Estrogen Regulation of the 15-LOX/LXA_4 Protective Lipid Circuit in the Corneal Inflammatory-Reparative Response

by

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Abstract

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The cornea is a transparent, avascular tissue at the ocular surface that refracts light and protects the eye from microbial infection. Inflammation, while a necessary and beneficial part of wound repair, can be detrimental if left unchecked especially in the cornea where transparency is necessary for vision. The cornea has thus evolved tightly regulated circuits to promote wound healing, maintain host defense, and control inflammation. One such pathway that plays a key role in the cornea is the 15-lipoxygenase(LOX)/lipoxin(LX) A_4 lipid circuit that is present endogenously in the uninjured cornea. LXA_4 is a lipid mediator derived from dietary fatty acids that displays potent anti-inflammatory and pro-resolving bioactions. Previous results have demonstrated a key role for LXA_4 in a murine model of corneal epithelial abrasion where 15-LOX (and subsequent LXA_4) deficiency impairs rate of wound repair while topical administration of LXA_4 restores corneal wound healing.

Sex-specific differences have long been observed in an inflammatory and/or autoimmune setting. Females have a higher incidence of many inflammatory diseases including rheumatoid arthritis, systemic lupus erythematosus, and Sjogren's syndrome. Furthermore, there is a sex-specific difference in rate of dermal wound repair that changes post-menopause, showing that inflammation and wound healing are intimately linked. In the eye, Dry Eye Syndrome is a highly widespread inflammatory-based disease that affects predominantly post-menopausal women. Androgen deficiency has been implicated in Dry Eye disease progression. On the other hand, estrogen's role in the eye has been largely ignored despite the presence of estrogen and its two traditional receptors in leukocytes and most major ocular tissues. An epidemiological study of forty thousand female health professionals revealed a correlation between estrogen replacement therapy and an increase in reported dry eye symptoms, implicating estrogen in inflammatory regulation.

The overarching aim of this dissertation was to determine if sex-specific differences are driven by estrogen regulation of the corneal inflammatory reparative response; more specifically, to establish a link, if any, between estrogen signaling and the 15-LOX/LXA_4 lipid circuit. Given the potent protective bioactions of LXA_4 in corneal wound healing, an understanding of sex-steroid regulation of the 15-LOX/LXA_4 circuit may have important implications in ocular therapy and inflammatory disease progression. The following hypothesis was tested: is there a sex-specific difference in the corneal inflammatory reparative response and, if so, what role does estrogen play in the establishment of such differences?

An estrogen-driven sex-specific difference in corneal wound healing was demonstrated for the first time. Estrogen receptor (ER) β signaling downregulated corneal epithelial 15-LOX expression and subsequent LXA_4 formation, the first study to link sex-specific differences and the action of estradiol to the protective anti-inflammatory 15-LOX/LXA_4 circuit. Furthermore, a sex-specific difference in corneal inflammatory tone and leukocyte population upon re-injury was established. *In vitro* assays of phagocytosis demonstrated for the first time that phagocytosis is a potent stimulus for LXA_4 formation, establishing a positive feedforward loop for LXA_4 action in leukocytes whereby LXA_4 stimulates phagocytosis and subsequent phagocytosis stimulates LXA_4 production. Estrogen signaling via ERβ was also shown to upregulate neutrophil retention and delay clearance by inhibiting the LXA_4-induced stimulation of phagocytosis. Taken together, these results provide evidence for an ERβ-driven upregulation of the inflammatory response and downregulation of the protective 15-LOX/LXA_4 lipid pathway in the corneal wound healing response. Elucidation of these sex-specific and sex steroid-driven differences provides insights into the high female-dominated incidence of inflammatory diseases. Estrogen primes females for an amplified inflammatory response upon a disruption to the ocular surface. This has important ramifications in the cornea where chronic inflammation can lead to blindness.

To my parents, Cindy and Zack Wang, for their loving support

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Chapter 1:

Introduction and Background

1.1 The Cornea

The cornea is a transparent tissue on the ocular surface, serving as a junction between the outside world and the rest of the eye. It is comprised of three main layers: the outermost corneal epithelium that in itself contains five to seven layers of epithelial cells, the stroma, and the corneal endothelium, a single layer of hexagonal cells attached to the cornea via the acellular, collagen-rich Descemet's membrane. The distinct layers of the cornea are all highly organized by necessity. The stroma, which represents approximately 90% of the entire human cornea by thickness, is a 0.5 mm thick layer made up of roughly 200 layers of type I collagen fibrils arranged in a lattice [1]. Interspersed within the collagen fibers are fibroblast-like cells called keratocytes (~10,000 cells/mm^3 at the central cornea) that secrete collagen and other extracellular matrix components to maintain stromal intensity [2]. Although corneal endothelial cells are incapable of regeneration, the cells in the corneal epithelium are continuously being replenished by stem cells located at the corneal/conjunctival border (aka limbus), with an estimated turnover rate of 3 to 5 days in humans [3, 4]. When the epithelium is injured, adjacent cells will quickly migrate to cover the wound area, which is then followed by a proliferative phase to regain normal epithelial thickness. This does not occur in isolation from the rest of the cornea. At the same time of epithelial injury, keratocytes directly beneath the wound area will undergo apoptosis, activating surrounding keratocytes. Cytokines, chemokines, lipid mediators, and growth factors all mediate signaling between the corneal epithelium and stroma.

Functionally, the cornea contributes two-thirds of the eye's total refractive power; therefore, maintaining corneal transparency is essential for sight. The cornea has several strategies in which it does so, setting it apart from most other tissues in the body. It is avascular – devoid of blood vessels as well as lymph vessels [5, 6]. The presence of MHCII-positive antigen-presenting cells (i.e. macrophages and dendritic cells) is largely nonexistent though MHCII-negative macrophages and dendritic cells are present in a normal cornea and are capable of expressing MHCII after corneal insult [7-9]. This contributes to the notion of the cornea as an immune-privileged site, which is defined operationally as any site in the body where foreign tissue grafts can survive for extended periods of time. The cornea achieves anterior-chamber-associated immune deviation (ACAID) via peripheral tolerance of eye-derived antigens as well as development of an intraocular immunosuppressive microenvironment (reviewed in [10]). Strategies to modify the innate and adaptive immune response to prevent sight-destroying inflammation have functional relevance in more arenas than just transplant surgery. The cornea faces a plethora of inflammatory stressors daily. It is constantly exposed to the shearing stress of eyelid motion as well as extended periods of hypoxia during sleep. Its position at the surface of the eye puts it in contact with environmental pathogens and irritants yet very rarely does it mount a prolonged inflammatory response. Highly developed circuits to control inflammation, maintain host defenses, and promote wound healing within the cornea have emerged in recent years as therapeutic targets for preventing chronic inflammation, angiogenesis, and vision loss.

1.2 The Inflammatory Response

Inflammation, derived from the Latin word 'inflammo' or 'I ignite, set alight', is a series of tightly regulated temporal events mounted by the host tissue in response to injury, microbial infection, or chemical stimuli. Its progression can be monitored by cell dynamics. Initiation of inflammation consists of the rapid influx of neutrophils followed by monocytes that differentiate into inflammatory macrophages. These cells act within the site of injury/infection to promote microbial/apoptotic cell clearance and wound healing. They also produce chemokines and cytokines that further the inflammatory response. Chemokines, such as interleukin(IL)-8, produced by leukocytes as well as pro-inflammatory lipid mediators like leukotriene B_4 activate and serve as potent chemoattractants of neutrophils. Nitric oxides and prostaglandins are vasodilators, increasing endothelial permeability and blood flow. As a result, inflammation as a whole is characterized by pain, redness/swelling, and heat.

The balance of these cell-derived signals ultimately determines tissue fate. There are two general outcomes to an inflammatory response: 1) prolonged, or chronic, inflammation that leads to host tissue damage and possible recruitment of the adaptive immune response or 2) acute inflammation that is characterized by inflammatory resolution. Though it was long thought that inflammatory resolution is a passive process resulting from abating inflammatory signals, it is now known that there exists classes of lipid mediators that actively regulate inflammatory resolution.

1.3 The LXA_4 Biosynthetic Pathway

Lipoxins (lipoxygenase interaction products) were first described in 1984 as weak agonists for superoxide anion production and degranulation of neutrophils [11, 12]. Subsequently, their true bioaction as potent anti-inflammatory and pro-resolving mediators was characterized, becoming the first lipid mediators to be attributed with such bioactions. Lipoxins (LX) are trihydroxytetraenes formed via lipoxygenase (LOX) metabolism of the polyunsaturated fatty acid, arachidonic acid (AA). LXA_4 (5*S*,6*R*,15*S*-trihydroxy-7,9,13-*trans*-11-*cis*-eicosatetraenoic acid) is one of the principal species of LX formed in mammals. Two major biosynthetic routes (vascular vs. mucosal) for LXA_4 formation have been established (reviewed in [13-15]) (Fig 1.1). In the vascular route, platelet-leukocyte interaction results in 12-LOX conversion of the leukocyte 5-LOX epoxide product leukotriene A_4 into LXA_4. The mucosal route involves the sequential actions of epithelial cell, monocyte, or eosinophil 15-LOX and neutrophil 5-LOX on AA. 15-LOX inserts molecular oxygen at the C-15 position of AA, generating 15S-H(p)ETE (15S-hydroperoxy-5Z,8Z,11Z,13E-eicosatetraenoic acid) that can be reduced to 15S-HETE (15-hydroxy-5Z,8Z,11Z,13E-eicosatetraenoic acid) by peroxidases. Subsequent 5-LOX activity on 15-H(p)ETE results in 5S-hydroperoxy,15S-hydro(peroxy)-DiH(p)ETE, which is rapidly converted to 5,6-epoxytetraene. LXA_4 is then formed from the action of LXA_4 hydrolase on 5,6-epoxytetraene. Experimental models show that cell-cell interactions are critical for LXA_4 biosynthesis, which was then coined transcellular biosynthesis. Human platelets must be adherent to neutrophils to produce LXA_4 [15]. Similarly, macrophage-neutrophil interaction (i.e. during macrophage phagocytosis of apoptotic neutrophils) results in markedly higher production of LXA_4 while cultured macrophages alone produce no

detectable LXA_4(Fig 3.5) [16]. Interestingly, there is evidence that primed leukocytes isolated from peripheral blood of individuals with inflammatory disorders as well as thrombin and fMLP (formyl-methionyl-leucyl-phenylalanine) primed neutrophils are capable of generating LXA_4 on their own *in vitro* through disease-induced esterification and storage of LXA_4 precursors in cell membranes, raising an interesting question about eicosanoid formation during the course of chronic inflammation [17-19]. Indeed, human peripheral neutrophils that were exposed to proinflammatory prostaglandin E_2 exhibited a lipid mediator 'class switch' from generating pro-inflammatory leukotrienes to formation of LXA_4, illustrating how lipid mediators generated during the early inflammatory phase can reprogram neutrophils for resolution [20].

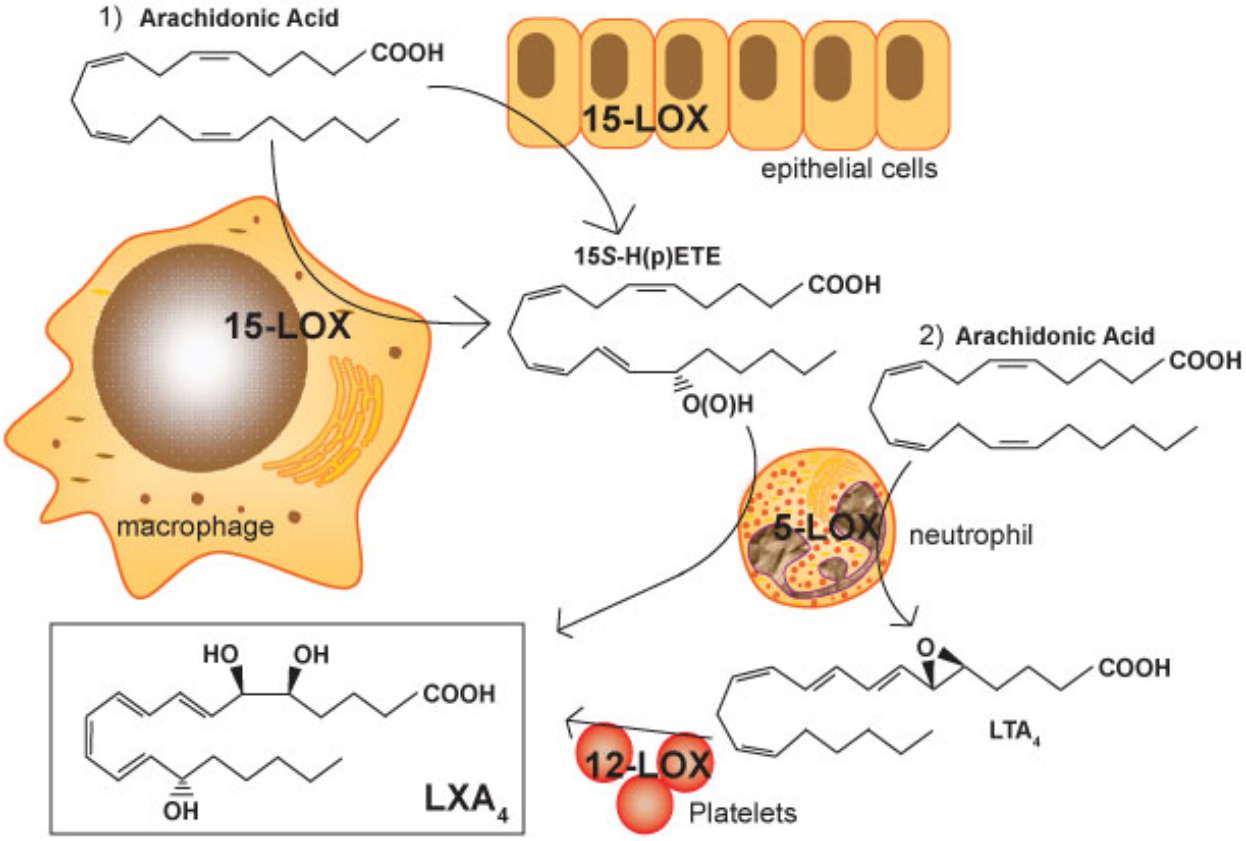

Fig 1.1. LXA_4 biosynthetic pathways. Two main lipoxin-generating pathways involve the sequential transcellular interaction between 1) 15-LOX containing epithelial cells or macrophages and 5-LOX containing neutrophils or 2) 5-LOX containing neutrophils and 12-LOX containing platelets

1.4 ALX: a specific LXA_4 Receptor

Binding sites for LXA_4 were first characterized using [11,12-^{3}H]-LXA_4 to demonstrate specific and reversible binding in human neutrophils[21]. The subsequent receptor identified, ALX, was transfected into Chinese hamster ovary (CHO) cells and determined to have a K_d value of 1.7 nM for [^{3}H]LXA_4, which is comparable to established K_d values for LXA_4 of 0.7 nM and 0.8 nM in

isolated human plasma membrane and granule membrane-enriched fractions, respectively [22, 23]. ALX (also known as FPRL1 or FPR2), is a member of the seven-transmembrane rhodopsin superfamily of G-protein-coupled receptors (GPCRs) that belongs to a class of chemoattractant peptide receptors that include fMLP receptors, leukotriene B_4 receptors, as well as ChemR23, the newly discovered receptor for the omega-3 derived resolvin E1 [24]. It is a multirecognition receptor capable of interacting with a wide panel of peptides (reviewed in [22]). Non-lipid ligands of ALX include synthetic peptides, serum amyloid A, and N-formyl hexapeptides, which typically bind to ALX with lower affinity than LXA_4 and are linked to signaling pathways distinct from LXA_4 activation [25-27]. For example, while LXA_4 inhibits neutrophil chemotaxis, synthetic peptide ligands act in the opposite manner and stimulate neutrophil chemotaxis [26]. Evidence has arisen suggesting that N-glycosylation of ALX may play a role in determining ligand specificity and receptor function within the local inflammatory response [25].

ALX has been detected in resident tissue cells such as intestinal and bronchial epithelial cells, synovial fibroblasts, and renal mesangial cells as well as in inflammatory cells such as neutrophils, monocytes/macrophages, and T-cells. ALX expression is dynamically regulated in colonic epithelial cells by IL-13 and interferon γ [28]. Furthermore, the receptor has been identified and cloned in humans, rats, and mice [23, 29, 30]. Mouse homologues of ALX include *Fprl1* (ALX1), which encodes a functional receptor for LXA_4, and *Fpr-rs2* (ALX2) [30, 31]. Both human and mouse ALX cDNA contain an open reading frame of 1053 nucleotides, encoding for a protein 351 amino acids long, and share an overall homology of 74% in nucleotides and 65% in amino acid sequences. The second intracellular loop and the sixth transmembrane domain exhibit the highest homology (100% and 97%, respectively). To determine regions necessary for ligand recognition, chimeric receptors were constructed using receptors with opposing functions, namely ALX and the leukotriene B_4 receptor BLT_1. The seventh transmembrane domain along with its adjacent regions was determined to be essential for LXA_4 recognition while other regions such as extracellular loops bound peptide ligands, providing the first evidence for distinct interaction sites within a single GPCR [25].

1.5 Bioactions of LXA_4

Several lines of evidence in animal models of inflammation as well as in humans implicate the 15-LOX/LXA_4 circuit as being an essential protective lipid pathway. Endogenous LXA_4 production was detected and upregulated in ischemia/reperfusion models of the hind limb, focal cerebellum, and myocardium [15, 32]. Furthermore, LXA_4 was generated following microbial infection by *Toxoplasma gondii* [33]. In humans, altered levels of LXA_4 formation was detected in patients with chronic liver disease, leukemia, localized juvenile periodontitis, cystic fibrosis, and asthma [15, 34-36].

These animal models of disease also revealed specific protective/anti-inflammatory bioactions for LXA_4. In a murine model of dermal inflammation, topical application of LXA_4 inhibited neutrophil recruitment to the site of inflammation (i.e. the ear skin) while preventing vasodilation [37]. This LXA_4 induced inhibition of neutrophil chemotaxis was correlated by studies *in vitro* as well as *in vivo* in models of peritonitis, glomerulonephritis, and cystic fibrosis (reviewed in [22]). Overexpressing

the human 15-LOX enzyme, a key enzyme involved in LXA_4 synthesis, resulted in enhanced LXA_4 production and conferred protection against onset of glomerulonephritis as well as against leukocyte-mediated bone destruction in a model of acute periodontitis [38, 39]. The protective actions of LXA_4 is both ligand and receptor specific since transgenic animals overexpressing the human LXA_4 receptor, ALX, exhibited a sharp decrease in neutrophil infiltration in a model of zymosan-induced peritonitis [40]. Directly overexpressing ALX on leukocytes led to decreased pulmonary and tissue infiltration while ALX transgenic mice were protected against acute lung injury [41, 42].

On a cellular level, LXA_4 regulates anti-inflammatory gene expression (i.e. NAB1, HO-1) as well as NF-$\varkappa$B activation[43, 44]. LXA_4 also inhibits pro-inflammatory cytokine production in a variety of cell types: IL-12 in dendritic cells, IL-1β induction of IL-6 and IL-8 in fibroblasts, IL-8 in neutrophils, IL-5 in eosinophils, pathogen stimulated IL-8 release in gastrointestinal epithelial cells [28, 45-48]. In addition to counter-regulating pro-inflammatory signals, LXA_4 also actively promotes inflammatory resolution on numerous key levels. LXA_4 regulation of monocytes and monocyte-derived macrophages is multidimensional. LXA_4 stimulates monocyte chemotaxis and adherence in a nonphlogistic manner, i.e. LXA_4 upregulates monocyte recruitment to the site of inflammation [15]. Once in the tissue, the monocytes differentiate into one of two general macrophage subtypes depending on the local inflammatory environment: the classically activated, pro-inflammatory M1 macrophage or the alternatively activated, anti-inflammatory M2 macrophage (reviewed in [49]). LXA_4 can reprogram M1 macrophages into the anti-inflammatory M2 subtype. Furthermore, LXA_4 has been shown to upregulate macrophage phagocytosis of apoptotic neutrophils, both *in vivo* and *in vitro,* in a murine model of thioglycollate-induced peritonitis [50, 51].

The cornea is one of a few tissues in the human body that contains endogenous basal levels of LXA_4, a novel concept first reported in 2005 [52]. Immunohistochemical analysis of the eye reveals startling quantities of 15-LOX concentrated in the intact corneal epithelium (Fig 1.2). Given the evolutionary necessity to tightly regulate and limit the corneal inflammatory response to prevent debilitating vision loss, the protective and anti-inflammatory 15-LOX/LXA_4 circuit may play a key role in the cornea and establishment of ACAID. Indeed, in a model of epithelial abrasion where the corneal epithelium is mechanically removed to elicit an inflammatory reparative response, LXA_4 has significant effects on wound outcome [52]. Consistent with immunohistochemistry results showing significant 15-LOX expression in corneal epithelial cells, epithelial debridement effectively removes both 15-LOX and ALX expression from the cornea, which is associated with a 54% decrease in endogenous LXA_4 formation. The levels of gene expression as well as lipid formation return to basal levels as the epithelium heals. Mice deficient in 15-LOX exhibited a 65% decrease in rate of wound healing compared to wild-type counterparts 24 hours after injury, which is associated with a 43% decrease in LXA_4 formation. Addition of LXA_4 in the form of topical eye drops throughout the course of the wound healing response significantly increased corneal wound re-epithelialization. Interestingly, the wound healing actions of LXA_4 was separate from previously reported inhibitory effects of neutrophil recruitment as 15-LOX deficient eyes exhibited decreased neutrophil infiltration and eyes that received LXA_4 contained greater neutrophil numbers.

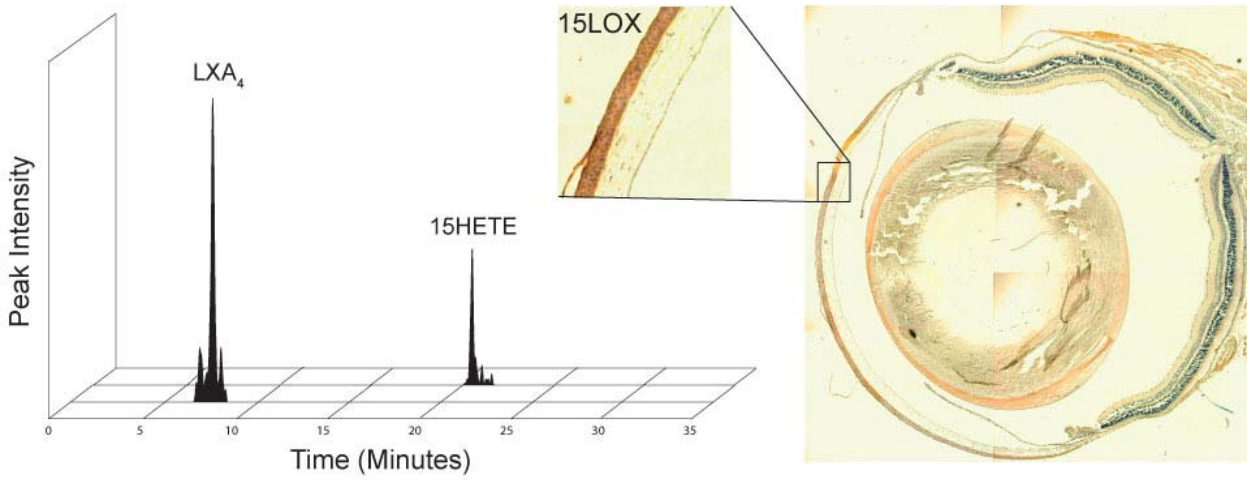

Fig 1.2 Endogenous LXA_4 formation in the cornea. The presence of LXA_4 and its precursor, 15-HETE, is detectable in the uninjured Balb/C cornea by liquid chromotography (LC)/mass spectrometry (MS)/MS based lipidomics. Representative multiple reaction monitoring (MRM) peaks are shown. The presence of the enzyme 15-LOX in the intact murine cornea is shown in brown in the inset. 15-LOX is an essential enzyme for LXA_4 formation.

1.6 Sex-Specific Differences in Inflammatory-Based Diseases

Anecdotal evidence about disparities between females and males with regard to incidence of autoimmune/inflammatory diseases has existed for well over a century. There are over 80 autoimmune diseases recognized by the National Institute of Health. These together affect roughly 5% of the US population, for which immunosuppression therapies serve as a stop-gap measure and not a cure [53]. These diseases tend to be chronic, debilitating, and life threatening over time. Females have a higher incidence of many autoimmune diseases. Patients with Sjogren's syndrome, systemic lupus erythematosus, autoimmune thyroid disease, rheumatoid arthritis, and multiple sclerosis are predominantly female accounting for, strikingly, over 80% of all patients with autoimmune diseases [53]. This is especially serious when one realizes that, taken as a whole, autoimmune diseases are among the top ten leading causes of death among females younger than 65 in the US [54].

To understand the basis behind sex-specific differences in autoimmune diseases, it is necessary to first have an understanding of sex-specific differences in the typical immune/inflammatory response. Work in animal models revealed an enhanced T-cell response with greater antibody production in mice [55]. While the picture is less clear in humans, women do have greater numbers of $CD4^+$ T-cells, which might also partly account for female conferred protection against sepsis induced mortality and postsurgery infections [56]. Women also have higher plasma IgM levels and tend to secrete more IL-1, IL-4, and interferon γ [56, 57]. The peritoneal cavity of naïve female mice contain more T and B lymphocytes and macrophages than their male counterparts and female mice produced more inflammatory cytokines in response to a hepatic ischemia/reperfusion injury, all implicating the presence of a sexual dichotomy in the innate immune response [58]. Yet, sex-specific

differences in ocular inflammatory/autoimmune diseases are poorly defined beyond observations of disease prevalence.

1.7 Estrogen and the Estrogen Receptors

Estrogens are a group of primary female sex steroids found in all vertebrates. The group includes estrones, estradiols, and estriols, of which 17β-estradiol is the most predominant form of estrogen found in females during the reproductive years. In females, the major source of estrogen production is the ovaries with tissues such as liver, breast cells, and adipose tissue serving as secondary sources. Estrogen is metabolized from cholesterol in a sequence of events that involves first the theca interna cells of the ovaries followed by the granulosa cells. In a series of steps, cholesterol is metabolized into androgen which is then converted into estradiol by the enzyme aromatase (Fig 1.3).

Classic estrogen signaling occurs via nuclear receptors present in a variety of cell types that mediate female and male reproductive development, lipid metabolism, and cardiovascular tone (Fig 1.3) (reviewed in [59, 60]). The two estrogen receptors (ERs) known in humans, ERα and ERβ, are encoded by distinct genes on separate chromosomes: the 6q25.1 locus on chromosome 6 for ERα and 14q23.2 on chromosome 14 for ERβ [61-64]. They are intracellular transcription factors that, when unliganded, exist in the cytoplasm in monomeric form associated with heat shock proteins. Binding to estrogen induces a major conformational change that completes the folding of the protein. ER will then dissociate from the heat shock protein, dimerize, and move into the nucleus where it will bind directly to estrogen-responsive elements of select genes and recruit coregulators to regulate genetic transcription. While many estrogen-responsive elements share the consensus sequence GGTCAnnnTGACC, other estrogen-responsive elements deviate substantially from this sequence, making it difficult to determine estrogen-responsive elements within genes [65]. ERs are also capable of eliciting changes in genes without specific estrogen-responsive elements by interacting with other transcription factors like activating protein-1 and stimulating protein-1 [59].

ERα and ERβ share high homology within their DNA binding domain (96%) and the ligand binding domain (58%) and bind 17β-estradiol with similar affinities (~IC_{50}=0.9 nM); however, they show different expression patterns in tissue distribution and bioaction. ERα but not ERβ is present in liver, kidney, spleen, aorta, and skeletal muscles. Conversely, ERβ is highly expressed (3:1 ERβ:ERα) in lung tissues while both ERs are present in male and female reproductive tissues [66]. In breast cancer cells, a substantial focus in ER research, ERβ antagonizes the tumor proliferative actions of ERα [67]. An isoform of ERβ, ERβcx, appears to preferentially dimerize with ERα, inhibiting binding of ERα to DNA [68]. Both ERs are expressed in leukocytes and all major ocular tissues, including corneal epithelial cells [69]. Furthermore, estrogen is present in the tear film and aromatase, the enzyme that catalyzes tissue estrogen synthesis, in the cornea, yet the *in vivo* roles of estrogen and the ERs in the eye are not defined [70, 71].

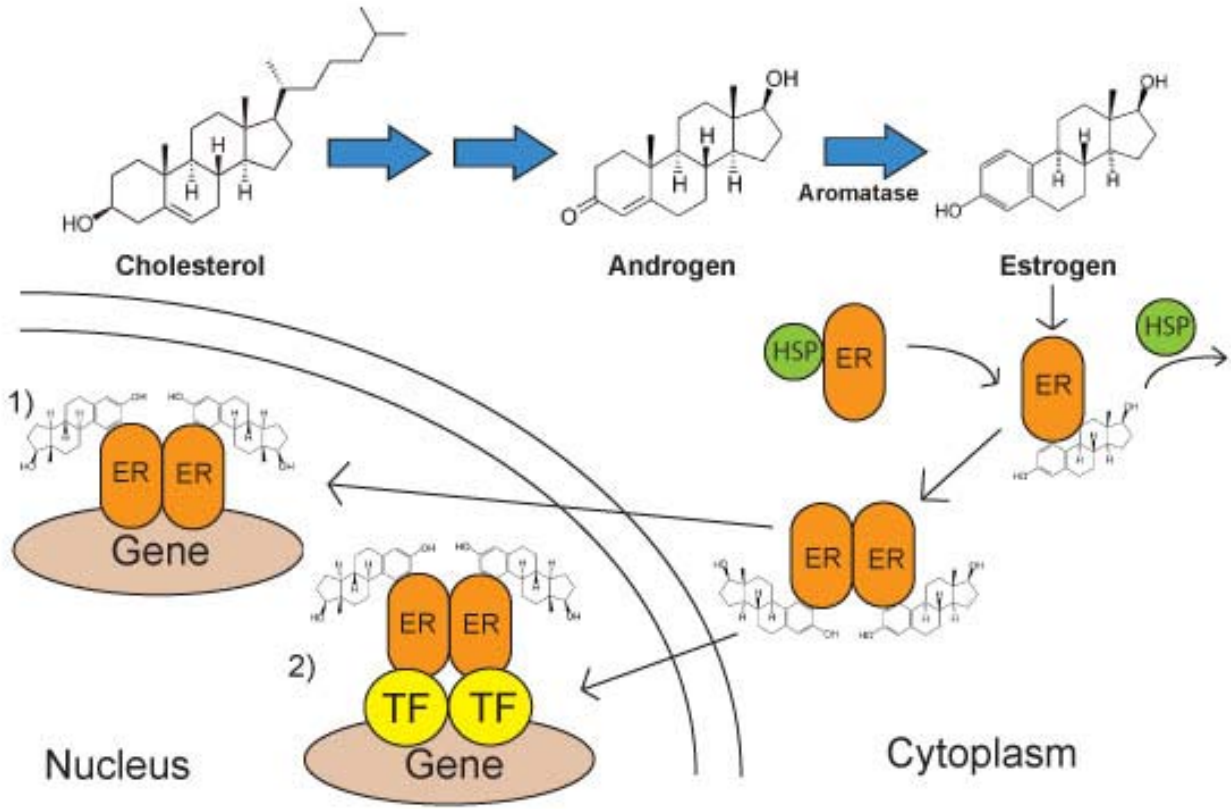

Fig 1.3 Schematic of classical routes of estrogen signaling. Estrogen is converted from cholesterol via an androgen intermediate. Upon binding to estrogen, estrogen receptor (ER) dissociates from heat shock proteins (HSP), dimerizes, and translocates to the nucleus where it 1) directly binds to genes or 2) interacts with other transcription factors (TF), regulating gene transcription.

1.8 Estrogen Modulation of Inflammation

As sex-specific differences in autoimmune diseases and the immune response have gained more prominence, attention on the sex steroids as effector molecules has also increased. Interest in the immunoregulatory actions of sex steroids is compounded by evidence showing inflammatory differences in females during known periods of hormone fluctuation (i.e. pregnancy and menopause). During the third trimester of pregnancy, estrogen and progesterone levels rise significantly then fall after parturition. In this same period, reported disease activity of both multiple sclerosis and rheumatoid arthritis see a sharp decrease followed by an increase with a fall in hormone levels *post-partum* [72]. The effects of sex hormones on inflammation are complicated by a variety of factors including tissue, cell type, and the presence of other sex steroids. Whether the environment favors a T_H1 or a T_H2 response also plays a role. While disease symptoms decrease during pregnancy for rheumatoid arthritis and multiple sclerosis (both T_H1 responses to central nervous system and joint antigens), systemic lupus erythematosus (SLE) – a T_H2 response – actually worsens [72-74]. Animal models of collagen-induced arthritis and experimental autoimmune encephalomyelitis all show altered disease symptoms during pregnancy [75, 76].

Menopause, the second event in a women's life that drastically affects circulating hormone levels, comes with its own immune changes. Patients with multiple sclerosis report a worsening of symptoms post-menopause with a favorable decrease following hormone replacement therapy [77]. Incidence rates of rheumatoid arthritis and scleroderma, autoimmunity against collagen tissues typically characterized by collagen buildup on the skin of the hands and face, all increase with onset of menopause while SLE disease activity decreases [78]. Menopause has also been shown to alter the more benign and beneficial inflammatory response in dermal wound healing. Pre-menopausal females have anecdotally been observed to heal faster than males while the onset of menopause drastically decreases wound healing. Hormone replacement therapy increased dermal wound healing in post-menopausal humans. In a mouse model of dermal injury, ovariectomized females displayed a slower rate of wound repair, which could be reversed with topical estrogen [79].

Treatment with estrogen (typically 17β-estradiol, the most prevalent form of estrogen in pre-menopausal women) and selective ER agonists in animal models of inflammation/autoimmune disease have demonstrated a key role for estrogen in the immune system. Moreover, *in vitro* experiments reveal estrogen has direct effects on immune cells. Estrogen-treated dendritic cells have better antigen-presenting functions and activate auto-reactive T- and B-cells [80]. Estrogen regulates inflammatory cytokine production by activated monocytes and macrophages and drives T-cells toward Th2, inhibiting Th1 and Th17 functions [81, 82]. In B-cells, estrogen induces polyclonal activation and upregulates IgG and IgM production [83]. In animal models, estrogen is associated with both pro- and anti-inflammatory functions that are tissue and disease dependent. While estrogen treatment accelerates dermal wound healing, ERβ signaling is anti-proliferative in breast cancer cells and vascular smooth muscle cells. Estrogen treatment of mice with experimental autoimmune encephomyelitis alleviates disease symptoms partly due to estrogen induced downregulation of dendritic cell recruitment and antigen-presenting functions [84, 85]. Conversely, estrogen treatment in systemic lupus erythematosus (SLE) and rheumatoid arthritis (RA) increases disease severity by upregulating cell proliferation (B-cells in SLE, macrophages and fibroblasts in RA) [86-88]. **Taken together, these studies demonstrate that estrogen is a clear immune modulator with cell, tissue, and receptor specific actions.**

Not much is known about estrogen's effects in the cornea. An epidemiological study of forty thousand female health professionals revealed a positive correlation between reported dry eye symptoms and estrogen replacement therapy [89, 90]. Estrogen treatment *in vitro* induced changes in expression of the pro-inflammatory cytokines IL-6 and IL-8 in immortalized and primary human corneal epithelial cells [91, 92]. However, little else is known about estrogen regulation of inflammatory circuits in the cornea despite the presence of estrogen in the tear film and the ERs in corneal epithelial cells [69-71].

1.9 Specific Aims

The goal of this project was to establish, for the first time, sex-specific differences in the corneal inflammatory reparative response and to investigate whether or not the sex steroid estrogen works in concert with the 15-LOX circuit and inflammatory cells to regulate the sequelae of corneal insult. The crucial anti-inflammatory and reparative actions of 15-LOX metabolites have been clearly demonstrated in the eye. Estrogen's importance in dermal wound healing is also well established and has been the subject of numerous studies. However, estrogen's impact on inflammation is complex, tissue specific and remains poorly defined. Estrogen's regulation of inflammatory resolution and anti-inflammatory lipid mediators remains poorly understood. Furthermore, nothing is known about sex-specific differences in corneal wound healing and, while sex steroids have been detected in the tear film and aromatase has been found in ocular tissues, their actions on the important ocular inflammatory balance necessary for the maintenance of sight is largely unknown. This project set out to address these gaps in knowledge through the completion of the following three aims driven by the hypothesis: Does estrogen drive sex-specific differences in the sequelae of corneal injury through regulation of the 15-LOX/LXA_4 regulatory circuit?

Aim 1. Establish that there are sex-specific differences in the corneal acute inflammatory reparative response

Aim 2. Elucidate how the two estrogen receptors, ERα and ERβ, regulate the execution of acute inflammatory reparative responses and epithelial wound healing in the cornea

Aim 3. Investigate whether ERα and/or ERβ modulates leukocyte phenotype and function in the cornea

Chapter 2:

Estrogen negatively regulates epithelial wound healing and protective lipid mediator circuits in the cornea

2.1 ABSTRACT

Estrogen receptors (ER) are expressed in leukocytes and in every ocular tissue. However, sex-specific differences and estradiol's role in ocular inflammatory/reparative responses are not well understood. We found that female mice exhibited delayed corneal epithelial wound closure and attenuated neutrophil responses, a phenotype recapitulated by estradiol treatment both *in vivo* (topically in male mice) and *in vitro* (corneal epithelial cell wound healing). The cornea expresses 15-lipoxygenase (15-LOX) and receptors for lipoxin A_4 (LXA_4), which have been implicated as an intrinsic lipid circuit that regulates corneal inflammation and wound healing. Delayed epithelial wound healing correlated with lower expression of 15-LOX in the regenerated epithelium of female mice. Estradiol *in vitro* and *in vivo* down-regulated epithelial 15-LOX expression and LXA_4 formation while estradiol abrogation of epithelial wound healing was completely reversed by treatment with LXA_4. More importantly, ERα and ERβ selectively regulated epithelial wound healing, neutrophil recruitment and activity of the intrinsic 15-LOX/LXA_4 circuit. Our results demonstrate for the first time a sex-specific difference in the corneal reparative response, which is mediated by ERα and ERβ selective regulation of the epithelial and neutrophil 15-LOX/LXA_4 circuit. These findings provide new insights into the etiology of sex-specific ocular inflammatory diseases.

2.2 INTRODUCTION

The role of sex steroids, especially estrogen, in inflammation is complex and highly tissue specific for a given inflammatory/immune response. Estrogen has well documented pro- and anti-inflammatory actions yet lacks general unifying bioactions in the pathogenesis of inflammatory diseases [93-96]. Several animal models have clearly shown that estrogen inhibits both acute and chronic inflammation and accelerates dermal wound healing [93, 95, 97]. In sharp contrast to these reparative anti-inflammatory functions, estrogen promotes B-lymphocyte-driven immune responses, has been linked to the pathogenesis of autoimmune diseases and has pro-inflammatory actions in prostatitis [94-96]. Receptors for estrogen are expressed in leukocytes and every major tissue in the eye [69], which is one of the few organs where estrogen has reported pro-inflammatory activities. An epidemiological analysis of 39,878 female health professionals demonstrated that estrogen replacement therapy is associated with a significant increase in dry eye symptoms [89, 90], a finding that has important implications as Dry Eye Syndrome primarily affects women. The etiology for this highly sex-specific inflammatory/immune disease is unknown but key features are chronic inflammation, irritation and epithelial injury [98-100].

Several lines of evidence indicate that a relative deficiency in androgens in menopausal women correlates with initiation of autoimmune responses [99-101]. However, the role of androgen in ocular surface diseases and inflammatory/reparative responses is far from clear despite an impressive body of work. The role of estrogen in ocular surface diseases and inflammatory/reparative responses has been largely ignored despite the fact that its receptors are expressed in every ocular tissue and most leukocytes and estrogen induces pro-inflammatory gene expression in corneal epithelial cells and meibomian glands [91, 92, 102, 103]. Pronounced changes in estrogen levels during pregnancy, menopause and hormone replacement therapy have been linked to striking effects on inflammatory/immune response in non-ocular tissues. Furthermore, animal and *in vitro* studies place estrogen and its nuclear receptors (ERα and ERβ) as key and selective regulators of wound healing and immune responses in these tissues [93-96].These studies provide a compelling argument and rationale for an important role of estrogen in ocular surface inflammation and wound healing. Dry Eye animal studies are carried out exclusively in females due to disease incidence in humans; however, there is a striking gap of knowledge regarding even basic sex-specific differences in inflammatory and wound healing responses in the eye.

A key feature of essential frequent inflammatory and wound healing responses is that they are acute and self-resolving by design. It is now recognized that dysregulation of inflammatory resolution is an early and critical event that leads to chronic inflammation and diseases [104-107]. Among the earliest inflammatory/immune regulators that are released in response to injury, infection or stress are lipid mediators such as eicosanoids. Specific eicosanoid circuits such as the lipoxin A_4 (LXA_4) and 15-lipoxygenase (15-LOX) circuit have emerged as key mediators of inflammatory resolution and anti-inflammation [105, 108]. The protective actions of LXA_4 are mediated by G-protein coupled receptors in both humans (ALX) and mice (ALX1, ALX2). Elegant studies [22, 44, 109-113] have demonstrated that LXA_4 is formed endogenously, regulates adaptive and innate immune responses,

pain and drives inflammatory resolution. We recently reported that the cornea expresses a unique 15-LOX/LXA_4 circuit that has essential roles in inflammatory responses of the eye and is critical for endowing the cornea with an amplified anti-inflammatory tone to ensure privileged injury responses [43, 52, 108, 114, 115]. LXA_4 is formed via the interaction of 5-LOX and 15-LOX, which are both expressed in corneal epithelial cells. This pathway is significantly amplified by the recruitment of specific neutrophil and macrophage populations that carry 5-LOX and/or 15-LOX, which sets in motion a temporally defined counter-regulatory program that drives inflammatory resolution. A striking feature in both mouse and human corneas is the high epithelial expression of 15-LOX [108, 116] and the expression of the ALX receptors [52, 114, 116, 117]. Acute and chronic inflammation selectively regulates expression of 15-LOX and ALX receptors. Genetic deletion of the LXA_4 biosynthetic pathway (15-LOX and 5-LOX) leads to a phenotype of delayed epithelial wound healing, impaired induction of cytoprotective genes and amplified chronic inflammation, which can be rescued by adding back topical LXA_4. The relevance of intrinsic protective lipid circuits in ocular health is underscored by recent reports that demonstrate that 5-LOX, 15-LOX and/or LXA_4 have key roles in pathological angiogenesis [114], uveitis [118], retinopathy [119, 120] and protection of retinal pigmented epithelial cell against oxidative stress [120, 121].

We set out to assess if acute inflammatory/reparative responses are regulated by estrogen in the cornea and if sex-specific differences in this fundamental response involves regulation of the intrinsic 15-LOX/LXA_4 circuit. Our findings demonstrate for the first time a female specific phenotype of delayed corneal epithelial wound healing that can be induced by topical treatment with estradiol. More importantly, sex-specific differences and the estradiol-mediated phenotype of delayed wound healing and attenuated neutrophil response is paralleled by concomitant and estrogen receptor-specific inhibition of 15-LOX expression and LXA_4 formation, while LXA_4 abrogates the epithelial actions of estradiol. In view of the intrinsic role of the 15-LOX/LXA_4 circuit in self-resolving inflammation and wound healing, these results have important implications for estrogen's role in regulating routine ocular inflammatory/reparative responses.

2.3 MATERIALS AND METHODS

Animal Experiments

All animal studies have been approved by the University of California, Berkeley in accordance with the NIH Guide for the Care and Use of Laboratory Animals and the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Age-matched (6 to 10 week old) Balb/c female and male mice were purchased from Jackson Laboratory (Bar Harbor, ME). Mice were maintained on a 12-hour day/night cycle and fed *ad libitum* a standard diet (Rat/Mouse diet LM-485, Harlan Tekland, Madison, WI).

Corneal Epithelial Abrasion

Mice were anesthetized with ketamine (50 mg/kg) and xylazine (20mg/kg) intraperitoneally. A drop of proparacaine hydrochloride ophthalmic solution 0.5% was applied to the eye to deliver local corneal anesthesia before injury. Epithelial abrasion was achieved using an Algerbrush II with a 0.5-mm corneal rust ring remover as previously described [43, 52, 122]. The corneal epithelium was mechanically removed up to the limbal border under a dissection microscope and full removal was verified using fluorescein stain as a direct marker of epithelial defect. A 2-3 epithelial cell layer (100% wound healing) was regenerated within 5 days post injury in both males and females [52]. Reinjury was initiated by complete re-abrasion of the regenerated epithelium 7 days after initial abrasion. Epithelial defect was visualized with fluorescein. Wound area was quantified using imaging software (ImagePro Express 6.0) as previously described [43, 52, 122].

Topical Treatments

17β-estradiol (Sigma Aldrich, St. Louis, MO), estrogen receptor α (ERα) specific agonist propylpyrazole triol (PPT) (Cayman Chemical, Ann Arbor, MI), and estrogen receptor β (ERβ) specific agonist diarylpropiolnitrile (DPN) (Cayman Chemical) were used in topical applications to the eye. Each compound was dissolved in ethanol and diluted to treatment concentrations of 1 μM in sterile Hank's balanced salt solution (HBSS) (<0.1% EtOH). The topical doses were selected based on published human clinical trials with 17β-estradiol eye drops in menopausal women with Dry Eye Syndrome [123]. 5 μL of solution was administered topically to each eye three times per day (*t.i.d.),* 24 hrs prior to abrasion and subsequent to injury for up to 5 days post reinjury. The control group received 5 μL of sterile HBSS containing <0.1% ethanol.

Sample Isolation

Mice were killed at 2 days or 7 days post injury and 2 days or 5 days post reinjury. Corneas were removed using a stainless steel surgical blade. All non-corneal tissue was cleaned from the cornea in sterile phosphate-buffered saline (on ice) under a dissecting microscope with sterile instruments. To isolate the epithelial sheet, cleaned corneas were incubated in 2% ethylenediaminetetraacetic acid (EDTA) for 30 minutes at 37°C. The epithelium was then lifted from the cornea using sterile instruments.

Histological Sections

Whole eyes were removed and embedded in Optical Cutting Temperature (OCT) compound. The samples were then allowed to set at -80°C for at least 2 hrs before being sectioned lengthwise into 5 μm thick slices [114, 124]. Hematoxylin and eosin (H&E) were used as a physical stain to distinguish cell types.

Assessment of Inflammation

Myeloperoxidase (MPO) activity was used as an index of tissue leukocyte infiltration [43, 52, 114]. Corneas (1 cornea/data point) were mechanically homogenized in a solution of 50 mM potassium phosphate buffer (pH 6.0) containing 0.5% hexadecyltrimethylammonium bromide, followed by three cycles of freeze-thaw and a ten second sonication. The homogenates were then centrifuged and supernatants collected. MPO activity was measured by spectrophotometry using o-dianisidine dihydrochloride reduction as a colorimetric indicator. MPO activity was converted into total neutrophil number using calibration curves established from neutrophils collected from zymosan A-induced peritonitis exudates in Balb/c mice.

Cell Culture

Immortalized human corneal epithelial cells (HCE) were a kind gift from Haydee Bazan at Louisiana State University Health Science Center and maintained in serum-free keratinocyte growth medium (KGM) (Lonza Walkersville Inc, Walkersville, MD) supplemented with the following growth factors and antibiotics: bovine pituitary extract, hEGF, insulin (bovine), hydrocortisone, GA-1000 (gentamicin, amphotericin-B), epinephrine, transferrin. For treatment, medium was removed from confluent HCE cells and replaced with fresh KGM without growth factors. After 24 hrs, HCE cells were treated with 1 pM of 17β-estradiol, ERα specific agonist PPT, or ERβ specific agonist DPN in medium for a period of 24 hrs. A concentration range of 1-100 pM of estradiol induces physiological responses in primary and immortalized HCE cells in culture [92]. Cells were rapidly harvested by mechanical scraping and collected by centrifugation at 4°C.

In vitro Scratch-Wound Assay

HCE cells were cultured on 35 x 10 mm tissue culture dishes and allowed to grow to 80% confluence. After an incubation period of 24 hours in growth factor free KGM, cells were wounded by making one horizontal scratch with a sterile 200 μL pipette tip. To ensure that the same areas were compared between time points, another scratch directly perpendicular to and bisecting the first mark was made. The cells were then washed (to remove detached cells) and incubated in fresh growth factor-free KGM (with and without 1 pM estradiol). Images of HCE monolayers were taken using a Zeiss confocal microscope and wound area quantified using ImagePro Express 6.0.

Gene Expression

mRNA from corneas, corneal epithelium, and cultured HCE cells was isolated using a RNA Easy Mini Kit (Qiagen Sciences, Maryland) and quantified via spectrophotometry. The mRNA was then

reverse transcribed using a High-Capacity cDNA Kit (Applied Biosystems, Foster City, CA) and each sample was diluted to 2.5 ng/μL cDNA. A total reaction volume of 20 μL/well included 10 μL of SYBR Green Master Mix (Applied Biosystems), a final primer concentration of 200 nM, and a cDNA template amount corresponding to 5 ng RNA. Real-time PCR was performed with a Step One Plus QPCR system (Applied Biosystems) as previously described [114, 124]. Briefly, samples were heated to 95°C for 10 min followed by 40 cycles of 95°C for 15 sec and 60°C for 1 min. In the final melt curve stage, samples were heated again to 95°C for 15 sec followed by 60°C for 1 min before a final step of 95°C for 15 sec. Primer pairs used are:

mouse:
5-LOX: sense 5'-ACTACATCTACCTCAGCCTCATT-3'; antisense 5'-GGTGACATCGTAGGAGTCCAC-3'
15-LOX: sense 5'-GCGACGCTGCCCAATCCTAATC-3'; antisense 5'-ATATGGCCACGCTGTTTTCTACC-3'
ALX1: sense 5'-CATTTGGTTGGTTCATGTGCAA-3'; antisense 5'-AATACAGCGGTCCAGTGCAAT-3'
ALX2: sense 5'-GCCAGG ACTTTCGTGGAGAGAT-3'; antisense 5'-GATGAACTGGTGCTTGAATCACT-3'
ERα: sense 5'-CGTGTGCAATGACTATGCCTC-3'; antisense 5'-TTTCATCATGCCCACTTCGTAA-3'
ERβ: sense 5'-CTGTGATGAACTACAGTGTTCCC-3'; antisense 5'-CACATTTGGGCTTGCAGTCTG-3');

human:
5-LOX: sense 5'-ACAAGCCCTTCTACAACGACT-3'; antisense 5'-AGCTGGATCTCGCCCAGTT-3'
15-LOX: sense 5'-GGGCAAGGAGACAGAACTCAA-3'; antisense 5'- GCACAGAGATCCAGTTGCAGAA-3'
ALX: sense 5'- AGTGTCCTATGAGTCTGCTGG-3', antisense 5'-GTAATGTGGCCGTGAAAGAAAAG-3'
ERα: sense 5'- CGTGGTGCCCCTCTATGAC-3', antisense 5'- CAAGTGGCTTTGGTCCGTC-3'
ERβ: sense 5'-CATGCGAGGGCAGAAAAGG-3', antisense 5'- ATCGTTGCTTCAGGCAAAAGA-3'

All were selected from the Harvard Primer Bank and verified by the NIH GenBank database. The ΔΔCT method was used to quantify mRNA expression levels using Step One Software version 2.0 (Applied Biosystems). mRNA expression is expressed as relative quantity (RQ) to the housekeeping gene, β-actin, and universal mouse reference RNA that was generated from mRNA obtained from pooled Balb/c kidney and spleen. Universal human reference mRNA was purchased from Stratagene (La Jolla, CA) and amplified according to manufacturer specifications. Amplifications were run in duplicate and efficiency curves for all primers were established.

Lipid Mediator Lipidomics

For lipid autacoid analysis [114, 119, 124], injured HCE monolayers or isolated mouse corneal stroma was incubated with 2 μM calcium ionophore (A23187, Fisher Bioreagents, Fair Lawn, NJ) in KGM without growth factors for 30 minutes (37°C). The reaction was then stopped by the addition of 66% methanol containing deuterated internal standards, prostaglandin (PG)E_2-d_4, 15(S)- HETE-d_8, and leukotriene B_4 (LTB_4)-d_4 (400 pg/each), to calculate the recovery of prostanoids or mono-hydroxy- and dihydroxy-containing fatty acids. Lipid autacoids were extracted by solid phase using Accubond ODS-C18 cartridges (Agilent Technologies, Santa Clara, CA). Eicosanoids were identified and quantified by liquid chromatography (LC)/mass spectrometry (MS)/MS-based

lipidomics. In brief, extracted samples were analyzed by a triple quadrupole linear ion trap LC/MS/MS system (MDS SCIEX 3200 QTRAP) equipped with a Kinetex C18 mini-bore column, using a mobile phase gradient of water/acetonitrile/acetic acid (72:28:0.01, v:v:v) and isopropanol/acetonitrile (60:40, v:v) with a 450 μl/min flow rate. MS/MS analyses were performed in negative ion mode and prominent fatty acid metabolites were quantified by multiple reaction monitoring (MRM mode) using established transitions for LXA_4 (351→115 m/z). Calibration curves (1 to 1000 pg) and specific LC retention times for LXA_4 were established with synthetic standards (Cayman Chemical, Ann Arbor, MI).

Statistics

One-tailed, unpaired student's *t*-test was used to evaluate the significance of differences between two groups. One-way ANOVA was performed on groups of three or greater followed by a Tukey-Kramer multiple comparisons post test for p values less than 0.05 using GraphPad InStat software. One-tailed Fisher's exact test (a variation of a chi-square test) was used to evaluate the significance of differences between groups in wound closure. A t-test was not appropriate due to the integer-based, non-continuous nature of the data. *P* values of less than 0.05 were considered significant. All data reported as mean ± SEM unless otherwise indicated.

2.4 RESULTS

To determine if there are inherent sex-specific differences in the corneal reparative response during an acute and self-resolving epithelial injury, age-matched female and male Balb/c mice were wounded by complete mechanical removal of the corneal epithelium up to the limbus border. Corneas of male mice consistently healed faster than corneas of female mice (Fig. 2.1A, B). At 2 and 3 days post epithelial injury 80% and 100% of the males (n=5) had achieved full epithelial wound healing compared to 0% at day 2 and 50% at day 3 for females (n=5, p=0.001). By day 7 both male and female corneas were 100% re-epithelialized. Histology (Fig 2.1C) demonstrated that the normal corneal epithelial layer in the uninjured healthy cornea of males and females contains 5-7 cell layers while this normal epithelium is only partially restored 7 days post injury (2-3 cell layers). More importantly, the stroma of healed corneas in both male and female mice contained significant numbers of remnant leukocytes from the acute inflammatory response. A pattern of increased H&E straining in the cornea 7 days post injury potentially indicates more infiltrating cells in males than in females. The most abundant infiltrating cells in the injured cornea are neutrophils. Quantification by MPO assay (Fig 2.2) clearly indicates that there are no sex-specific differences in corneal neutrophil content 7 days post injury. The identity of all H&E stained cells in the cornea cannot be determined by H&E staining and cell morphology alone. Cells in the tissue section may include dendritic cells, γδT cells and macrophages and their potential role in the sex-specific difference in epithelial wound healing are of interest but remain to be defined.

A key feature of corneal inflammatory diseases such as Dry Eye Syndrome is recurrent epithelial injury. To assess if sex-specific differences in wound healing persist during recurrent epithelial injury, the regenerated corneal epithelium was re-abraded 7 days post injury. Despite re-injury of the healing epithelium and inflamed cornea, the second injury induced a rapid and accelerated wound healing response with 88% of male mice achieving full epithelial wound closure by day 2. In sharp contrast only 48% of females achieved full epithelial wound closure 2 days post re-injury (p=0.026). These findings provide the first evidence for a sex-specific difference in the rate of epithelial wound healing in corneas of female mice.

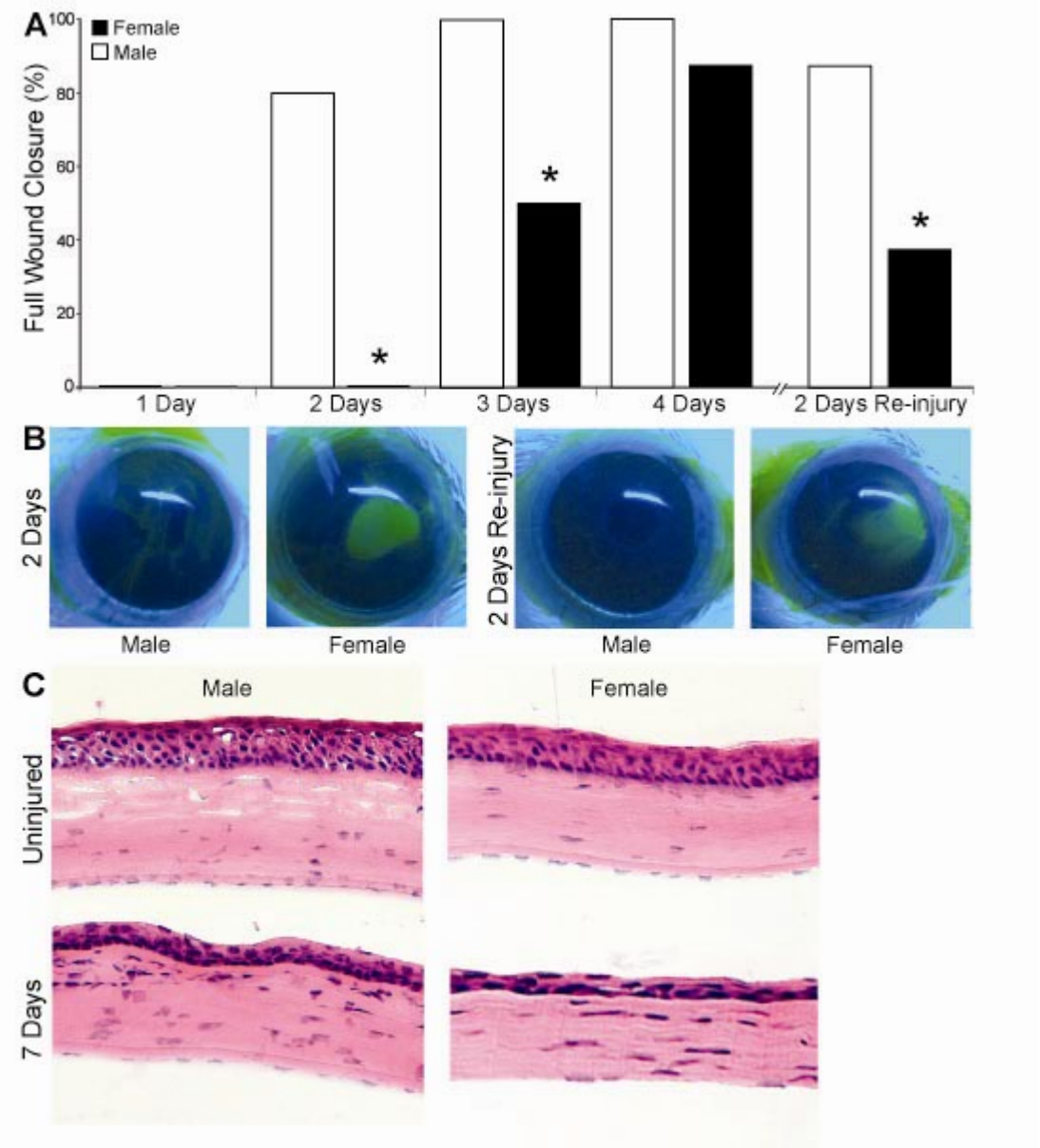

Figure 2.1. **Corneal epithelial wound healing is delayed in female mice**. Corneas of age-matched Balb/c male and female mice were injured by complete epithelial abrasion. In the re-injury group the regenerated epithelium was removed 7 days post initial epithelial injury. Wound healing was quantified via flourescein stain 1-4 days after initial injury and 2 days after re-injury. **A)** Wound healing is expressed as percent of corneas with complete epithelial wound closure. Significant differences were assessed by Fisher's Exact Test (n=5, *$p<0.05$ vs Males). **B)** Representative images of male and female fluorescein stained eyes 2 days after initial injury and 2 days post re-injury. **C)** Representative H&E images of 5 μm cross-sections of uninjured corneas and healed corneas 7 days post injury.

Inflammation is intimately linked to wound healing. In the corneal reparative response to acute epithelial abrasion injury, neutrophil infiltration is an essential and beneficial factor that drives corneal re-epithelialization [52, 125-127]. Hence, we next assessed if sex-specific differences in wound healing correlate with a difference in levels of recruited neutrophils in male and female corneas. Despite a marked difference in epithelial wound closure 2 days post injury, corneal neutrophil content did not differ in injured corneas of males and females nor was neutrophil resolution delayed in the re-epithelialized cornea at day 7 (Fig 2.2). However, epithelial re-injury induced markedly amplified recruitment of neutrophils to the cornea 2 days after a second abrasion when directly compared to the initial abrasion injury. Specifically, a second epithelial abrasion induced more than 7.6-fold increase in the male neutrophil response (302,800±46,284 cells/cornea) than was observed with the initial 2 day epithelial injury (39,920±13,541 cells/cornea). Amplified inflammation in response to a recurrent epithelial injury also revealed a marked sex-specific difference as female corneas had an average of 166,443 fewer neutrophil per cornea (55% lower response) when directly compared to age-matched males (Fig 2.2). This correlates with a significant delay in full wound closure in females (36% females with full wound closure versus 88% males with full wound closure, Fig 2.1A). These results provide strong evidence for a specific intrinsic difference in the corneal inflammatory/reparative response of male and female mice.

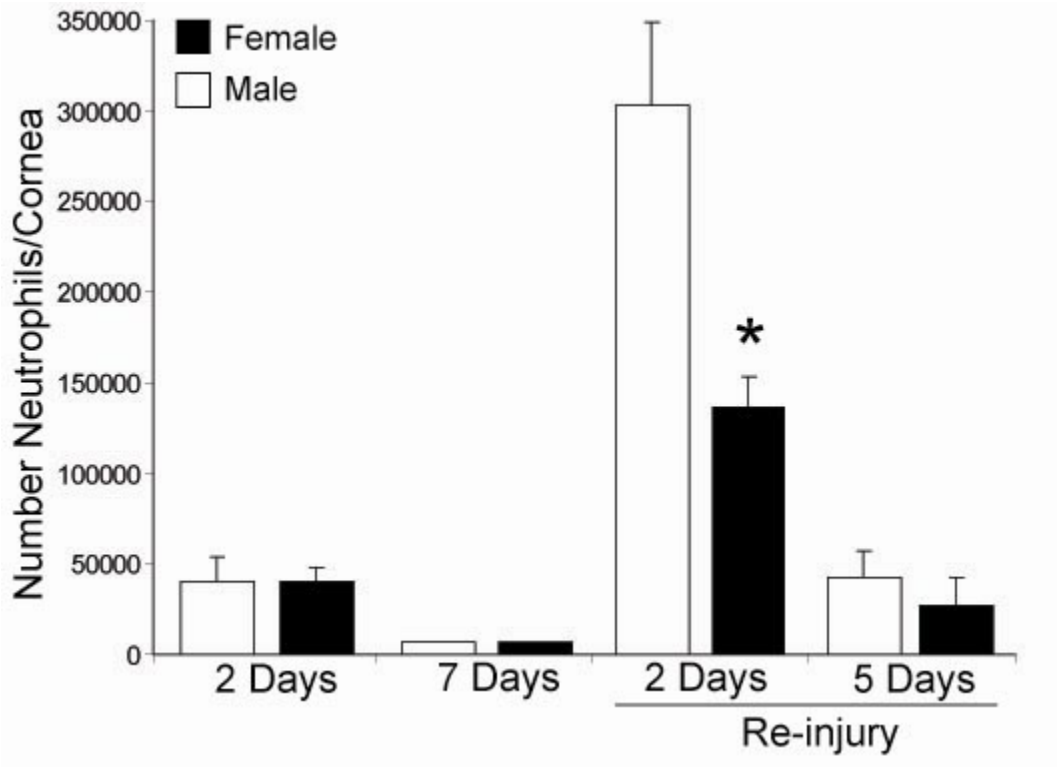

Figure 2.2. Attenuated neutrophil response to recurrent epithelial injury in females. Corneas from male and female mice were harvested at indicated time points post injury and tissue myeloperoxidase (MPO) activity was quantified (n=5-8; *p<0.05 vs males). MPO activity is expressed as number neutrophils/cornea based on calibration curves that were established with isolated peritoneal neutrophils.

Estrogen has been shown to regulate and promote cutaneous wound healing in rodent models [79, 97], which may explain reported differences in healing abilities between sexes and between post-menopausal and pre-menopausal females [93, 94, 96]. The role of estrogen in ocular reparative responses is unknown, even though estrogen is present in the tear film [71] and its biosynthetic enzyme (aromatase) and receptors (ERα and ERβ) are expressed in the cornea [70, 128], Fig 2.6A). To assess the *in vivo* action of estrogen on epithelial wound healing, we treated male mice topically with estradiol (17β-estradiol) throughout the wound healing response. The action of estradiol on the inflammatory/reparative response was assessed 2 days post re-injury as this time point exhibits marked sex-specific differences in both epithelial wound healing and the neutrophil response to injury. Treatment of males with estradiol recapitulated the female phenotype of delayed corneal re-epithelialization and attenuated neutrophil recruitment (Fig 2.3). Specifically, topical estradiol reduced the number of mice that achieved complete re-epithelialization by 56% (Fig 2.3A, B), which correlated with a 71% reduction (Fig 2.3C) in the amplified neutrophil infiltration that is a consequence of recurrent epithelial injury.

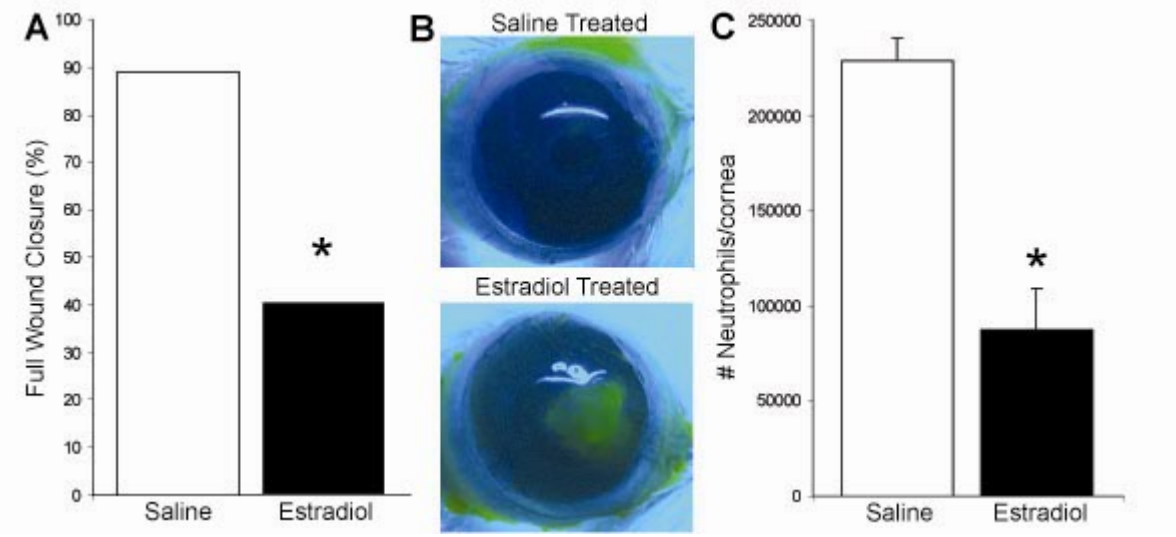

Figure 2.3. Estradiol treatment induces a female specific wound healing and neutrophil response in male mice. Eyes of male mice were treated topically with 17β-estradiol (1 μM, 5 μL drop/eye, *t.i.d.*) or saline alone from day 0-9 and full wound closure was quantified 2 days post epithelial re-injury. A) Percent of eyes in each group with full epithelial wound closure (Fisher's Exact Test n=5, *p<0.05 vs saline alone). B) Representative flourescein stained corneas 2 days post re-injury. C) Estradiol regulation of the amplified neutrophil response to re-injury. Corneas were harvested 2 days post re-injury and MPO activity quantified. MPO activity is expressed as number of neutrophils per cornea; (n=5-8, *p<0.05 vs saline group).

Corneal epithelial cells have a critical function in defending the ocular surface while also expressing high levels of 15-LOX, a key enzyme for the formation of LXA_4 and regulator of epithelial wound healing in the cornea [52, 129, 130]. Hence, we next assessed if 15-LOX (Alox15) expression in male and female corneas exhibits sex-specific differences 2 days after epithelial re-injury. The average mRNA level of 15-LOX was lower in the cornea of females (Fig 2.4A). However, this difference did not reach statistical significance, which is likely due to the presence of distinct 15-LOX expressing leukocyte populations in the stroma of the healing cornea. To focus on the sex-specific differences in epithelial 15-LOX expression, we isolated corneal epithelial sheets 2 days after re-injury from both male and female mice. QPCR analysis demonstrated a significant 47% lower expression of 15-LOX in the regenerated epithelium of females when directly compared to males (Fig 2.4B). Topical treatment of male eyes with estradiol throughout the wound healing response reduced the expression of 15-LOX in the regenerated male epithelium to levels measured in the epithelium of females. These findings provide the first evidence for sex-specific differences in the expression of corneal epithelial 15-LOX and for the ability of estrogens to downregulate intrinsic epithelial 15-LOX expression.

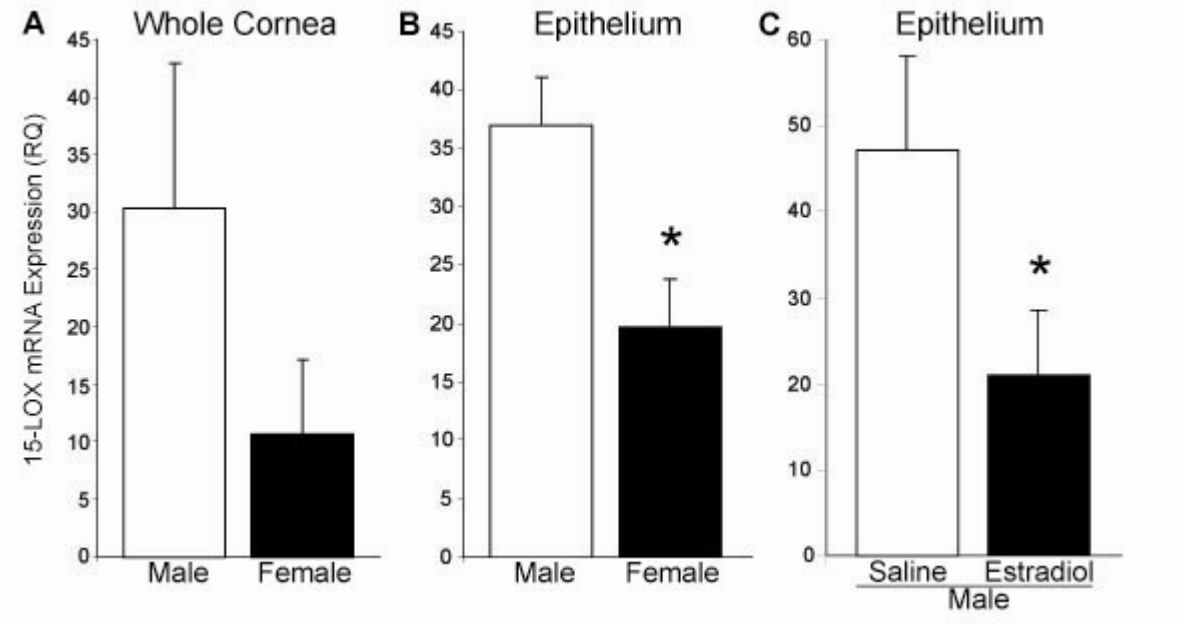

Figure 2.4. Regenerated corneal epithelium has sex-specific 15-LOX expression that is induced by estradiol. A) Whole corneas were isolated from male and female mice at 2 days after re-injury and 15-LOX mRNA expression was quantified by QPCR (p-value=0.14, n=4). Normalized 15-LOX mRNA expression is expressed as relative quantity (RQ) to β-actin and a positive control generated from mouse kidney and spleen. **B)** Corneal epithelial sheets were isolated from corneas 2 days post re-injury and 15-LOX mRNA expression quantified by QPCR in male and female mice (n=3-4, *p<0.05 vs male). **C)** Male mice were treated topically with saline alone or 17β-estradiol (1 μM, *t.i.d.*, 5 μL drop/eye) from day 0-9, epithelial sheets isolated 2 days post re-injury and 15-LOX expression quantified by QPCR (n=3-4, *p<0.05 vs saline)

To assess the direct action of estrogen on epithelial wound healing and functional expression of the LXA_4 circuit, we used human corneal epithelial cells in an established *in vitro* scratch-injury model. Uninjured confluent human epithelial monolayers, like mouse corneal epithelial cells [52], functionally express both 5-LOX and 15-LOX as evidenced by mRNA expression (Fig 2.5A) and generate LXA_4 (16.7 ± 3.3 pg/ml) following calcium ionophore activation (Fig 2.5B). Activation of epithelial cells with a low concentration of estradiol (1 pM) significantly reduced expression of 15-LOX and 5-LOX by 47% and 38% (Fig 2.5A), respectively, after 24 hrs. More importantly, estradiol inhibited endogenous LXA_4 formation by ionophore activated epithelial cells by 64% (Fig 2.5B). A defined scratch injury to the epithelial monolayer results in rapid re-epithelialization of the denuded area. 8 hrs after injury, 30.4 ± 2.1% of the wound area was re-epithelialized (Fig 2.5C, D). Activation of epithelial cells with a low concentration of LXA_4 (1 nM) 24 hrs prior to the epithelial injury significantly increased epithelial wound healing by 31%, which is consistent with our previous findings that topical LXA_4 in mice increases the rate of epithelial wound healing in injured corneas 24 hrs and 48 hrs after injury [52]. In sharp contrast, treatment with estradiol (1 pM) 24 hrs prior to injury reduced the normal rate of epithelial wound healing by 93% to 2.4 ± 0.3% (Fig 2.5C, D). LXA_4 was able to rescue epithelial cells from the estradiol effect as the wound healing rate of epithelial monolayers treated with a combined dose of estradiol and LXA_4 24 hrs prior to injury was not significantly different from untreated cells (Fig 2.5C, D). These results indicate that estradiol downregulates biosynthetic pathways for LXA_4 formation in epithelial cells and that this inhibition directly correlates with impaired epithelial wound healing.

The bioactions of estradiol are primarily mediated by two nuclear hormone receptors, namely ERα and ERβ. Both of these receptors are expressed in immortalized human corneal epithelial cell lines (Fig 2.6C) and mouse and human corneas [91, 128, 131]. QPCR analysis of gene expression in freshly isolated corneal epithelial sheets demonstrated that murine expression of both ERα and ERβ is markedly higher in females than in males (Fig 2.6A). Based on relative RNA expression, ERα is the most abundant estrogen receptor in the corneal epithelium; its RNA expression levels are approximately 5 fold higher than ERβ in both sexes. The wound healing response in female epithelium markedly upregulated the expression ratio of ERβ:ERα from 0.21±0.07 to 1.43±0.65 two days after re-injury (Fig 2.6B). Hence, corneal injury in females results in sex-specific upregulation of epithelial ERβ, which is of interest as each ER regulates distinct but yet to be defined gene cassettes [95, 132]. Estradiol (1 pM) significantly upregulates both ERα and ERβ expression (Fig 2.6C) in HCE cells within 24 hrs, suggesting a feed-forward loop for the action of estradiol in these cells.

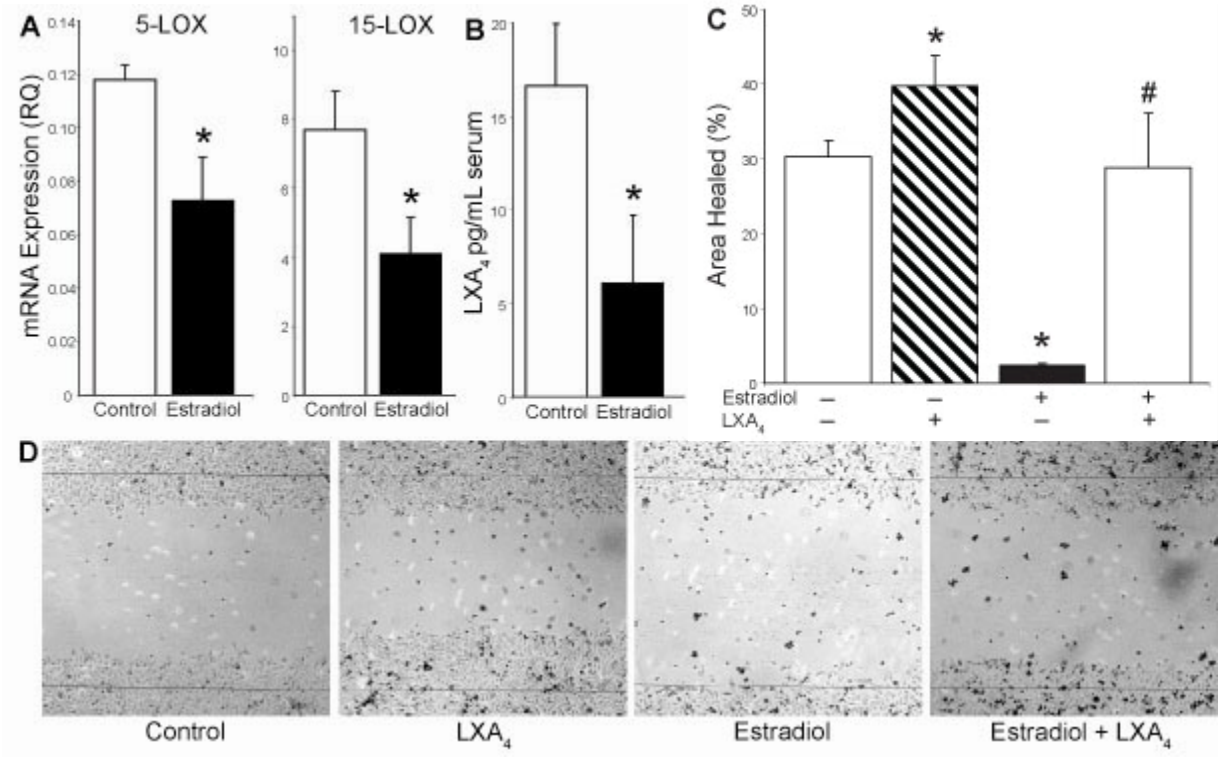

Figure 2.5. Estradiol regulates epithelial wound healing and LXA_4 formation. A) Human corneal epithelial cell (HCE) monolayers were treated with 1 pM estradiol for 24 hrs. Cells were harvested and 5-LOX and 15-LOX mRNA expression quantified by QPCR (n=6, *p<0.05 vs saline). **B)** Epithelial LXA_4 formation. HCE were treated with estradiol (1 pM) or pure media for 24 hrs, cells were activated with calcium ionophore for 15 mins and media collected for LC/MS/MS quantification of endogenous LXA_4 formation (n=6, *p<0.05 vs control). **C)** *In vitro* epithelial wound healing. Confluent cells were incubated with or without LXA_4 (1 nM) in the presence or absence of estradiol (1 pM) for 24 hours prior to scratch wounding. Wound healing was assessed 8 hrs post injury and is expressed as percent area healed (n=5, p<0.05 for * vs. no treatment and # vs. estradiol treatment, ANOVA). **D)** Representative image of epithelium 8 hrs after scratch injury taken using bright field illumination. Lines indicate initial wound area.

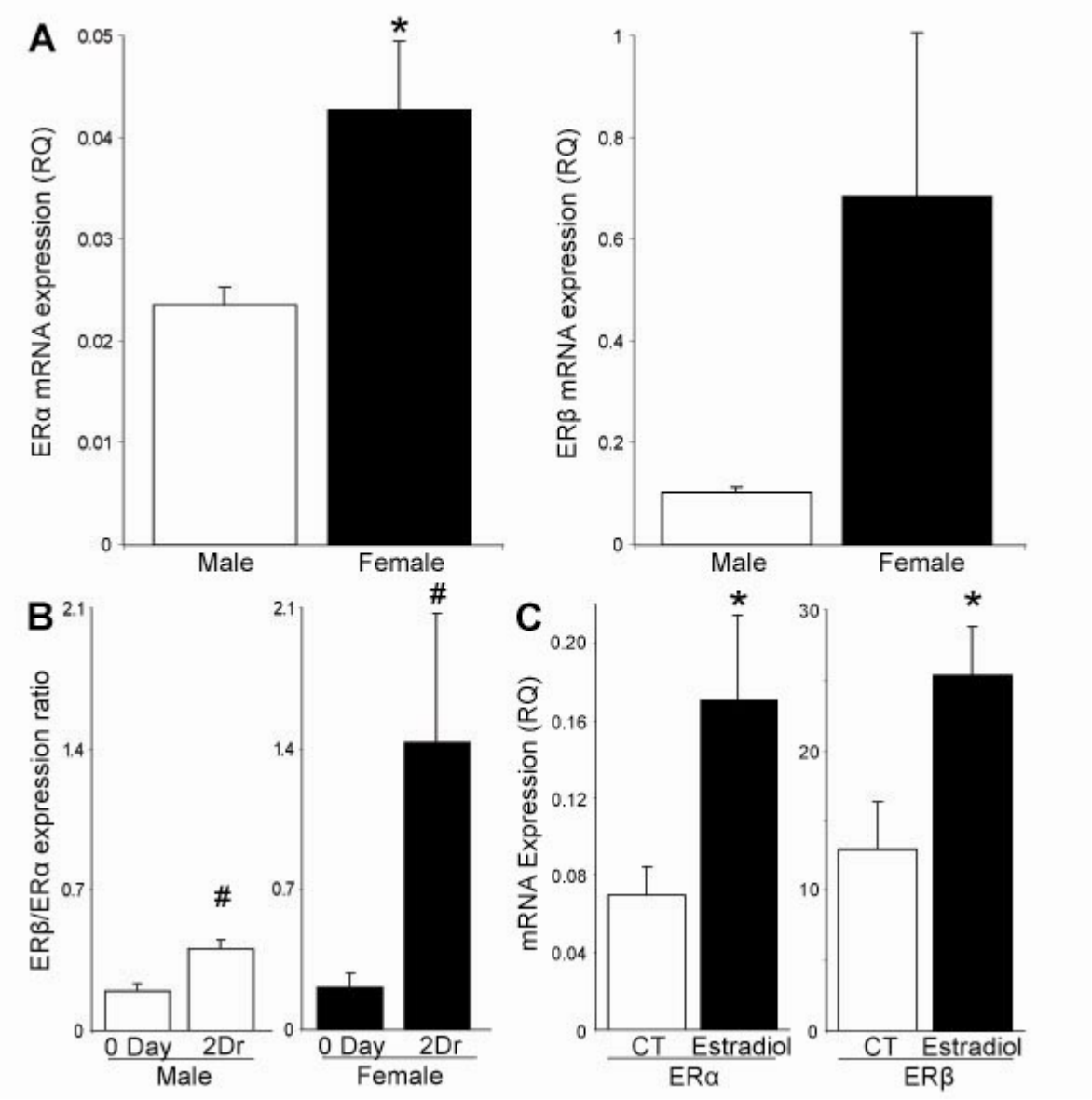

Figure 2.6. **Corneal inflammatory-reparative response regulates epithelial ERα and ERβ expression. A)** Corneas from uninjured male and female mice were removed and epithelial sheets isolated for QPCR analysis of ERα and ERβ mRNA expression. Normalized mRNA quantity is expressed as relative quantity (RQ) to β-actin and a positive control derived from mouse kidney and spleen (n=5, *p<0.05 vs males). **B)** Expression ratio of ERβ to ERα. Epithelial sheets were collected from uninjured corneas and corneas 2 days after re-injury and relative expression of ERβ:ERα assessed by QPCR (n=5, #p<0.05 vs uninjured). **C)** Estradiol regulation of ERα and ERβ. Human corneal epithelial cells were treated with estradiol (1 pM) or media alone (CT) for 24 hrs, collected and expression of ERα and ERβ quantified by QPCR (n=5, *p<0.05 vs control group).

To assess the *in vivo* action of ERα and ERβ, we treated male mouse eyes topically with selective and established receptor agonists (1 μM, 5 μL drop, *t.i.d.*) throughout the duration of the inflammatory-reparative response. The therapeutic doses were selected based on a clinical trial in menopausal women that used an ophthalmic topical dose of 1 μM estradiol [123]. Diarylpropiolnitrile (DPN) was used as an ERβ-specific agonist, which has a 70-fold higher binding affinity for ERβ, and propylpyrazole triol (PPT) was used as an ERα-specific agonist, which has a 410-fold higher binding affinity for ERα. Topical treatment with both DPN and PPT reduced neutrophil content two days after re-injury by 58-62% in male mice, effectively inducing a female neutrophil response to recurrent epithelial injury (Fig 2.7A). Reduced neutrophil infiltration correlated with a >50% reduction in the rate of full wound healing in the ERβ treatment group (Fig 2.7B). Even though there was a trend, the ERα agonist PPT did not significantly delay full re-epthelialization of the corneal wound, suggesting that estrogen's epithelial and leukocyte targeted action are receptor specific.

To directly assess the epithelial action of the ER agonists, we employed the *in vitro* epithelial injury model. Consistent with experiments in Fig 3, estradiol abrogated re-epithelialization of denuded area (94% inhibition, Fig 2.7C). The epithelial action of estradiol was selectively mediated by ERβ as DPN reduced wound healing from 33±6% to 3±1%. In contrast, the ERα selective agonist PPT did not affect the epithelial wound healing rate (Fig 2.7C). Next, we assessed the selective *in vivo* action of ERα and ERβ on the intrinsic expression of the 15-LOX/ALX circuit in the regenerated corneal epithelium. Eyes were treated topically with DPN, PPT or saline throughout the initial wound healing response (Day 0-7) and the recurrent epithelial injury (Day 7-9). Expression of 5-LOX in the regenerated epithelial sheets was not affected by treatment with either ER agonist. In contrast, ERβ activation during the *in vivo* reparative response reduced 15-LOX expression in the regenerated epithelium by 63% while the ERα agonist PPT did not change epithelial 15-LOX expression. This indicates that estradiol's ability to markedly reduce epithelial 15-LOX expression (Fig 2.4b, 2.5B) and endogenous LXA_4 formation (Fig 2.5C) is selectively mediated by the epithelial ERβ receptor. The expression levels of the two mouse LXA_4 receptors, ALX1 and ALX2, showed trends for reduced expression following topical treatment with either ERα or ERβ agonist but these differences did not reach significance (ANOVA, $p>0.05$, $n=5$) due to variability in the saline treatment group (Fig 2.7E). Functional expression of the LXA_4 circuit in the cornea depends on LXA_4 formation, which is driven by both epithelial cells and leukocytes during inflammatory responses. Hence, we next assessed if the leukocyte rich stroma 2 days post epithelial re-injury is capable of generating LXA_4 in the absence of epithelial cells and if ERα and/or ERβ receptors regulate leukocyte dependent LXA_4 formation. Upon activation with calcium ionophore, the isolated leukocyte rich stroma generated significant levels of LXA_4 (43±4 pg/cornea, $n=4$). More importantly, endogenous LXA_4 formation was significantly impaired in mice treated with either the ERα (PPT, 65% inhibition, 15±1 pg/cornea) or ERβ (DPN, 52% inhibition, 21±6 pg/cornea) selective agonist.

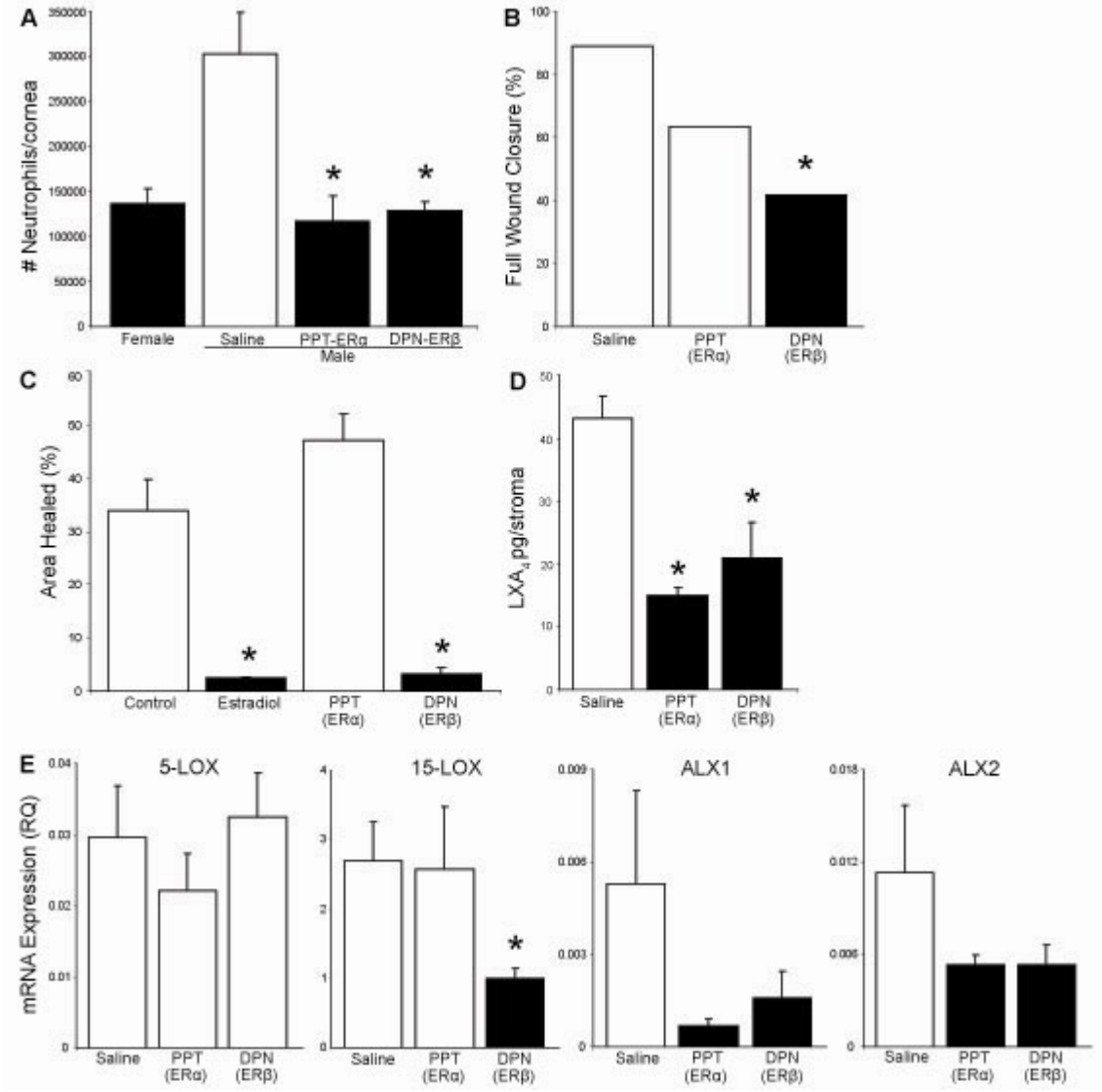

Figure 2.7. Estrogen receptor specific regulation of the neutrophil response, wound healing and 15-LOX/LXA_4 circuit. **A)** ER regulation of neutrophil recruitment. Male mice were treated topically with PPT, DPN or saline alone (1 μM, 5 μL *t.i.d.*, day 0-9) and were compared to females. Corneal neutrophil content 2 days post re-injury was measured by quantifying MPO activity. (n=4, *p<0.05 vs saline). **B)** ERβ regulation of epithelial wound healing. Wound closure was assessed 2 days post re-injury in males treated topically with PPT, DPN or saline alone. (n=4, *p<0.05 vs saline). **C)** ERβ regulation of epithelial wound healing *in vitro*. HCE were incubated with 1 pM estradiol, PPT or DPN 24 hrs prior to scratch wounding. Wound area was quantified by image analysis 8 hrs post injury. (n=4-5, *p<0.05 vs control). **D)** ER regulation of LXA_4 in the leukocyte-rich stroma. Corneas were isolated from saline, PPT or DPN treated male mice 2 days after re-injury, the corneal epithelium was removed and the leukocyte rich stroma was activated with calcium ionophore. Released LXA_4 in the media was quantified by LC/MS/MS (n=5, *p<0.05 vs saline) **E)** Expression of the LXA_4 circuit. Male mice were treated topically with saline, PPT or DPN and mRNA expression was quantified by QPCR in the isolated epithelial sheets (n=5, *p<0.05 vs saline).

2.5 Discussion

To the best of our knowledge, this study provides the first findings demonstrating a sex-specific difference in ocular reparative/inflammatory responses. The clinical relevance of our findings in mice is underscored by results from a post hoc analysis of a trial of 120 patients that received treatment for fungal corneal ulcers [133]. The analysis demonstrated sex-specific differences in the clinical outcome of corneal ulcers. Specifically, re-epithelialization of corneal wounds was significantly delayed in female patients, suggesting worse prognoses for ocular surface wound healing in women. Even though the sex-specific prevalence and incidence of ocular diseases are carefully tracked, no studies have reported sex-specific differences in ocular surface wound healing or the clinical outcome of ocular inflammatory or immune events. Ocular diseases are not triggered by single events but likely by the recurrent dysregulation of intrinsic circuits essential for executing routine tissue maintenance, wound healing and innate or adaptive immune responses. Sex-specific differences in the dynamic execution of routine epithelial wound healing in the cornea identifies a potential factor in the etiology of ocular surfaces diseases in females as recurrent epithelial injury is a hallmark of dry eye syndrome. Wound healing and inflammation are fundamental and intimately linked responses. Hence, delays in wound healing may contribute to shifting healthy ocular inflammation during the reparative response to disease causing inflammation and adaptive immune responses if epithelial injury is recurrent or in the presence of other stress factors such as deficient tear film, infection or hypoxia.

Our results demonstrate that recurrent epithelial injury, which resulted in greatly amplified neutrophil infiltration into the stroma, correlated with accelerated re-epithelialization. Furthermore, the magnitude of the increased neutrophil response to recurrent epithelial injury was markedly reduced in female mice (>50%) compared to male, which correlated with a significant delay in wound closure in females. These findings seem counter-intuitive based on epidermal wound healing studies that indicate that neutrophils in general impair wound healing. However, it is important to recognize that a unique feature of corneal epithelial wound healing responses to self-resolving and minor epithelial injuries is the requirement of inflammation, neutrophil infiltration into the stroma and platelet activation at the vascular border [52, 125-127, 134]. Reduced neutrophil infiltration directly correlates with delayed re-epithelialization of corneal wounds that normally heal within 2-5 days. This unique privileged injury response dictates that redundant circuits are in place to tightly control the precarious activation and recruitment of beneficial neutrophils and is highly evolved in the cornea [115]. Consistent with the beneficial role of neutrophils in mild and self-resolving corneal injury responses, we have previously demonstrated that genetic disruption of the protective LXA_4 biosynthetic pathway decreases, while topical treatment with LXA_4 significantly increases, the neutrophil content of the cornea, which correlates with decreased or increased epithelial wound healing, respectively [52]. In sharp contrast, LXA_4 in chronic or severe corneal injury responses [43, 114] and in other reported severe inflammatory models [22] inhibits neutrophil infiltration. This *in vivo* attenuation of neutrophil content in inflamed tissues by LXA_4 is likely indirectly mediated by decreased expression of chemotactic gradients and increased phagocytosis by macrophages. Results

from the rapidly self-resolving epithelial injury model point towards a novel injury-specific and neutrophil-targeted bioaction of LXA_4 that is the subject of future studies.

Sex-specific differences in corneal neutrophil content were only observed after recurrent epithelial injury while the rate of wound closure was delayed in females both in the initial and recurrent reparative response. This suggests that both epithelial and leukocyte responses to injury exhibit sex-specific differences. The notion of sex-specific regulation of epithelial and leukocyte function in the cornea is indirectly supported by the expression of distinct estrogen receptors in these cell types [93, 95, 131] and the presence of estrogen and estrogen biosynthetic enzymes in the cornea [70, 71].

17β-estradiol, the most prominent human estrogen, induced a female phenotype of wound healing in corneas of male mice that was mirrored by concomitant decrease in the neutrophil response to recurrent epithelial injury and expression of epithelial 15-LOX. These data provide evidence that topical estradiol is sufficient to induce a female reparative/inflammatory response in male mice and provides further evidence for the critical role of neutrophils and epithelial 15-LOX in acute wound healing responses. The fact that estradiol at concentrations as low as 1 pM delayed wound healing in monolayers of human corneal epithelial cells suggests that estradiol has direct and physiologically relevant epithelial actions. More importantly, estradiol significantly reduced the constitutive expression of 5-LOX and 15-LOX in human corneal epithelial cells. These two LOX enzymes are required for the formation of LXA_4 in the cornea and are expressed in mouse and human corneal epithelial cells [52, 108]. LXA_4 alone was able to increase the rate of epithelial wound healing *in vitro*, which is consistent with previous studies that have demonstrated that LXA_4 and 15-LOX promote epithelial wound healing *in vivo* [43, 52] and that the epidermal growth factor (EGF) mediates its epithelial effects in part through LXA_4 [129]. Epithelial cells that were treated with LXA_4 and estradiol retained normal wound healing responses. Further experiments *in vivo* showing estradiol downregulation of wound healing and the 15-LOX/LXA_4 pathway suggest that this effect is not due to estrogen binding to LXA_4. This add-back experiment provides strong evidence that estradiol's actions on epithelial proliferation/migration are mediated in part by inhibition of the constitutive LXA_4 biosynthetic pathway in corneal epithelial cells. A recent study [135] demonstrated that LXA_4 can bind to ERs and can regulate estradiol-targeted gene expression in endometrial epithelial cells. Estradiol's and LXA_4's bioactions are likely distinct for tissue specific epithelial cell types. However, regulation of estradiol-targeted genes offers a potential mechanism for LXA_4's ability to retain normal wound healing in estrogen treated epithelial cells. Expression of 15-LOX is markedly lower in the regenerated epithelium of female mice and topical estradiol reduces expression of 15-LOX in male to the levels measured in the corneal epithelium of females. The mechanism for estrogen regulation of 15-LOX or 5-LOX expression remains to be defined. Gene regulation by estrogen receptors is complicated and is often not via response elements in the promoter region of a target gene [95, 132]. Taken together, these multiples lines of *in vivo* and *in vitro* evidence link the sex-specific differences in the corneal reparative/inflammatory response to regulation of the intrinsic epithelial LXA_4 biosynthetic pathway.

Estrogens regulate expression of distinct gene targets by two nuclear hormone receptors, ERα and ERβ [93, 95, 132]. The expression of both receptors in the cornea and corneal epithelium has been established in both males and females [69, 128, 131] though expression of both receptors is markedly higher in females (Fig 5). Our results demonstrate for the first time that epithelial expression of estrogen receptors is regulated by the corneal inflammatory/reparative response, as the receptor expression ratio shifts from predominantly ERα in the resident epithelium to ERβ in the regenerated epithelium. ERβ is of particular interest as this receptor has been linked to many of estrogen's immune regulatory functions [96]. Activation of either ERα or ERβ in the cornea resulted in reduced neutrophil infiltration in response to epithelial re-injury. By contrast, only ERβ activation significantly delayed epithelial wound healing *in vivo* in mice and *in vitro* in human corneal epithelial cells. These findings identify ERβ as the specific receptor that mediates estradiol's epithelial-targeted bioactions and indicate that, by either direct or indirect actions, both ERα and ERβ can regulate neutrophil recruitment to the injured cornea. Consistent with ERβ specific regulation of epithelial wound healing, only ERβ markedly down-regulated expression of 15-LOX in the regenerated epithelium of re-injured corneas. Neither ERα nor ERβ altered the expression of 5-LOX in the regenerated epithelium. This is in contrast to estradiol's ability to markedly reduce constitutive 5-LOX expression *in vitro* in human corneal epithelial cells. These differences are likely due to distinct differences between cultured human epithelial cells and *in vivo* regenerated mouse epithelium as well as multiple tissue specific factors that regulate 5-LOX expression *in vivo* in the inflamed cornea. Epithelial re-injury induces profound upregulation of neutrophil migration into the stroma that correlates with rapid epithelial wound healing. Even though the corneal epithelium expresses low levels of functional 5-LOX, the predominant source of 5-LOX activity in the inflamed cornea are leukocytes, especially neutrophils and macrophages. Consistent with the ability of both ERα and ERβ to markedly attenuate neutrophil infiltration, activation of either receptor markedly reduced endogenous formation of LXA_4 by the leukocyte rich stroma. Transcellular biosynthesis is the predominant endogenous route for LXA_4 formation [107, 108, 112, 113]. During healthy inflammation, epithelial cells, which highly express 15-LOX, interact with 5-LOX expressing leukocytes to set in place a temporal amplification loop. This intrinsic feed forward loop is likely an important factor in the cornea and counter-balances pro-inflammatory circuits, stimulates non-inflammatory activation of macrophages and drives normal execution of inflammatory/reparative responses. Estradiol attenuates functional expression of this intrinsic corneal circuit at multiple levels by receptor specific regulation in both epithelial cells and leukocytes, which correlates with marked sex-specific differences in the inflammatory and wound healing response.

In summary, our results provide the first evidence linking sex-specific differences and the action of estradiol to the 15-LOX/LXA_4 circuit. Specifically, ERβ downregulates epithelial expression and function of key enzymes in the formation of LXA_4 while both ERβ and ERα markedly reduce neutrophil infiltration and leukocyte-dependent LXA_4 formation. LXA_4, its receptor (ALX) and biosynthetic enzymes (15-LOX and 5-LOX) have emerged as intrinsic lipid circuits across the visual axis that are critical to the routine execution of healthy inflammatory/reparative responses and the counter-regulation of circuits that lead to dysregulated or chronic inflammation [107, 108, 114, 136]. Our finding that estrogen negatively regulates the intrinsic LXA_4 circuits, especially ERβ specific

regulation of epithelial 15-LOX, may provide novel insights into the etiology or pathogenesis of sex-specific ocular inflammatory diseases.

Chapter 3:

ERβ Regulates Macrophage and Neutrophil Functional Responses and Drives Sex-Specific Differences in Acute Corneal Inflammation

Abstract

The ability to remove apoptotic neutrophils from the inflammatory loci has important ramifications in many disease models including wound healing. In the tissue, macrophage phagocytosis is one major route of apoptotic neutrophil clearance. Previously, we reported a sex-specific difference in rate of corneal wound repair mediated by estrogen regulation of the anti-inflammatory 15-lipoxygenase (15-LOX)/lipoxin A_4 (LXA_4) circuit in corneal epithelial cells. LXA_4 is a potent anti-inflammatory and pro-resolving lipid mediator formed endogenously in the cornea. We discovered that neutrophil amplitude is markedly upregulated in female corneas at two days after re-injury due to increased neutrophil retention in an estrogen receptor (ER) β-specific manner. Examination of corneal macrophage content revealed similar numbers of $F4/80^+/Ly6G^-$ cells between sexes though further analysis revealed a higher proportion of M1-like macrophages in female corneas. This correlated with an increased pro-inflammatory tone (prostaglandin E_2 and thromboxane B_2) in female corneas at two days after re-injury while formation of the anti-inflammatory and pro-resolving LXA_4 was downregulated. We show for the first time that phagocytosis is a major stimulus for LXA_4 formation. 17β-estradiol inhibited LXA_4-induced stimulation of phagocytosis, an effect that was lost in ERβ deficient macrophages. 17β-estradiol also delayed neutrophil apoptosis. Taken together, these findings show that females exhibit a greater inflammatory response to recurrent injury with an ERβ-dependent upregulation of neutrophil retention. Estrogen regulation of macrophage and neutrophil functional response might prime females, lowering the threshold for a shift from an acute healthy inflammation to chronic disease-causing inflammation, which may explain the high female-dominated incidence of inflammatory diseases.

Introduction

Neutrophil clearance plays an important role in inflammatory resolution. In the cornea, unlike in dermal tissues, neutrophils are considered beneficial for wound healing. Indeed, downregulation of corneal neutrophil recruitment was correlated with impaired wound healing in a model of corneal epithelial injury [52]. However, while leukocytes are early and crucial responders to tissue injury, their continued presence in tissues can exacerbate the local inflammatory response and hinder wound healing [137]. Furthermore, the state of neutrophil activation dictates their role in the cornea [43]. Addition of lipoxin A_4 (LXA_4) to injured corneas promotes wound healing and an amplified neutrophil response (i.e. beneficial for wound healing). Conversely, the amplified neutrophil response in injured corneas exposed to endotoxin from *Pseudomonas aeruginosa,* a clinically relevant pathogen associated with bacterial keratitis, is associated with a decrease in rate of wound healing. In this setting, LXA_4 markedly reduced endotoxin-induced neutrophil recruitment and increased wound healing. An amplified neutrophil response in the cornea can therefore tilt the ocular immune response towards sight-threatening chronic inflammation upon further inflammatory stimulus.

Professional phagocytes such as macrophages facilitate neutrophil clearance by phagocytosing cells in the tissue [138-141]. In the classic understanding of phagocytosis, cells that undergo programmed cell death, or apoptosis, display 'find me' and 'eat me' markers on their cell surface. These markers are recognized by cell surface receptors on macrophages, which readily phagocytose and clear cells from the tissue. In addition to removing cells before they undergo lysis, phagocytosis also serves as a signal for the end of inflammation and the beginning of resolution (reviewed in [142]). Interactions between macrophages and neutrophils produce significant quantities of endogenous lipid mediators that have emerged in recent years as key and essential regulators of inflammatory resolution [16]. This includes LXA_4, one of the first lipid mediators characterized as having both anti-inflammatory and pro-resolution actions, which is produced from the transcellular actions of 5- and 15-LOX on dietary arachidonic acid (AA) [14, 113]. However, not much is known about LXA_4 formation during the process of phagocytosis. Previous studies indicated that production of other transcellular LOX lipid products of AA including LXB_4 and docosahexanoic acid-derived resolvins is altered upon macrophage phagocytosis of apoptotic neutrophils [16].

In the previous chapter, we reported a sex-specific difference in the expression and formation of the 15-LOX/LXA_4 pathway following an acute corneal epithelial injury, the first report to our knowledge linking sex hormones to lipid mediators of inflammation [143]. Estrogen directly downregulates, in an estrogen receptor (ER) β-specific manner, epithelial 15-LOX expression and subsequent formation of LXA_4, impairing epithelial wound healing. Of note, LXA_4 levels in the stroma, which contains the vast majority of recruited leukocytes, also exhibited marked sex-specific differences. Beyond accelerating epithelial wound healing, LXA_4 also has characterized pro-resolving actions on leukocytes [22]. LXA_4 inhibits both neutrophil and eosinophil chemotaxis while stimulating monocyte chemotaxis and adherence [45, 47, 144, 145]. LXA_4 and its analogs have been shown to enhance macrophage nonphlogistic phagocytosis of apoptotic neutrophils both *in vitro* and *in vivo* [50, 146]. Macrophage polarization can be skewed by LXA_4 and its DHA-derived analogue,

resolvin D1 [147-149]. We thus wondered if there is a sex-specific, estrogen-modulated difference in leukocyte dynamics following an acute inflammatory injury. Estrogen regulation of macrophage and neutrophil function can help explain the strong female bias in incidence rate of many autoimmune/inflammatory diseases.

We thus set out to assess if there is a sex-specific difference in the corneal inflammatory response and if estrogen drives such sex-specific differences by regulating macrophage and neutrophil function. Using a model of corneal epithelial injury, we reveal a distinct sex-specific difference in corneal inflammatory tone that correlated with the expression of more pro-inflammatory M1-like macrophages and increased neutrophil retention. We demonstrate for that first time that macrophage phagocytosis of apoptotic neutrophils is a potent stimulus for LXA_4 formation. We also show that 17β-estradiol delays neutrophil apoptosis and desensitizes macrophages to LXA_4 stimulation of phagocytosis in an ERβ-dependent manner, contributing to enhanced neutrophil content in females. These results provide evidence for estrogen regulation of the inflammatory response in the cornea, which may have important implications when considering greater female susceptibility to inflammatory diseases.

3.3 Materials and Methods

Animal Experiments

All animal studies have been approved by the University of California, Berkeley in accordance with the NIH Guide for the Care and Use of Laboratory Animals and the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Age-matched (6 to 10 week old) female, ovariectomized female, and male c57BL/6J (stock#00064) mice and ERβKO (B6.129P2-*Esr2*^{tm1Unc}/J) males were purchased from Jackson Laboratory (Bar Harbor, ME). Mice were maintained on a 12-hour light/dark cycle and fed *ad libitum* a standard diet (Rat/Mouse diet LM-485, Harlan Tekland, Madison, WI).

Corneal Epithelial Abrasion

Mice were anesthetized with ketamine (50 mg/kg) and xylazine (20 mg/kg) intraperitoneally. A drop of proparacaine hydrochloride ophthalmic solution 0.5% was applied to the eye to deliver local corneal anesthesia before injury. Epithelial abrasion was achieved using an Algerbrush II with a 0.5-mm corneal rust ring remover as previously described [43, 52, 122]. The corneal epithelium was mechanically removed up to the limbal border under a dissection microscope and full removal was verified using fluorescein stain as a direct marker of epithelial defect. A 2-3 epithelial cell layer (100% wound healing) was regenerated within 5 days post injury in both males and females [52]. Reinjury was initiated by complete re-abrasion of the regenerated epithelium 7 days after initial abrasion. Epithelial defect was visualized with fluorescein. Wound area was quantified using imaging software (ImagePro Express 6.0) as previously described [43, 52, 122].

Assessment of Neutrophil Content

Myeloperoxidase (MPO) activity was used as an index of tissue leukocyte infiltration [43, 52, 114]. Corneas (1 cornea/data point) were mechanically homogenized in a solution of 50mM potassium phosphate buffer (pH 6.0) containing 0.5% hexadecyltrimethylammonium bromide, followed by three cycles of freeze-thaw and a ten second sonication. The homogenates were then centrifuged and supernatants collected. MPO activity was measured by spectrophotometry using o-dianisidine dihydrochloride reduction as a colorimetric indicator. MPO activity was converted into total neutrophil number using calibration curves established from neutrophils collected from zymosan A-induced peritonitis exudates in c57BL/6J mice.

Bone marrow macrophage isolation

Macrophages were derived from bone marrow of c57BL/6 mice according to [150, 151]. Briefly, mice were killed and their femurs and tibias removed. Bone marrow was isolated mechanically followed by a 1 minute spin at 500 rpm at room temperature. Cells were plated in 20 mm dishes with complete media (15 mL RPMI 1640 containing 10% fetal bovine serum (FBS), 10% macrophage-colony stimulating factor (L929 cells), 1% penicllin/streptomycin, and 1% L-glutamine). On the third day, 2 mL of the complete media was added to each plate. Upon

confluence, cells were removed by incubation in cold HBSS for 10 minutes at 4° followed by mechanical scraping. Cells were then counted and plated in 10 mm dishes in 2 mL complete media. After an overnight incubation, bone marrow derived macrophages (BMMØ) were then polarized to M1 or M2 phenotype by a 24 hour incubation with 100 ng/mL lipopolysacharide (LPS) (Sigma-Aldrich, St Louis, MO) or 10 ng/mL interleukin(IL)-4 and 10 ng/mL IL-13 (R&D Systems, Minneapolis, MN), respectively. Expression of established M1 and M2 cell surface markers and arginase activity was used to confirm successful polarization. 17β-estradiol treated cells were first treated with 1 nM of 17β-estradiol (E2758, Sigma-Aldrich, St. Louis, MO) overnight before incubation with either LPS or IL4 and IL13.

In vitro studies were conducted with macrophages derived from both male and female c57BL/6J mice. Expression levels of the two classic estrogen receptors, ERα and ERβ, were similar in both groups while phagocytic capacity of macrophages displayed no marked differences between sexes. Therefore, we concluded that bone marrow derived macrophages exhibit no sex specific differences *in vitro* before exposure to sex steroids and, for the remainder of this study, will not be distinguished based on the sex of the donor animal.

Determination of Phagocytic Capacity

Analysis of phagocytic capacity was conducted with minor modifications from [50]. Leukocytess were collected from the peritoneal cavity of C57BL/6J mice 24 hours after zymosan-A injection (1 mg/mL/mouse). Leukocytes were incubated for 90 minutes in RPMI-1640 at 37°C and media containing non-adherent cells (i.e. neutrophils) was collected. The neutrophils were then allowed to undergo spontaneous apoptosis for 24 hours in RPMI-1640 containing 10% FBS and 1% penicillin/streptomycin. 500,000 apoptotic neutrophils were co-incubated with 1 million BMMØ for 30 minutes at 37°C. BMMØ in the LXA_4 treatment group were exposed to 1 nM LXA_4 for 15 minutes prior to co-incubation. After 30 minutes, all non-adherent cells were removed by two washes with phosphate buffered saline (PBS). Cells were then collected and processed for myeloperoxidase activity. Briefly, adherent cells were incubated in 450 μL 50 mM potassium phosphate buffer (pH 6.0) containing 0.5% hexadecyltrimethylammonium bromide for 10 minutes at 4°C. Cells were mechanically lifted and sonicated for ten seconds prior to three cycles of freeze-thaw. The homogenates were centrifuged and supernatant collected for analysis of MPO activity as described above.

Determination of Arginase Activity

For BMMØ, 1 million cells were mechanically lifted in 200 μL PBS then centrifuged at 200 rcf for 10 minutes at 4°C. Pellets were resuspended in 100 μL PBS and sonicated for 10 seconds before undergoing a freeze/thaw cycle. The samples were sonicated again then centrifuged at 16,100 rcf for 10 minutes at 4°C. Supernatant was taken for analysis of arginase activity following QuantiChrom Arginase Assay Kit (BioAssay Systems, Hayward, CA) specifications. For *in vivo* studies, corneas were isolated from mice at two days after re-injury and homogenized in 200 μL PBS before undergoing sonication and centrifugation.

Flow Cytometry

BMMØ were collected for flow cytometry analysis by incubation in cold PBS for 10 minutes at 4°C followed by mechanical scraping. For corneal samples, 4 corneas were pooled, mechanically diced and digested in 1:100 2.5 mg/mL Liberase TH (Roche) for 2 hours at 37°C. Cells were then washed four times in PBS containing 0.2% Tween-20 before a 15 minutes block with 5% bovine serum albumin (BSA) followed by a 30 minutes incubation with the following antibodies: 1:100 FITC-F4/80 (eBioscience, San Diego, CA), 1:100 PE-Ly6G (BD Biosciences, San Jose, CA), 1:10 PE-CD206 (Santa Cruz Biotechnology, Santa Cruz, CA), 1:10 PE/Cy7-CD86 (Abcam, Cambridge, MA) in 3% BSA/PBS. The cells were then washed once in PBS containing 0.2% Tween-20 before fixation with 4% paraformaldehyde for 10 minutes at 4°C. After a four time wash in PBS containing 0.2% Tween-20, cells were resuspended in PBS containing 10% fetal bovine serum before analysis with a Beckmann-Coulter FC-500.

Immunohistochemistry

BMMØ were allowed to adhere to a glass coverslip overnight before treatment with LPS (100 ng/mL) or IL-4 and IL-13 (10 ng/mL) and 17β-estradiol (1 nM). For assessment of apoptosis, an ApopTag Red In Situ Apoptosis Detection Kit (Millipore, Billerica, MA) was used. The coverslips were processed according to manufacturer instructions before mounting with Vectashield containing DAPI (Vector Laboratories, Burlingame, CA). Apoptotic cell number was manually counted using ImageJ software and compared to number of total cells. For visualization of phagocytosis, apoptotic neutrophils were incubated with PE-Ly6G (BD Biosciences) before introduction to macrophages. After phagocytosis, non-adherent cells were washed off and macrophages were fixed with 4% paraformaldehyde for 5 minutes on ice before permeabilization with 0.2% Triton X-100/PBS for 15 minutes. Cells were then blocked for 30 minutes with 0.2% Triton X-100/10% goat serum/PBS followed by incubation with anti-15-LOX (Abcam) antibody (1:100 in 0.2% Triton X-100/5% goat/PBS) for 30 minutes. A 30 minute incubation with FITC anti-rabbit secondary (BioLegend, San Diego, CA) followed. All above incubations were done at room temperature.

Gene Expression

RNA from corneas and cultured cells was isolated using a RNA Easy Mini Kit (Qiagen Sciences, Maryland) and quantified via spectrophotometry. The RNA was then reverse transcribed using a High-Capacity cDNA Kit (Applied Biosystems, Foster City, CA) and each sample was diluted to 2.5 ng/μL cDNA. and each sample was diluted to 2.5 ng/μL cDNA. A total reaction volume of 20 μL/well included 10 μL of SYBR Green Master Mix (Applied Biosystems), a final primer concentration of 200 nM, and a cDNA template amount corresponding to 5 ng RNA. Real-time PCR was performed with a Step One Plus QPCR system (Applied Biosystems) as previously described [114, 124]. Briefly, samples were heated to 95°C for 10 min followed by 40 cycles of 95°C for 15 sec and 60°C for 1 min. In the final melt curve stage, samples were heated again to 95°C for 15 sec followed by 60°C for 1 min before a final step of 95°C for 15 sec. The following primer pairs were used:

CD68: sense, 5'-GGACCCACAACTGTCACTCAT-3', antisense, 5'-AAGCCCCACTTTAGCTTTACC-3',
CD206: sense, 5'-GCTGAATCCCAGAAATTCCGC-3', antisense, 5'-ATCACAGGCATACAGGGTGAC-3'
CD86: sense, 5'-TGTTTCCGTGGAGACGCAAG-3', antisense, 5'-TTGAGCCTTTGTAAATGGGCA-3'
Fprl-1: sense, 5'-CATTTGGTTGGTTCATGTGCAA-3', antisense, 5'-AATACAGCGGTCCAGTGCAAT-3'
Fpr-rs2: sense,5'-GCCAGGACTTTCGTGGAGAGAT-3', antisense,5'-GATGAACTGGTGCTTGAATCACT-3'
All were selected from the Harvard Primer Bank and verified by the NIH GenBank database. . The ΔΔCT method was used to quantify mRNA expression levels using Step One Software version 2.0 (Applied Biosystems). mRNA quantity is expressed as relative quantity (RQ) to the housekeeping gene, β-actin, and an universal mouse reference RNA that was generated from mRNA obtained from pooled Balb/c kidney and spleen. Amplifications were run in duplicate and efficiency curves for all primers were established.

Lipid Mediator Lipidomics

Lipid autacoid analysis was performed as previously described [114, 124, 150]. Briefly, samples (1 cornea/sample) were homogenized in 66% methanol containing deuterated internal standards, prostaglandin (PG)E_2-d_4, LXA_4-d_5, leukotriene B_4 (LTB_4)-d_4 , 15(S)- HETE-d_8, arachidonic acid(AA)-d_8, and docosahexanoic acid(DHA)-d_5 (400 pg/each), to calculate the recovery of prostanoids or mono-hydroxy- and dihydroxy-containing fatty acids. Lipid autacoids were extracted by solid phase using Accubond ODS-C18 cartridges (Agilent Technologies, Santa Clara, CA). Eicosanoids were identified and quantified by liquid chromatography (LC)/mass spectrometry (MS)/MS-based lipidomics. In brief, extracted samples were analyzed by a triple quadrupole linear ion trap LC/MS/MS system (MDS SCIEX 3200 QTRAP) equipped with a Kinetex C18 mini-bore column, using a mobile phase gradient of water/acetonitrile/acetic acid (72:28:0.01, v:v:v) and isopropanol/acetonitrile (60:40, v:v) with a 450 μl/min flow rate. MS/MS analyses were performed in negative ion mode and prominent fatty acid metabolites were quantified by multiple reaction monitoring (MRM mode). Calibration curves (1 to 1000 pg) and specific LC retention times for each compound were established with synthetic standards (Cayman Chemical, Ann Arbor, MI).

Statistics

One-tailed, unpaired student's t-test was used to evaluate the significance of differences between two groups. One-tailed Fisher's exact test (a variation of a chi-square test) was used to evaluate the significance of differences between groups in wound closure. A t-test was not appropriate due to the integer-based, non-continuous nature of the data. P values of less than 0.05 were considered significant. All data reported as mean ± SEM unless otherwise indicated.

3.4 Results

To elicit an acute inflammatory injury, age-matched female and male mice underwent full corneal abrasion. The epithelial layer of the cornea was mechanically removed up to the limbal border and allowed to heal for seven days at which time it was re-injured to elicit an amplified inflammatory response. During both initial injury and re-injury, female eyes healed slower than males. 48 hours after full corneal epithelial abrasion, only 50% of female eyes had fully healed compared to 90% of their male counterparts. At two days after re-injury, 57% of male eyes achieved full wound closure, exhibiting a 490% increase in wound closure compared to females (Fig 3.1A).

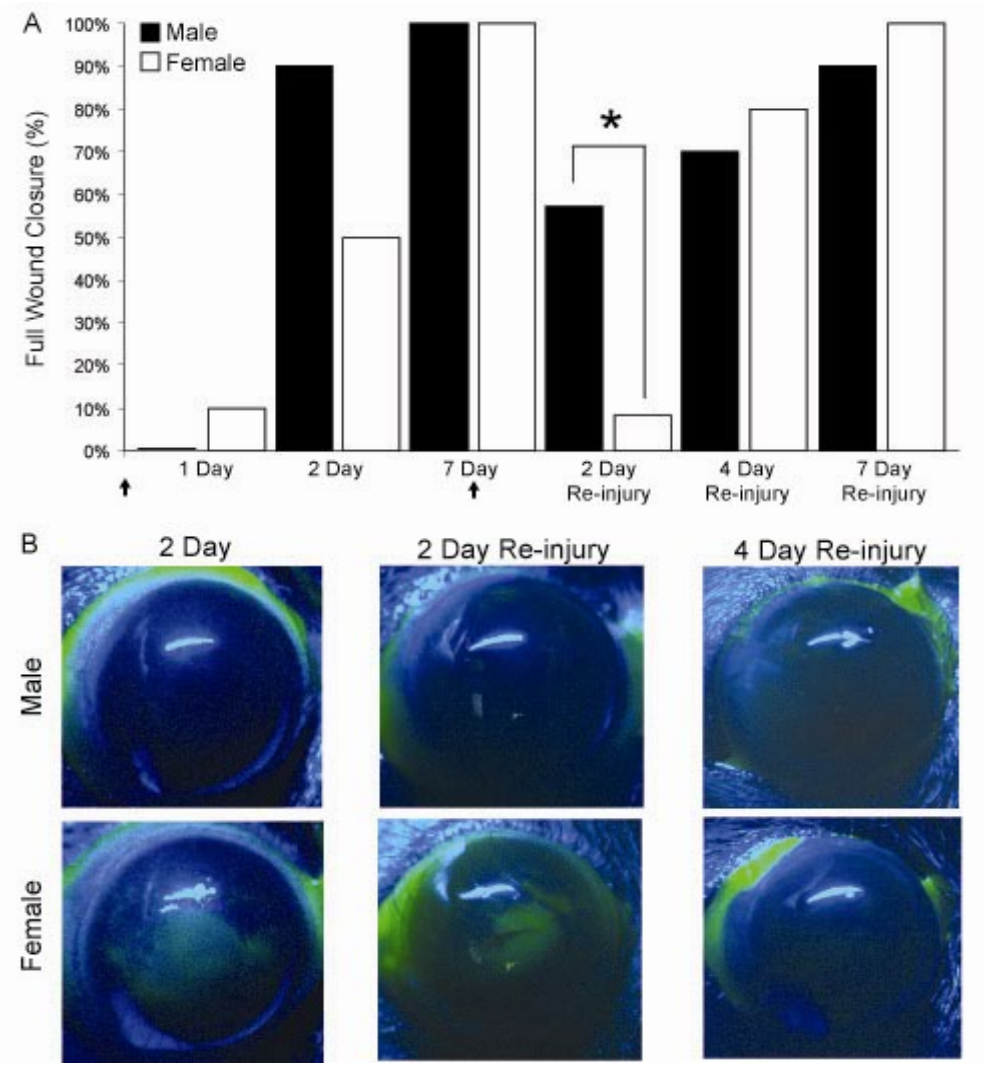

Figure 3.1. **Sex-specific differences in corneal wound healing**. Corneas of age-matched male and female mice underwent complete epithelial abrasion. At 7 days after injury, corneas were re-injured to elicit a more robust inflammatory response. Arrows indicate times of injury. **A)** Wound healing was quantified as percent of eyes that had achieved full wound closure. Full wound closure was determined by a lack of fluorescein staining on the corneas. **B)** Representative images of eyes at 2 days after injury and 2 and 4 days after re-injury (n=5, *$p<0.05$ vs males)

Interestingly, neutrophil dynamics during this time course also revealed striking sex-specific differences. In female mice, neutrophil infiltration peaked at 52,638 cells/cornea 24 hours after initial injury, resolving to 4,698 cells/cornea at 7 days, while neutrophil levels in males peaked 48 hours after injury at 69,992 cells/cornea (Fig 3.2). Re-injury elicited an amplified neutrophil response (170,339 cells/cornea) in females yet failed to do the same in males (22,114 cells/cornea) (Fig 3.2). This amplified female neutrophil response did not result in a failure in inflammatory resolution. At four days after re-injury neutrophil levels in both males and females returned to near normal numbers, reflected in 80% and 82% of eyes that had achieved full wound closure in males and females, respectively (Fig 3.2).

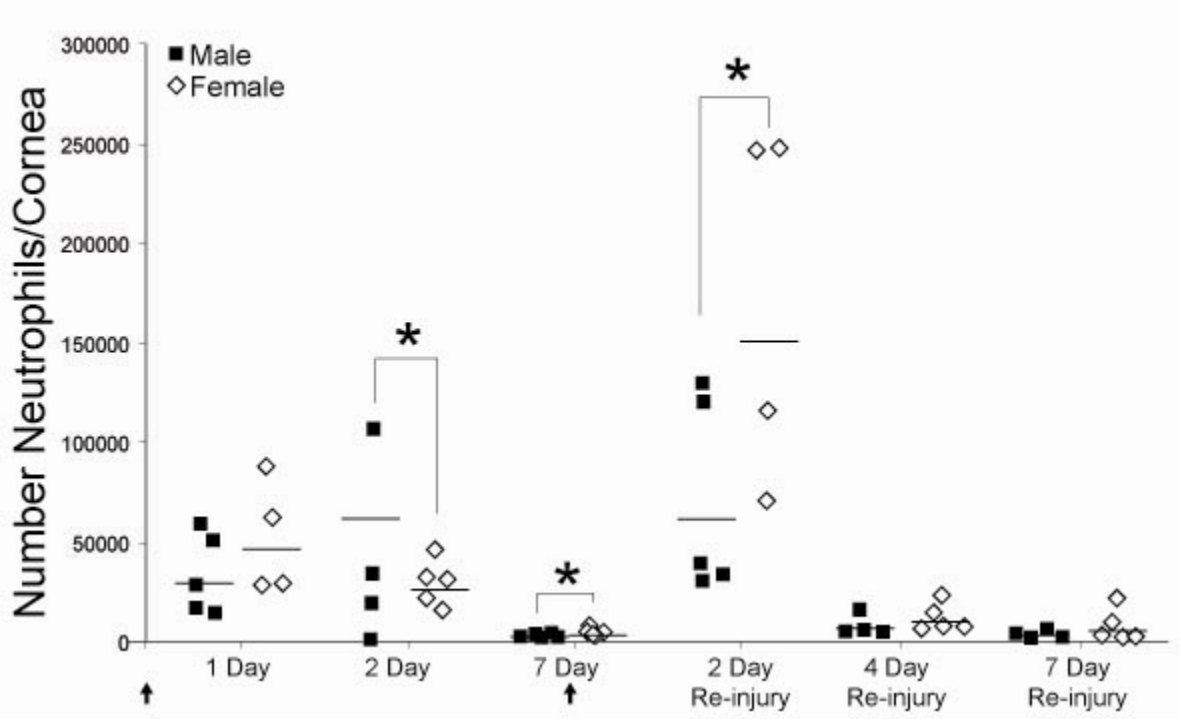

Fig 3.2. Amplitude in neutrophil response to recurrent injury is upregulated in females. Mice were killed at 1, 2, and 7 days after initial injury and 2,4, and 7 days after re-injury and corneas taken for neutrophil quantification. Number of total neutrophils within the cornea was assessed using myeloperoxidase as an indicator of neutrophil activity. (n=4-5, *$p<0.05$ vs males).

The increased amplitude of the neutrophil response in females at two days after re-injury may be a result of a temporary imbalance between neutrophil clearance and recruitment. A major source of neutrophil clearance in tissues is macrophage phagocytosis of apoptotic neutrophils. Macrophage numbers within the cornea were thus assessed for any sex-specific differences. FACS analysis of corneas at 2 days after re-injury revealed that macrophages ($F4/80^+/Ly6G^-$ cells) comprised 50% of leukocytes ($F4/80^+/Ly6G^-$ and $F4/80^-/Ly6G^+$ cells) within the male cornea compared to 30% in females ($p=0.05$) (Fig 3B). Absolute macrophage numbers showed no sex-specific difference ($p=0.5$). Female corneas contain the same amount of macrophages after epithelial injury as males despite a 2-fold increase in neutrophils (Fig 3C,D).

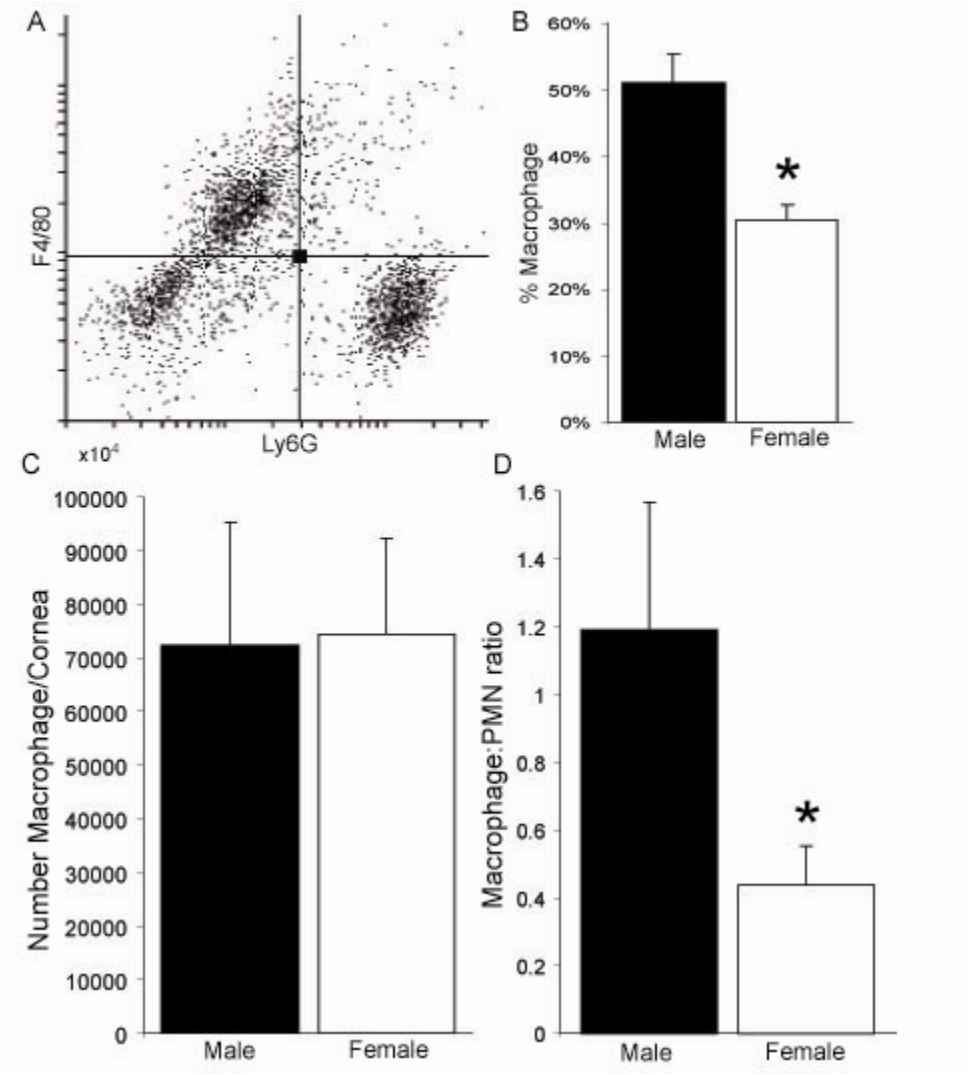

Fig 3.3 Neutrophil/macrophage ratios exhibit marked sex-specific differences. A) 4 corneas/sample were pooled for flow cytometry analysis. Macrophages were characterized as being F4/80$^+$/Ly6G$^-$ and neutrophils F4/80$^-$/Ly6G$^+$. **B)** Percent of total leukocytes that are F4/80$^+$/Ly6G$^-$. **C)** Total number of macrophages and **D)** Ratio of macrophage to neutrophils (PMN) per cornea were calculated via myeloperoxidase activity and flow cytometry. (n=3-5; *$p<0.05$ vs male).

Further analysis of the F4/80$^+$/Ly6G$^-$ population revealed sex-specific differences in macrophage subtype. Female corneas contained more cells expressing the M1 marker, CD86, while CD206, a M2 marker, remained unchanged at two days after re-injury(Fig 3.4A). At two days after re-injury, arginase activity, a marker of M2 macrophages, was markedly decreased compared to males (Fig 3.4B). mRNA analysis of male and female corneas at 2 days after initial injury, 2 days after re-injury, and 4 days after re-injury when both sexes have achieved inflammatory resolution revealed similar results. CD68, a common macrophage marker, showed no sex specific differences at any of the three time points, indicating that total macrophage numbers, but not neutrophil to macrophage

ratio, within the cornea remained unaltered between males and females. Similarly, expression levels of the M2 marker, CD206, exhibited no marked sex-specific difference at 2 days after initial injury and 4 days after re-injury while females had higher expression levels of CD206 at 2 days after re-injury. CD86, the M1 marker, remained consistently higher in females at all three time points (Fig 3.5).

Consistent with evidence for a higher proportion of M1-like proinflammatory macrophages in female corneas, formation of inflammatory lipid mediators also exhibited sex-specific differences. Levels of arachidonic acid (AA) were markedly increased in female corneas at two days after re-injury, a sign of increased inflammatory activation. 15-HETE formation was also upregulated and production of pro-inflammatory prostaglandin (PG) E_2 was increased 63% in female corneas compared to males. Thromboxane B_2 (TxB_2) was significantly higher in females (133.2±12.8 pg/cornea) compared to males (75.1±6.9 pg/cornea) (Fig 3.6B). Taken together, there is evidence for a sex-specific difference in inflammatory tone and macrophage population.

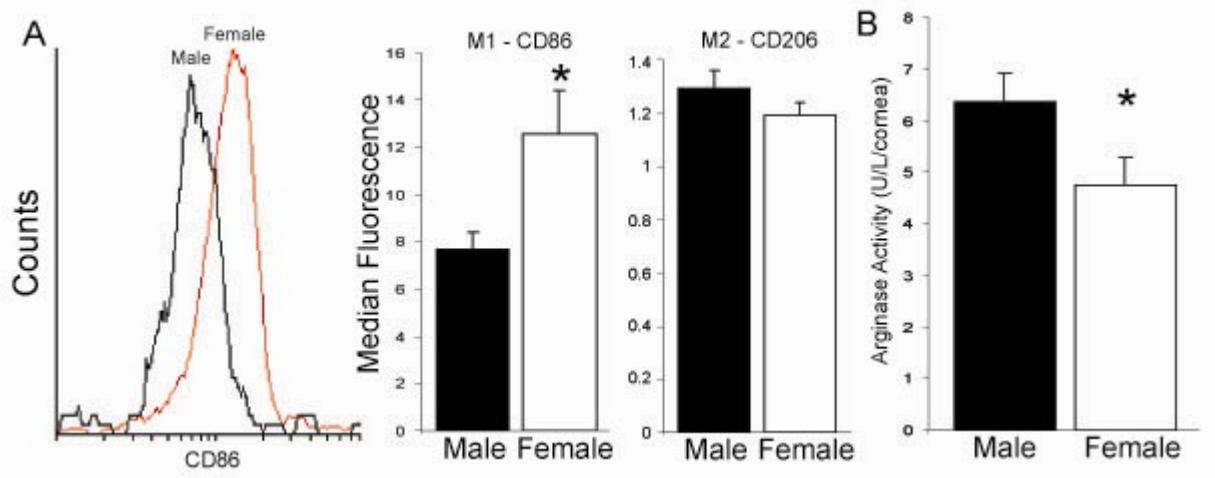

Fig 3.4 Females express more M1-like macrophage markers 2 days after re-injury. Corneas of male and female mice were isolated at 2 days after re-injury. **A)** Number of CD86+ and CD206+ cells were analyzed using flow cytometry from the population of F4/80+/Ly6G- cells within the cornea (4 corneas/sample). **B)** Arginase activity within the cornea was determined using a colorimetric assay. (n=3-5, *p<0.05 vs males).

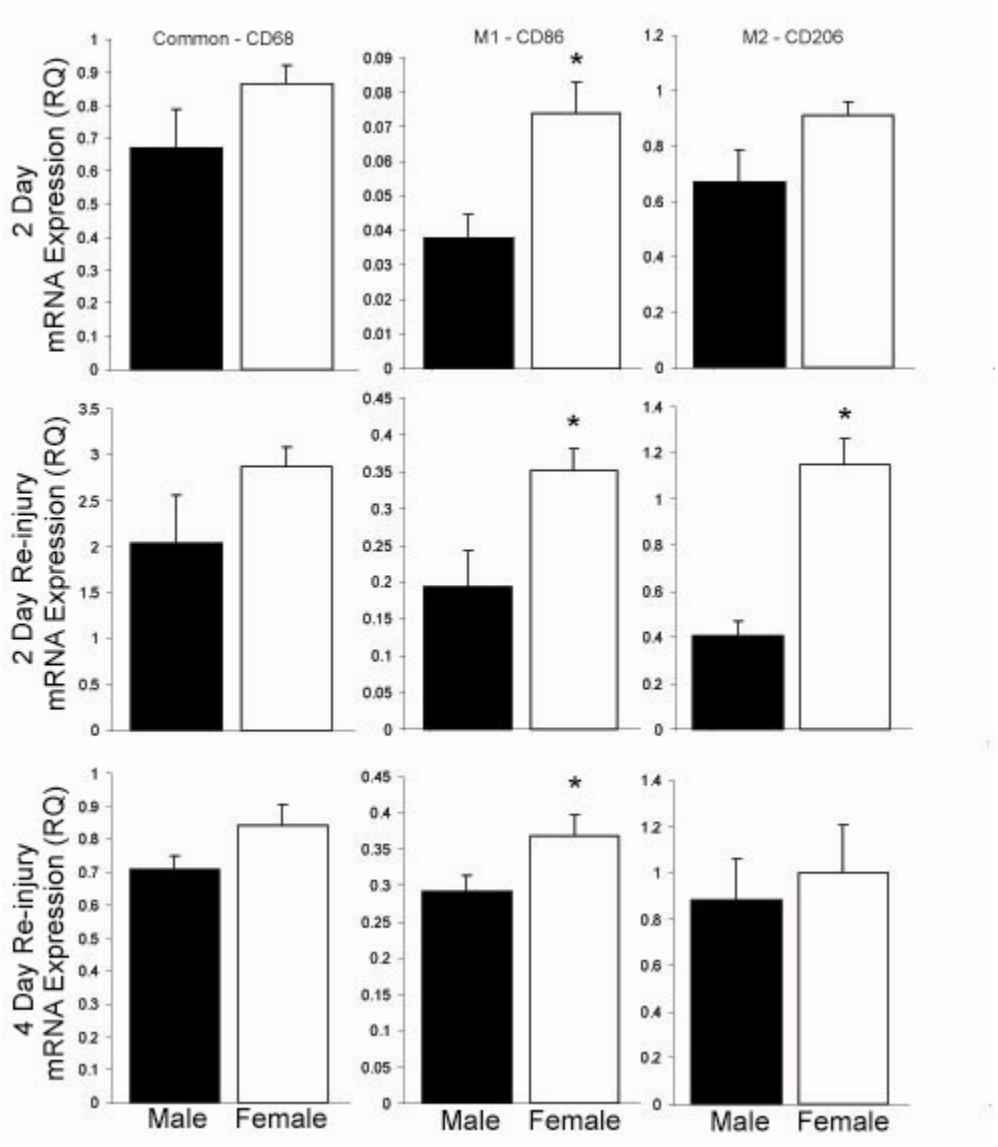

Fig 3.5 Expression of macrophage markers during the corneal inflammatory reparative response. Corneas of male and female mice were taken at 2 days after initial injury and 2 and 4 days after re-injury and RNA isolated. Expression levels of specific macrophage markers was quantified using QPCR and expressed as relative quantities (RQ) to an endogenous mouse β-actin control. (n=4-5, *$p<0.05$)

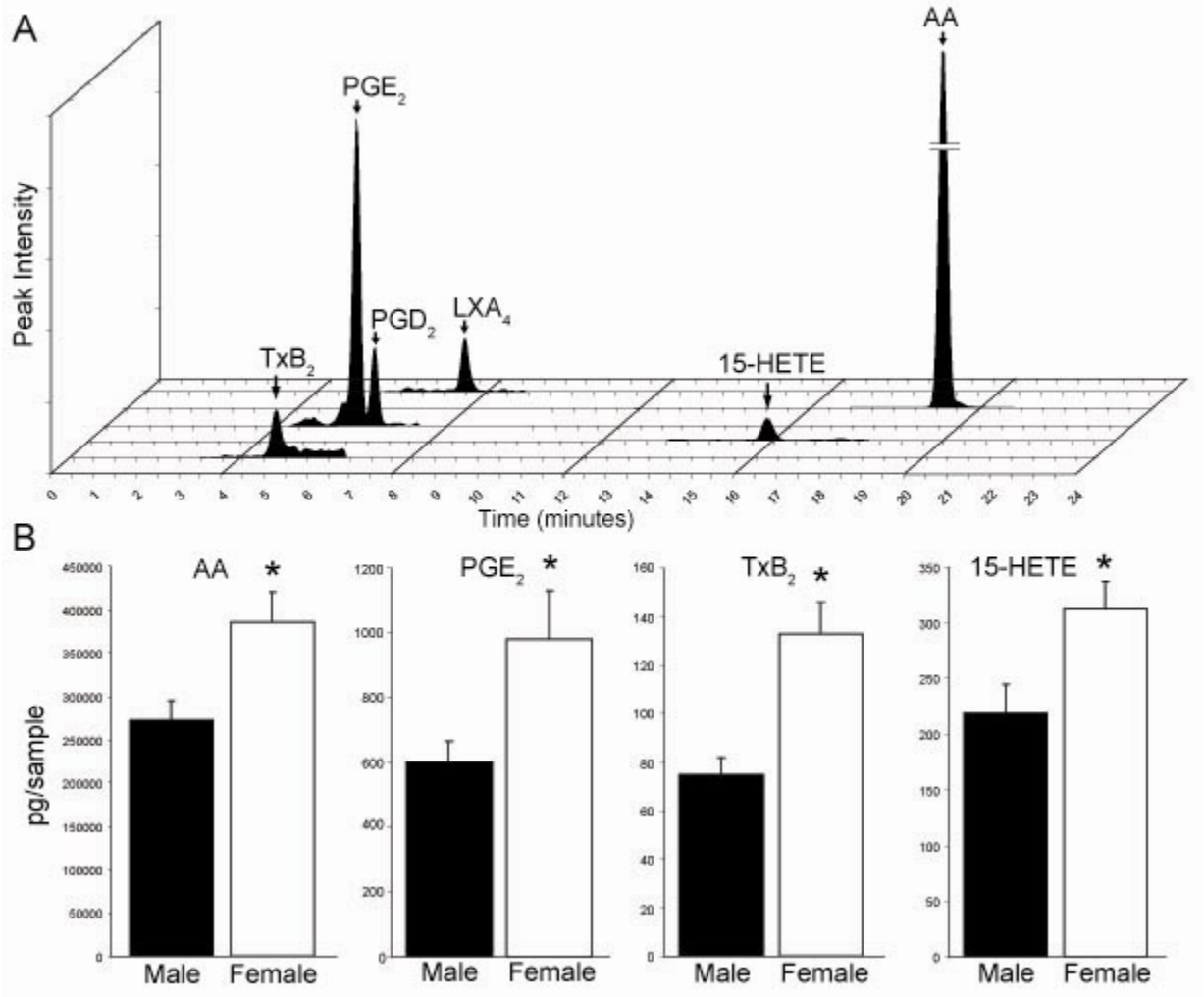

Fig 3.6 Pro-inflammatory lipid mediator production is upregulated in females. Corneas were isolated at 2 days after re-injury and analyzed for select lipid mediators by LC/MS/MS-based lipidomics. **A)** Representative multiple reaction monitoring (MRM) chromatograms of selected ion pairs for AA, 15-HETE, PGE_2, LXA_4, and TxB_2. **B)** Quantification of lipid mediator content (n=4; *$p<0.05$ vs males).

To assess formation of LXA_4 during the macrophage-neutrophil interaction of phagocytosis, an *in vitro* phagocytosis assay was used. Apoptotic neutrophils were introduced to bone marrow derived macrophages and amount of phagocytosed neutrophils was quantitated after a 30 minutes co-incubation. Phagocytosis is a potent agonist for LXA_4 production (Fig 3.7). While stimulation with calcium ionophore, a non-physiological agonist used to assess maximal production, resulted in the formation of 133.1±13.6 pg (M1) and 66.7±10.8pg (M2) of LXA_4, phagocytosis produced significantly greater amounts of LXA_4 (212.1±14.7 pg, M1 and 133.4±14.0 pg, M2), showing that phagocytosis is a selective stimulus for LXA_4 formation (Fig 3.7A). Immunohistochemistry revealed that macrophages containing 15-LOX, the enzyme critical for the formation of LXA_4, phagocytosed apoptotic neutrophils (Fig 3.7B). Furthermore, LXA_4 production in corneas increased by 313%

(males) and 189% (females) at 2 days after re-injury compared to uninjured corneas where minimal phagocytosis is taking place (Fig 3.7C). It is striking that female corneas at two days after re-injury produced significantly less LXA_4 than males (p=0.02), suggesting that phagocytosis is downregulated in female corneas.

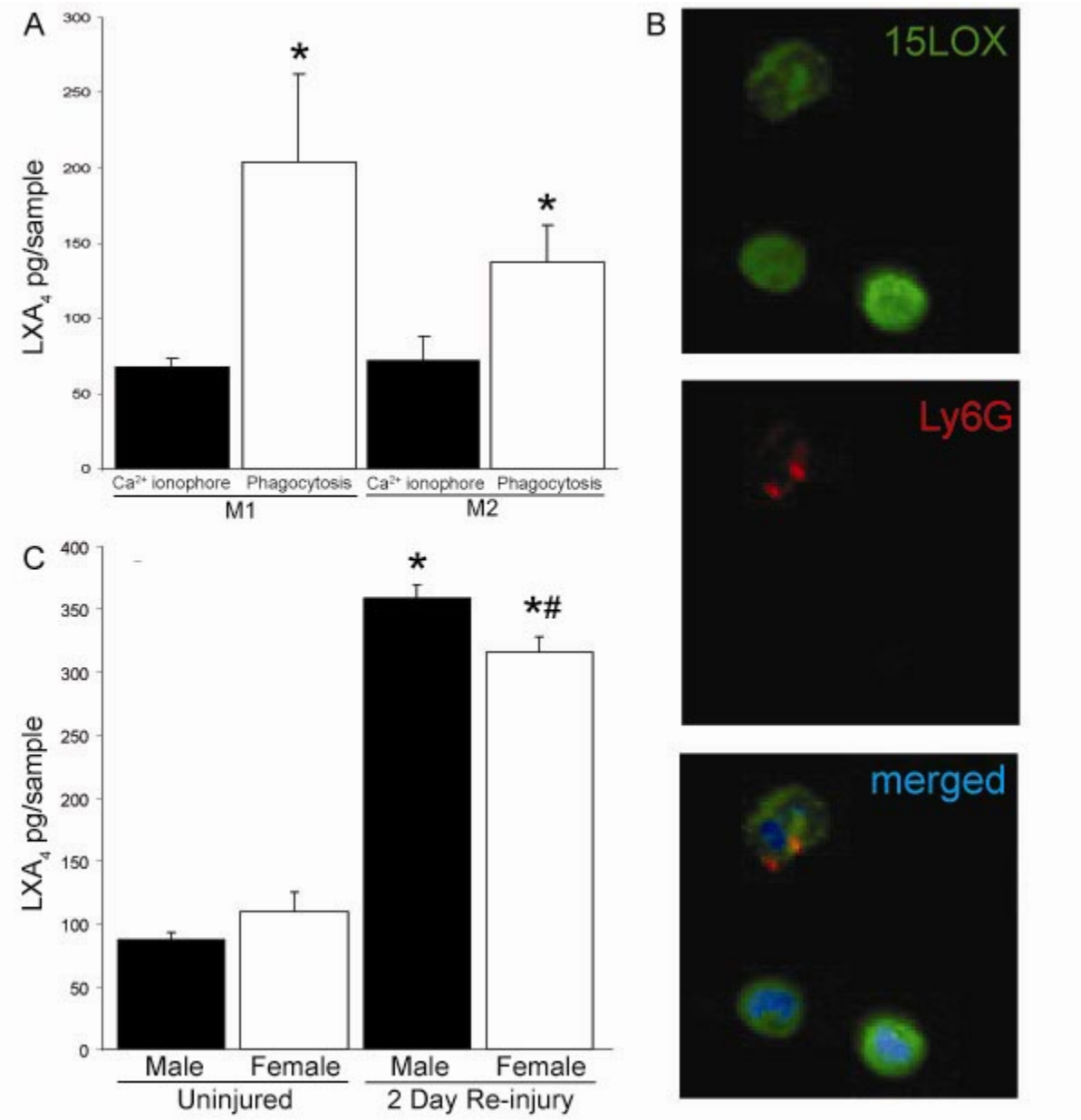

Fig 3.7 Phagocytosis is a potent agonist for LXA_4 formation. **A and B)** Bone marrow derived macrophages were polarized to M1 or M2 phenotypes using LPS or IL-4 and IL-13, respectively. Apoptotic neutrophils were co-incubated with macrophages for 30 minutes. A) Media from phagocytosis and Ca^+ ionophore (2 μM for 30 mins) stimulated macrophages was collected for lipidomic analysis (n=5, *p<0.05 vs Ca^+ ionophore stimulation). **B)** Representative image of 15-LOX^+ macrophage phagocytosis of $Ly6G^+$ neutrophils. **C)** Uninjured and 2 day re-injury corneas were collected for lipidomic analysis (n=5; *p<0.05 vs uninjured, #p<0.05 vs male 2 day re-injury).

To determine estrogen regulation of macrophage phagocytosis, bone marrow derived macrophages were treated with 17β-estradiol prior to exposure to apoptotic neutrophils. Macrophages treated with estrogen did not exhibit any difference in total phagocytic capacity compared to macrophages treated with vehicle (0.8 M1 and 0.7 M2 fold change) (Fig 3.9A). However, estrogen inhibited LXA_4 stimulation of phagocytosis. In a feed forward loop, macrophages pre-exposed to 1 nM of LXA_4 15 minutes prior to introduction of apoptotic neutrophils exhibited a 1.6-fold (M1) and 2.1-fold (M2) increase in phagocytic capacity (Fig 3.8A). Interestingly, 17β-estradiol inhibited this LXA_4 induced upregulation of phagocytosis. Macrophages that were treated with 17β-estadiol before stimulation with LXA_4 failed to exhibit any increase in phagocytic capacity (Fig 3.8A). To determine if estrogen signaling via the two classic estrogen receptors (ER), ERα and ERβ, was responsible for this estrogenic inhibition of LXA_4-induced stimulation of phagocytosis, bone marrow macrophages from ERβ knockout mice were used. Macrophages derived from ERβ knockouts responded equally to LXA_4 treatment irrespective of pre-exposure to 17β-estradiol, indicating that estrogen inhibition of LXA_4 stimulated phagocytosis is ERβ dependent (Fig 3.8B).

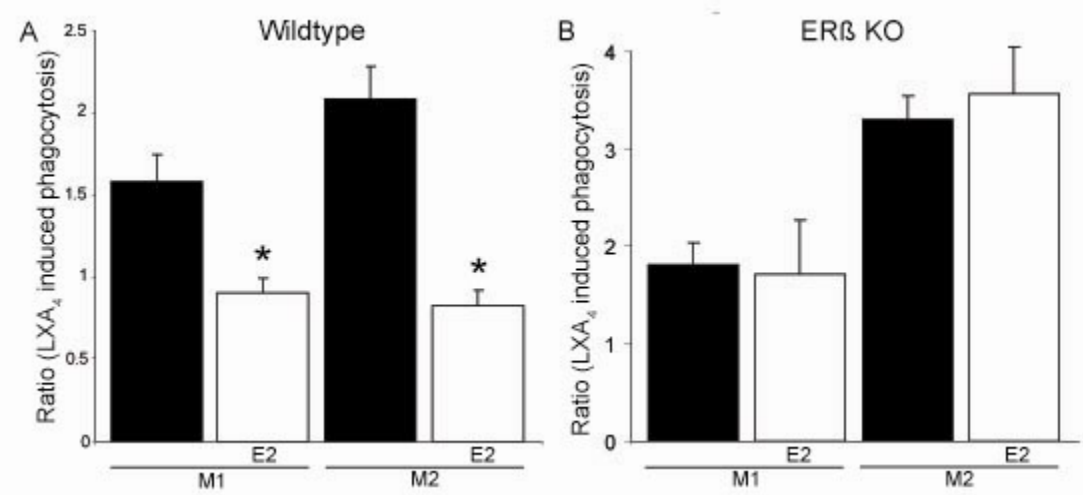

Fig 3.8 Estrogen desensitizes macrophages to LXA_4 stimulation via ERβ. Bone marrow derived macrophages (BMMØ) were differentiated into M1 and M2 subtypes using LPS (100 ng/mL) and IL4 and IL13 (10 ng/mL), respectively. Estrogen treated cells were subsequently given 1 nM 17β-estradiol for 24 hours. LXA_4 stimulated BMMØ were treated with 1 nM LXA_4 15 minutes prior to co-incubation with apoptotic neutrophils. **A)** The ratio of phagocytic capacity following LXA_4 stimulation to phagocytic capacity without LXA_4 stimulation was assessed for both estrogen-treated and untreated macrophages (n=3-4; *$p<0.05$ vs untreated). **B)** Phagocytic capacity of BMMØ derived from ERβ deficient mice was assessed (n=3-6;*$p<0.05$ vs untreated).

Since both macrophages and neutrophils come into contact with estrogen *in vivo* during the course of the inflammatory response in females, neutrophils were also incubated with 17β-estradiol for 24 hours prior to introduction to macrophages. 17β-estradiol delayed neutrophil apoptosis. After 24 hours, 71.4±5.2% of untreated neutrophils were apoptotic while only 52.0±3.3% of 17β-estradiol treated neutrophils had undergone apoptosis (Fig 3.9A). This delay in apoptosis also accounted for a

difference in phagocytic capacity. Macrophages incubated with 17β-estradiol-treated neutrophils phagocytosed 132,170 cells after a 30 minute co-incubation compared with 680,514 cells phagocytosed when co-incubated with untreated neutrophils, resulting in a 5.2-fold (M1) and 2.7-fold decrease (M2) in phagocytic capacity (Fig 3.9B).

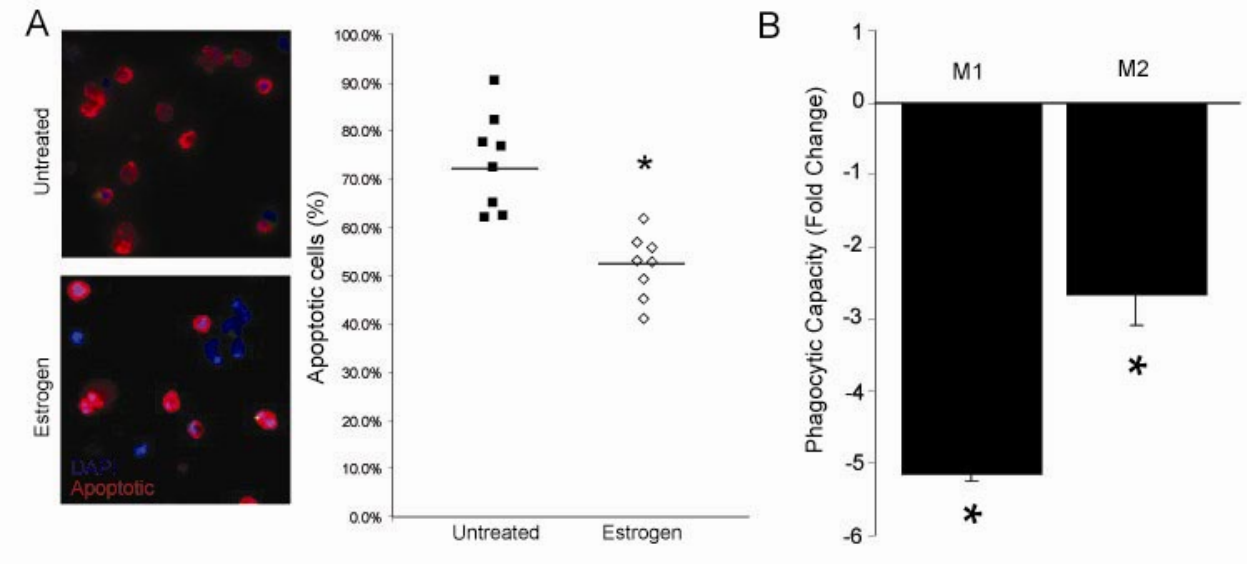

Fig 3.9 Estrogen delays neutrophil apoptosis and clearance by macrophages.
A) Zymosan-induced peritoneal neutrophils were incubated with 1 nM 17β-estradiol for 24 hours in RPMI-1640. An ApopTag Red In Situ Apoptosis Detection Kit (Millipore, Billerica, MA) was used to detect neutrophil apoptosis. Apoptotic cell number was manually counted using ImageJ software and compared to number of total cells. Representative images are shown (n=4, *p<0.05 vs untreated neutrophils). **B)** Estrogen-treated neutrophils were co-incubated with M1 and M2 polarized bone marrow derived macrophages. Phagocytic capacity is shown as fold change of total phagocytosis of estrogen-treated neutrophils vs phagocytosis of untreated neutrophils (n=5, *p<0.05 vs phagocytosis of untreated neutrophils).

The combined effects of estrogen-induced inhibition of LXA_4 stimulated phagocytosis and estrogen-induced delay in neutrophil apoptosis may result in decreased phagocytosis in female corneas and increased neutrophil retention, contributing to a higher neutrophil amplitude in female corneas at 2 days after re-injury. To determine if estrogen and ERβ signaling *in vivo* drives sex-specific differences in corneal neutrophil responses, ERβ knockout males were given topical 17β-estradiol (1 μM/eye *t.i.d.*) for the duration of the wound healing process. Topical treatment of 17β-estradiol to wild-type male eyes elicited a 3.2-fold increase in corneal neutrophil amplitude at 2 days post re-injury (p=0.002 vs saline treated males), recapitulating the female phenotype of increased neutrophil amplitude. This estrogenic response in males was abrogated by deletion of ERβ. 17β-estradiol failed to upregulate neutrophil retention in corneas of ERβ knockout males (58,191 ± 2,6137 cells/cornea, 17β-treated ERβ knockouts vs 40,264 ± 8,202 cells/cornea, saline-treated wild-types). Furthermore, ovariectomized females exhibited a marked decrease in neutrophil numbers

(p=0.004 vs normal females), implicating estrogen signaling in the sex-specific difference in corneal neutrophil amplitude (Fig 3.10).

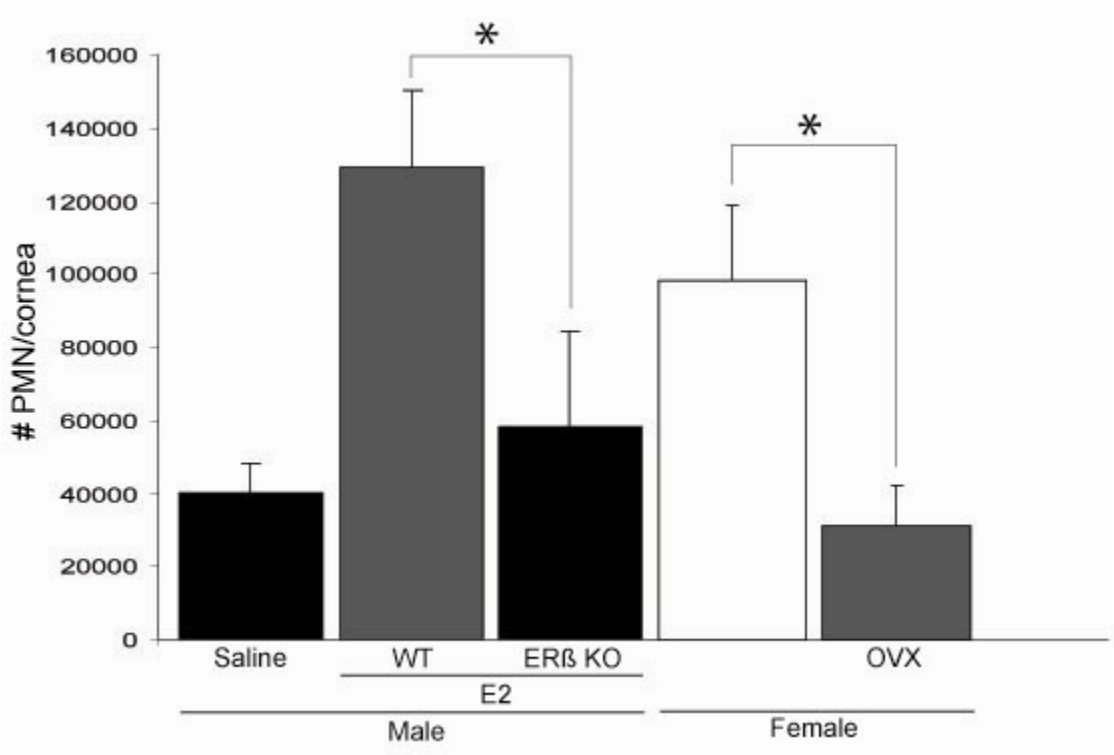

Fig 3.10 Estrogen signaling via ERβ is essential for the female phenotype of enhanced neutrophil amplitude. Ovariectomized females, intact females, ERβ knockout males, and wild-type males all underwent full corneal epithelial abrasion. ERβ knockout and wild-type males were treated topically with 17β-estradiol (1μM/eye, *t.i.d*) for the entire duration of the wound healing process. Saline was used as the vehicle control. Corneas were collected at 2 days after re-injury and processed for myeloperoxidase activity to determine corneal neutrophil content. (n=5; *p<0.05 vs indicated).

Discussion

Estrogen has been implicated in immune regulation in a variety of tissues. In the dermis, estrogen is considered beneficial for wound healing. Post-menopausal women heal slower than their pre-menopausal counterparts, estrogen replacement therapy promotes dermal wound healing, and estrogen upregulates anti-inflammatory cytokines like macrophage inhibitory factor (MIF) in the dermis. However, there exists a body of evidence suggesting that estrogen has the opposite effect in ocular tissues. Estrogen upregulates pro-inflammatory cytokines in murine meibomian and lacrimal glands. Estrogen regulation of cytokines in corneal epithelial cells is more complex with conflicting data between immortalized and primary human corneal epithelial cells. A *post hoc* analysis of forty thousand female health professionals revealed a correlation between estrogen replacement therapy with an increase in reported Dry Eye symptoms. A wide body of work in *Pseudomonas aeruginosa* infection, Dry Eye Syndrome, and herpetic stromal keratitis provides evidence that the corneal inflammatory response is largely Th1 driven [152-154]. c57BL/6J mice have a Th1 polarized innate immune response and were selected for the studies to recapitulate features of clinical ocular surface innate immune responses [155]. We previously reported a female-specific delay in corneal wound healing following epithelial injury in Balb/c mice consistent with an estrogen-induced downregulation of epithelial 15-LOX expression and anti-inflammatory LXA_4 formation. The Balb/c strain exhibits a Th2 polarized innate immune response however, despite fundamental strain specific differences in innate immune responses in both c57BL/6J and Balb/c mice, the female-phenotype of delayed wound healing is consistent between strains.

In our study, the corneas of female mice exhibited significantly higher levels of neutrophils at 2 days after re-injury compared to their male counterparts. Interestingly, we previously reported the opposite trend in Balb/c mice. Balb/c males had 222% more neutrophils than females and 435% more neutrophils than c57BL/6J males, illustrating a strain specific difference in the neutrophil immune response that is most likely driven by the Th1 vs Th2 responses in c57BL/6J vs Balb/c mice, respectively. However, given the large body of evidence indicating a primarily Th1 driven inflammatory response in ocular tissues, the c57BL/6J innate immune response more closely resembles clinical ocular surface innate immune responses compared to Balb/c.

Correlating with delayed wound healing, there was also an increased inflammatory tone in female corneas. AA levels were higher in females, indicating greater phospholipase A_2 activity and higher inflammation. The pro-inflammatory lipid mediators PGE_2, 15-HETE, and TxB_2 were upregulated and the anti-inflammatory LXA_4 downregulated in female corneas at two days after re-injury, implicating a pro-inflammatory role for estrogen in the corneal inflammatory reparative response, which is in sharp contrast to the reported primarily anti-inflammatory nature of estrogen in other tissues. However, it is clear that the estrogen effect is cell- and tissue- specific. Females are protected against cardiovascular disease, which is attributed to the anti-inflammatory role of 17β-estradiol on the vasculature. Recent studies by Nadkarni *et al* indicate that 17β-estradiol mediates its vasculoprotective actions by mobilizing the anti-inflammatory ALX ligand, annexin A1, in human neutrophils, contributing to inhibited neutrophil endothelial adhesion [156]. That 17β-estradiol

induces a pro-inflammatory tone in corneas is striking and can be attributed to many factors. Although ERα and ERβ share overall high homology in binding domains, they exhibit overlapping but distinct ligand-binding properties, differing tissue distribution, and distinct bioactions. The protective effects of 17β-estradiol in the central nervous system is dependent on ERα, not ERβ [157]. In the cornea, ERβ is the primary estrogen receptor involved in the inflammatory reparative response: ERβ, but not ERα, is markedly upregulated upon corneal epithelial injury [158]. Furthermore, recent evidence has emerged for a pro-inflammatory role of 17β-estradiol in microglia, involving inhibition of 5-androsten-3β,17β-diol (ADIOL) binding to ERβ and antagonizing ADIOL-induced ERβ-mediated repression of inflammation [159]. It is thus possible that 17β-estradiol-induced upregulation of corneal inflammatory tone is not due to enhanced upregulation of pro-inflammatory genes by 17β-estradiol bound ERs but rather a result of decreased ADIOL-stimulated inflammatory suppression.

The upregulation of pro-inflammatory signals, with a lack of increased anti-inflammatory signals to act as a counterbalance, can lead to a temporary imbalance between neutrophil clearance and recruitment. Lipid mediators of inflammation also have a demonstrated association with macrophage polarization. LXA_4 and its DHA-derived analogue, RvD1, shift macrophages to a M2-like phenotype [147, 148]. Consistent with a greater corneal inflammatory tone, females expressed a higher proportion of pro-inflammatory M1-like macrophages as evidenced by upregulated CD86 expression and decreased arginase activity. Both M1 and M2-like macrophages phagocytose neutrophils but M1s have a greater phagocytic capacity. It was therefore interesting that female corneas exhibited greater neutrophil content at two days after re-injury despite the presence of more M1-like macrophages. The increased neutrophil to macrophage ratio in females suggests an imbalance between neutrophil recruitment and clearance.

We show within this study that phagocytosis is a major stimulus for LXA_4 formation. Previous pathways for LXA_4 biosynthesis include the sequential transcellular action of 15-LOX containing macrophages and 5-LOX containing neutrophils. However, this is the first evidence showing that phagocytosis is a potent stimulus for LXA_4 production. LXA_4 had previously been demonstrated to have a stimulatory effect on macrophage phagocytosis of apoptotic neutrophils [50]. This result reveals a feedforward mechanism where LXA_4 stimulates macrophage phagocytic capacity and subsequent phagocytosis results in the upregulation of LXA_4 formation, promoting inflammatory resolution and clearance. Estrogen disrupts this loop by inhibiting LXA_4-induced upregulation of macrophage phagocytosis. Macrophages pre-exposed to 17β-estradiol were unable to respond to LXA_4 stimulation of phagocytosis, which results in lowered phagocytosis and downregulation of LXA_4 formation compared to un-exposed macrophages. Thus, in females, where 17β-estradiol is present in the circulatory and ocular tissues, downregulation of the LXA_4-phagocytic loop can result in increased corneal neutrophil retention, leading to a skewed ratio of recruitment and clearance. Uninjured corneas with little to no phagocytosis produced markedly less LXA_4 than injured corneas rich with leukocytes, suggesting that phagocytosis is a potent stimulus of LXA_4 production during the corneal inflammatory reparative response. Decreased LXA_4 formation in female corneas at 2

days after re-injury was coupled to downregulation of phagocytosis in females, contributing to increased neutrophil retention.

Estrogen therefore promotes neutrophil retention by downregulating phagocytosis in two manners: one, by acting on neutrophils to delay apoptosis and two, by rendering macrophages insensitive to the LXA_4-induced stimulation of phagocytosis in an ERβ-dependent manner. The importance of estrogen signaling in the enhanced neutrophil signature in female corneas at two days after re-injury is illustrated by the observation that topical 17β-estradiol treatment in males recapitulates the female phenotype of increased neutrophil content. Removal of estrogen in ovariectomized females resulted in a shift in the balance between neutrophil recruitment and clearance, leading to similar neutrophil levels in ovariectomized females as in saline-treated males.

In summary, females exhibit a greater inflammatory response to recurrent injury with an ERβ-dependent upregulation of neutrophil retention. An increased pro-inflammatory tone concurrent with a higher proportion of M1-like macrophages and increased neutrophil amplitude may prime female eyes, leading to greater susceptibility to dysregulated and exacerbated inflammation that could shift or trigger chronic disease upon disruption to the ocular surface. Also of interest is estrogen regulation of the neutrophil and macrophage functional responses since neutrophils and macrophages are critical effector cells in innate immune responses in the ocular surface. Given the high prevalence of female-linked incidence rates of autoimmune/inflammatory diseases, estrogen-induced macrophage insensitivity to anti-inflammatory mediators like LXA_4 may have key ramifications for therapeutic strategies for modulating inflammatory/immune diseases in women [160].

Chapter 4:

Conclusion

Estrogen has a diverse and complex immunomodulatory role in many settings. The ocular surface is one tissue where estrogen appears to promote pro-inflammatory signals. *In vitro* estrogen treatment of immortalized human corneal epithelial cells resulted in the upregulation of pro-inflammatory cytokines including IL-1β. IL-6, and IL-8 [91]. Similarly, estrogen upregulates pro-inflammatory cytokines in mouse meibomian glands [102]. In post-menopausal women, estrogen replacement therapy has been linked to increased dry eye symptoms; however, not much else is known about estrogen's role in the corneal inflammatory reparative response [89, 90]. Estrogen has a well established beneficial role in dermal wound healing, but not in the cornea. Not only is estrogen pro-inflammatory in the cornea, but neutrophil infiltration is also correlated with increased wound healing (i.e. beneficial), which is different from the dermis where neutrophil accumulation is associated with collateral tissue damage. This beneficial role in wound healing, however, is dependent on the state of neutrophil activation. Increased neutrophil recruitment was correlated with delayed wound healing in a model of corneal epithelial injury with LPS [43]. The hypothesis explored in this project was: is there a sex-specific difference in the corneal inflammatory reparative response and, if so, does estrogen regulate the intrinsic protective lipid mediator circuit 15-LOX/LXA_4 present on epithelial cells, macrophages, and neutrophils?

Sex-specific differences were established for the first time in corneal wound healing following full corneal epithelial abrasion. Females healed markedly slower than age-matched male counterparts at both two days following initial injury and two days after re-injury, demonstrating a female-specific delay in wound healing. This evidence as well as the presence of estrogen in the tear film and the two classic estrogen receptors (ERα and ERβ) in ocular tissues suggested that estrogen signaling via one or both ERs may downregulate corneal epithelial wound healing. Indeed, topical treatment with 17β-estradiol of male eyes throughout the entire course of the inflammatory reparative response recapitulated the female phenotype of slower wound healing. This is at odds with previous reports in dermal wound healing where evidence points to a protective role for estrogen. However, the results are in agreement with other studies suggesting that estrogen has a largely pro-inflammatory role in the cornea, demonstrating that estrogen has distinct tissue and cell specific bioactions. That estrogen amplifies inflammation in the cornea is concerning as any shift in the corneal inflammatory response toward a more pro-inflammatory, chronic inflammatory response can affect the delicate visual axis and compromise vision.

The protective anti-inflammatory 15-LOX/LXA_4 lipid mediator circuit was previously demonstrated to promote corneal epithelial wound healing and represents a novel component of the unique corneal Anterior Chamber-Associated Immune Deviation (ACAID) [52]. The results presented herein were the first evidence demonstrating estrogen regulation of 15-LOX/LXA_4. Estrogen downregulates corneal expression of 15-LOX, correlating with decreased anti-inflammatory and pro-resolving LXA_4 formation. Immunohistochemistry and lipidomic analysis revealed that corneal epithelial cells express high levels of 15-LOX, a key and critical enzyme for AA-derived LXA_4 production. I showed, using *in vitro* wound healing assays with immortalized human corneal epithelial cells, that estrogen directly downregulates the 15-LOX/LXA_4 pathway in corneal epithelial cells, delaying wound healing. Furthermore, the estrogen effect of delayed wound healing can be

rescued by addition of LXA_4, a key mediator of inflammatory resolution that helps establish an anti-inflammatory tone in the cornea.

Further analysis of estrogen signaling demonstrated a specific role for ERβ in the corneal inflammatory reparative response. QPCR analysis revealed that ERβ, but not ERα, is markedly upregulated following corneal insult, highlighting ERβ as a receptor of interest in the corneal inflammatory reparative response. The distinct bioactions of the ERs was corroborated by use of established and selective agonists for ERα and ERβ. Activation of ERβ but not ERα significantly downregulated corneal epithelial cell 15-LOX expression *in vivo* and epithelial wound healing *in vivo* and *in vitro*. Since the discovery of the second estrogen receptor, ERβ, in 1996, separate, nonredundant and occasionally even opposing bioactions for the two ERs have been reported in the immune, skeletal, cardiovascular, and central nervous systems [61]. ERβ has gained attention for its role in promoting growth arrest in animal models of cancer where ERα promotes tumor growth while the immune regulatory roles of the two ERs are dependent on their expression ratio within the tissue and disease models. These results demonstrating an ERβ-specific injury response in the cornea highlights ERβ as a potential therapeutic target in ocular inflammation.

Estrogen also regulates corneal inflammatory tone and leukocyte function. Following epithelial injury, the cornea exhibits sex-specific differences in amplitude of leukocyte response. At two days after re-injury, which elicits a more robust inflammatory response than initial injury, females exhibited a higher neutrophil to macrophage ratio with increased pro-inflammatory M1-like macrophages. This correlated with an enhanced pro-inflammatory tone in females, illustrating again the female-specific upregulation of pro-inflammatory signals in the corneal inflammatory response. The enhanced pro-inflammatory tone in female corneas may contribute to an imbalance between neutrophil recruitment and clearance, leading to a higher neutrophil profile at two days after re-injury. This further illustrates that females have a more amplified inflammatory response than males to the same stimulus. Furthermore, estrogen signaling via ERβ was found to regulate 15-LOX expression and LXA_4 formation in corneal epithelial cells.

Phagocytosis was shown for the first time to be a potent stimulus for LXA_4 production. LXA_4 upregulates macrophage phagocytosis of apoptotic neutrophils [50]. This new evidence established a feedforward loop for LXA_4 bioaction during phagocytosis: LXA_4 upregulates macrophage phagocytosis of apoptotic neutrophils and the macrophage-neutrophil interaction during phagocytosis elicites an upregulation of LXA_4 production. This feedforward loop results in increased production of anti-inflammatory signals and greater inflammatory resolution. In female corneas, however, estrogen inhibits this loop. Macrophages pre-exposed to 17β-estradiol were rendered insensitive to LXA_4 stimulation of phagocytic capacity. The inability to respond to LXA_4-induced upregulation of phagocytosis in addition to an estrogenic delay in neutrophil apoptosis may contribute to a greater ratio of neutrophil recruitment to clearance, causing a temporary amplified neutrophil profile in female corneas. Estrogen regulation of leukocyte function is again ERβ-dependent, demonstrating the importance of ERβ in the cornea.

This enhanced pro-inflammatory response does not result in a failure of resolution in a tightly controlled wound healing response. The abrasion injury elicits a mild rapidly resolving response in contrast to clinical ocular surface inflammation which requires intervention. Taken together this evidence suggests that females are primed for a greater response to clinical ocular surface inflammation. The female response to the same inflammatory stimulus results in a greater pro-inflammatory tone than in males with enhanced corneal neutrophil retention and downregulation of the protective 15-LOX/LXA_4 circuit in corneal epithelial cells as well as leukocytes. This implies that females require a smaller shift to move from an acute inflammatory reparative response to chronic disease causing inflammation that may be detrimental to maintaining corneal transparency necessary for good vision. The lifelong consequence to female ocular health can be significant. A lifetime of acute inflammatory responses can lead to a lowered threshold for eliciting an adaptive immune response due an estrogen-mediated accumulation of inflammatory signals in the cornea and/or downregulation of the 15-LOX/LXA_4 circuit and, as a consequence, ACAID. Females have an enhanced T-cell response with greater antibody production [55]. Neutrophils can activate T-cells and the adaptive immune response [162]. The accumulation of these factors in females, and not in males, over time may tilt the ocular response to an inflammatory stimulus toward activation of the adaptive immune response, partly explaining the high female-dominated prevalence of autoimmune/inflammatory-based diseases such as Dry Eye.

These results reported in this dissertation pinpoint some therapeutic targets for corneal inflammation. ERβ was demonstrated to be a key and essential receptor in the corneal inflammatory reparative response. 17β-estradiol signaling via ERβ is essential for downregulating 15-LOX in corneal epithelial cells and inhibiting LXA_4-stimulation of phagocytosis in macrophages. ERβ-deficient males were protected from the delay in wound healing and enhanced neutrophil amplitude induced by 17β-estradiol treatment in wild-type males. Targeting ERβ in females during a corneal inflammatory response may be protective and promote inflammatory resolution. Alternatively, addition of LXA_4 can counter-regulate estrogen downregulation of the 15-LOX/LXA_4 pathway and promote wound healing while counter-balancing the estrogen-induced pro-inflammatory corneal tone.

In summary, sex-specific differences in the corneal reparative response were established. These differences were driven by estrogen regulation of the 15-LOX/LXA_4 circuit in corneal epithelial cells and leukocyte functional responses. These results provide evidence for a lowered threshold in females to developing ocular surface inflammation. Given the strong female dominated incidence of autoimmune/inflammatory diseases, an understanding of this female-specific threshold shift is vital for developing therapies against sight-threatening inflammatory diseases in the female cornea.

Chapter 5:

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