneas results in inhibition of platelet recruitment; a similar level of inhibition is also seen for neutrophils. Collectively, these data suggest IL-20 plays a beneficial and direct role in corneal wound healing while negatively regulating neutrophil and platelet infiltration.

Wolk et al. reported that monocytes are the only leukocytes known to express IL-20 (Wolk et al., 2002). Since we found the majority of limbal macrophages occupy a perivascular position, where they may interact with infiltrating leukocytes and release inflammatory mediators following an epithelial abrasion injury, we hypothesized IL-20 may be expressed in corneal macrophages and contribute to wound healing. However,

immunostaining shows corneal macrophages are negative for IL-20 staining while distinct staining is evident in corneal epithelial cells and keratocytes. Moreover, staining for the IL-20 receptor subunit, IL-20R1, is readily detectable on corneal basal epithelial cells. Receptors for IL-20 exist as heterodimers as IL-20R1/IL-20R2 and IL-22R1/IL-20R2 (Dumoutier et al., 2001). Since the corneal epithelium stains positively for IL-22R1 (Li et al., 2011b), there is the potential for two IL-20 receptors within the mouse corneal epithelium. Unexpectedly, we didn't see IL-20R2 staining. IL-20R2 gene expression is detectable in skin by RT-PCR, while IL-20R2 staining was not detected (Blumberg et al., 2001). We've used three different IL-20R2 antibodies conjugated with various fluorochromes and purchased from different antibody companies, but none showed positive staining in mouse cornea. Li *et al.* reported IL-22R1 is expressed on corneal epithelial cells (Li et al., 2011b) however, they didn't show IL-20R2 immunostaining. Since IL-22 functions through binding to the IL-22R1/IL-20R2 heterodimer and IL-22 clearly promotes corneal epithelial wound healing (Li et al., 2011b), we assume IL-20R2 is present on corneal epithelial cells but not accessible to antibody detection. In vitro, binding of IL-20 to its receptor activates a STAT3-dependent pathway and promotes cell proliferation in a human keratinocyte cell line (Blumberg et al., 2001) and a glioblastoma cell line (Chen and Chang, 2009). In vivo, IL-20 induces keratinocyte proliferation and differentiation in normal skin (Blumberg et al., 2001; Roupe et al., 2010).

After wounding, a 2mm diameter circular region of central corneal epithelium was totally removed and this was associated with central anterior keratocyte death beneath the wounded area. Epithelial cells and keratocytes are the sources of IL-20 and many of these

cells are lost during wounding. The fact that IL-20 expression didn't drop after wounding suggests IL-20 expression increases in the surviving epithelial cells and keratocytes.

In the present study, IL-20 clearly has functional effects in the cornea since antibody neutralization of IL-20 delays corneal wound healing while addition of rIL-20 promotes wound healing. Interestingly, application of rIL-20 inhibits neutrophil infiltration by 60% while antibody neutralization of IL-20 enhances neutrophil infiltration by 35%. These observations are in marked contrast to the published effects of IL-22 on neutrophil recruitment in the injured cornea, a somewhat surprising finding since both cytokines belong to the IL-10 superfamily and share receptor subunit overlap. IL-22 indirectly promotes neutrophil infiltration by inducing epithelial CXCL1 expression (Li et al., 2011b), a potent neutrophil chemoattractant, whereas IL-20 inhibits CXCL1 expression. Thus, IL-20 may be an important cytokine that counterbalances the effect of IL-22.

The beneficial effect of neutrophil infiltration appears to be linked to the release of neutrophil-derived VEGF (Li et al., 2011a) which promotes epithelial wound closure and nerve recovery. Typically, when neutrophil recruitment is dysregulated (too few or too many neutrophils) corneal wound healing is delayed. In wildtype mice, rIL-20 clearly diminishes neutrophil recruitment yet corneal wound healing parameters (wound closure rate, cell division and nerve recovery) seem unaffected, suggesting rIL-20 also has an additional direct positive effect on wound healing, distinct from its inhibitory effect on neutrophil recruitment. This suggestion is supported by the observation that rIL-20 restores corneal wound healing in three specific mouse models (TCR-/-, CD11a-/- and neutropenic mice) known to have markedly reduced neutrophil infiltration and impaired

corneal wound healing. The fact that rIL-20 inhibits neutrophil recruitment while promoting corneal wound healing is of potential clinical significance.

Thus, IL-20 promotes wound healing without exacerbating inflammation. In fact, it inhibits neutrophil and platelet recruitment and therefore, reduces the inflammatory response. Although eliminating foreign pathogen is vital for host defense, damage to the host eye because of excessive numbers of neutrophils can be devastating and lead to loss of corneal transparency. In the clinic, IL-20 might be useful in the treatment of inflammatory eye diseases involving injury to the cornea.

Since rIL-20 inhibits neutrophil recruitment, we anticipated that it would not induce production of the potent neutrophil chemoattractant, CXCL1, as occurs when rIL-22 is topically applied to the injured cornea (Li et al., 2011b). Interestingly, even though the two cytokines share the same receptor, IL-20 can also bind to a second distinct receptor that possibly accounts for the ELISA data showing IL-20 does not induce CXCL1 production. In fact, it appears to inhibit the production of CXCL1 following a corneal abrasion injury. Hence, the second function of IL-20 (in addition to its positive effects on corneal wound healing) may be to limit or regulate neutrophil recruitment into the injured cornea by limiting the levels of the CXCL1 chemokine.

Platelet recruitment after corneal abrasion is tightly linked to neutrophil recruitment (Li et al., 2006c). This likely explains why rIL-20 inhibits not only neutrophil recruitment (through diminished CXCL1 levels) but also platelets. While platelets are generally considered to be non-motile, there is one publication suggesting they are capable of limited random movement when evaluated in a Boyden chamber under the stimulus of carbachol (Valone et al., 1974). The experimental conditions required to observe platelet

random migration are very artificial and require non-physiologic low levels of calcium and magnesium, otherwise the platelets aggregate rather than migrate. Hence, in vivo, where divalent cation concentrations are likely to be higher, it seems unlikely that platelets would migrate under their own power, and this is consistent with a requirement for neutrophils to assist in their migration. The molecular explanation for why recruitment of neutrophils and platelets to the injured cornea is co-dependent remains to be determined and is the subject of active investigation.

In conclusion, the data show that IL-20 plays a beneficial and direct role in corneal wound healing while negatively regulating neutrophil and platelet infiltration. To our knowledge, the finding that efficient corneal wound healing is dependent on expression of IL-20 is novel and worthy of further investigation. The ability of IL-20 to promote corneal wound healing without exacerbating neutrophil recruitment makes it a promising therapeutic for the treatment of corneal injuries in the clinic.

Chapter 4- Aim 3: To determine if oncomodulin plays a beneficial role in corneal wound healing

4.1 Introduction

The long standing dogma that the central nerve system can't regenerate after injury has recently been challenged. Leon and colleagues found retinal ganglion cells are able to regenerate axons following optic nerve or peripheral nerve grafting following lens injury both in cell culture and in vivo in the rat (Fischer et al., 2001; Fischer et al., 2000; Leon et al., 2000). Additional experiments in the mouse and cat showed the recruitment and activation of macrophages in response to injury was necessary for retinal ganglion cell axon regeneration following optic nerve implantation (Leon et al., 2000; Lorber et al., 2005; Okada et al., 2005; Yin et al., 2003). Collectively, these studies suggest macrophages may secrete neurotrophic factors that promote axon regeneration.

While macrophages are able to secrete various neurotrophic factors like brain-derived neutrophic factor (BDNF), platelet-derived growth factor (PDGF), glial cell line-derived neutrophic factor (GDNF), and interleukin-6 (IL-6), none of these factors appear to stimulate retinal ganglion cell axon outgrowth (Yin et al., 2003). More recently, in vitro and in vivo studies demonstrated that macrophages and neutrophils can express oncomodulin and that oncomodulin supports central and periphery nerve regeneration (Kurimoto et al., 2013; Yin et al., 2009; Yin et al., 2006). Adding activated macrophages (more oncomodulin production) to cultured retinal ganglion cells stimulates axon regeneration (Yin et al., 2003). In vivo, antibody depletion of neutrophils, which leads to decreased oncomodulin release at the site of injury, or blocking oncomodulin function

using a peptide inhibitor, diminished axon regeneration in the rat retina (Kurimoto et al., 2013).

Limbal corneal macrophages occupy perivascular positions while neutrophils migrate into the cornea from limbal vessels after injury. Documenting oncomodulin staining in these two cell types is the first step to linking leukocyte-derived oncomodulin with corneal epithelial nerve regeneration after abrasion. However, whether oncomodulin promotes nerve recovery in the injured mouse cornea and whether it has any effect on epithelial wound healing is unknown. After corneal epithelial injury, rapid wound healing involves not only epithelial wound closure, but also nerve recovery. We hypothesize oncomodulin released by corneal macrophages and neutrophils plays a beneficial role in corneal nerve regeneration. The purpose of this study was two-fold. First, to determine if corneal macrophages and infiltrating neutrophils stain positively for oncomodulin. Second, to determine if oncomodulin contributes to corneal epithelial nerve regeneration following a central abrasion.

4.2 Materials and Methods

Animal model

Studies were carried out at Baylor College of Medicine (Houston, TX) and all mice for use were 8-12 weeks old and were treated following the guidelines in the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Eight female C57BL/6J wildtype mice were purchased from Jackson Laboratory (Bar Harbor, ME). The central corneal wound was made following an established protocol (Li et al., 2006b; Li et al., 2006c). Briefly, mice were anesthetized by intraperitoneal injection of

pentobarbital sodium solution (50mg/kg) and a 2mm diameter central corneal epithelial region was marked by a trephine and mechanically debrided with a golf club spud under a dissecting microscope.

Immunohistology

Wildtype unwounded and 18h wounded female C57BL/6J mice were sacrificed and dissected corneas (including limbus) were fixed for 30 mins in phosphate buffered saline (PBS, pH 7.2) containing 2% paraformaldehyde. Fixed corneas were washed in PBS 3 times, 5 mins each and then permeabilized with 0.1% Triton X-100 for 30 mins. Corneas were then blocked for 30 min with 1% bovine serum albumin. Dissected corneas were incubated with anti-oncomodulin antibody (a kind gift from Dr. Larry Benowitz) together with Alexafluor 488-conjugated anti-CD301 for macrophages (AbD Serotec, Raleigh, NC), FITC-conjugated anti-Ly6G for neutrophils (BD Pharmingen, San Jose, CA) and /or FITC-avidin for mast cells (Invitrogen, Grand Island, NY). Other antibodies used were: PE or APC-conjugated anti-CD31(BD Pharmingen, San Jose, CA) for detection of blood vessels; PE-conjugated anti-β-III tubulin (R&D Systems, Minneapolis, MN) for detection of nerves and FITC-conjugated monoclonal anti-α-tubulin (Sigma, St. Louis, MO) for detection of mitotic spindles. Appropriate fluorchrome labeled non-immune isotype matched antibodies served as controls for non-specific staining. Unwounded corneas were used for comparison with injured corneas. All immunostained corneas were imaged as wholemounts using a 40X oil immersion lens mounted on a DeltaVison Core Spectris microscope system (Applied Precision, Issequah, WA).

Local effect of oncomodulin-specific blocking peptide

Yin *et al.* reported the existence of oncomodulin receptor on retinal ganglion cells surface for observation of high-affinity binding of oncomodulin to those cells (Yin et al., 2006). Although the specific oncomodulin receptor is still unknown, an oncomodulin-specific blocking peptide P1 was developed to interfere with oncomodulin binding. P1 is a 24 amino acid peptide that corresponds to the N-termius of oncomodulin and has been shown to inhibit oncomodulin binding (Yin et al., 2009). To study the local effect of oncomodulin on corneal epithelial wound healing, oncomodulin function was blocked by topical application of P1 (a kind gift from Dr. Benowitz). Briefly, 8 wildtype mice received corneal epithelial abrasions and were divided into 2 groups. In the first group, 4 mice received a topical application (5µl) of oncomodulin-specific blocking peptide P1dissolved in PBS (100ng/5ul). The process was repeated every 6 hours for 24 hours. In the other group of 4 mice, a control peptide P3 (a kind gift from Dr. Benowitz) dissolved in PBS (100ng/5ul) was topically applied and used as control. P3 is a peptide containing amino acids 25-48 from the N-termius of oncomodulin. P3 does not inhibit oncomodulin binding (Yin et al., 2009). The rate of corneal wound closure was determined using an established protocol (Li et al., 2006a, b; Li et al., 2006c). Briefly, sodium fluorescein was applied to the wounded cornea every 6 hours beginning at wounding and up to 24 hours. Photographs were taken right after sodium fluorescein application and the peptides were given after respectively. The extent of fluorescein staining demarcates the size of the open wound and images were photographed with a digital camera and analyzed by Optimus 6.2 software (Media Cybernetics). The relationship of wound size at various time points establishes the rate of wound closure after injury. At 24h post-injury, epithelial nerve

recovery was analyzed in fixed corneal wholemounts at 4 regions (PL, PW, W and WC). Nerves were imaged after staining with anti-β-III tublin antibody and serial epithelial images were collected using a 40X oil immersion lens at 0.2μm Z-steps through the entire thickness of the epithelium, deconvolved and saved as a maximum projected image. The projected images were analyzed using a custom nerve tracing program (Matlab) that quantifies nerve density. Dividing basal epithelial cells, where mitotic spindles stained positively with anti-α-tubulin antibody, were counted from L to WC and compared between P1 and P3 treated groups.

Statistical analysis

Data were analyzed using a two-way analysis of variance (ANOVA) with a Tukey post-test for pair-wise multiple comparisions. Data are expressed as means±SEM and P values ≤ 0.05 were considered statistically significant.

4.3 Results

Oncomodulin staining was detected in corneal mast cells and neutrophils

Immunostaining showed oncomodulin staining was clearly evident in a sub-population of mast cell granules both before and after injury (Figure 26). Less intense staining for oncomodulin was observed in infiltrating neutrophils (Figure 27). Surprisingly, oncomodulin staining was not detected in CD301+ macrophages (data not shown).

Blocking oncomodulin function diminished corneal nerve regeneration

To study the local effect of blocking oncomodulin function on corneal epithelial wound healing, a blocking peptide P1 or control peptide P3 was topically applied on an injured wildtype mouse cornea every 6 hours up to 24 hours after injury. No significant difference was detected for wound closure rates at all time points after injury after topical application of blocking peptide P1 when compared to control peptide P3 (Figure 28). However, after blocking oncomodulin function with P1 peptide, nerve regeneration at 24h post-injury was markedly diminished ($P \leq 0.05$) at the PL and W (Figure 29).

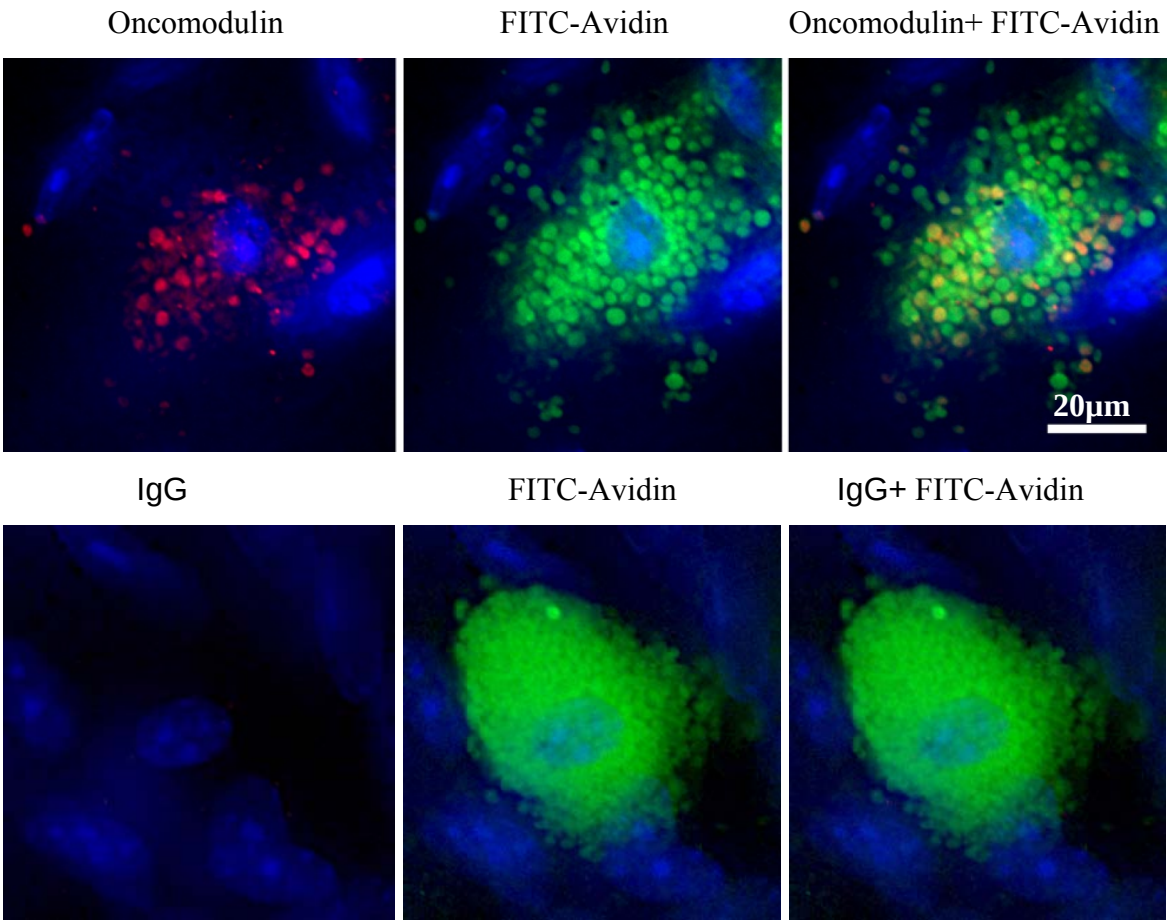


Figure 26 - Oncomodulin staining was observed in mast cell granules.

Oncomodulin staining (red) was seen in FITC-avidin stained (green) mast cell granules 24h after wounding which show orange colocalization in the third image in top row.

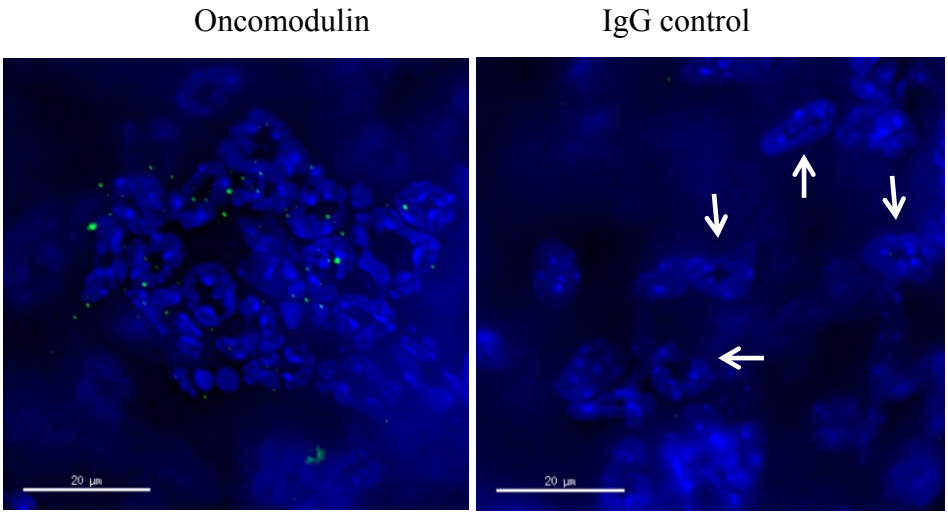


Figure 27 - Oncomodulin staining was detected in infiltrating neutrophils.

Oncomodulin staining (green) was detected in infiltrating neutrophils after wounding, while such staining was not seen in non-immune IgG control images (white arrows point to infiltrating neutrophils in IgG control mouse cornea image).

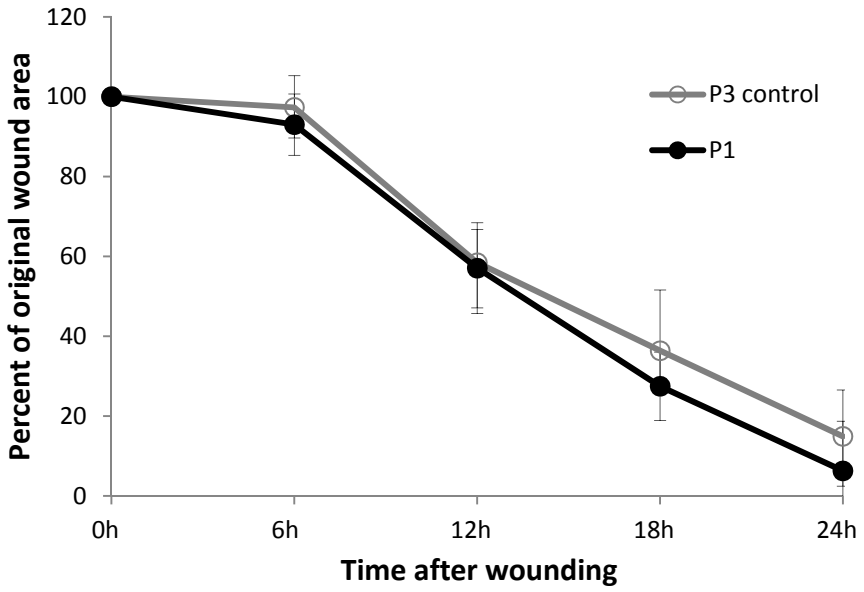


Figure 28 - Blocking oncomodulin function had no effect on epithelial wound closure. Topical application of blocking peptide P1 had no effect on the rate of wound closure and was not different from control peptide P3.

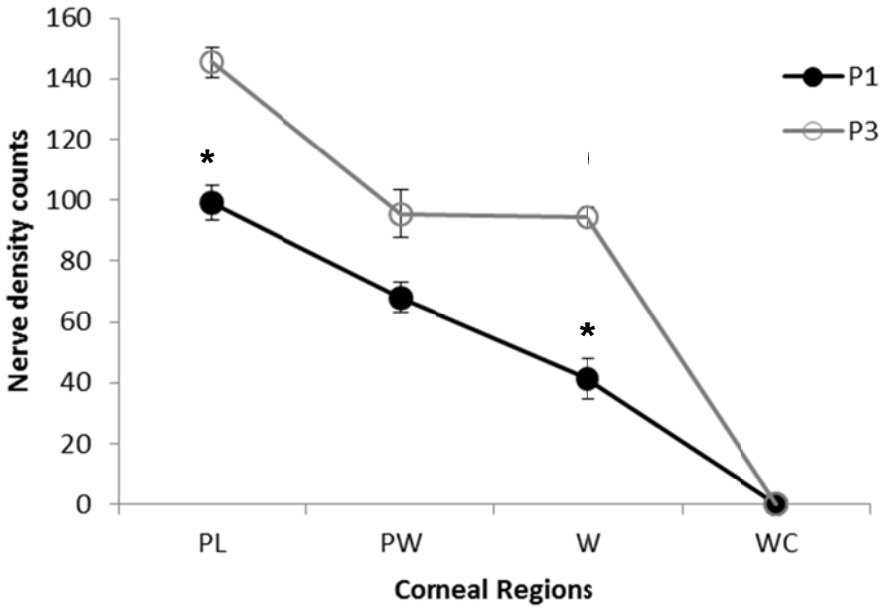


Figure 29 - Blocking oncomodulin function inhibited nerve regeneration.

Blocking oncomodulin function with peptide P1 significantly diminished nerve regeneration in wildtype mice at PL and W after wounding when compared to control peptide P3. * = P≤0.05 compared to P3.

4.4 Discussion

The purpose of this study was two-fold. First, to determine if corneal macrophages and infiltrating neutrophils stain positively for oncomodulin. Second, to determine if oncomodulin contributes to corneal epithelial nerve regeneration following a central abrasion. The data show limbal mast cells and infiltrating neutrophils stain positively for oncomodulin and that blocking onocomodulin function has no effect on wound closure rates after abrasion but nerve regeneration is significantly delayed. Surprisingly, oncomodulin staining was not detected in corneal macrophages.

Yin and colleagues reported oncomodulin is expressed in macrophages and neutrophils in the injured rat retina based on immunostaining of cells collected from vitrious humor in posterior chamber (Kurimoto et al., 2013; Yin et al., 2009; Yin et al., 2006). In the present study, the cellular sources of oncomodulin appear to be mast cells and neutrophils. It is unclear why corneal CD301+ macrophages failed to stain for oncomodulin. It is not because of technical limitations (antigen fixation sensitivity, incorrect antibody concentration, incorrect antibody incubation conditions, limited antibody tissue penetration, etc.) because distinct staining was also observed in mast cells and, to a lesser extent, in neutrophils.

Oncomodulin is a novel calcium binding protein that was detected in 1979 and was originally thought to be expressed only in fetal and cancerous tissues (MacManus, 1979). It is now clear that oncomodulin can also be expressed in adult tissue, as evidenced by immunostaining in the outer hair cells in the organ of Corti in a variety of vertebrates where it is thought to be involved in mediation of cholinergic stimulation (Sakaguchi et al., 1998). Oncomoculin belongs to the parvalbumin family which is composed of α and β

sublineages (Goodman and Pechere, 1977). Early studies referred to oncomodulin as β-parvalbumin, which is different than mammalian muscle α-parvalbumin based on amino acids composition (MacManus et al., 1983a, b). While the structure of oncomodulin has been explored, its function is less well understood.

Recently oncomodulin was shown to play a beneficial role in optic nerve and peripheral nerve regeneration in vitro and in vivo in the rat retinal tissue (Yin et al., 2003; Yin et al., 2006). Oncomodulin production from activated macrophages following intravitreal injection of zymosan stimulates axon regeneration in retinal ganglion cells (Yin et al., 2003), while oncomodulin neutralization or decreased oncomodulin production following neutrophil depletion diminishes axon regeneration in rat retina (Kurimoto et al., 2013; Kurimoto et al., 2010). In the present study, after corneal epithelial injury, rapid wound healing involves wound closure and nerve regeneration. Our data suggest a role for oncomodulin in nerve regeneration but not epithelial recovery.

Mast cells reside at the limbus and conjunctiva in the mouse (this study), human, and rat (Allansmith et al., 1980; Iwamoto and Smelser, 1965; McMenamin et al., 1996; Smith, 1963). In other tissues, mast cells play important roles in allergy and anaphylaxis because they are a rich source of histamine (Dachman et al., 1994; Hirasawa and Ohuchi, 2011; Sawaguchi et al., 2012; Shin et al., 2009). Mast cells are also involved in wound healing such as re-epithelialization, revascularization of injured tissue, collagen deposition and matrix remodeling (Artuc et al., 1999; Gottwald et al., 1998). They are also a source of various of growth factors (e.g., VEGF, PDGF, and NGF) (Abe et al., 2000; Noli and Miolo, 2001; Weller et al., 2006). Based on our present immunostaining results, we now show for the first time that a sub-population of mast cells granules stain positively for

oncomodulin. This observation suggests mast cells may contribute to corneal nerve regeneration through oncomodulin secretion.

Neutrophils appear to contribute to corneal epithelial nerve regeneration after injury by releasing the growth factor VEGF. Supporting evidence shows that prior to corneal injury, antibody depletion of neutrophils markedly decreases corneal VEGF levels after abrasion and this is associated with delayed and diminished nerve recovery. In a separate set of experiments, antibody blockade of VEGF function also diminished nerve recovery (Li et al., 2011a). It is well established that after injury, a large number of neutrophils infiltrate the wounded cornea. Although oncomodulin staining in neutrophils is less intense when compared to mast cell staining, the large number of neutrophils that enter the injured cornea far exceeds the resident number of mast cells at the limbus and suggests corneal neutrophils are a major source of oncomodulin.

P1 is a designed blocking peptide representing the first 24 amino acids from the N-terminus of oncomodulin which are thought to be necessary in receptor binding. P3 control peptide contains the next 24 amino acids of oncomodulin (25-48) and it does not inhibit oncomodulin binding (Yin et al., 2009). Topical application of P1 did not affect the rate of wound closure after corneal epithelial injury when compared to control peptide P3. However, corneal epithelial nerve regeneration is significantly diminished after blocking oncomodulin function. These results suggest oncomodulin plays an important role in corneal wound healing by promoting nerve regeneration. Since neutrophils can release both VEGF and oncomodulin, further experiments are warranted to determine the relative contributions of these two growth factors to epithelial nerve regeneration. Since mast cell granules are also a potential source for oncomodulin, and mast cells reside at the limbus,

mast cells are ideally positioned to release oncomodulin at the peripheral cornea and it is the peripheral cornea where nerve regeneration begins following a large (2 mm) central abrasion in the mouse cornea. Determining the relative contribution of mast cell-derived oncomodulin and neutrophil-derived oncomodulin to corneal nerve regeneration warrants further investigation as it may be a potential therapeutic growth factor for corneal nerve regeneration after injury.

Chapter 5- Summary and future directions

5.1 Summary

The cornea works as a refractive medium and protective barrier for the eye. Thus, maintaining corneal transparency is of vital importance to human health. After corneal epithelial injury, inflammation is beneficial to wound healing because it promotes efficient epithelial wound closure and nerve regeneration. However, even though macrophages are known to reside within the mouse cornea, nothing is known regarding the distribution of macrophage phenotypes and how macrophages respond to corneal injury with respect to wound healing. This dissertation focused on macrophages and sought to determine if macrophage-derived IL-20 and oncomodulin contribute to corneal epithelial wound healing after injury.

The significance of this dissertation is: (1) a specific macrophage phenotype CD301+ M2 was detected in mouse cornea and these macrophages were able to infiltrate the cornea after wounding with a biphasic pattern. (2) IL-20 and oncomodulin staining was found in the cornea and both were found to be capable of promoting corneal epithelial wound healing. What is more, IL-20 has anti-inflammatory properties that limit excessive inflammation by decreasing neutrophil and platelets infiltration.

Chapter 1 reviewed corneal development and anatomy in human and mouse. The cascade of corneal inflammation and epithelial wound healing was also addressed in which neutrophils, dendritic cells and NK cells all play important roles for maintaining corneal integrity based on our previous publications. Yet little is known regarding the distribution of macrophage phenotypes and if macrophages contribute to the inflammatory cascade during corneal wound healing.

Thus, in Aim 1, we hypothesized that corneal macrophages can be characterized as M1 (pro-inflammatory) and M2 (anti-inflammatory) phenotypes and their number and distribution within the cornea was expected to change after epithelial abrasion. By using fluorescently tagged antibodies raised against mouse macrophage markers together with known M1 and M2 markers to identify and localize macrophages, the majority of macrophages were found to be of the M2 phenotype as evidenced by positive staining for CD301 (a specific M2 macrophage marker) and F4/80, CD115 and CD206 (three well known M2 macrophage markers). M2 macrophages are located primarily in the peripheral cornea and limbus and most of them occupy a perivascular position (Figure 30). After wounding, CD301+ macrophages show a biphasic infiltration pattern and 91% of the infiltrating macrophages are confined to the anterior stroma (Figure 30). The perivascular location of the M2 macrophages and the ability of these cells to infiltrate the wounded cornea suggests CD301+ macrophages may be able to influence corneal inflammation and wound healing. Unexpectedly, M1 phenotype macrophages were not found in this study. While M1 macrophages stain with CD80 and CD86 (two M1 macrophage markers) in other tissues, CD80+ cells in the cornea are negative for CD86 as well as the traditional macrophage marker F4/80. Further investigation revealed CD80+ cells co-stain with FITC-avidin and this confirmed their identity as mast cells (Figure 30). At the moment, it is unclear why M1 macrophages were not detected in the mouse cornea.

To investigate the function of CD301+ macrophages during corneal epithelial wound healing, two potential macrophage-derived growth factors, IL-20 and oncomodulin, were studied in Aim 2 and Aim 3. Surprisingly, corneal macrophages showed no immunostaining for IL-20 or oncomodulin before or after corneal abrasion.

Instead, IL-20 staining was found in corneal epithelial cells and keratocytes before and after injury, while oncomodulin staining was detected in corneal mast cells and neutrophils (Figure 30). IL-20 is commonly viewed as a pro-inflammatory cytokine based on studies of inflammatory disorders like rheumatoid arthritis and lupus nephritis (Hsu et al., 2006; Wei and Chang, 2008). In my study of the cornea, IL-20 shows a distinct anti-inflammatory characteristic based on the fact that it inhibits CXCL1 (a potent neutrophil chemoattractant) upregulation thereby suppressing neutrophil infiltration. Platelet recruitment is also inhibited since it is tightly linked to neutrophil recruitment (Figure 30). We previously reported that neutrophils and platelets are beneficial for corneal epithelial wound healing. Delayed wound healing is commonly found when neutrophil and platelet recruitment is dysregulated (Li et al., 2011a; Li et al., 2006c). If IL-20 negatively regulates neutrophil and platelet recruitment, predictably, corneal wound healing would be delayed. However, IL-20 clearly supports corneal epithelial wound healing since antibody neutralization of IL-20 delays wound healing while addition of rIL-20 sustains wound healing when neutrophil/platelet infiltration is blunted. Thus, IL-20 plays a beneficial and direct role in corneal wound healing while negatively regulating neutrophil and platelet infiltration. At the moment, it is unclear why IL-20 has anti-inflammatory properties in the cornea, but it suggests IL-20 effects on inflammation (pro- or anti-) may be tissue specific.

Oncomodulin is a novel calcium binding protein that was shown to play a beneficial role in optic nerve and peripheral nerve regeneration in rat (Yin et al., 2003; Yin et al., 2006). Our study shows neutrophils stain positively for oncomodulin and, for the first time, that mast cell granules also stain positively for oncomodulin (Figure 30). Our data suggest a role for oncomodulin in nerve regeneration based on the fact that blocking

oncomodulin function diminished corneal nerve regeneration during the first 24h after injury. Whether oncomodulin promotes long term nerve recovery will require further study.

Given that IL-20 and oncomodulin have the potential to enhance corneal epithelial wound healing, especially nerve regeneration, the combined application of IL-20 and oncomodulin may further improve wound healing. Neutrophil derived VEGF promotes epithelial wound closure and nerve recovery (Li et al., 2011a). However, VEGF can potentially cause corneal angiogenesis (Kvanta et al., 2000). IL-20 negatively regulates neutrophil infiltration thus diminishing VEGF expression while still maintaining epithelial wound healing. Thus IL-20 is a better therapeutic choice than VEGF when it comes to designing treatments for corneal nerve regeneration. Since IL-20 production in the cornea is relatively low, topical rIL-20 combined with oncomodulin, after corneal epithelial injury, should promote efficient corneal epithelial wound healing without exacerbating inflammation and neovascularization. In the clinic, the combination of IL-20 and oncomodulin would be beneficial for efficient epithelial wound healing for normal human eye, especially for type II diabetes. Diabetic keratopathy is a well-known ocular complication associated with neuropathy, delayed wound healing, and high risk of infection (Schultz et al., 1981). IL-20 and oncomodulin may promote corneal nerve regeneration, epithelial wound healing and lower the risk of infection and thus help prevent corneal ulcers. Therefore, IL-20 and oncomodulin may be promising therapeutics for the treatment of corneal injuries of diabetic patients in the clinic.

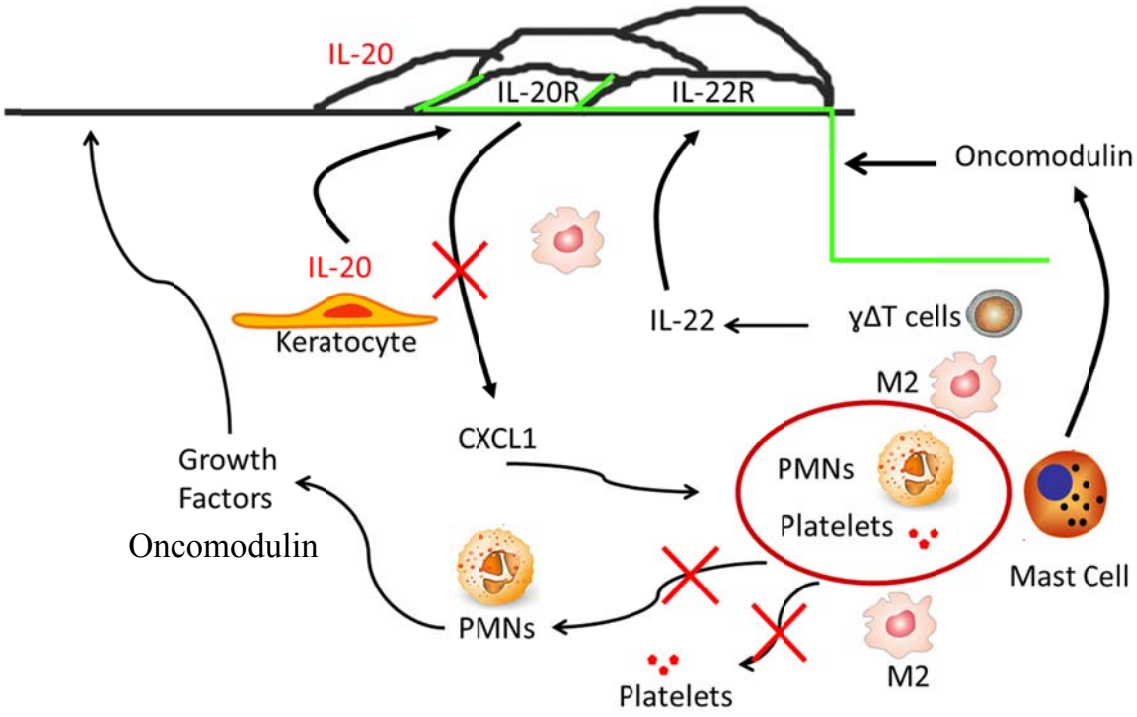


Figure 30 – Summary diagram of macrophage (M2) distribution, role of IL-20 and role of oncomodulin in corneal epithelial wound healing.

IL-20 inhibits epithelial CXCL1 production which limits neutrophil (PMN) and platelet recruitment (see red X's). Nerves (green lines) and blood vessel (red oval) are also shown.

5.2 Future directions

This dissertation showed that a large number of mast cells are located adjacent to the limbal vessels. Previous reports show mast cells are involved in ocular allergy (Fukuda et al., 2009; Irani and Schwartz, 1994; Miyazaki et al., 2008) and wound healing (reviewed by (Asboe-Hansen, 1968)). Whether mast cells contribute to corneal epithelial wound healing is unknown, but through the use of mast cell deficient mice (c-kit), it will be possible to evaluate wound healing parameters (e.g., wound closure rate and epithelial nerve recovery) to determine if mast cells are involved in corneal epithelial wound healing.

Two novel growth factors, IL-20 and oncomodulin, are found in this study and each one enhances corneal epithelial wound healing. IL-20 negatively regulates neutrophil and platelet recruitment while sustaining wound healing and oncomodulin promotes corneal nerve regeneration that is critical for corneal wound healing. These findings are novel and worthy of future investigation.

The corneal epithelium and keratocytes are the cellular sources of IL-20. Following corneal abrasion, keratocyte recovery is incomplete and only reaches 72% of uninjured baseline numbers up to 28 days post-injury (Gagen, thesis). Even so, platelet infiltration is critical for this recovery. When platelet recruitment is inhibited (antibody blockade, P-selectin deficiency), keratocyte recovery worsens. Hence, before IL-20 can be considered as a useful therapeutic for corneal wound healing, it must be determined that the reduction in platelet recruitment observed after rIL-20 treatment does not negatively impact keratocyte recovery. If keratocytes express the IL-20 receptor, IL-20 may

sustain/promote keratocyte recovery in the presence of reduced platelet infiltration. Hence, more work is needed to evaluate IL-20 and its effects on keratocyte recovery.

Nerves are critical for maintaining corneal epithelial integrity and release growth factors and neurochemicals, such as substance P (Nagano et al., 2003; Nakamura et al., 2003; Nakamura et al., 1997; Reid et al., 1993), neuropeptide Y (Jones and Marfurt, 1996; Shigeri and Fujimoto, 1993). Damage to the corneal epithelium also results in damaged corneal nerves. Thus regeneration of corneal nerves is vital to the restoration of corneal integrity and function. Oncomodulin plays a beneficial role in optic nerve and peripheral nerve regeneration in rat (Yin et al., 2003; Yin et al., 2006). In our current study, blocking oncomodulin function significantly decreased corneal epithelial nerve regeneration, which suggests a beneficial role for oncomodulin in corneal epithelial wound healing. In future studies, recombinant oncomodulin should be applicated to wounded mouse cornea to investigate its effect on corneal epithelial nerve regeneration. Since mast cells and neutrophils are sources of oncomodulin in mouse cornea, using mast cell and neutrophil double depleted mice to observe whether oncomodulin is necessary for corneal wound healing would be a critical experiment.

Our study hypothesized a therapeutic effect of using IL-20 and oncomodulin together for the treatment of corneal injuries. A way to test this is to apply this combination to the wounded wildtype mouse cornea and observe the effect on wound healing (corneal wound closure rate, dividing epithelial cell numbers, and epithelial nerve regeneration). However, the dosage of each growth factor in the combination would need to be carefully determined.

In conclusion, this dissertation suggests IL-20 and oncomodulin are potential therapeutic candidates for corneal wound healing and as such, the proposed future studies merit consideration as there may be significant clinical benefits for patients suffering from corneal injuries and disorders (e.g. type II diabetes).

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