Title
Sentinels of Homeostasis: Lipid Mediators in Immune Regulation and Neuroprotection

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Publication Date
2019

Peer reviewed|Thesis/dissertation

Sentinels of Homeostasis:
Lipid Mediators in Immune Regulation and Neuroprotection

By

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A dissertation submitted in partial satisfaction of the

requirements for the degree of

Doctor of Philosophy

in

Vision Science

in the

Graduate Division

of the

University of California, Berkeley

Committee in charge:

Professor Karsten Gronert, Chair
Professor Lu Chen
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Spring 2019

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Abstract

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Karsten Gronert, Chair

Lipid mediators are fatty acid-derived small signaling molecules that are critical in vascular responses and leukocyte trafficking during inflammation. Eicosanoids prostaglandin E_2 (PGE_2) induces pain and fever responses while leukotriene B_4 (LTB_4) recruits neutrophils to initiate inflammation, the recruited neutrophils subsequently produce lipoxin A_4 (LXA_4) to limit the magnitude and duration of acute inflammation. Eicosanoid regulation of innate immune cells is well established, but eicosanoid regulation of adaptive immunity is less understood. Moreover, the bioactions of eicosanoids are often context-dependent. Therefore to fully understand how lipid mediators modulate immune responses and homeostasis, it is vital to assess their immunological mechanisms in cell type- and disease-specific settings.

My dissertation aims to address whether and how LXA_4 mediates adaptive immune responses in posterior autoimmune uveitis. In the first Chapter I will introduce arachidonic acid-derived eicosanoids PGE_2, LTB_4, LXA_4 and lipoxin B_4 (LXB_4) and discuss their multifaceted bioactions in various inflammatory settings, with the focus of their regulation of lymphocytes. In Chapter Two I will provide an overview on the therapeutic effects of special proresolving mediators (SPMs) and LXA_4 in ocular diseases and briefly highlight two collaborations that revealed the roles of PGE_2 and LTB_4 in enhancing inflammatory neutrophil function in peritonitis, and the mechanism in which PGE_2 contributes to pruritic responses in atopic dermatitis.

Chapter Three will present my thesis aims in identifying whether LXA_4 plays a role during autoimmune uveitis pathogenesis and dissecting the mechanism in which LXA_4 modulates disease. Data from my thesis project showed *in vivo* generation of LXA_4 was significantly downregulated in the draining lymph nodes at peak uveitis. Mice lacking 5-lipoxygenase (5-LOX), the rate-limiting enzyme for LXA_4 production, exhibited aggravated disease. Ccr7 expression was downregulated in $CD4^+$ T cells of LXA_4 deficient mice while glycolytic responses of these T cells were enhanced, resulting in elevated IFN-γ production and migration of effector $CD4^+$ T cells to the eye. Conversely, LXA_4 treatment of immunized mice ameliorated disease. The findings underscore the importance of the LXA_4 circuit in guiding adaptive T cell function in autoimmunity, and uncover a novel mechanism of LXA_4 regulation in T cell effector function and trafficking from peripheral lymph nodes to the site of inflammation.

Chapter Four demonstrates a novel neuroprotective role of astrocyte-secreted lipoxins A_4 and B_4 in retinal neurodegeneration. Lipoxins' anti-inflammatory functions are well established, but they have not been shown to impact neuronal survival. In this project, we determined that LXA_4 and LXB_4 are synthesized in the inner retina in health, but their endogenous formation becomes reduced following injury. Lipoxin treatment provided neuroprotection following acute injury, while inhibition of the LXA_4 pathway exacerbated injury-induced neuronal death. These results identified lipoxins as important neuroprotective factors that maintain neuronal homeostasis and inflammation in the central nervous system (CNS).

In the last chapter, I will conclude with the main findings from my projects and suggestions for future studies on lipid mediators. In this dissertation, we demonstrate the indispensable roles of lipoxins in maintaining homeostasis and mediating inflammatory responses, and their abilities in fine-tuning cellular responses to control the duration and amplitude of inflammation.

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Acknowledgements

It is only appropriate to begin by thanking Karsten for giving me the opportunity and freedom to pursue the projects I wanted. I came to Berkeley with the goals of studying T cells and glaucoma, and I accomplished both because of your open mindedness and support despite not actually being funded on either topic. The open communication style was invaluable for maneuvering through some of the grad school malarkey. I leave the Gronert lab with many stories to tell for years to come.

Many thanks to my thesis committee members: Russell, for challenging my brain power and the exceptionally fast email responses. Lu and Nancy, for the trainings during my rotations and developing my understanding of the eye. Thank you Michael, for lending an ear during some decision-making times and help with letters of recommendation. My graduate school experience was enriched through collaborations with Izzy from the Flanagan/ Sivak lab, Jacques from the Barton lab, and Josh from the Julius lab. Thank you for expanding my scientific horizons and involving me in your stories, I truly enjoyed the teamwork. The dedication and hard work from the undergraduate students Victoria, Allison, Kimberly, Nicole and many others made living in the lab more manageable.

This dissertation could not have been completed without the enormous amount of help I received in the Caspi lab. Thank you Rachel, for adopting me into your lab and providing guidance and scientific rigor to ensure the success of the project. Reiko, a big thank you for putting up with my incessant questions and giving me advice for experiments and beyond. Mary, I'm grateful for your emotional and scientific support at all hours of the day. Ryan, it was great to have you normalize my experiences and help with experiments, I will always remember that special morning during mouse sac. Ed, thank you for all the weekend funduscopies and help with the nonstop immunizations. I must also thank all other members of the Caspi lab and the greater immunology community at the NIH, I developed into a better scientist from your input and discussions.

My Bay Area compadres were instrumental in the preservation of my sanity. Robi, you have been there for me at my worst and my best, I am immensely fortunate to have you as my sounding board. Somehow you can make me laugh when it didn't seem possible. Leo, thank you for being the most loyal friend, you always come through and selflessly give to others in your craziest way. Cécile, you are the most thoughtful and helpful person that I strive to be one day, I'm sorry (but not sorry?) that my swearing habits rubbed off on you. Kelly, I aspire to your unrelenting positivity and support for everyone around you. Our quals cram sessions and trips were among my best experiences during the last six years. Diana, truly grateful for your immeasurable kindness and generosity as I transformed into a workaholic.

The kindred spirits from Seattle had been my anchor. David, I look forward to every annual rendezvous because you are such a breath of fresh air, let's stay on the

same wavelength infinitely. Alice, you were my pillars during the transition back to school, thank you for believing in me before I did myself. Scott, the one constant in my life that responds to my random texts at weird hours of the day providing mental and comic relief. Caroline, how great that we went back to school the same year, the PhD commiseration, life rumination mixed with encouragements from across the ocean were precious. We made it! To the best mentors Nadeem and James, thank you for recognizing my potential, I would never have embarked on this journey if it wasn't for your encouragement and support.

My dissertation writing companion Pepper has been clutch in fueling extra motivation and discipline, simply by perching next to my computer and watching me type, or parking in my lap to ensure I stay in the chair focused during what felt like endless thesaurus searches for synonyms of "regulate" and "define". To my mom, for being supportive the best way you know how. The reminders of focusing on the big picture and my health were great pressure relief. Zacc, your unwavering patience and empathy, going the extra mile to alleviate my dissertitis and laughing off the crank were more than I could ever ask for.

More gratitude to people I crossed paths with during the last six years for the laughter, hugs, conversations, coffee, adventures, texts, noms, cat memes and videos, serotonin, perspectives and understanding.

Chapter One: Lipid Mediators in Inflammation

1.1. Intrinsic Lipid Mediator Signaling in the Immune System

The immune system comprises an intricate network of continuous signaling and communication where cells regulate one another in a complementary and interlocking manner for stratified defense and response. Acute inflammation elicits a tissue-specific immune program through the activation of tissue-resident cells and innate immune cells, which secrete signaling molecules to enlist and attune adaptive immune cells equipped with an enhanced and specific response to previously encountered antigens. Lipoxygenase- and cyclooxygenase-derived lipid mediators (LMs), eicosanoids and special proresolving mediators (SPMs), are early-response signaling molecules generated as an essential response to inflammatory triggers and regulators of leukocyte-mediated inflammation. Eicosanoids and SPMs are tissue-specific autocrine and paracrine signals that regulate the activation, amplitude, and resolution of acute inflammation (Bennett and Gilroy, 2016; Serhan and Levy, 2018). The production of eicosanoids and SPMs is temporally defined, and in acute inflammation they often have opposing and counter-regulatory actions. Every human cell type and tissue expresses one or more cyclooxygenase and lipoxygenase enzymes and G protein-coupled receptors for prostaglandins, leukotrienes, and/or SPMs. A large body of work has established the integral roles of these LMs in innate immune cell function that drives inflammation and its resolution (Alvarez et al., 2010; Dennis and Norris, 2015; Serhan and Levy, 2018). However, current understanding of the direct actions of eicosanoids and SPMs on lymphocytes remains limited. In this chapter, I present an overview of direct regulation of T cells by SPMs and eicosanoids (Fig. 1) (Wei and Gronert, 2019) to preface my thesis project that investigated whether LXA_4 regulates T cell responses in an autoimmune setting.

1.2. Lipoxin A_4 and B_4 Synthesis, Bioaction, and Lymphocyte Regulation

Lipoxins A_4 and B_4 were first identified in 1984 and defined as immune modulatory molecules produced to resolve inflammation, followed in the 2000s by the discovery of a large superfamily of DHA-, EPA-, and DPA-derived SPMs. *In vivo* generation of lipoxins requires the double oxygenation of arachidonic acid by 5-lipoxygenase (5-LOX) and 12/15-lipoxygenase (12/15-LOX) through transcellular biosynthesis, where two cells carrying the lipoxygenase enzymes have to interact in coordination to metabolize arachidonic acid to the final lipoxin product. 5-LOX is widely expressed by immune cells including neutrophils, macrophages, mast cells, monocytes, eosinophils and dendritic cells, while 12/15-LOX is expressed by macrophages, neutrophils, fibroblasts, and epithelial cells at high levels. The LXA_4 cognate receptor formyl peptide receptor 2 (ALX/FPR2) is widely expressed on neutrophils, eosinophils, macrophages, T cells, fibroblasts, epithelial cells among many tissues. Hallmark bioactions of lipoxins (that also define some SPMs) are the abilities to inhibit vascular neutrophil migration, enhance macrophage efferocytosis, and downregulate proinflammatory cytokines, chemokines, and cell adhesion molecules, all of which

reduce the amplitude of inflammation and drive active resolution (Godson et al., 2000). Initially, LXA_4 bioactions on lymphocyte function in inflammatory disease models were considered secondary, since ALX/FPR2 was originally discovered in myeloid cells (Fiore et al., 1994) as the first non-prostanoid eicosanoid receptor. In addition, SPMs including LXA_4 demonstrated potent bioaction in controlling innate immune cell function and antigen presenting cell (APC) activation. This paradigm shifted with the discovery of ALX/FPR2 on T cells and the ability of LXA_4 stable mimetics to inhibit TNF-α secretion in activated human T cells (Ariel et al., 2003). SPM receptors have recently been discovered in conjunction with SPM proresolving functions, and a second LXA_4 receptor, GPR32, was identified in humans (Dalli and Serhan, 2018).

Recent reports have uncovered the important roles of endogenous and tissue-resident LXA_4 circuits in T cell function. The immune regulatory function of LXA_4 on T cells was investigated *in vivo* in an immune-driven dry eye model where neutrophil-derived LXA_4 was a critical resident signal in controlling pathogenic T helper cell type 1 (Th1) and T helper cell type 17 (Th17) effector cells and increased the number of T regulatory cells (Tregs) in the eye draining lymph nodes (Gao et al., 2015; Gao et al., 2018). More importantly, sex-specific regulation of the LXA_4 circuit in resident lymph nodes was identified as a key factor that drives female-specific immune-driven dry eye disease. The amplified adaptive immune response in females to routine ocular surface stress was rescued by treatment with LXA_4. An *in vitro* study showed that LXA_4 promotes the differentiation of naïve T cells into T follicular cells, which in turn induces B cells to form germinal centers (Nagaya et al., 2017), demonstrating that LXA_4 mediates cellular signaling among lymphocytes. Direct LXA_4 regulation of B cells has also been established. *In vitro* LXA_4 treatment reduces IgG and IgM production from B cells and decreases memory B cell proliferation by an ALX/FPR2 receptor-dependent mechanism (Ramon et al., 2014) and *in vivo* LXA_4 treatment protects against LPS-induced sepsis by promoting the generation and migration of splenic B cells (Cheng et al., 2016). ALX/FPR2 receptor expression has been identified in human natural killer (NK) cells (Kim et al., 2009) and LXA_4 can induce protective functions of NK cells during airway inflammation. NK cells from asthma patients treated with LXA_4 *ex vivo* maintain functional killing responses (Duvall et al., 2017), alleviate airway inflammation by increasing NK cell-mediated eosinophil apoptosis, and reduce interleukin-13 (IL-13) release by group 2 innate lymphoid cells (ILC2s) (Barnig et al., 2013).

Though biosynthesized through the same biochemical pathway and enzymes as LXA_4, LXB_4 is a largely neglected lipoxin due to the lack of an identified receptor. LXB_4 demonstrates more potent bioactions in resolving allergic inflammation (Karra et al., 2015) and promoting neuronal cell survival (Livne-Bar et al., 2017) than LXA_4. However the only direct bioaction of LXB_4 on lymphocytes shown to date is LXB_4 enhancement of human memory B cell activation and antibody production *in vitro* (Kim et al., 2018).

Multiple studies have demonstrated that lipoxins are not only formed during inflammation and the resolution phase of inflammation, but are also part of normal signaling in healthy tissues and actively regulate homeostasis and the threshold for activation of immune responses in the cornea, lymph nodes, lacrimal glands, and retina

(Gao et al., 2015; Gao et al., 2018; Livne-Bar et al., 2017; Wei and Gronert, 2017). Regulation and therapeutic amplification of this homeostatic SPM circuit in health and disease is the focus of several NIH-funded projects.

1.3. EPA- and DHA- Derived SPM Regulation of Lymphoid-Derived Cells

Although SPMs are not within the research scope of this dissertation, their bioactions are worth noting due to their functional similarity to lipoxins. The field of SPMs emerged from the discovery of distinct EPA- and DHA-derived mediators that shared some of the basic pro-resolving and protective actions of lipoxins and displayed potent bioactions in several inflammatory disease models. Distinct SPM receptors that were originally identified in innate leukocytes are also expressed in lymphocytes (Dalli and Serhan, 2018; Serhan, 2014): ALX/FPR2 for LXA_4 and resolvin D1 (RvD1); G protein-coupled receptor 32 (GPR32) for LXA_4 and RvD1; G protein-coupled receptor 18 (GPR18) for RvD2; chemokine-like receptor 1 (ChemR23) for resolvin E1 (RvE1) (Yoshimura and Oppenheim, 2011).

Identification of SPM receptors on lymphocytes spurred efforts to investigate direct lymphocyte regulation by SPMs. *In vitro*, RvD1 and RvE1 downregulate Th1 and Th17 differentiation, cytokine production and expression of T cell lineage transcription factors T-bet, GATA3 and RORc, as well as promote *de novo* induced Treg (iTreg) generation (Chiurchiu et al., 2016). Several reports have also demonstrated *in vivo* lymphocyte regulation by RvD1 in inflammation and infection models. RvD1 treatment in LPS-induced uveitis reduces infiltration of $CD4^+$ T cells, $CD8^+$ T cells, B cells and $CD11b^+$ cells in the eye (Rossi et al., 2015a; Settimio et al., 2012). Consistent with its protective function in inflammation, RvD1 increases local Treg cell counts in the inflamed tissue in experimental autoimmune neuritis (Luo et al., 2016). It is important to note that the DHA-derived RvD1 is a structural homolog of LXA_4 and mediates its action via the same two receptors (ALX/FPR2 and GPR32) as LXA_4. Hence, it is expected that LXA_4 and RvD1 have similar direct actions on lymphocytes.

As a treatment, the RvD1 epimer 17R-RvD1 can quell infection by reducing the number of Th1 and Th17 T cells and inhibiting the production of proinflammatory cytokines in stromal keratitis (Rajasagi et al., 2017). RvD1, like LXA_4, also has direct actions on human B cells by suppressing IgE production and differentiation of naïve B cells (Kim et al., 2016). In a follow up study, RvD1 reduces IgE production by B cells in asthma patients treated with low dose steroids (Kim et al., 2018). Other members of the SPM family such as maresin-1 (MaR1) also demonstrated its therapeutic and protective effects *in vivo* by restraining IL-13 cytokine production from ILCs and increasing *de novo* generation of iTregs to resolve lung inflammation (Krishnamoorthy et al., 2015). A receptor for MaR1 has yet to be identified, therefore it is unclear if these are direct or indirect actions on lymphocytes.

Consistent with their broad protective actions in acute inflammation, SPMs downregulate effector T cell and B cell function. Hence, they are attractive potential therapeutic targets for controlling dysregulated innate and adaptive immune responses.

1.4. Leukotriene B_4 Synthesis, Bioaction, and Lymphocyte Regulation

Initially identified as a potent proinflammatory neutrophil chemoattractant, LTB_4 is now known to exhibit versatile functionalities in host defense and inflammation. LTB_4 also mediates T cells trafficking, releases antimicrobial peptides, and promotes macrophage phagocytosis. LTB_4 is generated *in vivo* in a two-step process by 5-LOX and LTA_4 hydrolase metabolizing the arachidonic acid substrate. In mice, LTB_4 binds with high affinity to its receptor leukotriene B_4 receptor 1 (BLT1) that's widely expressed on leukocytes, and BLT1 expression becomes highly upregulated during inflammation (Tager and Luster, 2003).

Expression of BLT1 was identified on T cells in 2003 (Goodarzi et al., 2003; Tager et al., 2003), and the initial findings on LTB_4-mediated T cell response were investigated using allergic lung inflammation models (Gelfand, 2017). These experiments established LTB_4-mediated T cell recruitment, and implicated $CD8^+$ T cells as the main pathogenic cell type driving allergic airway inflammation. BLT1 expression was higher on T cells of human asthma patients than healthy individuals, which corresponded to disease severity and confirmed a role of the BLT1-LTB_4 axis in pathogenic T cell recruitment in asthma (Chung et al., 2014).

In vitro, LTB_4 has dichotomous effects on T lymphocytes. In T cell differentiation assays, LTB_4 inhibits *de novo* iTreg generation and increases interleukin-17 (IL-17) cytokine production (Chen et al., 2009), whereas LTB_4-activated T cells inhibit proliferation of Epstein-barr virus-infected B cells (Liu et al., 2008), demonstrating that LTB_4, like LXA_4, can mediate cellular interactions among T cells and B cells.

Recent work has provided evidence of LTB_4 regulating migration of various lymphoid-derived cell types. In an experimental autoimmune encephalomyelitis model, LTB_4 guides the migration of Th17 cells into the central nervous system and induces pathology (Lee et al., 2015). In contact dermatitis, inhibition of the LTB_4-BLT1 axis ameliorates disease by preventing neutrophil and $CD8^+$ T cell recruitment (Lv et al., 2015). Although eicosanoid and SPM generation by the different lymphoid cell types is not well defined, it has been shown that virus-infected human $CD4^+$ T cells can secrete LTB_4 to further recruit T cells and propagate virus infection, and inhibition of LTB_4 synthesis reduces viral load (Percher et al., 2017). The LTB_4-BLT1 axis also directs γδ T cell migration in murine pleural cavities in an LPS inflammation model (Costa et al., 2010), and induces NK cell chemotaxis *in vitro* in a BLT1 receptor-specific manner (Wang et al., 2015a).

Most recently, BLT1 and the cysteinyl leukotriene receptor 1 (CysLT1R) were identified on ILC2s (Doherty et al., 2013; von Moltke et al., 2017), and LTB_4 was shown to activate ILC2s and the downstream T helper cell type 2 cytokine production in a NFAT-dependent manner during lung inflammation (von Moltke et al., 2017). Thus LTB_4 may indirectly regulate T cell proliferation and differentiation through NFAT-mediated IL-2 production.

Current state of the field indicates that LTB_4 has pleiotropic actions on lymphocytes to dynamically regulate the immune response in a cell type- and context-dependent manner. The underlying mechanisms warrant further investigation, especially since many drugs that target the LTB_4 pathways and BLT1 are in clinical trials or FDA-approved.

1.5. Prostaglandin E_2 Synthesis, Bioaction, and Lymphocyte Regulation

Prostaglandin E_2 is the most abundant eicosanoid produced *in vivo*, during homeostasis and upon encountering inflammatory stimuli, and is a target for the ubiquitous nonsteroidal anti-inflammatory drugs. The constitutively expressed cyclooxygenase 1 (COX-1) or the inflammation induced cyclooxygenase 2 (COX-2) metabolizes arachidonic acid, and through unstable intermediates PGG_2 and PGH_2, PGE synthase catalyzes the formation of the final product PGE_2. PGE_2 exerts complex and multidimensional actions due to having four distinct receptors, EP1, EP2, EP3, and EP4, expressed in many cell types. PGE_2 is essential in maintaining homeostasis such as gut barrier function, but is also a key mediator of acute inflammation, autoimmune disorders and many immune driven diseases. Thus, its pleiotropic nature requires careful consideration when administering drugs targeting this pathway.

The immunomodulatory actions of PGE_2 on T lymphocytes have been studied extensively. PGE_2 exerts many physiological actions on T cells, including thymic T cell development, T helper cell differentiation, migration, and cytokine production (Kawahara et al., 2015; Nicolaou et al., 2014). The main prostaglandin receptor expressed on T effector cells are EP2, involved in Th17 cytokine production, and EP4 that regulates IFN-γ and interleukin-10 (IL-10) production (Boniface et al., 2009).

The contrasting effects of PGE_2 on T cell activation and cytokine production are in part due to different expression of co-stimulation molecules and the state of activation of T cells (Kickler et al., 2012). More importantly, the diverse and often contrasting actions PGE_2 also stem from the heterogeneous cellular sources of PGE_2 and cell type-specific interaction with T effector cells. Macrophage-produced PGE_2 enhances IFN-γ and IL-17A production by $CD4^+$ T cells (Sato et al., 2014), thereby augmenting T effector functions. In contrast, multipotent adult progenitor cell-produced PGE_2 upregulates suppressor of cytokine signaling-2 (SOCS2) and growth arrest and DNA-damage-inducible protein alpha (GADD45A) expression in T cells, thereby preventing T effector cell expansion (Reading et al., 2015). However, PGE_2 appears to downregulate T effector cell function *in vivo* (Zaslona et al., 2014). The intrinsic role of PGE_2 in regulating lymphocyte function is underscored by the discovery that activated human $CD4^+$ T cells generate PGE_2, which in turn serves as an autocrine signal to further upregulate EP2 and EP4 expression in T cells (Sreeramkumar et al., 2016). More importantly, T cell-secreted PGE_2 is a determinant for T cell cytokine response and polarization to Treg, Th1 or Th17 phenotypes (Maseda et al., 2018).

PGE_2 has conflicting roles in T cell differentiation. Keratinocyte-produced PGE_2 inhibits T helper cell proliferation in a psoriasis model. *In vitro*, PGE_2 can upregulate Foxp3, a lineage specification marker expressed by Treg (Kopfnagel et al., 2011), consistent with cancer models where PGE_2 produced in the tumor microenvironment can polarize T cells toward the iTreg phenotype to suppress anti-tumor responses (Shimizu et al., 2017; Whiteside, 2014). However, in another *in vitro* study, PGE_2 was shown inhibit iTreg differentiation via EP2 the receptor (Li et al., 2017), emphasizing differential roles that depend on the tissue environment.

Direct bioactions of PGE_2 on NK cells and the regulation of cognate EP receptors have been elucidated mostly *in vitro*. Tissue-secreted PGE_2 suppresses NK cell activation (Noone et al., 2013; Zhang et al., 2014), suggesting protection against cytotoxic cell damage. All four EP receptors are functionally expressed on NK cells but PGE_2 primarily mediates its suppressive action via EP2 and EP4. PGE_2 regulation of NK cells leads to loss of function by blocking cell migration, inhibiting NK cell-mediated cytotoxicity and IFN-γ and TNF-α cytokine production (Dupuy et al., 2012; Holt et al., 2012; Martinet et al., 2010). Protective functions of PGE_2 also include direct regulation of ILCs, which express the EP4 receptor. In a systemic inflammation mouse model, PGE_2 maintains gut barrier homeostasis by triggering interleukin-22 released by type 3 innate lymphoid cells (ILC3s) (Duffin et al., 2016). PGE_2 can also directly inhibit ILC2 function in allergic airway inflammation by reducing eosinophilia, interleukin-5 and cytokine production (Zhou et al., 2018).

PGE_2 is a complex signaling molecule with disparate functions that are contingent on the cell type, tissue, EP receptor signaling and immune response. It is clear that PGE_2 and EP receptors are an integral part of lymphocyte responses; however, PGE_2 as a therapeutic target so far has largely been overlooked by the immunology field, likely due to its complex and diverse actions in and health and disease.

1.6. Dissertation Overview

When eicosanoids were first identified, each molecule was categorized as pro-inflammatory or anti-inflammatory based on the context in which they were discovered and investigated. As our knowledge expands with increasing research efforts to understand eicosanoids in various inflammatory settings, it has become clear that these small signaling molecules are not functionally one-dimensional. On the contrary, eicosanoids LTB_4 and PGE_2 could either amplify or reduce inflammation depending on the tissue microenvironment and pathology, while LXA_4 supports homeostasis not just in the context of inflammation but also normal cellular function.

In Chapter Two, I will present the current knowledge on therapeutic effects of SPMs and LXA_4 in ocular diseases, which provides a rationale in investigating the role of LXA_4 in posterior autoimmune uveitis as the topic has yet to be explored. I will also briefly discuss how PGE_2 and LTB_4 participate in the amplification of inflammatory responses through findings from collaborations to highlight the diverse bioactions of

eicosanoids in different disease settings. In Chapter Three I will present my thesis project that shows LXA_4 can regulate T cell responses in autoimmune setting, adding another line of evidence to LXA_4 regulation of $CD4^+$ T effector cells. Extending beyond the immune system in Chapter Four, we uncovered a novel neuroprotective function of astrocyte-derived lipoxins A_4 and B_4 that promote retinal ganglion cell survival in acute neuronal injury and chronic elevated intraocular pressure glaucoma models.

In addition to presenting the essential role of tissue resident LXA_4 in maintaining routine immune responses and healthy physiology, this dissertation highlights new clinically relevant targets and unexpected roles of lipoxins. In the final chapter I will address clinical implications of lipoxins that can be drawn from my research.

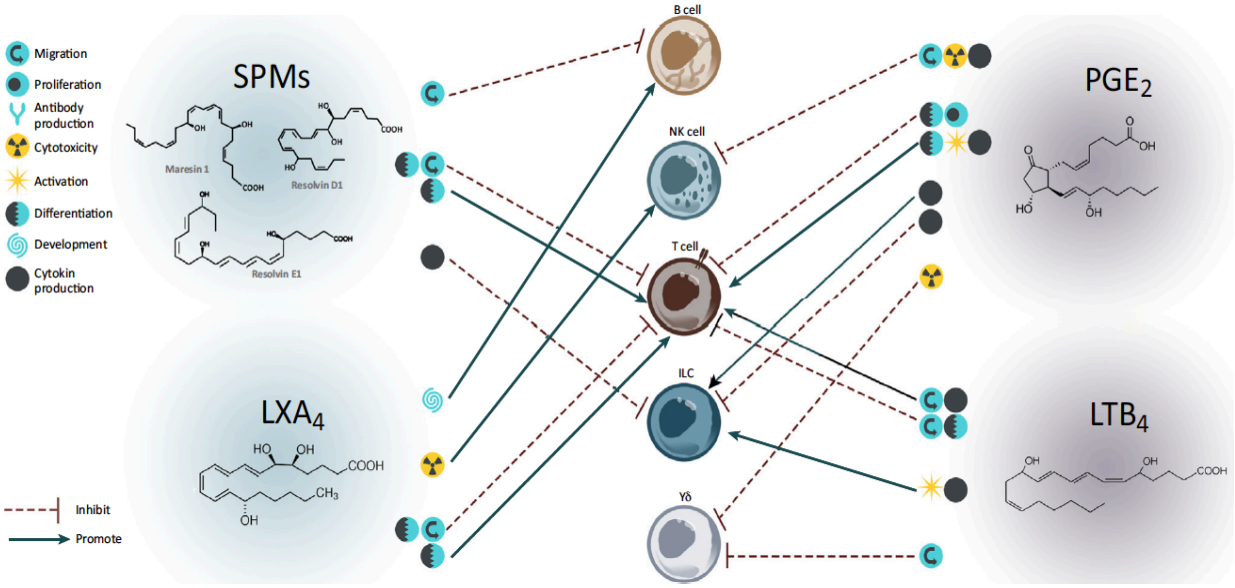


Figure 1. Direct immune regulation of lymphoid cells by specialized proresolving mediators (SPMs) and eicosanoids during inflammation. Lipid mediators and inflammation are conceptually and functionally closely intertwined. On cellular activation, lipid mediators are synthesized de novo locally and rapidly metabolized at the site of inflammation to modulate the immune response. SPMs and eicosanoids can directly inhibit or promote lymphocyte functions as depicted by symbols representing migration, proliferation, antibody production, cytotoxicity, activation, differentiation, development, and cytokine production. Eicosanoids and SPMs mediate intracellular communication in a cell-specific approach (Wei and Gronert, 2019).

Chapter Two: Context-dependent Lipid Mediator Bioactions

2.1. Therapeutic Effects of SPMs in Ocular Diseases

Chapters One introduced PGE_2, LTB_4 and LXA_4 and their direct regulation of lymphocyte function. In this chapter, I will present the most current literature on the therapeutic effects of SPMs and LXA_4 in ocular inflammatory diseases, and briefly highlight the inflammatory roles of PGE_2 and LTB_4 through two collaboration projects using models of peritonitis and atopic dermatitis.

2.1.1. Lipoxygenase expression and SPMs in the eye

The tissue graft experiments by Peter Medawar launched the basic principles of ocular immune privilege in the 1940s, with the eye being one of the special organs able to tolerate foreign antigens without rejecting the donor tissue. The eye's unique ability to manage foreign antigen exposure without mounting an inflammatory response likely evolved from the ocular surface's constant contact with the environment, the recruitment of immune cells via the dense retinal vasculature and the evolutionary pressure to preserve vision as an essential primary sense. To provide visual function, the cornea, aqueous humor, vitreous humor and the fovea are avascular in order to undergo phototransduction and convert photons into electric signals for transmission to the visual cortex in the brain. The necessity of protecting the delicate visual axis is underscored by the fact that a large portion of cerebral cortex function is dedicated to visual analysis and perception (Felleman and Van Essen, 1991). Hence, a case can be made that innate, adaptive and reparative immune responses in the visual axis are among the most highly evolved. Sight threatening inflammation could be triggered by routine exposure to microbes, pathogens, irritants, neutrophils (PMN) of nocturnal tears (Gorbet et al., 2015) at the ocular surface, or through aberrant immune responses resulting from injury or diseases in the neural retina. However, the transparent visual axis has evolved sophisticated mechanisms to tightly control inflammation, raise the threshold for triggering immune responses, and maintain active immune tolerance. Experiments from the past few decades have established immune privilege as active surveillance where immunosuppression and regulatory cells are actively deployed to avert full-fledged innate or adaptive immune responses (de Andrade et al., 2016; Stein-Streilein and Streilein, 2002). In addition, the eye effectively minimizes immune responses through physical barriers at two sites, the corneal epithelium and the blood retinal barrier. In the eye, specialized pro-resolving mediators (SPMs) lipoxin A_4 (LXA_4) and neuroprotectin D1 (NPD1/PD1) were first identified as endogenous lipid mediators in the immune privileged cornea (Gronert, 2005). Ongoing studies have identified SPMs as essential endogenous factors for maintaining immune homeostasis in the visual axis, uphold an elevated basal anti-inflammatory tone, amplify wound healing, control leukocyte functional responses, downregulate inflammatory responses and drive nerve regeneration and neuroprotection (Bazan, 2007; Bazan et al., 2010; Gronert, 2005; Kenchegowda and Bazan, 2010).

Photoreceptors have the highest DHA content and retention in comparison to all cells in the body (Rice et al., 2015), providing an abundant substrate for 5- and 15-lipoxygenase (5-, 15-LOX) to potentially generate most of the ω-3 SPMs resolvins, protectins and maresins in the retina. Several lines of evidence have established protection against macular degeneration and retinopathy with DHA-enriched diets (Connor et al., 2007; Tuo et al., 2009), which underscore the importance of endogenous levels of DHA-derived SPMs in the eye. SPM circuits have provided new targets to combat dysregulated ocular innate and adaptive immune responses as current treatment option are limited and aimed at broad immune suppression. Treatment with resolvin E1 (RvE1), RvE1 analogs, LXA_4, NPD1/PD1 and resolvin D1 (RvD1) in murine and *in vitro* models of ocular diseases have established the efficacy of SPMs as potential treatment options to limit inflammatory pathogenesis in the cornea and conjunctiva, prevent adaptive immune responses and protect against retinopathy due to injury or oxidative stress.

5- and 15-LOX are the rate-limiting enzymes responsible for generating SPMs, especially the eicosanoid LXA_4 generated in many tissues of the eye. A striking feature of the cornea is the high expression of 15-LOX in human and mouse corneal epithelial cells. 15-LOX-LXA_4-ALX/FPR2 circuit has been identified as an important resident circuit that controls wound healing and immune responses at the ocular surface. Two 15-LOX enzymes (*ALOX15, ALOX15B*) have been identified in the human cornea (Brash et al., 1997; Chang et al., 2005; Liminga et al., 1994) and the mouse homolog 12/15-LOX (*Alox15*) is expressed in the corneal epithelium, retinal pigment epithelium (RPE) and lens (Bazan, 2009; Liclican and Gronert, 2010). Knockdown of 15-LOX (*ALOX15*) in the human RPE increased susceptibility to oxidative stress induced apoptosis (Calandria et al., 2009). More importantly, 12/15-LOX knockout mice exhibit a phenotype of impaired LXA_4 formation and induction of cytoprotective heme oxygenase-1 (HO-1), which correlated with exacerbated inflammation and delayed wound healing (Biteman et al., 2007; Seta et al., 2006). 5- and 15-LOX dependent formation of LXA_4 and/or NPD1 has been established in the cornea, retina and ocular draining lymph nodes and 15-LOX has a key role in preventing RPE cell death during oxidative stress and pathological angiogenesis in the avascular cornea (Bazan, 2009; Gronert, 2005).

SPMs exert their actions through G-protein coupled receptors (GPCR) that are widely expressed in the eye. ALX/FPR2, the GPCR for LXA_4 and its DHA-homolog RvD1, is expressed in the corneal epithelium, RPE, PMN, macrophages, T cells (Chiang et al., 2006; Gao et al., 2015; Gronert et al., 1998) and conjunctiva (Hodges et al., 2016). In humans, GPR32 has been identified as a second receptor for LXA_4 and RvD1 (Serhan and Chiang, 2013) and its expression has been established in conjunctival goblet cells (Li et al., 2013) and human retinal endothelial cells (Shi et al., 2016).

Emerging evidence has identified endogenous SPM circuits in the ocular surface, retina and draining lymph nodes of the eye. These intrinsic protective circuits have key roles in immune regulation, injury responses, infection, inflammatory neovascularization, neuropathy and autoimmunity. The concept of SPMs as promising therapeutic targets

has been validated in pre-clinical animal models of ocular diseases and early clinical trials with RvE1 analogs in Dry Eye Disease (DED), which have established the efficacy and broad protective action of local and systemic treatment with SPMs (Cortina and Bazan, 2011; Gronert, 2010; He and Bazan, 2010). This chapter aims to provide an overview of the current knowledge on the endogenous role of SPMs in ocular trauma and diseases (Fig. 2).

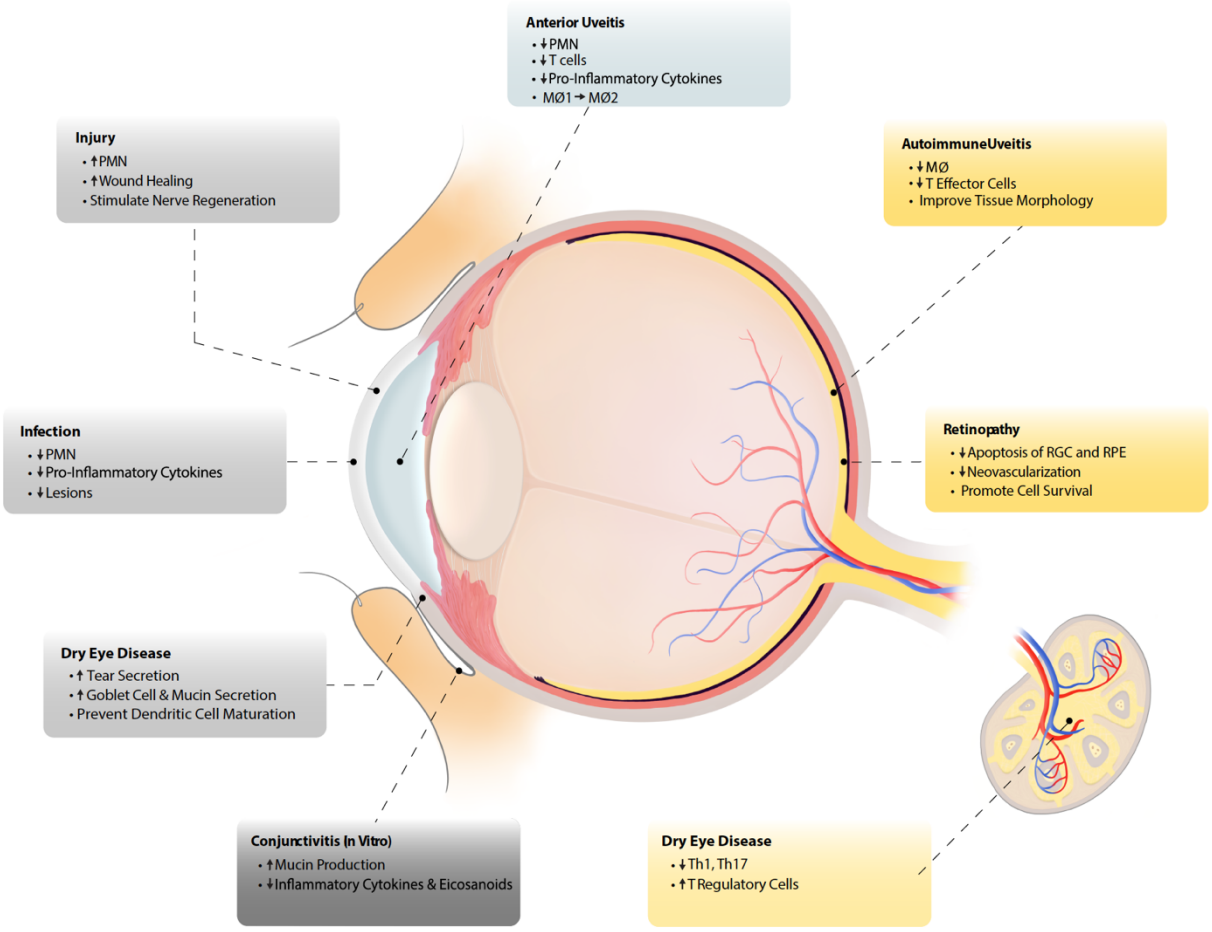


Figure 2. The anti-inflammatory bioactions of SPMs in ocular tissues and the cervical draining lymph node. MØ= macrophages, Th1= T helper 1 cells, Th17= T helper 17 cells, PMN= polymorphonuclear cells, RGC= retinal ganglion cells, RPE= retinal pigmented epithelium (Wei and Gronert, 2017).

2.1.2. Specialized pro-resolving mediators in ocular diseases

2.1.2.1. Corneal injury and wound healing

The cornea is predominantly composed of the corneal epithelium, the stroma and a monolayer of corneal endothelium lining the inner surface. The corneal epithelium maintains tight junctions that prevent microbes, viruses and irritants from penetrating the cornea. The essential role of SPM circuits in the cornea is supported by the high expression of 12/15-LOX circuit in the corneal epithelium (Brash et al., 1997; Chang et al., 2005; Liminga et al., 1994), and both human and mouse corneal epithelial cells can generate LXA_4. Mouse models of acute corneal injury have demonstrated that loss of epithelial cells or genetic deletion of 12/15-LOX abrogated LXA_4 formation in the cornea and led to impaired epithelial wound healing, while topical treatment with LXA_4, NPD1 as well as 17-HDHA, a metabolic precursor for several members of the DHA-resolvin family, accelerated re-epithelialization in acute corneal abrasions (Gronert, 2005; Gronert et al., 2005).

Contrary to LXA_4's established role of inhibiting PMN recruitment in various disease models, both topically administered and endogenously formed LXA_4 and NPD1 increase PMN numbers after epithelial injury in the cornea (Gronert et al., 2005). The requirement of PMN for normal corneal wound healing and nerve regeneration in acute and self-resolving epithelial injury responses is well established (Li et al., 2006a; Li et al., 2006b). Multiple lines of evidence also suggest that these wound healing PMN as well as nocturnal tear PMN are a non-inflammatory phenotype (Gorbet et al., 2015) that is distinct from stereotypical vascular inflammatory PMN. Unlike LXA_4's endogenous role in healthy and self-resolving corneal inflammatory-reparative responses, topically added or endogenous LXA_4 in chronic or infectious injuries inhibit recruitment of inflammatory PMN, formation of inflammatory cytokines and chemokines, platelet activating factor and pathological angiogenesis (Biteman et al., 2007; Kakazu et al., 2012; Kenchegowda et al., 2011; Leedom et al., 2010). The protective actions of the 15-LOX-LXA_4 circuit in the cornea are in part mediated by the cytoprotective hemeoxygenase-1 (HO-1) system. In a positive feedback mechanism, HO-1 expression and 15-LOX activity depend on one another to maintain an elevated anti-inflammatory tone that raises the threshold for triggering full inflammatory responses in the immune privileged cornea (Biteman et al., 2007; Seta et al., 2006). In view of endogenous formation of several SPM pathway markers and expression of their receptors (ALX/FPR2, ChemR23), it is likely that in addition to NPD1 and LXA_4, other SPMs are formed in the ocular surface and have roles in executing healthy inflammatory and reparative responses in the cornea. A notion that is supported by a recent lipidomic analysis that document the presence of SPMs in human tears, namely RvD1, RvD2, RvD5, NPD1, LXA_4 and aspirin-triggered LXA_4 (English et al., 2017).

The cause for sex-specific differences in innate and adaptive immune responses and the striking higher prevalence of autoimmune disease in females compared to males is largely unknown. A 2011 post-hoc analysis of a prospective clinical trial identified for the first time a marked sex-specific difference in the outcome of wound

healing in the cornea. Namely, after correcting for confounding factors, corneal wounds in men healed two times faster than in women (Krishnan et al., 2012). Employing the mouse model of self-resolving corneal injury, it was discovered that estrogen regulates the 15-LOX-LXA_4-ALX circuit via the epithelial ERβ receptor (Wang et al., 2012). Estrogen downregulated 15-LOX and ALX expression and LXA_4 formation in the cornea that resulted in a female-specific phenotype of delayed re-epithelialization, reduced recruitment of wound healing PMN and an amplified inflammatory response; a phenotype that can be reversed by LXA_4 treatment (Wang et al., 2012). This study identified regulation of SPM circuits by estrogen as a potential factor in the etiology or pathogenesis of sex-specific ocular surface inflammatory diseases. Recent lipidomic analysis of human tears demonstrated that women have lower SPM levels than men (English et al., 2017), emphasizing the clinical relevance of animal models that have identified sex-specific regulation of SPM circuits.

The cornea is the most innervated tissue in the human body, and corneal innervation serves a vital role in maintaining homeostasis and normal injury responses. In a lamellar keratectomy model that severs corneal nerves, NPD1 treatment markedly increased nerve regeneration (Cortina et al., 2010; Cortina et al., 2013; Kenchegowda et al., 2013), a bioaction that is consistent with the originally defined role of NPD1 as a neuroprotective SPMs in the retina and in mouse models of stroke (Bazan, 2007; Bazan et al., 2013)

Several SPMs have been assessed as treatment options for corneal diseases in pre-clinical animal models of infection, graft survival, inflammatory angiogenesis and keratectomy. Both NPD1 and RvE1 treatment after herpes simplex virus infection decreased pro-inflammatory cytokines, virus-induced lesions and angiogenesis (Rajasagi et al., 2013; Rajasagi et al., 2011), while RvE1 and LXA_4 also reduced the severity of LPS-induced stromal keratitis (Biteman et al., 2007; Lee et al., 2015). In a mouse model of corneal transplantation RvD1 treatment prevented dendritic cell maturation by downregulating MHCII, CD40 and IL-12 expression on dendritic cells, which led to suppressed alloimmunity and enhanced graft survival (Hua et al., 2014). Local or topical treatment with LXA_4, LXA_4 analogs, RvD1 or RvE1 markedly reduced pathological angiogenesis by inhibiting VEGF, VEGFR2, VEGFR3, TNF-α and IL-1 (Jin et al., 2009; Leedom et al., 2010). In a rabbit model of keratectomy, the RvE1 analog RX-10045 reduced corneal opacity after photorefractive keratectomy (Torricelli et al., 2014). These *in vivo* studies demonstrated that consistent with established bioactions of SPMs in other organ systems, SPMs are promising drug targets for corneal diseases, infections and graft survival. However, regulation of SPM circuits and sex-specific differences in corneal health and diseases and cellular mechanisms of action still remain to be defined.

2.1.2.2. Conjunctivitis

The conjunctiva is a tissue composed of stratified epithelium, goblet cells, resident mast cell, lymphocytes and antigen presenting cells that covers the inner eyelids and anterior sclera. As a result of being highly vascularized, the conjunctiva is responsible for

immune surveillance and cell trafficking of the ocular surface but has been largely ignored as the primary tissue for initiating ocular surface immune responses. Goblet cells serve as a physical fortification by producing mucin to keep the eye lubricated and to clear microbes and irritants. Conjunctivitis is a common ocular surface disease caused by viral or bacterial infection, allergies or autoimmune diseases. SPM actions and signaling mechanisms have been studied at length *in vitro* using isolated human or rat goblet cells. *In vitro* models that recapitulate goblet cell responses in DED and allergic conjunctivitis have demonstrated that RvD1 and RvE1 can block inflammatory eicosanoids or histamine activation of ERK and intracellular Ca^{2+} mobilization in goblet cells (Dartt et al., 2011; Li et al., 2013). LXA_4 has unique actions on isolated conjunctival goblet cells as it can stimulate mucin secretion via activation of phospholipases, ERK and downstream protein kinases (Hodges et al., 2016). The *in vivo* expression, endogenous formation, and therapeutic actions of SPMs have not been determined in the healthy or inflamed conjunctiva or animal models of conjunctivitis. However, *in vitro* data with goblet cells and mouse models of inflammation in other mucosal tissues (i.e. lung and colon) have established the protective therapeutic actions of SPMs, inferring that treatment with SPMs in general should attenuate the pathogenesis of conjunctivitis.

2.1.2.3. Retinopathy

SPM's endogenous protective roles extend beyond the ocular surface to the posterior segment of the eye, especially the retina. The neural retina is composed of densely packed photoreceptors and neurons responsible for converting light to electrical signals that are transmitted to the visual cortex, where they are decoded and interpreted to provide visual perception. Unlike the self-renewal capabilities of the corneal epithelium, cells in the retina are not equipped with regenerative mechanisms thus damage to the retina could lead to irreversible vision loss. The RPE is comprised of a monolayer of highly specialized cells that are vital in maintaining the health of retina. The RPE phagocytoses the outer segments of the photoreceptors, facilitates the visual cycle, upholds the blood-retinal barrier and expresses immunosuppressive molecules. Injury to RPE or dysregulation of homeostatic function of RPE is a key factor in the etiology of retinopathy.

NPD1 is of particular interest in retinopathy due to highly enriched levels of DHA in the retina and the pathway for NPD1 formation only requires 15-LOX. A large body of work has shown that NPD1 promotes cell survival of human RPE cells upon exposure to oxidative stress and that 15-LOX is the rate-limiting enzyme for endogenous formation (Calandria et al., 2012; Mukherjee et al., 2007; Mukherjee et al., 2004). Stereospecific binding sites for NPD1 have been identified in RPE cells and PMN (Marcheselli et al., 2010) but the receptor remains to be identified. NPD1 mediates protection against RPE apoptosis in an autocrine fashion, suggesting a critical role for the 15-LOX-NPD1 circuit in maintaining RPE homeostasis (Calandria et al., 2009). The protective and anti-apoptotic bioactivity of RPE-released NPD1 are mediated by upregulation of anti-apoptotic Bcl-2 and Bcl-xL, as well as reduction of pro-apoptotic Bax expression and caspase-3 activation (Antony et al., 2010; Calandria et al., 2012; Mukherjee et al., 2007). In addition, NPD1 promotes RPE cell survival against oxidative

stress by increased phosphorylation of PI3K/Akt and mTOR/p70S6K signaling pathways (Faghiri and Bazan, 2010; Halapin and Bazan, 2010).

In addition to maintaining RPE homeostasis, NPD1 also inhibits inflammatory responses in the retina by exerting direct neuroprotective actions. In laser-induced choroidal neovascularization (CNV) that recapitulates macular degeneration, NPD1 attenuated neovascularization and shifted microglia toward a non-injury inducing phenotype *in vivo* (Sheets et al., 2013; Sheets et al., 2010). NPD1 also has direct actions on neuron cell survival and apoptosis. Retinal ganglion cells are neurons that relay action potentials from photoreceptors through the optic nerve, making cells along this path indispensable for processing visual information. Treatment with NPD1 after optical nerve transection in rats markedly delayed retinal ganglion cell death (Qin et al., 2008). *In vitro* data has demonstrated endogenous formation of NPD1 by cone photoreceptor cell lines (Kanan et al., 2015)indicating that NPD1 formation in the retina is likely not restricted to RPE cells.

Inflammation is now recognized as a prominent feature of many retinal diseases and the role of SPMs in resolution of inflammation has sparked research efforts to explore their therapeutic potential in retinopathies. A leading cause of blindness is diabetic retinopathy triggered by glucose-induced inflammation of the choroid and/or the retina, characterized by dysfunction and/or abnormal growth of retinal blood vessels. Inflammatory cells are subsequently recruited and facilitate further destruction. *In vitro* experiments have established that RvE1and RvD1 are formed during retinal endothelial-PMN interactions and inhibit PMN transmigration and pro-inflammatory cytokines production (Tian et al., 2009). A potential role of SPM circuits in human diabetic retinopathy is underscored by marked reduction of LXA_4 in the serum and vitreous humor with a concurrent increase of IL-6 in patients with diabetic retinopathy (Kaviarasan et al., 2015). RvD1 formation has also been demonstrated in human endothelial cells in an *in vitro* model of diabetic retinopathy. Endothelial formation of RvD1 is reduced in high glucose conditions and restored to normal by vasoactive intestinal peptide, suggesting that RvD1 has a role in maintaining retinal endothelial cell homeostasis (Shi et al., 2016). A preclinical model of retinopathy of prematurity emphasizes the important role of enzymes in the formation of SPM circuits. Dietary DHA is protective in this model but inhibition of pathogenic angiogenesis by the dietary supplement required 5-LOX (Sapieha et al., 2011). It is important to recognize that 5-LOX is the key enzyme for amplifying and initiating inflammation but is also the rate-limiting and required enzyme for formation of the majority of SPMs. Potential roles of SPM circuits in controlling innate immune responses in the retina are suggested by *in vitro* experiments that demonstrated LXA_4's ability to inhibit LPS induced IL-6 production in human RPE cells (Kaviarasan et al., 2015).

The retina has the highest metabolic rate, oxygen demand and DHA content of any tissue and thus evolved robust mechanisms to prevent photoreceptor and neuronal cell death. NPD1 was the first identified *in vivo* retinal SPMs (Qin et al., 2008) and its neuroprotective actions in the retina and central nervous system after injury and oxidative stress are well established (Bazan, 2007; Bazan et al., 2010). Emerging

evidence has identified formation and bioactions of other SPMs in the retina such as RvD1 and LXA_4. However, the endogenous roles of SPMs in neuroprotection, retinopathy or maintaining homeostasis still remain to be explored.

2.1.2.4. Endotoxin-induced anterior uveitis

Uveitis is characterized by inflammation of the uveal layer, which includes the iris, ciliary body and the choroid. Inflammation of the middle layer of the eye can be further categorized into anterior, posterior or pan uveitis depending on the tissues affected. Anterior uveitis is mediated by PMN, macrophage and T cell infiltration, modeled by endotoxin induced uveitis (EIU) in rats and is primarily an acute and self-resolving inflammatory response. Consistent with the anti-inflammatory and pro-resolving actions of SPMs, treatment with LXA_4 or LXA_4 analogs reduced the clinical inflammation score, PMN cell counts and protein levels in the aqueous humor in rats with EIU (Karim et al., 2009). RvD1, the structural DHA homolog of LXA_4, mediates its action via the same shared receptors. Consistent with LXA_4's action in EIU, tail vein injection of RvD1 upon LPS-induced uveitis led to a dose-dependent reduction of MPO activity along with PMN and T cell infiltration into the eye (Settimio et al., 2012), while intravitreal injection of RvD1 decreased TNF-α, chemokine MIP1-a, ubiquitin proteasome (Karim et al., 2009) and caspase 3 (Rossi et al., 2015b). Macrophage profiles also shifted from the M1 phenotype to high expression of M2 upon RvD1 administration (Rossi et al., 2015a). Endogenous roles for LXA_4 or RvD1 in EIU have not been reported. However, the established therapeutic actions of SPMs in many inflammatory disease models and the marked inhibition of inflammation with LXA_4 and RvD1 treatment in uveitis models provide strong evidence for SPMs as promising treatment options for anterior uveitis.

2.1.2.5. Immune-driven dry eye disease

The role of eicosanoids and SPMs in the etiology of ocular and other autoimmune diseases is relatively unexplored. Autoimmunity occurs when the host immune cells mistakenly recognize and/or triggers T effector cell-driven responses against self-proteins and tissues. Autoimmune DED is a debilitating disease that inflicts working-age adults with unknown triggers. This T cell-driven chronic disease targets the lacrimal gland, goblet cells in the conjunctiva and ocular surface leading to impaired tear formation, inflammation and epithelial defects. DED is multifactorial and affects approximately 4 million people in the United States, and of which 3.2 million are women (Gayton, 2009). The cause for this striking sex-specific prevalence in women is unknown.

Current primary treatment options are limited to corticosteroids, the immunosuppressant cyclosporine A and artificial tears. A recent study identified resident LXA_4 circuits in the ocular surface and draining lymph nodes of the eye, which demonstrated striking sex-specific differences and demonstrated LXA_4 as a key regulator of T_H1 and T_H17 effector cells and T regulatory cells (Tregs). In a mouse model that recapitulates human T cell driven DED, a population of regulatory PMN that

highly expresses 15-LOX was identified. The regulatory PMN generate LXA_4 in the lacrimal grand, lymph nodes and ocular surface. The loss of LXA_4 formation in the lymph nodes upon resident regulatory PMN depletion during desiccating stress was female-specific, and caused amplified T_H1 and T_H17 responses and reduction of Tregs. The exacerbated adaptive immune response in females was reversed by LXA_4 treatment, which reduced the number of T effector cells and increased Tregs in the draining lymph nodes and reduced dry eye pathogenesis (Gao et al., 2015). This provided the first evidence that LXA_4 and regulatory PMN are a resident circuit in draining lymph nodes and key factors that control T_H1, T_H17 and Tregs.

The therapeutic potential of amplifying endogenous SPM circuits is underscored by studies that examined the pharmacological effects of SPM treatment in experimental autoimmune DED. RvE1 increased tear production and corneal epithelium density while inhibiting macrophage infiltration in a mouse model of immune DED (Li et al., 2010) and an analog of RvE1 applied at the initiation of DED was able to maintain goblet cell density (de Paiva et al., 2012). Based on promising data form pre-clinical animal models synthetic RvE1 analogs were tested in Phase I and Phase II clinical trials for chronic DED and initial results demonstrated significant improvements (Hessen and Akpek, 2014). New emerging data provides compelling evidence that lipoxins (Gao et al., 2015) as well as their DHA structural homologs, RvD1 and RvD2 (Chiurchiu et al., 2016) have direct actions with T cells and regulate adaptive immune responses, which warrants further research to define the cellular mechanism of action, signaling pathways and direct T cell-targeted actions.

2.1.3. Summary

The anti-inflammatory and pro-resolving therapeutic actions of SPMs are well established in many organ systems and pre-clinical inflammatory diseases models. However, there is a clear gap of knowledge regarding SPM's distinct cellular mechanisms of action, their endogenous regulation and role in disease etiology and health. The unique properties of the eye, such as highly conserved primary sense with immune-privileged tissues, neural tissues that are the direct outgrowth of the central nervous system and an exceptional high metabolic rate and DHA content, have uncovered novel bioactions and insights into the endogenous roles of SPM circuits, which extend well beyond their traditional roles of regulating acute inflammation or resolution.

Enzymes for SPM formation are highly expressed in corneal and retinal epithelial cells and tissue resident leukocytes as well as receptors for LXA_4, RvD1, NPD1 and RvE1. Studies in the eye have discovered that SPMs have novel intrinsic roles in driving corneal wound healing, preventing RPE and ganglion cell apoptosis, promoting survival of photoreceptors and controlling T effector cell responses in adaptive immune responses. More importantly, endogenous formation of LXA_4 and NPD1 in the eye is not limited to the resolution phase of inflammation (Table 1). These SPM circuits have fundamental roles in maintaining healthy homeostasis in the cornea, retina and draining lymph nodes in the absence of acute inflammation. Of particular interest is the sex-

specific regulation of the intrinsic LXA_4 circuit in the ocular surface and draining lymph nodes, which has emerged as a factor in driving female-specific adaptive immune response and indicate that SPM circuits have direct actions on lymphocytes. *In vitro* studies and animal models have established the therapeutic potential of SPMs on limiting inflammatory ocular diseases. Consistent with inflammatory disease models in other organ systems, SPM circuits are validated therapeutic targets for inflammatory diseases in the eye. However, many questions remain to be answered regarding the regulation of SPM circuits in health, disease and their role or dysregulation in the etiology of autoimmunity, retinopathy and neurodegeneration. Thus my projects in Chapters Two and Three aim to fill the gap in knowledge regarding the roles of lipoxins in autoimmunity and neurodegeneration.

In Vivo + *In Vitro* ★	LXA_4		NPD1		RvD1, 2, 5	
Corneal Epithelium	+,★		+			
Corneal Stroma	★					
Vitreous Humor	+					
Retina	+		+			
Retinal Pigmented Epithelium			★			
Draining Lymph Nodes	+					
Tears	+		+		+	

Table 1. Formation of SPMs in ocular tissues, tears and cervical draining lymph nodes *in vivo* and *in vitro*. LXA_4= lipoxin A_4, NPD1= neuroprotectin D1, RvD1, 2, 5= resolvin D1, resolvin D2, resolvin D5. *In vivo* or *in vitro* formation indicated in grey columns. Adapted from (Wei and Gronert, 2017).

2.2. Pro-Inflammatory Functions of PGE_2 and LTB_4 Mediated by Innate Immune Cells

2.2.1. Neutrophil licensing by TNF-α increases lipid mediator production

Neutrophils are now known to be more dynamic than just pro-inflammatory first responders to acute inflammation. To fend of bacteria and pathogens, neutrophils can phagocytose, produce superoxide, degranulate or form neutrophil extracellular traps. Another primary function of neutrophils is to generate eicosanoids such as leukotrienes and prostaglandins to initiate and amplify acute inflammation and induce leukocyte trafficking to the site of inflammation. Recent studies suggest that neutrophils are able to adapt its functionality based on the local environment in tumors or during chronic

inflammation (Fridlender et al., 2009), however whether local cues can also affect neutrophil function at the onset of inflammation is unknown.

The Barton lab compared the functional properties of murine neutrophils recruited to different types of peritoneal inflammation and found that neutrophils recruited by microbial ligands displayed increased cytokine production compared with naive neutrophils, and identified that TNF-α paracrine signaling is essential in neutrophil licensing. From RNA expression data (not shown), licensed neutrophils that received microbial stimulation also appeared to upregulate Ptgs2, the gene that encodes COX-2, which drives the production of inflammatory prostaglandins. This data provided a rationale in investigating the role of lipid mediators in neutrophil licensing since activated neutrophils can produce lipid mediators at high levels, and LTB_4 is a known potent chemoattractant of neutrophils.

To assess whether treatment with TNF-α could also modulate eicosanoid production, we measured lipid mediator production by neutrophils treated with TLR ligand Pam3CSK4 with or without TNF-α treatment. TNF-α or TLR stimulation alone of neutrophils did not induce significant production of LTB_4 and PGE_2; however, these two stimuli synergistically induced significant levels of LTB_4 and PGE_2 release. From this collaboration, we found that licensed neutrophils produce more PGE_2 and LTB_4 in the inflammatory local milieu (Deguine et al., 2017), in parallel to what was observed with cytokine production. However, TNF-α treatment alone does not induce cytokine and lipid mediator production, showing that within an inflammatory environment, the licensing of neutrophils can only occur after TLR engagement. These results suggest that TNF treatment directly modulates the outcome of TLR signaling in neutrophils, namely licensing TLRs to trigger the release of LTB_4 and PGE_2, which are potent mediators of neutrophil and effector T cell recruitment.

2.2.2. Keratinocyte-derived PGE_2 recruits mast cells that induce pruritic response in atopic dermatitis

Deletion of *Tmem79* in mice recapitulates human atopic dermatitis with skin-barrier defects, eczematous skin lesions, and severe scratching. Prostaglandins and leukotrienes are produced by keratinocytes in response to cellular oxidative stress (Ahn et al., 2002; Hu et al., 2017) and mediates mast cell chemotaxis (Halova et al., 2012). The Julius lab investigated whether these lipid mediators contribute to phenotypes associated with *Tmem79* deletion and found that daily administration of the 5-LOX inhibitor zileuton had no effect on scratching behavior, but treatment with COX inhibitor indomethacin abolished elevated scratching in *Tmem79*$^{-/-}$ mice. Indomethacin treatment also reduced dermal mast cell and $CD45^+$ immune cell counts, suggesting excessive production of prostaglandins induces itch behavior in *Tmem79*$^{-/-}$. Next, the PGE_2-EP3 axis was identified as the culprit in mast cell recruitment, as inhibition of EP3 reduced mast cell counts and itch responses.

The goal of the collaboration was to identify the cellular source of PGE_2 in *Tmem79*$^{-/-}$ mice skin samples. Lipidomic analyses confirmed an increase of PGE_2

production from *Tmem79*$^{-/-}$ keratinocytes with no change in PGE_2 metabolites 13,14-dihydro-15-keto-PGE_2, 13,14-dihydro-15 keto-PGA_2, or bicyclo-PGE_2 (Emrick et al., 2018), showing that the observed *Tmem79*$^{-/-}$ phenotype is attributed by PGE_2 and not other metabolites in the pathway. Although previous reports have implicated PGE_2 and PGD_2 as culprits in inducing itch responses, this is the first report of PGE_2 mediating itch responses in a gene deletion mouse model that recapitulates human Tmem79-mediated atopic dermatitis. The results also offer a potential therapeutic avenue of treating atopic dermatitis associated itch through EP3 antagonism.

2.2.3. Summary

Through collaborations, we revealed diverse eicosanoid functions beyond ocular diseases. We found that neutrophil licensing by TNF-α amplifies local inflammatory responses by increasing cytokine, PGE_2 and LTB_4 secretion, and the PGE_2-EP3 axis is responsible for recruiting mast cells that elicit itch responses in an atopic dermatitis mouse model. These results highlight the proinflammatory functions of these pleiotropic mediators and underscore the essential role of innate immune cell-secreted PGE_2 and LTB_4 in initiation of inflammation and leukocyte trafficking.

Chapter Three: Lipoxin A_4 Regulates Effector T Cell Function and Trafficking and Ameliorates Autoimmune Uveitis

3.1. Introduction

The defining functions of lipoxin A_4 (LXA_4) include inhibition of neutrophil infiltration to the site of inflammation, promotion of macrophage efferocytosis, and reduction of inflammatory cytokine and chemokine production (Serhan et al., 2008). *In vitro* studies have shown that LXA_4 and special pro-resolving mediators (SPMs) have a role in T follicular and helper cell differentiation (Chiurchiu et al., 2016; Nagaya et al., 2017), and neutrophil-derived LXA_4 was previously shown to inhibit T effector cell responses in lymph nodes in the immune-driven dry eye disease (DED) model (Gao et al., 2015). The LXA_4 receptor, ALX/FPR2, is highly expressed on T cells, providing a modality for LXA_4 regulation of T cells. However, the bioactions of LXA_4 on T lymphocytes has been minimally explored.

LXA_4 is an endogenous arachidonic acid-derived small signaling molecule that exerts anti-inflammatory and pro-resolving activities. To control inflammation in affected tissues, 5-lipoxygenase (5-LOX) and 12/15-lipoxygenase (12/15-LOX) enzymes oxygenate arachidonic acid in tandem to biosynthesize LXA_4 locally. 5-LOX contributes to the development of retinal axons (Campello-Costa et al., 2006) and inhibits neovascularization in retinopathy (Sapieha et al., 2011), and amplified 12/15-LOX expression confers retinal ganglion cell protection in an optic nerve transection model (Qin et al., 2008), thus establishing an important role of LXA_4 enzyme precursors in retinal homeostasis. Emerging evidence from animal disease models has identified a protective role for LXA_4 as an essential resident circuit that regulates immune responses in ocular inflammatory models including neovascularization, wound healing, and immune-mediated dry eye disease (DED) (Gao et al., 2015; Leedom et al., 2010; Wang et al., 2012). Hence, the LXA_4 circuit is critical *in vivo* not only as an inflammation-induced mediator to actively resolve inflammation, but also as a homeostatic regulator to maintain a balanced immune response.

While LXA_4 is well established as a key mediator of innate immune cell responses during inflammation, it is important to investigate whether LXA_4 can regulate adaptive immune responses in T cell-driven autoimmunity since the prevalence of autoimmune diseases has increased steadfastly in recent decades (Lerner and Matthias, 2015). Posterior autoimmune uveitis accounts for 10-15% of blindness in the Western world and primarily affects working age adults (Durrani et al., 2004; Gritz and Wong, 2004). Autoimmune uveitis is mediated by pathogenic Th1 and/or Th17 responses to retinal-specific antigens, such as interphotoreceptor retinoid-binding protein (IRBP) and retinal arrestin. The experimental autoimmune uveitis (EAU) mouse model closely recapitulates human disease. Upon immunization with retinal antigens in complete Freund's adjuvant (CFA), retina-specific T cells get primed in the inguinal lymph nodes that drains the immunization sites, traffic to the eye and elicit inflammatory responses which peak within 14-16 days (Caspi, 2010).

The aims of this thesis project were to determine whether the LXA_4 circuit is modulated during EAU pathogenesis, and to uncover whether and how LXA_4 regulates T effector cell function in autoimmune uveitis. We showed that gene expression of 5-LOX, 12/15-LOX and ALX/FPR2 is temporally defined during disease development, and *in vivo* generation of LXA_4 in response to EAU induction is tissue-specific. T cells from LXA_4 deficient mice (*Alox5*$^{-/-}$, a mutant mouse in the gene encoding 5-LOX) exhibited amplified T effector function with augmented effector cytokine production and metabolic responses that results in more severe disease than wild-type (WT) controls. By contrast, LXA_4 treatment in WT mice attenuated development of autoimmune uveitis by limiting pathogenic T cell infiltration into the eye. The data indicated that the LXA_4 circuit limits migration of T effector cells by upregulating their expression of T cell homing molecule Ccr7 and downregulating sphingosine-1 phosphate receptor 1 (*S1pr1*) in the peripheral lymph node, thereby preventing them from egress. Deficiency of tissue resident LXA_4 circuit also intensified T effector function through increase in T cell glycolysis and IFN-γ cytokine production. These findings uncovered a novel mechanism of LXA_4 regulation of T cell function and trafficking.

3.2. Materials and Methods

Mice, EAU induction and LXA_4 treatment: C57BL/6J mice between 8 and 12 weeks of age were purchased from the Jackson Laboratory. All experimental procedures were approved by the Animal Care and Use program at University of California, Berkeley, and the National Eye Institute at the National Institutes of Health. For the EAU model, each mouse was immunized with 150 or 300 ug $IRBP_{651-670}$ peptide (Genscript or BioBasic), as indicated in the figure legends, emulsified in CFA supplemented with 2.5mg/ml *Mycobacterium tuberculosis* (Sigma), and injected with 0.2ug pertussis toxin (Sigma) adjuvant. *Alox5*$^{-/-}$ mice (B6.129S2-*Alox5*^{tm1Fun}/J, stock number 004155), *TCR*$\beta^{-/-}$ mice (B6.129P2-*Tcrb*^{tm1Mom}/J, stock number 002118) were also purchased from the Jackson Laboratory. *Fpr2*$^{-/-}$ mice were a gift from Dr. Asma Nusrat at the University of Michigan. For assessing therapeutic effects of LXA_4, either 1ug/100ul of LXA_4 or 100ul of 10% EtOH in PBS was injected subcutaneously and intraperitoneally starting at day 0 of disease induction. IRBP-specific TCR transgenic R161H mice (Horai et al., 2013) generated on the congenic CD90.1 B10.RIII background and WT CD90.2 B10.RIII mice (Jackson Laboratory) were maintained in house in the Caspi lab.

Evaluation of EAU by funduscopy and histology: Funduscopic scoring system was established to evaluate activity of inflammation in the retina in Dr. Caspi's laboratory. Mice were anesthetized with Ketamine: Xylazine (7:3) and retinas were observed under a binocular microscope after pupil dilation with tropicamide and phenylephrine. Scores range from 0-4 were assigned to each eye (Horai and Caspi, 2011). For histological evaluation, eyes were enucleated and fixed in 4% glutaraldehyde for 1 hour and transferred to 10% buffered formalin, embedded in paraffin and processed by H&E staining. Scores were determined on a scale of 0-4 in half-point increments according to the EAU scoring criteria (Caspi, 2003).

Electroretinography and fundus imaging: Mice were dark-adapted overnight, and experiments were performed under dim red illumination. Electroretinograms were recorded using Espion E2 System (Diagnosys LLC) that generated and controlled light stimuli. A reference electrode was inserted above the tongue, corneal electrodes were placed at the center of corneas, and sterile lubricant eye gel was dropped on corneal electrodes. The amplitudes of a-wave and b-wave were analyzed and measured using Espion software. Fundus image was captured using Micron III retinal imaging microscope (Phoenix Research Labs) for small rodents.

Tissue harvest and processing: Eyes, lymph nodes and spleens were harvested from PBS and LXA_4 treated immunized mice 16 days post-immunization. After the perfusion, eyes were trimmed of lenses and connective tissues, minced and treated with 1mg/ml collagenase D for 40 minutes at 37°C. Lymph nodes and spleens were mashed, and cells filtered through 40um strainers and resuspended in single cell suspensions. Red blood cells from spleens were lysed with ACK lysis buffer.

Real-time quantitative PCR: RNA was isolated from mouse tissues using RNeasy isolation kit (Qiagen), and reverse transcribed with High Capacity cDNA kit (Applied Biosystems). Mouse β-actin was used as the endogenous reference gene. PCR was performed using Taqman gene expression assays (Thermo Fisher Scientific). *Alox5*: Mm01182747_m1, *Alox15*: Mm00507789_m1, *Fpr2*: Mm00484464_s1, *S1pr1*: Mm00514644_m1.

Identification and quantification of endogenous formation of eicosanoids: Whole lymph nodes and eyeballs were harvested from the mice immunized with $IRBP_{651-670}$ at peak inflammation from the mice immunized with $IRBP_{651-670}$. Tissues were homogenized with Bead Ruptor, and lipid mediators were extracted via solid phase C-18 columns. Lipid mediators were then quantified via liquid chromatography-tandem mass spectrometry (LC-MS/MS). Extraction recovery was calculated based on deuterated internal standards (PGE_2-d4, LTB_4-d4, 15-HETE-d8, LXA_4-d5, DHA-d5, AA-d8). The LC-MS/MS system is composed of Agilent 1200 series HPLC, Kinetex C18 minibore column (Phenomenex), and AB Sciex QTRAP 4500 mass spectrometer (SCIEX). Analyses were performed in negative ion mode with scheduled multiple reaction monitoring using 4-5 transition ions per lipid mediator.

Flow cytometry analyses: Single cell suspensions were prepared from isolated tissues, and ≤$1x10^6$ cells per sample were stained with anti-mouse fluorescence-conjugated antibodies with the following fluorochromes: BV421, BV510/V500, FITC, PE, PerCP-Cy5.5/PerCP-eF710, PE-Cy7, APC, APC Cy7/APC-eF780, BV650/SB645, BV785, UV450. Surface staining was performed using antibodies against: CD3, CD4, CD25, CD44, CD62L, CD69, and CCR7. Intracellular staining was performed after stimulating cells in high glucose DMEM with 10% FBS with 50 ng/ml PMA and 1 µg/ml Ionomycin (Millipore Sigma) in the presence of Brefeldin A (Golgi Plug™, BD Biosciences) for 4-6 hours at 37°C. Cells were fixed with 4% PFA and permeabilized with Triton buffer (0.5% Triton X-100 and 0.1% BSA in PBS). Anti-mouse fluorescence conjugated FoxP3, IL-17A, and IFN-γ were used for intracellular staining. Ghost Dye UV

450 or Red 780 (Tonbo) were used to exclude dead cells. Cells were acquired on BD LSR Fortessa (BD Biosciences), MACSQuant Analyzer (Miltenyi Biotec), and Cytoflex (Beckham Coulter). Fcs files were analyzed using FlowJo (TreeStar).

Immunohistochemistry and fluorescence quantitation: Tissues were embedded in OCT and cryosectioned into 7um thickness. Tissues sections were fixed in 4% PFA and blocked with serum prior to primary antibody staining. Primary antibodies against CD4 (Biolegend) and COX-2 (Abcam) were used. Secondary antibodies AlexaFluor 488 and AlexaFluor 568 (ThermoFisher Scientific) were used for immunofluorescent labeling. Cells were counterstained with DAPI at 1:3000 dilution (Sigma). Fluorescent images were acquired with a Zeiss AxioImager microscope at 10X and 20X. Fluorescence signals were quantified using macro in Fiji software. Particle mean intensity was measured in each fluorescent channel of each retina and choroid tissue section. Statistical significance was calculated on 3-7 mice per experimental group.

T cell proliferation assays: For antigen recall assays, $5x10^5$ cells resuspended in HL-1 media were plated in 96-well round bottom plates and stimulated with serial dilutions of $IRBP_{651-670}$ peptide. Cells were cultured for 2 days and 1uCi/well of [^{3}H] thymidine was added and incubated for 16 hours. Samples were harvested and counted by liquid scintillation (Perkin Elmer).

Adoptive transfer: 5x 10^6 $IRBP_{161-180}$ *in vitro*-activated cells from R161H CD90.1 mice were injected intraperitoneally into WT CD90.2 B10.RIII mice. LXA_4 was administered at the doses of 1 µg or 250 ng, respectively, every day throughout the disease course. Funduscopy was assessed starting on day 4 after cell transfer to track disease development.

T cell transfer and EAU induction: Donor C57BL/6 WT, *Alox5*$^{-/-}$ or *Fpr2*$^{-/-}$ mice were immunized with $IRBP_{651-670}$ peptide, and $CD3^+$ T cells purified with T cell enrichment columns (R&D Systems) from lymph nodes that drains immunization sites and spleens on day 11 post immunization. WT, *Alox5*$^{-/-}$ or *Fpr2*$^{-/-}$ T cells ($1x10^7$ each) were transferred intraperitoneally into *TCRβ*$^{-/-}$ recipients. Three weeks after the cell transfer to allow for homeostatic engraftment of donor T cells, these *TCRβ*$^{-/-}$ recipient mice were immunized with 150ug $IRBP_{651-670}$ in CFA and pertussis toxin as described above.

nanoString: RNA was extracted from freshly frozen tissues using RNeasy (Qiagen) and analyzed by nCounter Analysis Technology on mouse cancer immunology panel (nanoString). Data was normalized to housekeeping genes and analyzed using nSolver software. Gene expression threshold was set at 30 counts to eliminate the background.

Seahorse metabolic assays: Seahorse XFe96 Extracellular Flux Analyzer (Agilent Technologies) was used to measure rates of cellular oxygen consumption and glycolysis. $CD4^+$ T cells were harvested from WT or *Alox5*$^{-/-}$ mice immunized with $IRBP_{651-670}$ and seeded at $1x10^6$ per well. The extracellular acidification rate (ECAR) and the oxygen consumption rate (OCR) for each well were calculated while the cells were

subjected to XF Glycolytic Rate assays with the following concentrations of injected compounds: 50 mM 2-DG and 0.5 μM rotenone/antimycin A.

Transwell migration assay: $CD4^+$ T cells were isolated using T cell isolation kit (STEMCELL Technologies) from inguinal lymph nodes and spleens of mice immunized with $IRBP_{651-670}$, and stimulated with 2ug of anti-CD3 and 1ug of anti-CD28 for 18 hours. Stimulated T cells were seeded at 2x 10^5 per transwell insert (Costar) and allowed to migrate toward 100ng of CCL19 and 100ng of CCL21 chemokine ligands (R&D) in 2% FCS for four hours. Cells were harvested and counted on Cytoflex.

Statistical analysis: Statistics were performed using GraphPad Prism 8 (GraphPad, La Jolla, CA). Statistical differences were determined by ANOVA and student t tests for parametric variables and Mann-Whitney test for nonparametric variables. All error bars are standard error mean. P value < 0.05 was considered statistically significant and denoted with *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$.

3.3. Results

3.3.1. LXA_4 is generated *in vivo* in a tissue- and disease- dependent manner

Changes of SPMs in human blood have been proposed as diagnostic markers of health and disease outcome (Colas et al., 2014; Norris et al., 2018). To assess if LXA_4 serum concentration is altered during uveitis and specifically posterior uveitis, we analyzed serum samples from 69 healthy subjects and 87 uveitis patients (with diagnoses of anterior, pan, and posterior uveitis) using ELISA. Out of all the patients, 43 had diagnoses of posterior uveitis, including Birdshot Retinochoroidopathy and Vogt-Koyanagi-Harada disease, which all exhibit clinical manifestations of autoimmune uveitis in the posterior segment of the eye. LXA_4 serum concentration in posterior uveitis patients was significantly higher compared to healthy subjects or other (anterior and panuveitis) patients (Fig. 3.1), suggesting a compensatory systemic upregulation of the LXA_4 circuit specifically in posterior uveitis patients.

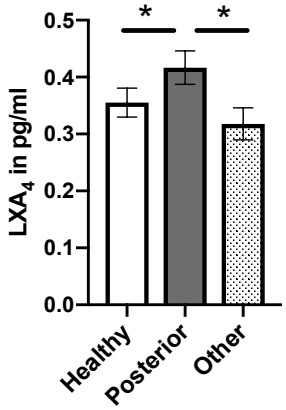
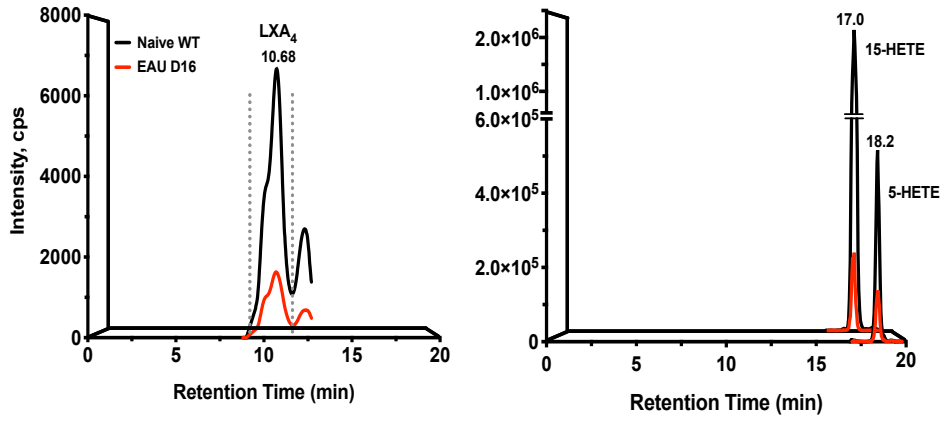


Figure 3.1. LXA_4 serum concentration is elevated in posterior autoimmune uveitis patients. Bar graph of LXA_4 concentration in pg/ml measured in human serum by ELISA. Healthy subjects, n=69; posterior uveitis patients, n=43; and other uveitis (anterior and panuveitis) patients, n=44. Posterior uveitis patients include those with diagnoses of Birdshot Retinochoroidopathy and Vogt-Koyanagi-Harada disease. * $p < 0.05$, One-way ANOVA.

The significant increase of the serum LXA_4 level in posterior uveitis patients prompts us to investigate the role of LXA_4 circuit in autoimmune uveitis using the mouse EAU model. As the lymph nodes are critical sites for initiating and regulating adaptive immune responses that would subsequently trigger ocular inflammation, we quantified LXA_4 and the lipoxygenase pathway-specific metabolites 5-HETE, 12-HETE and 15-HETE formation in the eye and various peripheral lymph nodes (Fig. 3.2A) of EAU-challenged mice using LC-MS/MS at the onset (day 10 post-immunization) and peak of uveitis (day 16 post-immunization). LC-MS/MS-based lipidomic analyses revealed that production of endogenous LXA_4, 5-LOX and 12/15-LOX pathway markers 5-HETE, 12-HETE and 15-HETE, respectively, in the eye were significantly elevated in comparison to naïve unimmunized controls at peak inflammation (Fig. 3.2B). By contrast, in the submandibular lymph nodes that drain the eye, as well as in axillary (distal) lymph nodes, the LXA_4 levels did not show a significant difference between naïve and EAU-challenged mice (Fig. 3.2B). However, LXA_4, 5-HETE, and 15-HETE levels were significantly downregulated at peak inflammation in the inguinal lymph nodes that drain the site of immunization (Fig. 3.2B), suggesting that the decrease in 5-HETE and 15-HETE levels directly leads to LXA_4 abrogation as EAU progresses to peak inflammation. These lipidomic data from the eye and peripheral lymph nodes indicate tissue-specific regulation of the LXA_4 circuit in response to EAU disease development.

A



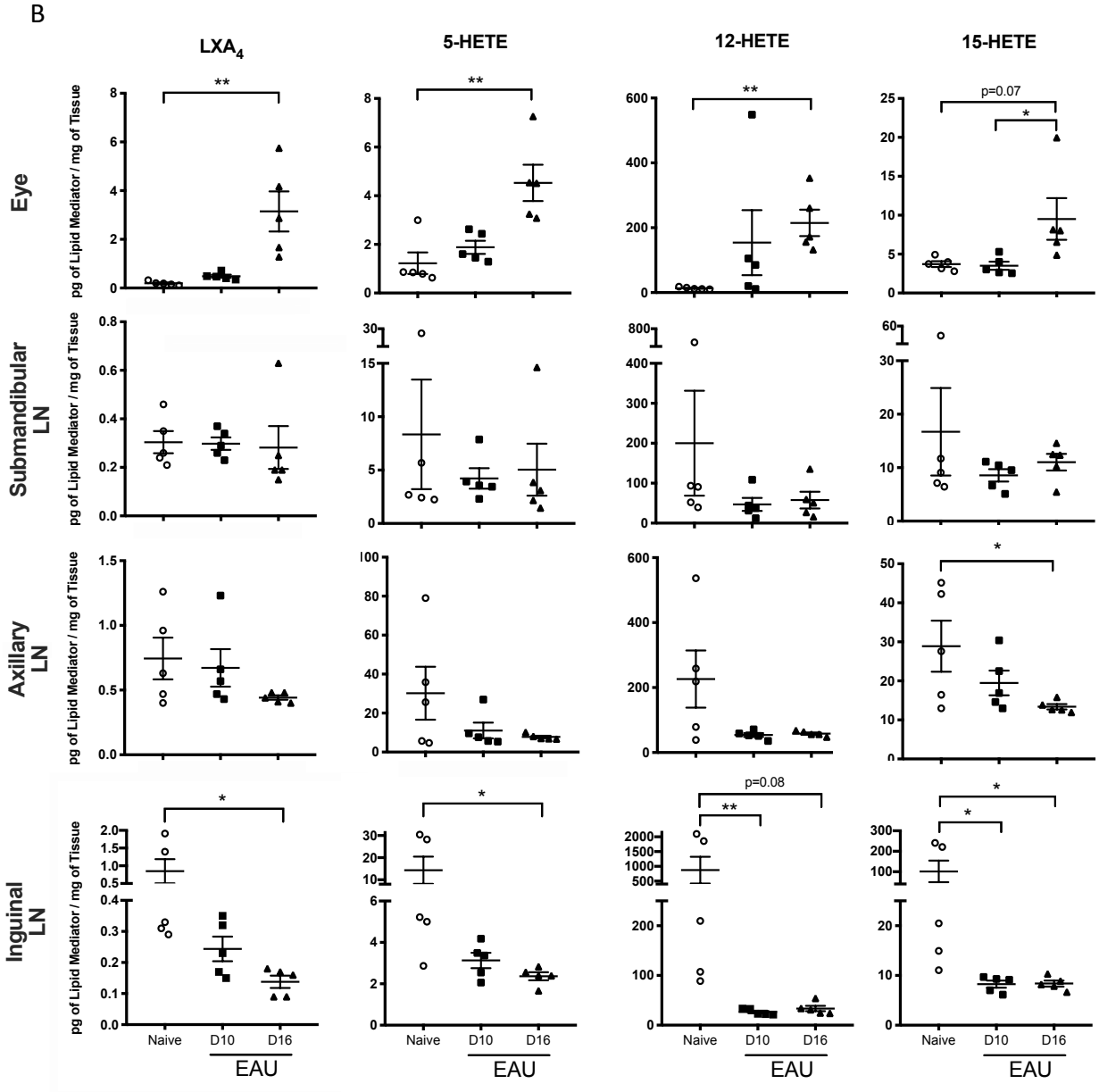


Figure 3.2. Lipidomic analysis demonstrates *in vivo* formation of LXA_4 is disease- and tissue- dependent. A) Representative multiple reaction monitoring LC/MS-MS chromatogram of LXA_4, 5-HETE, and 15-HETE levels in the inguinal lymph nodes from naïve mice and EAU-challenged mice on day 16. B) LXA_4 and its pathway markers in pg per mg of tissue in whole eye balls, submandibular lymph nodes, axillary lymph nodes, and inguinal lymph nodes quantified by LC-MS/MS on non-immunized naïve and EAU-challenged mice (days 10 and 16). n=5 per group. * $p < 0.05$, ** $p < 0.01$, One-way ANOVA.

To more closely assess the changes in the gene expression of lipoxygenases, 5-LOX and 12/15-LOX, and LXA_4 receptor ALX/FPR2 upon EAU induction, retinas and inguinal lymph nodes were harvested from immunized mice on day 3, day 7, and day 14 post-immunization. In the retina, *Alox5* expression was upregulated by 5-fold on day 14 post-immunization in comparison to other time points (Fig. 3.3A), which directly correlated with upregulation in LXA_4 production in the eye. However, *Alox15* expression remained at the healthy level while *Fpr2* showed a trend of upregulation in the retina. In the inguinal lymph nodes *Alox5* expression was significantly upregulated on day 3 and day 14 post-immunization, while Fpr2 expression was highly upregulated on day 3 post-immunization (Fig. 3.3B). Since EAU pathogenesis is mediated by retina-specific $CD4^+$ T cells, we evaluated Fpr2 expression on $CD4^+$ T cells isolated from inguinal lymph nodes and found that the receptor expression was significantly upregulated at peak disease in comparison to $CD4^+$ T cells isolated from inguinal lymph nodes of naïve mice (Fig. 3.3C). RNA expression of lipoxygenases and *Fpr2* indicates temporal modulation of the LXA_4 pathway during EAU, at a cellular level and in whole tissues.

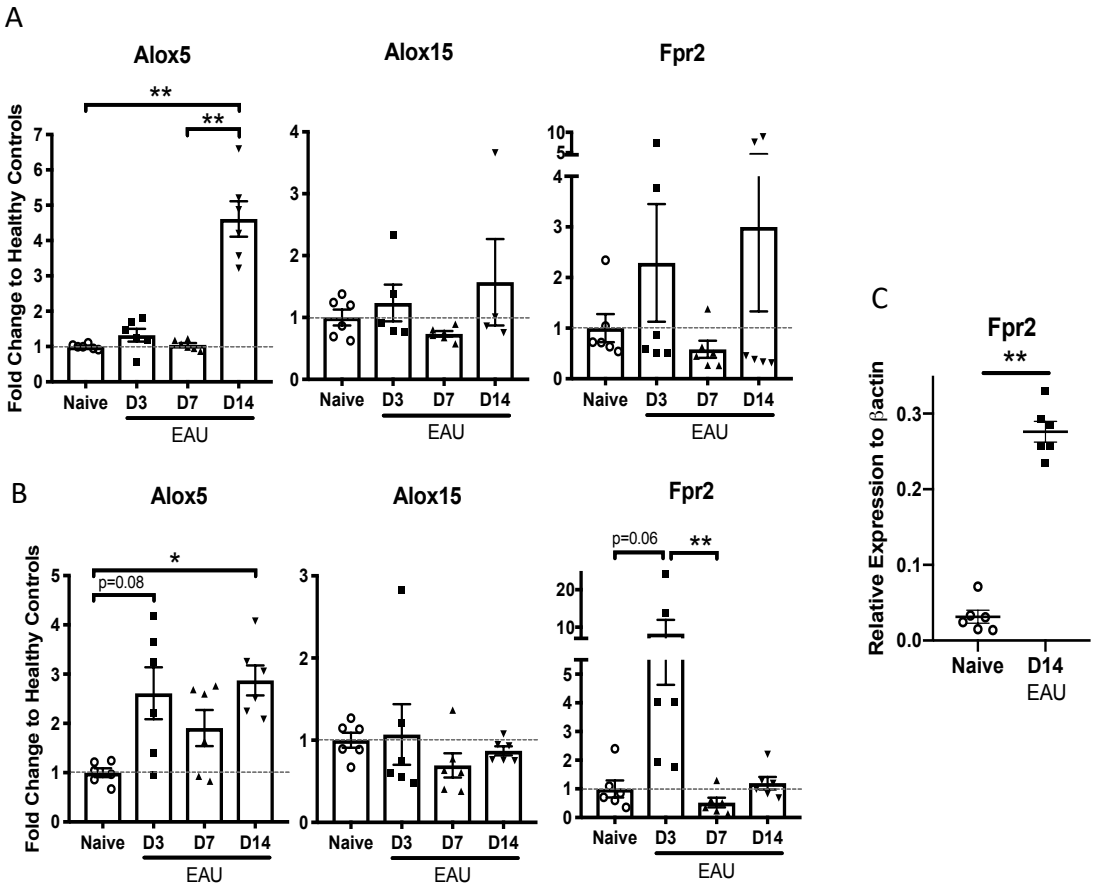


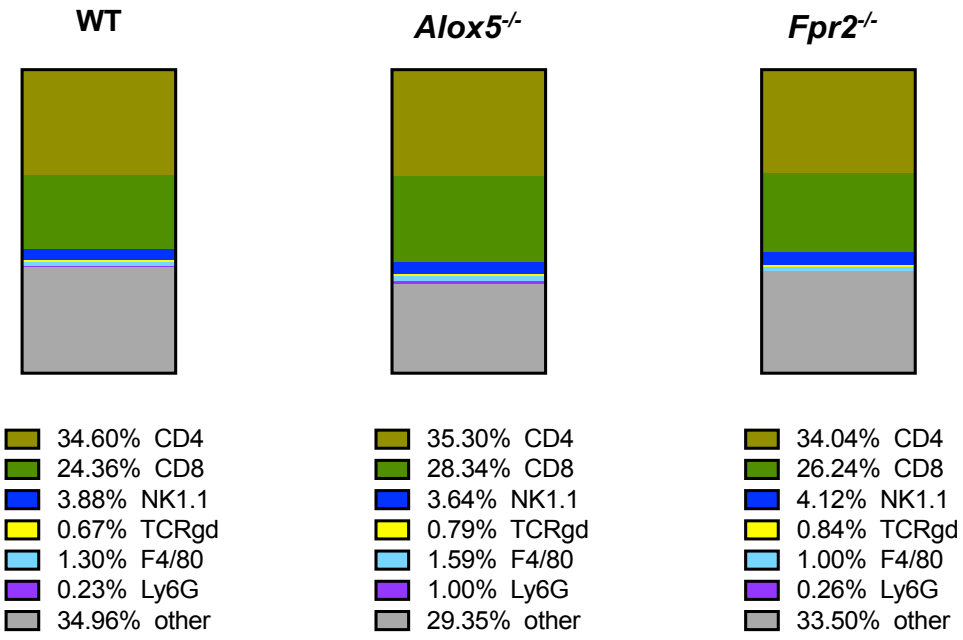
Figure 3.3. Temporal expression of *Alox5*, *Alox15* and *Fpr2* during EAU. Gene expression was assessed by qPCR in A) The retina, and B) inguinal lymph nodes harvested on days 3, 7, 14 post-immunization in comparison to non-immunized naïve mice. n=6 per group. C) Fpr2 expression on CD4+ T cells isolated from inguinal lymph

nodes of healthy and immunized mice on day 14 post-immunization. * $p < 0.05$, ** $p < 0.01$, One-way ANOVA.

3.3.2. Characterization of WT, *Fpr2*$^{-/-}$ and *Alox5*$^{-/-}$ mice

Before diving into the EAU disease model, we characterized naïve WT, LXA_4 receptor knockout (*Fpr2*$^{-/-}$), and 5-LOX knockout (*Alox5*$^{-/-}$) mice. Submandibular lymph nodes, inguinal lymph nodes and spleens were harvested from five age-matched mice per genotype, and stained for cell surface markers by flow cytometry. Interestingly, *Alox5*$^{-/-}$mice showed higher frequencies of $CD8^+$ T cells and neutrophils ($Ly6G^+$) across all tissues in comparison to WT and *Fpr2*$^{-/-}$ mice (Fig. 3.4A-C). However we did not observe difference in frequency in the $CD4^+$ T cell population between all three strains (Fig. 3.4A-C). The increase in neutrophil frequency of *Alox5*$^{-/-}$ mice is curious as neutrophils are high expressers of the 5-LOX enzyme, suggesting that there may be a compensatory mechanism in attempt to increase 5-LOX expression in the *Alox5* global knockout mice. No significant difference in frequencies was observed in NK cells (NK1.1), γδ T cells (TCRgd), macrophages (F4/80) between all three mouse strains.

A **Submandibular LN**



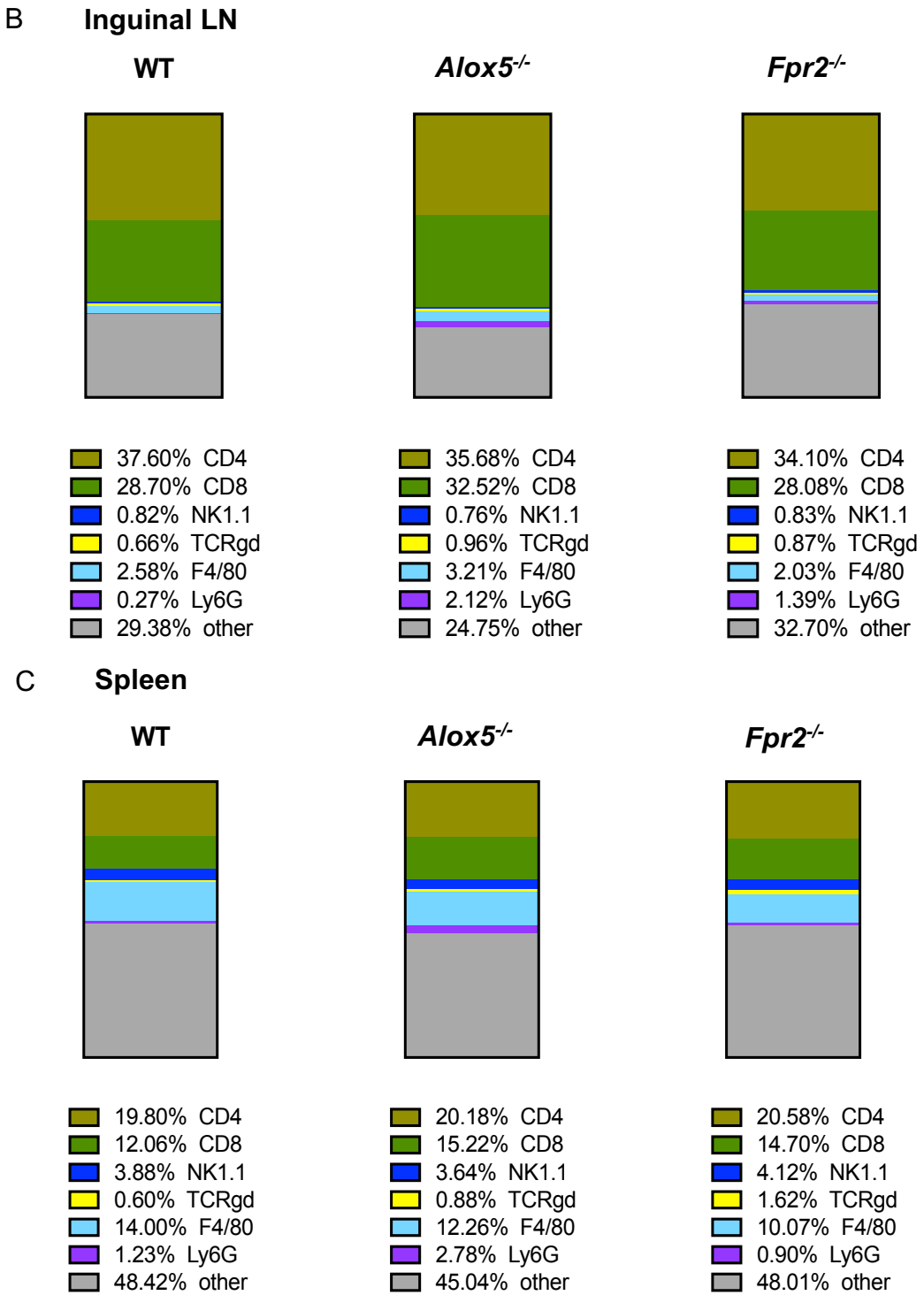


Figure 3.4. No difference in CD4$^+$ T cell frequency observed in the peripheral lymphoid tissues of naïve WT, *Fpr2*$^{-/-}$ and *Alox5*$^{-/-}$ mice. A-C) Peripheral lymphoid tissues were harvested from naïve mice and stained for cell surface markers by flow cytometry, n=5 per group. Frequency of immune cell populations in A) submandibular lymph nodes, B) inguinal lymph nodes, C) spleens.

To further assess the baseline CD4^{+} T cell phenotype, I analyzed the total number of cells, frequencies of CD4^{+}CD44^{hi}CD62L^{lo} memory T cell population and CD4^{+}CD44^{lo}CD62L^{hi} naïve T cell population in the peripheral lymphoid tissues of naïve mice. Across all tissues, WT and *Alox5*$^{-/-}$ mice exhibited similar total cell number and frequencies of these populations, except *Alox5*$^{-/-}$ mice had significant lower number of total cells in the inguinal lymph nodes compared to WT (Fig. 3.5 A-C). Cell populations in the naive *Fpr2*$^{-/-}$ mice appeared to deviate more from WT and *Alox5*$^{-/-}$, with significantly lower total cell number in the submandibular and inguinal lymph nodes (Fig. 3.5A), and significantly higher frequency of activated T cells in the submandibular lymph node and the spleen in comparison to naive WT mice (Fig. 3.5B), and significantly lower frequency of naïve T cells across all tissues in comparison to WT and *Alox5*$^{-/-}$ mice (Fig. 3.5C).

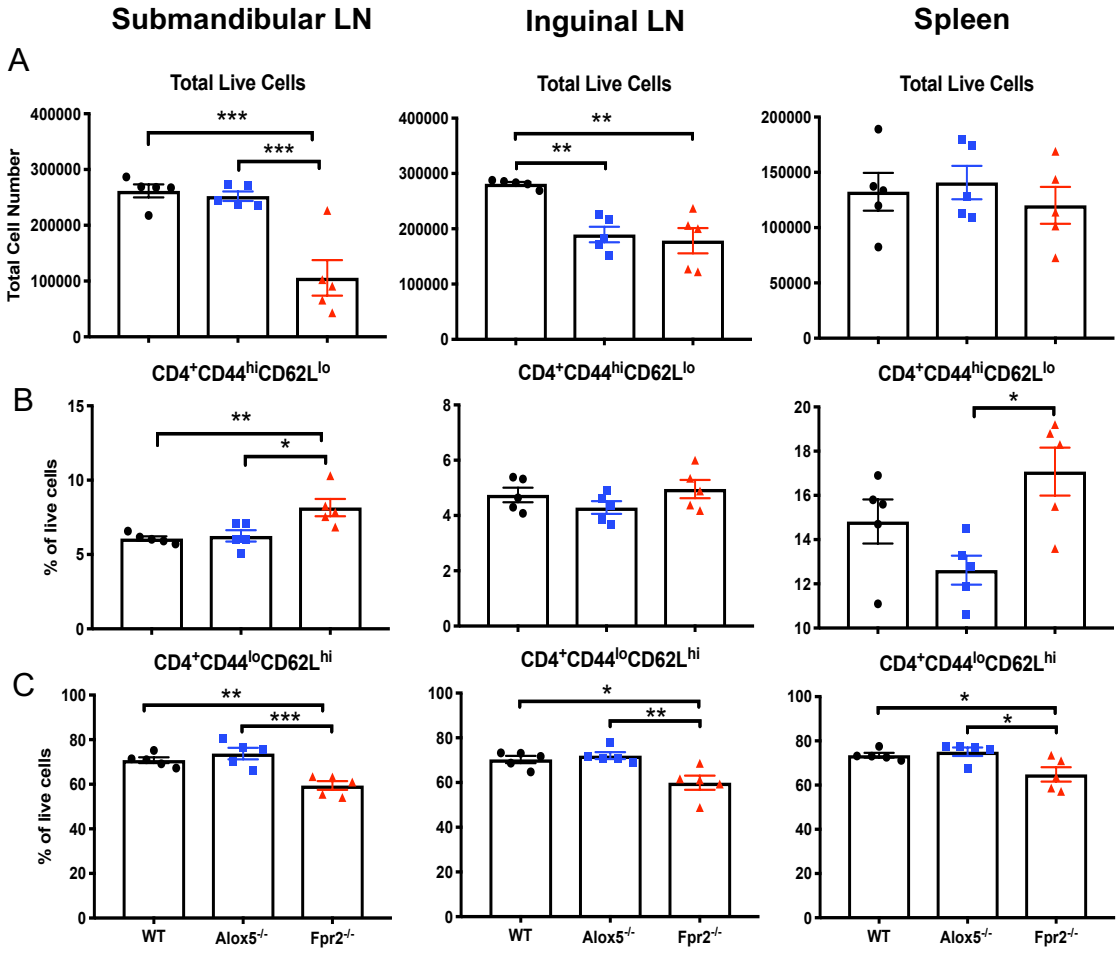


Figure 3.5. *Fpr2*$^{-/-}$ T cells are phenotypically distinct from WT and *Alox5*$^{-/-}$ T cells. A-C) Peripheral lymphoid tissues were harvested from naïve mice and stained for cell surface markers by flow cytometry, n=5 per group. A) Total cell number in submandibular lymph nodes, inguinal lymph nodes and spleens. B) Frequency of CD4+CD44hiCD62Llo T cells in submandibular lymph nodes, inguinal lymph nodes and spleens. C) Frequency

of CD4+CD44loCD62Lhi T cells in submandibular lymph nodes, inguinal lymph nodes and spleens.

Autoimmune uveitis is mediated by pathogenic Th1 and Th17 cell types. The phenotypic differences observed between the WT and *Alox5*$^{-/-}$ mice were in the neutrophil population, which do not play a major role in ocular infiltration and directly causing retina damage. However, cellular phenotypes in *Fpr2*$^{-/-}$ mice are more distinct from WT and *Alox5*$^{-/-}$, with more activated T cells in the lymph nodes. Based on the data from Fig. 3.1, we concluded that the LXA_4 circuit is significantly modulated during EAU and the data provided a rationale to pursue the second aim, where we investigate if LXA_4 can regulate T cell responses *in vivo* using the EAU model.

3.3.3. Experimental autoimmune uveitis induction in WT and knockout mice

In light of LXA_4 pathway modulation observed during EAU in Fig. 3.2, we induced EAU in age-matched LXA_4 receptor knockout mice (*Fpr2*$^{-/-}$), LXA_4 deficient mice (*Alox5*$^{-/-}$) and C57BL/6J wildtype (WT) mice by immunization with 150ug of $IRBP_{651-670}$ peptide emulsified in CFA. Surprisingly, clinical scores of retinal inflammation assessed by funduscopy of *Alox5*$^{-/-}$ were significantly higher than WT, while no difference was observed between *Fpr2*$^{-/-}$ and WT mice (Fig. 3.6). This result could be explained by several factors: both WT and *Alox5*$^{-/-}$ mice were purchased from Jackson Laboratory directly, and *Fpr2*$^{-/-}$ mice were transferred from University of Michigan. In the experiment, we did not use littermate controls and simply kept the homogenous knockout mice breeding program and used purchased age-matched C57BL/6J mice as controls. While the *Fpr2*$^{-/-}$ mice were developed on the B6 background, we cannot rule out the possibility of genetic drift or contamination of this line since we do not know the full history of how this mouse line was bred, what generation were the breeder mice we received, and when they were last backcrossed to the B6 mice to maintain genetic integrity. The *Fpr2*$^{-/-}$ had more activated T cell phenotype than the WT and *Alox5*$^{-/-}$ at naïve state, therefore it is possible that the threshold for *Fpr2*$^{-/-}$ T cells to become activated or pathogenic after EAU induction is higher than that of WT and *Alox5*$^{-/-}$ T cells.

Autoimmune uveitis is a relapse-remitting disease. In immunized WT mice, inflammation self resolves around day 21 post-immunization. Interestingly, both immunized *Alox5*$^{-/-}$ and *Fpr2*$^{-/-}$ mice exhibited a delay in resolution of ocular inflammation in comparison to immunized WT mice. This data suggests that impaired LXA_4 pathway impedes the resolution program of adaptive immune responses in EAU-challenged mice.

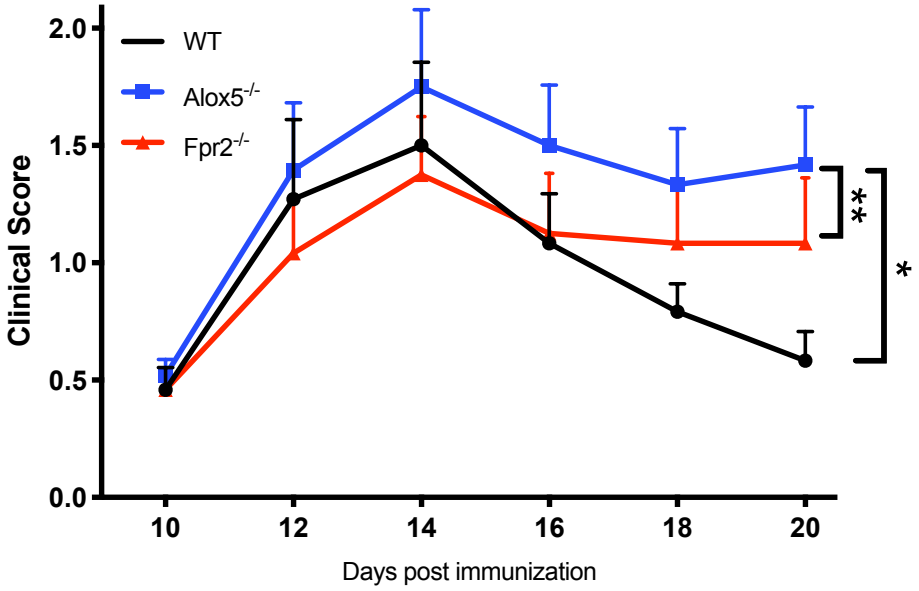


Figure 3.6. *Alox5*$^{-/-}$ mice develop worse EAU than WT. Funduscopy scores of WT, *Fpr2*$^{-/-}$, *Alox5*$^{-/-}$ mice immunized with 150ug of $IRBP_{651-670}$. n=6 per group, one representative experiment out of three. * $p < 0.05$, ** $p < 0.01$ Wilcoxon matched-pairs signed rank test.

3.3.4. LXA_4 deficiency exacerbates progression of autoimmune uveitis

Because of the disease phenotypic difference observed in Fig. 3.6, we decided to pursue the EAU model further only in WT and *Alox5*$^{-/-}$ mice. 5-LOX deletion results in LXA_4 deficiency *in vivo*, since 5-LOX is the rate limiting enzyme for LXA_4 generation (Fig. 3.7A). Age-matched *Alox5*$^{-/-}$ and WT mice were immunized with EAU. Disease scores assessed by funduscopy of *Alox5*$^{-/-}$ were significantly higher than WT starting on day 16 post-immunization, and the inflammation in *Alox5*$^{-/-}$ persisted while WT gradually resolved by day 20 post-immunization (Fig. 3.7B). Representative Micron III fundus images of WT and *Alox5*$^{-/-}$ mice depicted more severe inflammation in the *Alox5*$^{-/-}$ eye with retinal haze caused by cellular infiltration from the retina to the vitreous, as opposed to clear and defined retinal folds in the WT eye (Fig. 3.7C). Electroretinography showed that visual function of immunized *Alox5*$^{-/-}$ mice was significantly impaired compared to that of WT mice (Fig. 3.7D), correlating to more severe disease phenotype in *Alox5*$^{-/-}$ mice. Flow cytometric analyses of ocular infiltrating cells revealed that there were more viable cells, $CD4^+$, $CD4^+$ IFN-γ or $CD4^+$ IL-17A producing infiltrates in *Alox5*$^{-/-}$ mice on day 18 post-immunization than in WT mice (Fig. 3.8A, B). *Alox5*$^{-/-}$ exhibited higher antigen recall proliferation responses than WT as assessed by ^3H thymidine-incorporation assay of inguinal lymph node cells cultured in the presence of $IRBP_{651-670}$ peptide (Fig. 3.8C). These results suggest that uveitogenic T cells induce more severe EAU when the LXA_4 circuit is impaired *in vivo*.

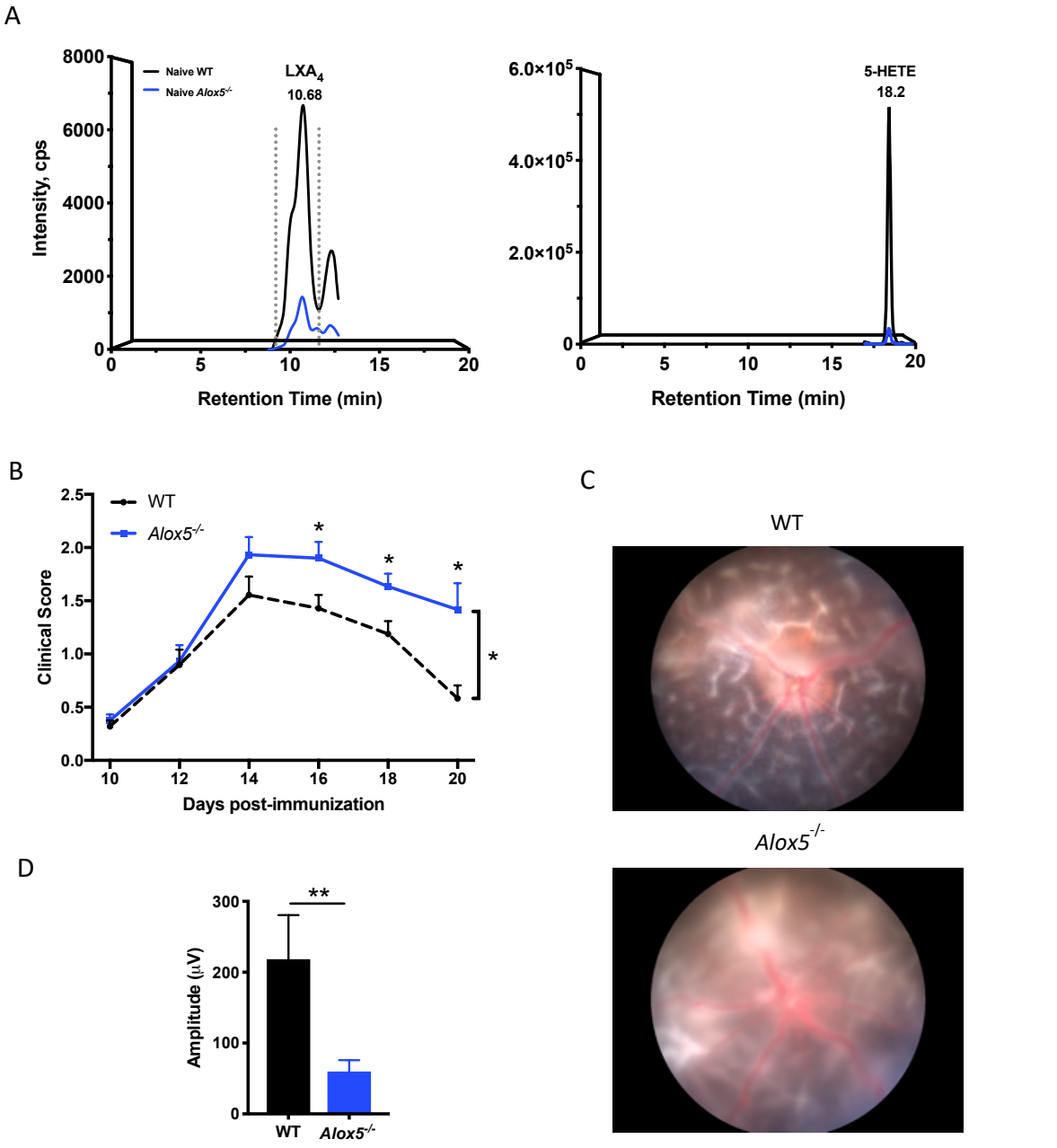


Figure 3.7. EAU-challenged LXA_4 deficient mice develop more severe EAU. A) Funduscopy scores of WT and *Alox5*$^{-/-}$ mice immunized with 150ug of $IRBP_{651-670}$. n=15 per group through day 18, n= 6-9 per group on day 20. Combined scores of two experiments. B) Representative Micron III fundus images of immunized WT and *Alox5*$^{-/-}$ mice taken on day 14 post-immunization. C) Electroretinography of b-wave of EAU-challenged WT and *Alox5*$^{-/-}$ mice measured on day 13 post-immunization following dark adaptation, n=10 eyes per group. * $p < 0.05$, ** $p < 0.01$ Wilcoxon matched-pairs signed rank test and Mann-Whitney test.

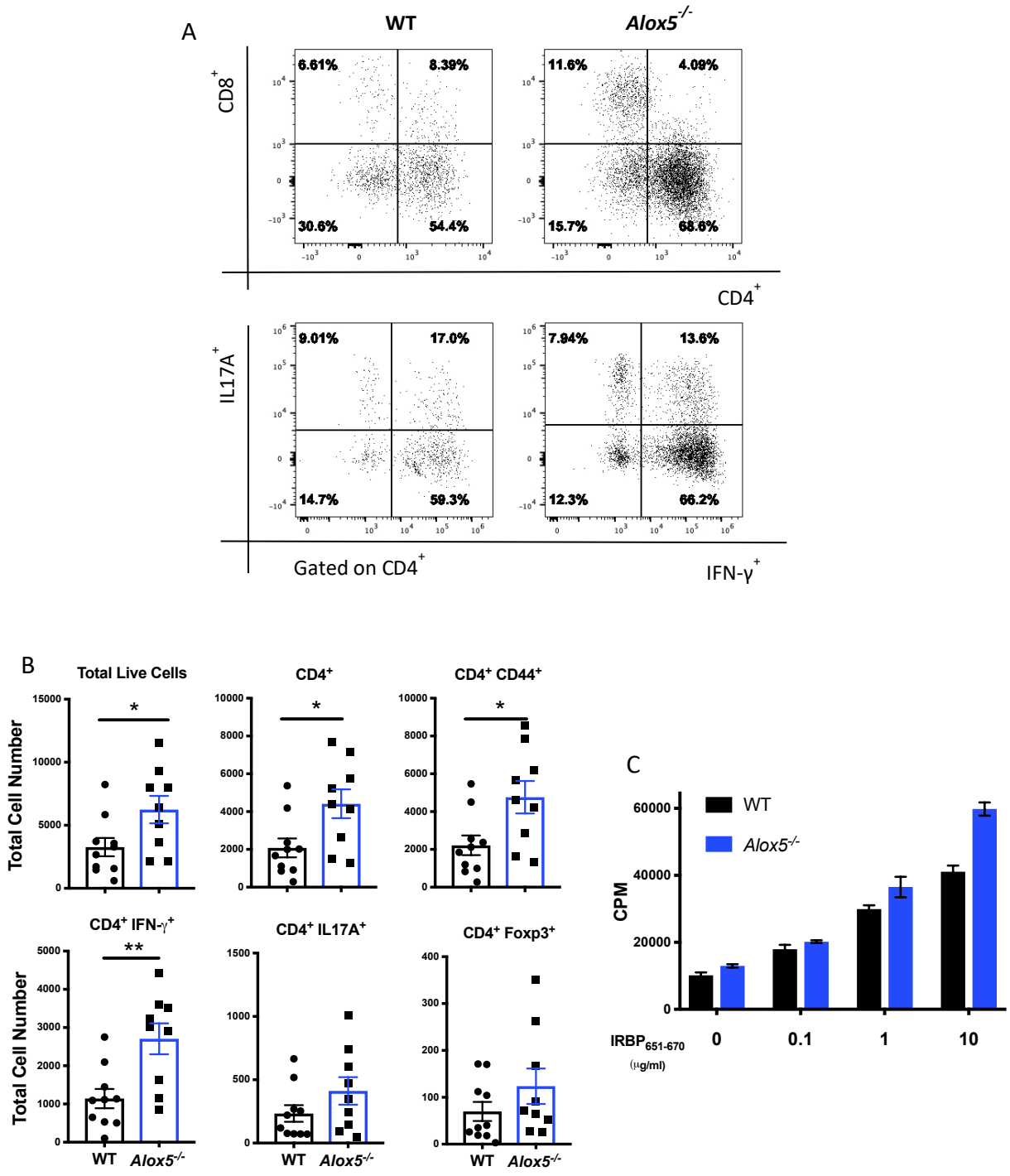
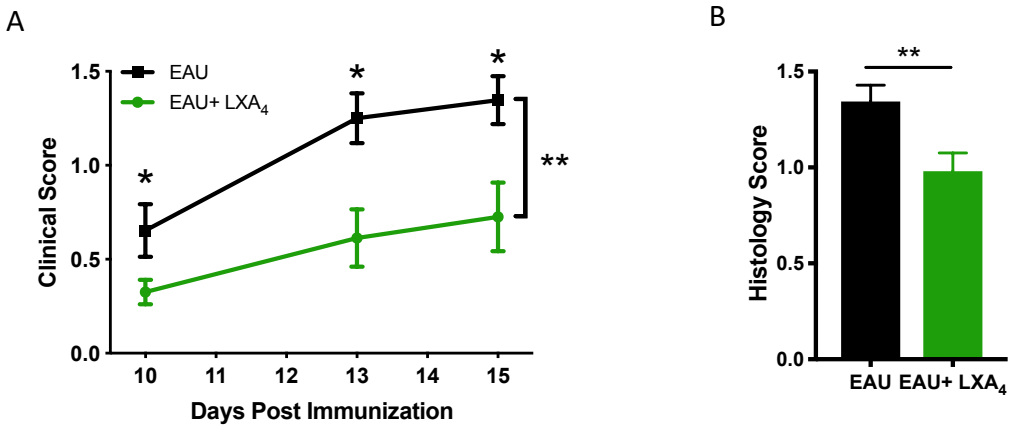


Figure 3.8. EAU-challenged LXA_4 deficient mice display significantly higher ocular infiltration and antigen-specific cell proliferation. A) Representative flow plots of $CD4^+$ vs. $CD8^+$ and IFN-γ vs. IL-17A producing $CD4^+$ cells in the eyes of WT and *Alox5*$^{-/-}$ mice. B) Flow cytometry analyses of total number of ocular infiltrates in EAU induced WT and *Alox5*$^{-/-}$ mice gated on live cells, $CD4^+$, $CD4^+$ $CD44^+$, $CD4^+$ IFN-γ^+, $CD4^+$ IL-17A$^+$ populations, n=9-10 per group. C) Representative bar graphs of tritiated thymidine

incorporation assay to measure antigen-specific cell proliferation performed on inguinal lymph nodes harvested at day 18 post-immunization from WT and *Alox5*$^{-/-}$ mice, pooled n=6-9 mice per group. Showing one representative from three repeat experiments with the same trend. * $p < 0.05$, ** $p < 0.01$, Mann-Whitney test.

3.3.5. LXA_4 treatment ameliorates the development of autoimmune uveitis

Since LXA_4 deficiency led to more severe disease, we next asked whether amplifying the LXA_4 circuit could limit EAU progression. To this end, WT mice were immunized and received 1ug of LXA_4 or vehicle control daily, starting on the day of immunization. LXA_4 treated immunized mice developed significantly less disease compared to the vehicle control treated group (Fig. 3.9A). Retinal histology assessed by H&E staining confirmed that the LXA_4 treated group had significantly less structural damage in comparison to vehicle control group (Fig. 3.9B). The eyes from the vehicle control treated group exhibited retinal barrier break down and cellular infiltration that contributed to retinal folds. In contrast, the posterior segment of the eye from LXA_4 treated immunized mice maintained retinal morphology similar to that of naïve unimmunized mice, with an exception of intravitreous cell infiltrates which are typical signs of mild uveitis (Fig. 3.9C). Prostaglandin E_2 (PGE_2) enzyme precursor cyclooxygenase-2 (COX-2) is widely associated with induced inflammation, therefore COX-2 was used as an inflammation marker in immunohistochemistry (IHC) for the retina. IHC revealed COX-2 localization in the outer plexiform, choroid, retinal pigmented epithelium and photoreceptor layers of the eye, while $CD4^+$ T cell infiltration was observed in the choroid, retinal pigment epithelium and into the photoreceptor layers (Fig. 3.9D). In immunized mice treated with LXA_4, the number of $CD4^+$ T cells and the level of COX-2 expression were significantly reduced in comparison to mice that received vehicle control, quantified by mean fluorescence intensity using Fiji software (Fig. 3.9E).



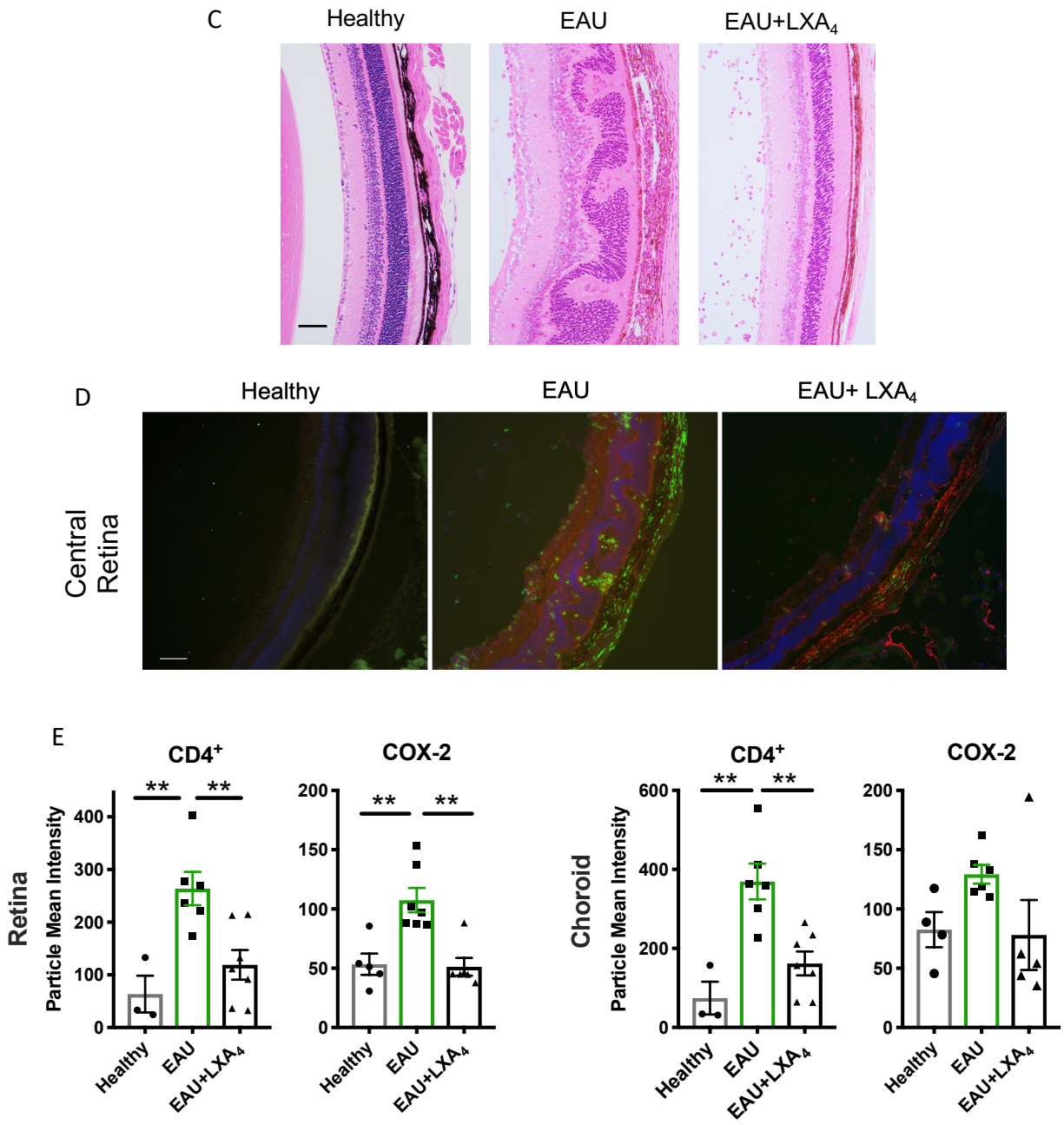


Figure 3.9. LXA_4 treatment ameliorates the development of uveitis. A) Mice were immunized with 300ug of $IRBP_{651-670}$ to induce EAU and treated with LXA_4 or vehicle control starting on the day of immunization. Clinical disease scores of retinal inflammation assessed by funduscopy, n=10 per group. B) Histology scores of eyes harvested from immunized mice treated with vehicle control or LXA_4 at peak inflammation (day 14-16 post-immunization), n=24 per group combined from 3 experiments. C) Representative H&E staining of the posterior segment of the eye from EAU-immunized mice that received vehicle control or LXA_4 treatment. 20X, scale bar= 50um. D) Representative immunohistochemistry images of eyes harvested at peak inflammation (day 14-16 post-immunization). Top row: central retina, bottom row:

peripheral retina. Green fluorescence: CD4-Alexa Fluor 488, red fluorescence: COX-2-Alexa Fluor 568, blue fluorescence: DAPI. 10X, scale bar= 100um. E) Fiji quantification of CD4 and COX-2 fluorescence particle intensity of immunohistochemistry images n=3-7 per group.

Th1 and Th17 are the pathogenic T cell subsets that contribute to EAU pathogenesis. Flow cytometry analyses of eyes harvested during peak inflammation confirmed that LXA_4 treatment significantly attenuated ocular infiltration, specifically pathogenic IFN-γ and IL-17A producing $CD4^+$ T cells (Fig. 3.10A, B).

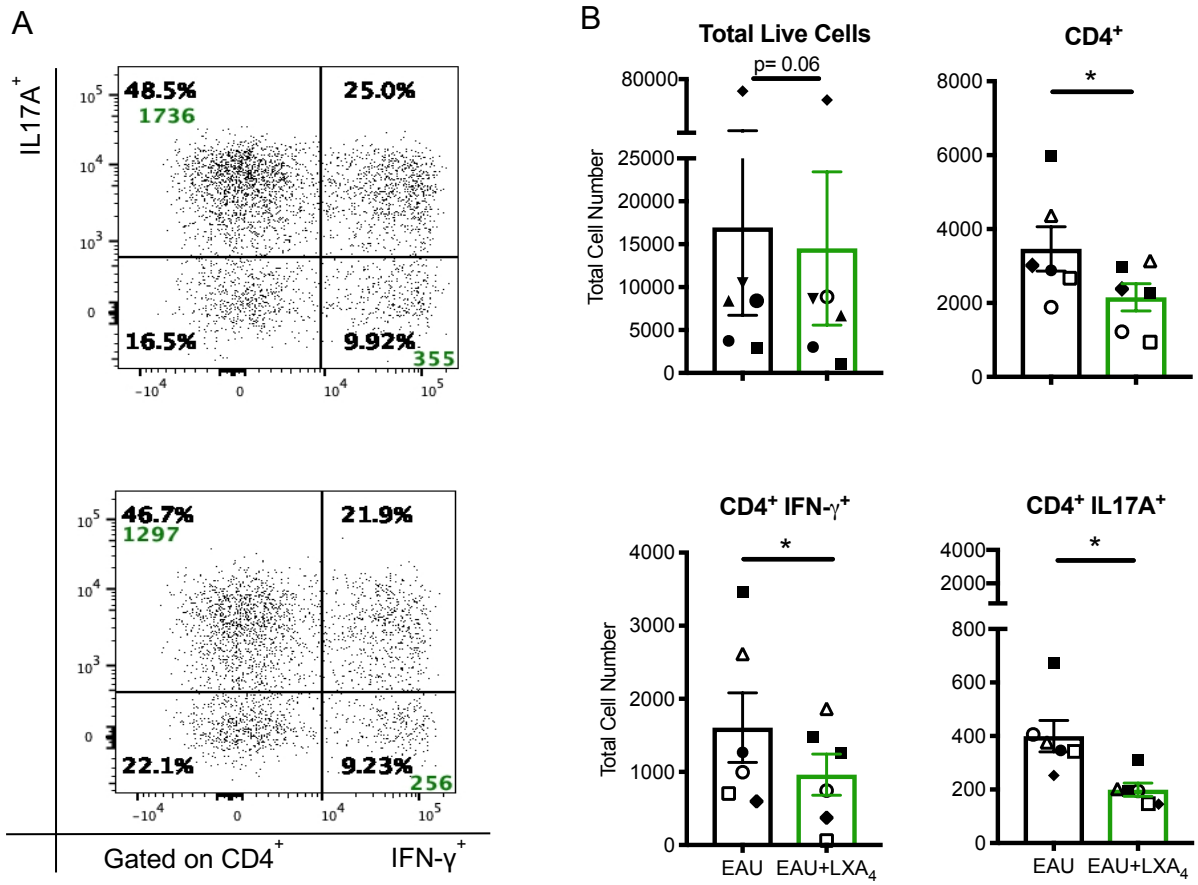


Figure 3.10. LXA_4 treatment reduces T cell infiltration into the eye. A) Representative flow plots of eyes harvested from immunized mice treated with vehicle control or LXA_4 at peak inflammation (day 16 post-immunization). Numbers in green are the total cell counts in the gated population. B) Total number of ocular infiltrates of indicated populations from EAU mice on day 14-16 post-immunization, treated with vehicle control or LXA_4, n=6 experiments (pooled 4-6 mice per group).

In accordance to disease severity, peripheral immune cells from the inguinal lymph nodes of vehicle treated controls proliferated dose-dependently to $IRBP_{651-670}$ peptide, while antigen recall responses from inguinal lymph nodes of LXA_4 treated mice were dampened (Fig. 3.11A). As LXA_4 treatment appeared to affect antigen recall

responses of T cells, we investigated whether LXA_4 could also regulate T cell responses during the effector phase using the T cell receptor transgenic mice specific for IRBP residues 161-180 (R161H) that develop spontaneous uveitis, with disease peaking around 2-3 months of age. Lymph node cells from CD90.1 congenic R161H mice were cultured with $IRBP_{161-180}$ peptide for three days *in vitro*, which induced about 70% of IFN-γ producing T cells (data not shown). 5x 10^6 $IRBP_{161-180}$ primed cells were injected intraperitoneally into WT CD90.2 recipient mice, that received LXA_4 or vehicle control treatment starting on the day of cell transfer. LXA_4 treated group developed significantly lower disease in a dose dependent manner, than vehicle control treated group (Fig. 3.11B), indicating that LXA_4 is able to deter ocular infiltration of antigen-primed cells, thus effectively employs its regulatory functions during the effector phase of the adaptive immune response.

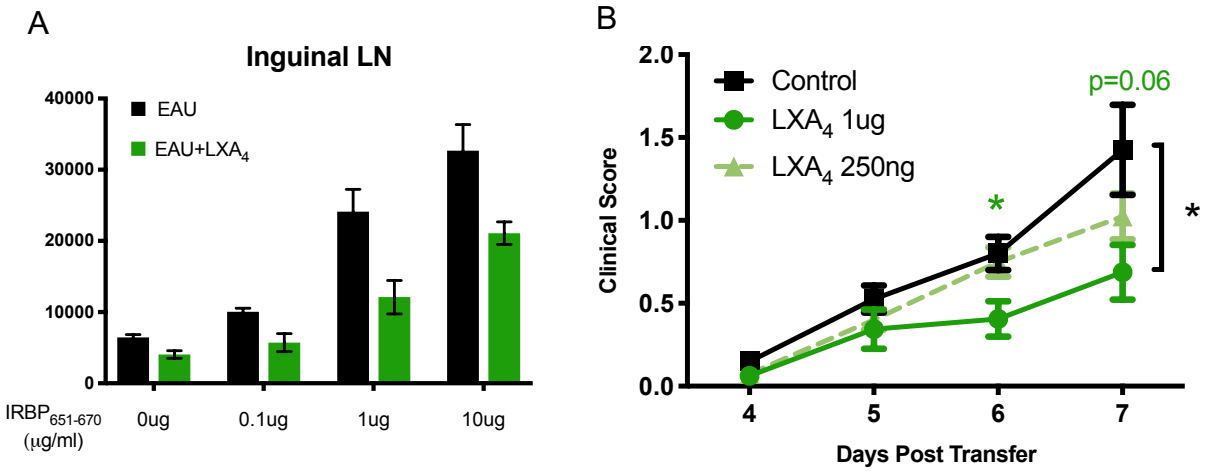


Figure 3.11. LXA_4 treatment dampens antigen-specific cell proliferation and effector T cell response. A) Bar graphs of tritiated thymidine incorporation assay performed on lymph nodes harvested on day 16 post-immunization from immunized mice treated with LXA_4 or vehicle, pooled n=6 mice per group. Representative assay of 3 separate experiments that showed the same trend. B) Funduscopy score of a representative adoptive transfer experiment of three. 5x10^6 R161H CD90.1 lymphocytes were activated with antigen $IRBP_{161-180}$ *in vitro* for 3 days and were transferred into WT CD90.1 recipients. Recipients received vehicle control or LXA_4 (1 μg or 250 ng) daily starting at the day of adoptive transfer. * $p < 0.05$, ** $p < 0.01$, Mann-Whitney test and One-way ANOVA.

3.3.6. LXA_4 modulates T cell trafficking marker expression in inguinal lymph nodes that drain the immunization site

To identify the genes that are potentially modulated by LXA_4 treatment, we compared RNA expression of the eye where the pathogenic T cell response is targeted to, and the inguinal lymph nodes where the immune response is initiated, from immunized mice treated with LXA_4 and vehicle control. RNA was extracted from tissues harvested on day 16 post-immunization and analyzed by nanoString. Upon EAU

induction, expression of inflammation markers, such as Icam1, Il1b, Mapk1, Mapk3, Nfkb1, Ptgs2, Tgfb, Tnfaip3 and Vcam1, were elevated locally in the eye. LXA_4 treatment downregulated expression of these genes. By contrast, expression of these inflammation markers was unchanged in the inguinal lymph nodes of LXA_4 or vehicle control treated EAU mice (Fig. 3.12A). These data suggest that systemic LXA_4 treatment reduces pathogenic T cell responses locally in the eye without affecting expression of inflammation-related genes in the periphery. As LXA_4 limits T cell migration into the eye (Fig. 3.9), we examined T cell homing marker C-C chemokine receptor type 7 (*Ccr7)*, its cognate ligands C-C motif chemokine ligand 19 (*Ccl19)* and 21 (*Ccl21a)*, as well as C-X-C motif chemokine receptor 3 (*Cxcr3),* Th1 chemokine receptor, and C-C chemokine receptor type 6 (*Ccr6),*Th17 chemokine receptor. In immunized mice that received LXA_4 treatment, *Ccr7* and *Ccl21a* were highly upregulated in the inguinal lymph nodes, where antigen-specific T cells are primed, in comparison to vehicle control treated group (Fig. 3.12B). In the eye, however, *Ccr7*, *Ccl19*, and *Cxcr3* were more upregulated in the vehicle control group compared to LXA_4 treated group (Fig. 3.12B). This correlated with higher number of $CD4^+$ and $CD4^+$ IFN-γ^+ infiltrates without LXA_4 treatment (Fig. 3.10). Difference in *Ccr6* expression was less discernable between the two groups. Supporting the data from Fig. 3.5 and Fig. 3.6 that show IFN-γ producing Th1 cells increased in the LXA_4 deficient immunized mice and decreased upon LXA_4 treatment, and that Th1 cells are the major populations infiltrating the eye as opposed to Th17 cells. We next assessed the gene expression of sphingosine-1 phosphate receptor 1 (*S1pr1*), which regulates lymphocyte egress from lymphoid tissues, by q-PCR to evaluate the effect of LXA_4 treatment on T cell trafficking. The expression of *S1pr1* was similar between LXA_4 and vehicle control treated groups in the eye, submandibular lymph nodes, and axillary lymph nodes (Fig. 3.12C). However, upon LXA_4 treatment the expression of *S1pr1* was downregulated in the inguinal lymph nodes (Fig. 3.12C). To examine whether downregulation of *S1pr1* is specifically detected in uveitis-relevant T cells, we assessed the *S1pr1* expression in $CD4^+$ T cells from inguinal lymph nodes of EAU-challenged *Alox5*$^{-/-}$ and WT mice by q-PCR. Although *S1pr1* expression appeared higher in $CD4^+$ T cells from LXA_4 deficient mice than WT mice (Fig. 3.12D), it did not reach statistical significance. Together, these data suggest that LXA_4 treatment upregulates *Ccr7* and *Ccl19* expression and downregulates *S1pr1* expression in the inguinal lymph nodes, thereby effectively prevents T cells from emigrating out of the lymph node that drains the sites of immunization into the target tissue.

Next, we asked whether LXA_4 deficiency would promote migratory functions of $CD4^+$ T cells from secondary lymphoid tissues. $CD4^+$ T cells were isolated from inguinal lymph nodes and spleens of EAU-challenged WT and *Alox5*$^{-/-}$ mice on day 13 post-immunization then *in vitro* stimulated with anti-CD3 and anti-CD28 antibodies for 18 hours prior to the transwell migration assay. Significantly higher number of $CD4^+$ T cells from *Alox5*$^{-/-}$ mice migrated toward CCL19 and CCL21 chemokine gradient than $CD4^+$ T cells from WT mice (Fig. 3.12E). These results indicate that absence of tissue resident LXA_4 enhances T cell migration out of disease priming lymph nodes and spleen, thereby amplifying inflammation in the eye (Fig. 3.7).

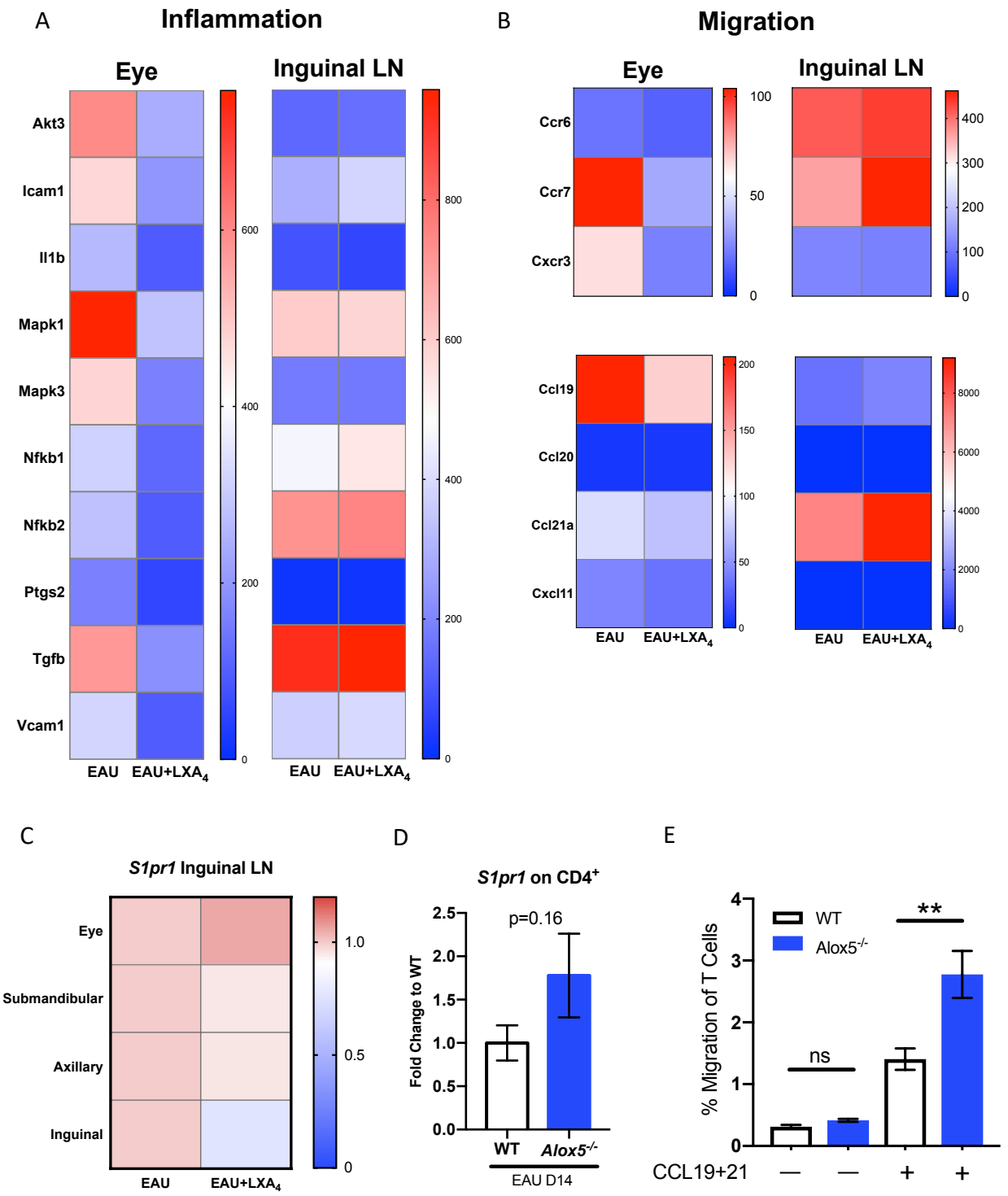


Figure 3.12. LXA_4 modulates T cell trafficking marker expression in inguinal lymph nodes that drain the immunization site. All tissues were collected from mice immunized with 150ug of $IRBP_{651-670}$. A-B) Heat maps showing gene expression of inflammation and cell trafficking markers in the eye and inguinal lymph node harvested

from EAU mice treated with vehicle control or LXA_4. (A) inflammation markers, and (B) chemokine receptors and ligands as trafficking markers analyzed by nanoString. Each sample was pooled from 3-4 mice on day 16 post-immunization. C) Relative expression of *S1pr1* from eyes and various lymph nodes of day 16 post-immunization mice treated with LXA_4 or vehicle control, pooled 3-4 mice per group from the same experiment in panels A and B. D) Relative expression of *S1pr1* in $CD4^+$ T cells isolated from WT and *Alox5*$^{-/-}$ mice on day 14 post-immunization. N=11 from two experiments combined. E) Transwell migration assay of $CD4^+$ T cells isolated from immunized WT and *Alox5*$^{-/-}$ mice on day 13 post-immunization. $CD4^+$ T cells were pre-stimulated with anti-CD3 and anti-CD28 antibodies for 18 hours, and transferred to transwell and incubated for 4 hours in the absence or presence of CCR7 ligands, CCL19 and 21. n=12 per group, one representative experiment from 4 experiments showing the same trend. ** $p < 0.01$, Mann-Whitney test.

3.3.7. Disease limiting effect of LXA_4 is T cell mediated

We employed the T cell adoptive transfer model to examine whether enhanced EAU in *Alox5*$^{-/-}$ mice is a T cell mediated effect. We immunized WT and *Alox5*$^{-/-}$ mice to expand IRBP-specific T cells, and purified $CD3^+$ T cells from inguinal lymph nodes and spleens on day 11 and respectively transferred into *TCRβ*$^{-/-}$ mice that are deficient of $CD4^+$ T cells. These *TCRβ*$^{-/-}$ recipient mice were immunized three weeks after cell transfer (Fig. 3.13A). Similar to the findings in active immunization-induced EAU in WT and *Alox5*$^{-/-}$ mice (Fig. 2.7), funduscopy scores revealed that recipients of *Alox5*$^{-/-}$ donor T cells developed more severe EAU than that of WT donor T cells (Fig. 3.13B). Retinal histology showed that recipients of *Alox5*$^{-/-}$ donor T cells had more granulomatous lesions and damage in the photoreceptor layer than that of WT donor T cells (Fig. 3.13C). To elucidate the T cell effector phenotype and the T cell trafficking marker expression in recipients of WT or *Alox5*$^{-/-}$ donor T cells, donor T cells were harvested from the eyes and inguinal lymph nodes of *TCRβ*$^{-/-}$ recipient mice on day 20 post-immunization and analyzed by flow cytometry. $CD4^+$ T cells from *Alox5*$^{-/-}$ donors, in particular IFN-γ producing $CD4^+$ T cells, infiltrated significantly more into the recipients' eyes than WT donor T cells (Fig. 3.14A), while there was no difference in trafficking marker expression between *Alox5*$^{-/-}$ and WT donor T cells once they infiltrated into the target tissue (Fig. 3.14A). In the inguinal lymph node, there was no difference in the frequency of $CD4^+$ T cells or in activation status of T cells ($CD4^+$ $CD44^+$) and the frequency of IL-17A producing $CD4^+$ effector T cells between WT and *Alox5*$^{-/-}$ donor T cells, however the frequency of IFN-γ producing $CD4^+$ effector T cells was higher in *Alox5*$^{-/-}$ donor T cells than WT donor T cells (Fig. 3.14B).

To confirm that the modulation of CCR7 observed in nanoString analyses could be attributed to T cells, we examined chemokine receptor expression on donor T cells from WT or LXA_4 deficient mice. In the eye, the frequency of activated T cells and chemokine receptor expression are similar between donor T cells from WT or LXA_4 deficient mice (Fig. 3.15A). However, in the inguinal lymph nodes the frequency of activated $CD4^+$ $CXCR3^+$ population was upregulated in T cells from LXA_4 deficient donors in comparison to T cells from WT donors while the frequency of activated $CD4^+$

CCR7^{+} was significantly lower on T cells from LXA_4 deficient donors (Fig. 3.15B). These results indicate that effector Th1 cells from LXA_4 deficient lymph nodes produce more IFN-γ cytokines and exhibit higher effector function due to downregulation in Ccr7 expression, therefore more efficient in migrating out of the lymph nodes and triggering inflammation in the eye in the absence of LXA_4 circuit.

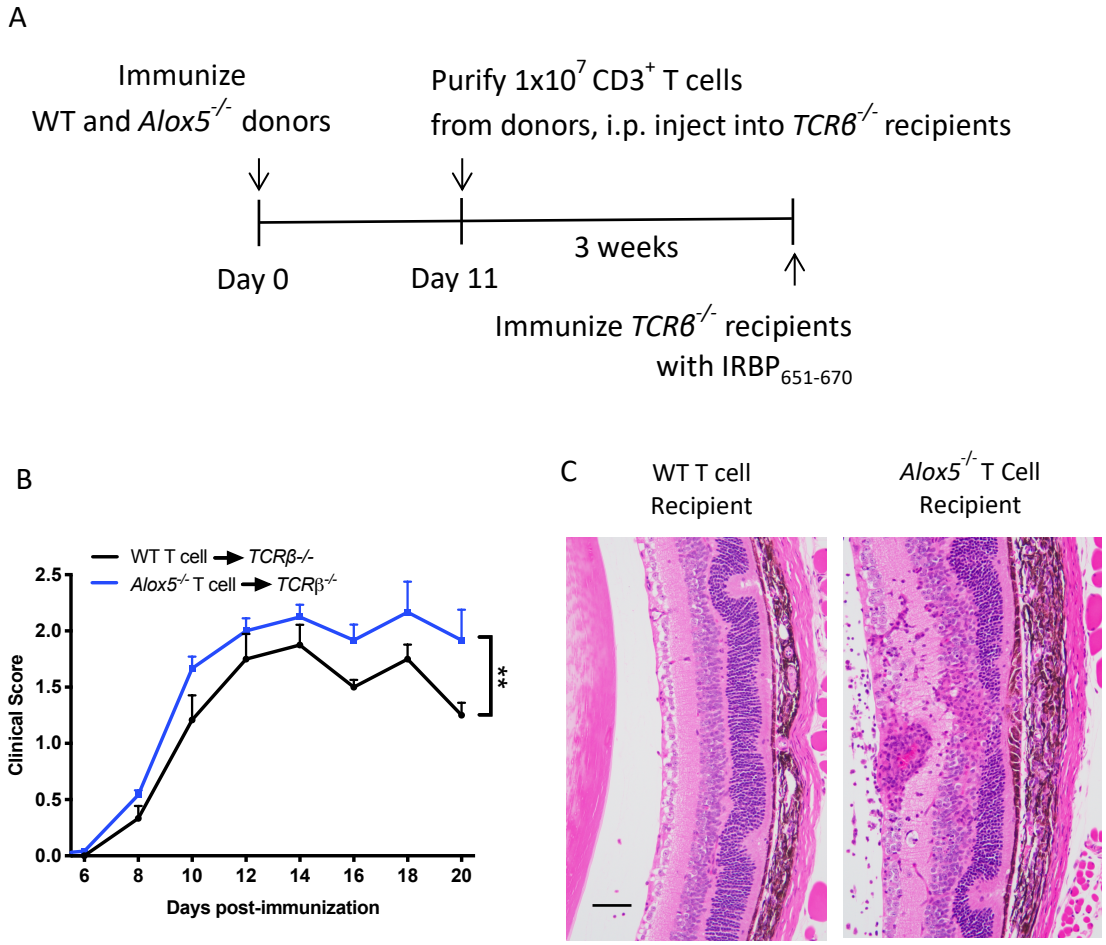


Figure 3.13. Disease limiting effect of LXA_4 is T cell mediated. A) Scheme of T cell transfer. Following EAU induction, 1x 10^7 CD3^{+} T cells were enriched from inguinal lymph nodes and spleens of WT or *Alox5*$^{-/-}$ mice and injected i.p. into *TCRβ*$^{-/-}$ mice. B) Funduscopy scores of WT or *Alox5*$^{-/-}$ T cell-transferred *TCRβ*$^{-/-}$ recipients immunized with EAU, n=6 mice per group. C) Representative H&E histology of the posterior segment of the eye from *TCRβ*$^{-/-}$ recipients of WT or *Alox5*$^{-/-}$ T cells, scale bar= 50uM. ** $p < 0.01$, Wilcoxon matched-pairs signed rank test.

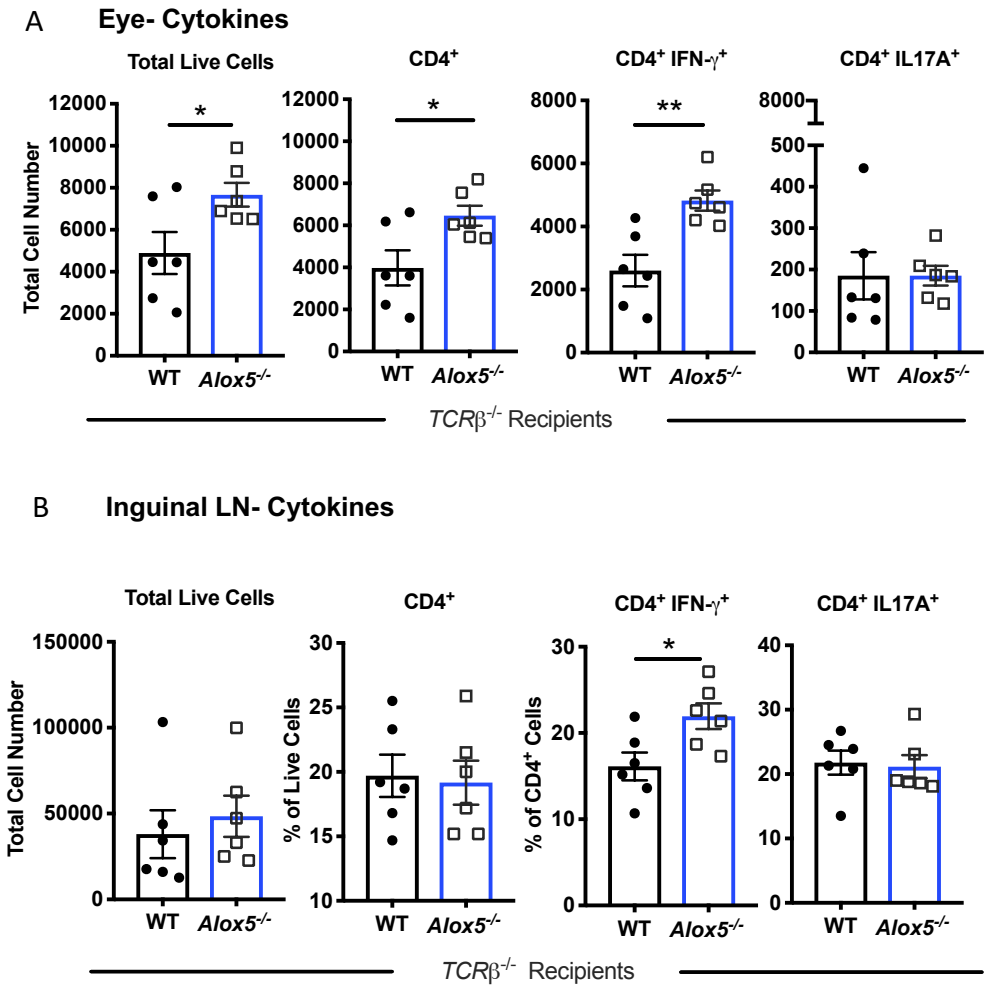


Figure 3.14. Recipients of T cells isolated from LXA_4 deficient mice have higher number of ocular infiltrates in comparison to WT T cell recipients. Flow cytometry analysis of total number of live cells and frequencies of $CD4^+$ T cell populations producing IFN-γ or IL-17A in A) eyes and B) inguinal lymph nodes of WT and *Alox5*$^{-/-}$ T cell recipients, n=6 per group. * $p < 0.05$, ** $p < 0.01$, unpaired Welch's t test.

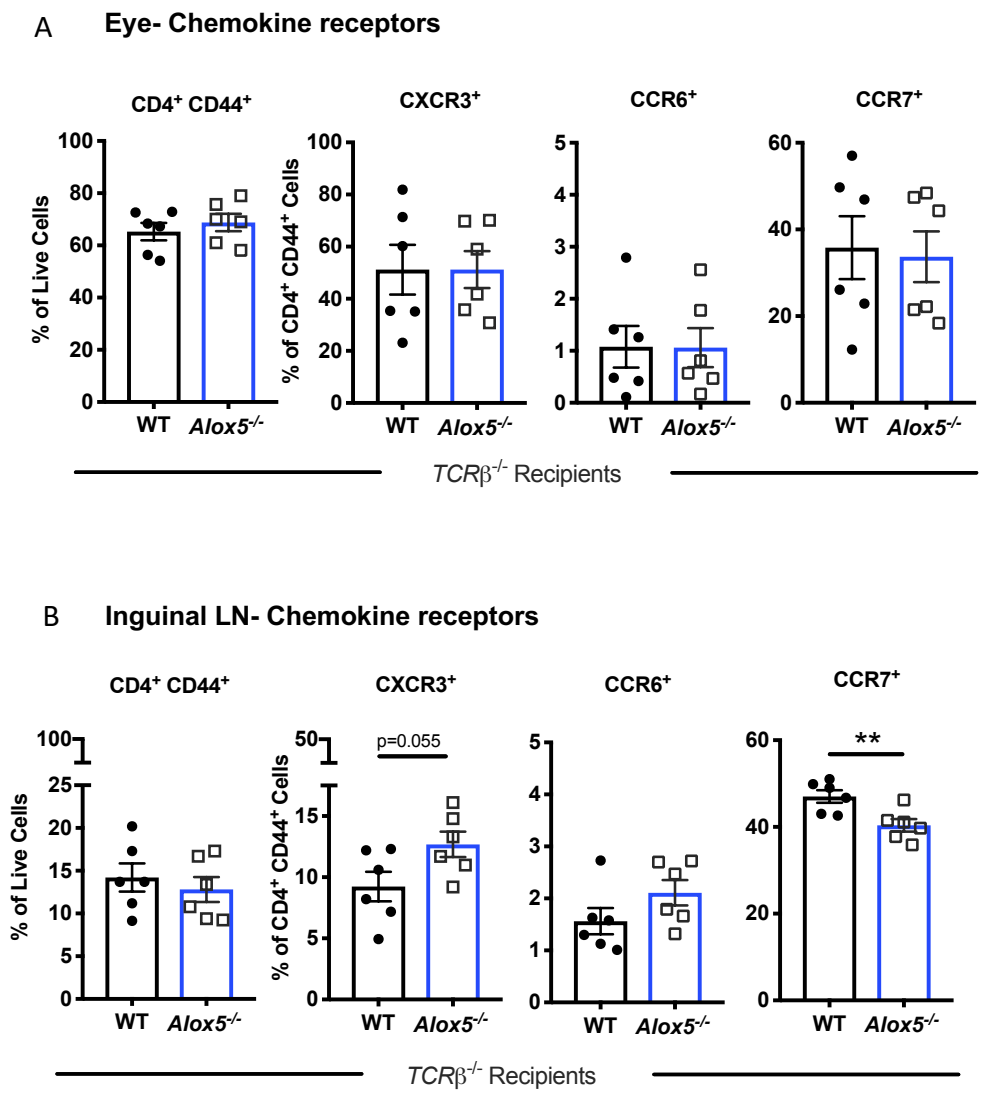
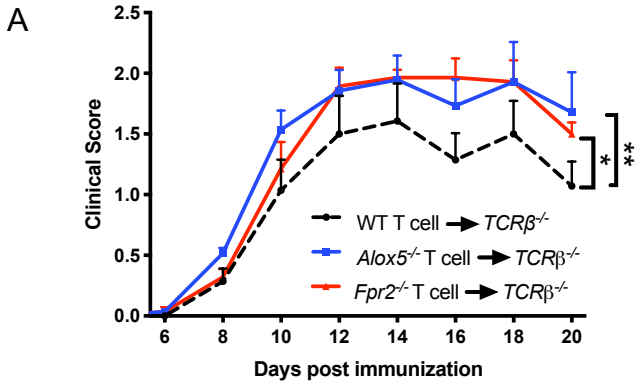
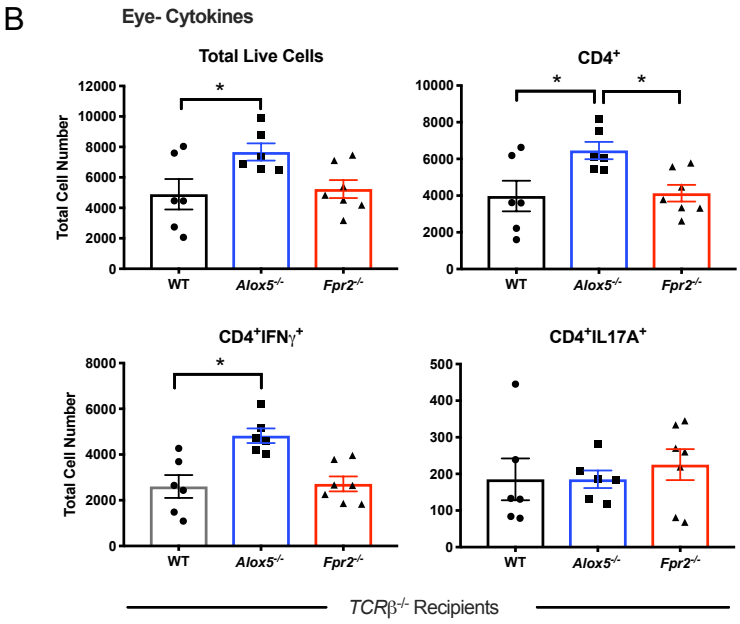


Figure 3.15. Ccr7 expression is downregulated on T cells from LXA_4 deficient donors. Flow cytometry analysis of chemokine receptor expression on activated $CD4^+$ T cells in A) the eye and B) inguinal lymph nodes of *TCRβ*$^{-/-}$ recipients of WT and *Alox5*$^{-/-}$ T cells, n=6 per group. ** $p < 0.01$, unpaired Welch's t test.

Using the same method in Fig. 3.13A, we also transferred *Fpr2*$^{-/-}$ T cells along with WT and *Alox5*$^{-/-}$ T cells into *TCRβ*$^{-/-}$ recipients to see whether T cells unable to receive LXA_4 signaling would induce more severe EAU. Funduscopy scores revealed that recipients *Fpr2*$^{-/-}$ donor T cells developed more severe EAU than that of WT donor T cells (Fig. 3.16A). However flow cytometry analysis showed that the disease phenotype observed in recipients of *Fpr2*$^{-/-}$ donor T cells was not mediated by T cells since the total cell numbers, number of $CD4^+$, $CD4^+$IFN-γ^+ and $CD4^+$IL-$17A^+$ cells in the eye are similar between WT and *Fpr2*$^{-/-}$ T cell recipients (Fig. 3.16B). There appeared to

be a slight increase, but not statistically significant, in the number of neutrophils and macrophages ocular infiltrates in *Fpr2*$^{-/-}$ T cell recipients in comparison to WT and *Alox5*$^{-/-}$ T cell recipients (Fig. 3.16C). This data suggests that increase in ocular inflammation of *Fpr2*$^{-/-}$ T cell recipients could potentialy be caused by more efficient recruitment of innate immune cells during EAU.





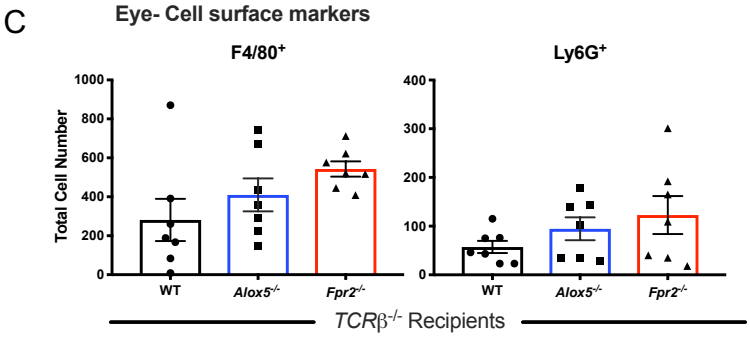


Figure 3.16. Disease phenotype in *Fpr2*$^{-/-}$ T cell recipients is not T cell mediated. A) Funduscopy scores of WT, *Alox5*$^{-/-}$ or *Fpr2*$^{-/-}$ T cell-transferred *TCRβ*$^{-/-}$ recipients immunized with EAU, n=6 mice per group. B) Flow cytometry analysis of total number of live cells and frequencies of CD4^{+} T cell populations producing IFN-γ or IL-17A in the eyes of WT, *Alox5*$^{-/-}$ and *Fpr2*$^{-/-}$ T cell recipients, n=6 per group. C) Flow cytometry analysis of macrophage and neutrophil populations in the eyes of WT, *Alox5*$^{-/-}$ and *Fpr2*$^{-/-}$ T cell recipients, n=6 per group. * p =0.05, ** p < 0.01, Wilcoxon matched-pairs signed rank test and One-way ANOVA.

3.3.8. The LXA_4 circuit regulates effector function and metabolism of CD4^{+} T cells

T cell metabolic programming closely shapes T cell development and responses. Upon TCR stimulation, activated T cells engage in aerobic glycolysis to support effector T cell growth and function (Almeida et al., 2016; Buck et al., 2015). Since our results show that LXA_4 regulates the migration, proliferation and effector function of CD4^{+} T cells, we investigated whether LXA_4 deficiency impacts T effector cell metabolism. To correlate the observed amplified effector function of T cells in absence of LXA_4 (Fig. 3.14) to glycolysis, CD4^{+} T cells were isolated from inguinal lymph nodes and spleens of EAU-challenged WT and *Alox5*$^{-/-}$ mice and stimulated for 18 hours with anti-CD3 and anti-CD28 antibodies, then subjected to Seahorse Glycolytic Rate Assay. CD4^{+} T cells from LXA_4 deficient mice achieved significantly higher glycolytic proton efflux rates (glycoPER) at baseline and after inhibiting the mitochondrial electron transport chain with rotenone and antimycin A (Rot/AA) (Fig. 3.17A). To accurately quantify glycolysis-derived ECAR, mitochondrial CO_2 contributed acidification was measured and subtracted, resulting in proton efflux rate specific to glycolysis (glycoPER). CD4^{+} T cells from inguinal lymph nodes deficient of LXA_4 exhibited higher basal glycoPER (Fig. 3.17B), and higher compensatory glycoPER, the maximum glycoPER following Rot/AA injection (Fig. 3.17C). To assess the direct effect of LXA_4 on T cell glycolysis, CD4^{+} T cells were isolated from inguinal lymph nodes and spleens from WT mice and treated *in vitro* with LXA_4 or vehicle control. The treated T cells were stimulated for 18 hours with anti-CD3 and anti-CD28 antibodies, then subjected to Seahorse Glycolytic Rate Assay. CD4^{+} T cells treated with LXA_4 had significantly lower glycoPER at base line and after mitochondrial inhibition (Fig. 3.17D), as well as lower basal (Fig. 3.17E) and compensatory (Fig. 3.17F) glycolysis. The higher glycolytic activities of T cells isolated from LXA_4 deficient environment and the reduction in T cell glycolysis after *in vitro* treatment with LXA_4 indicate that the LXA_4 circuit plays an important role in metabolically reprogramming glycolysis and regulating activated effector T functions.

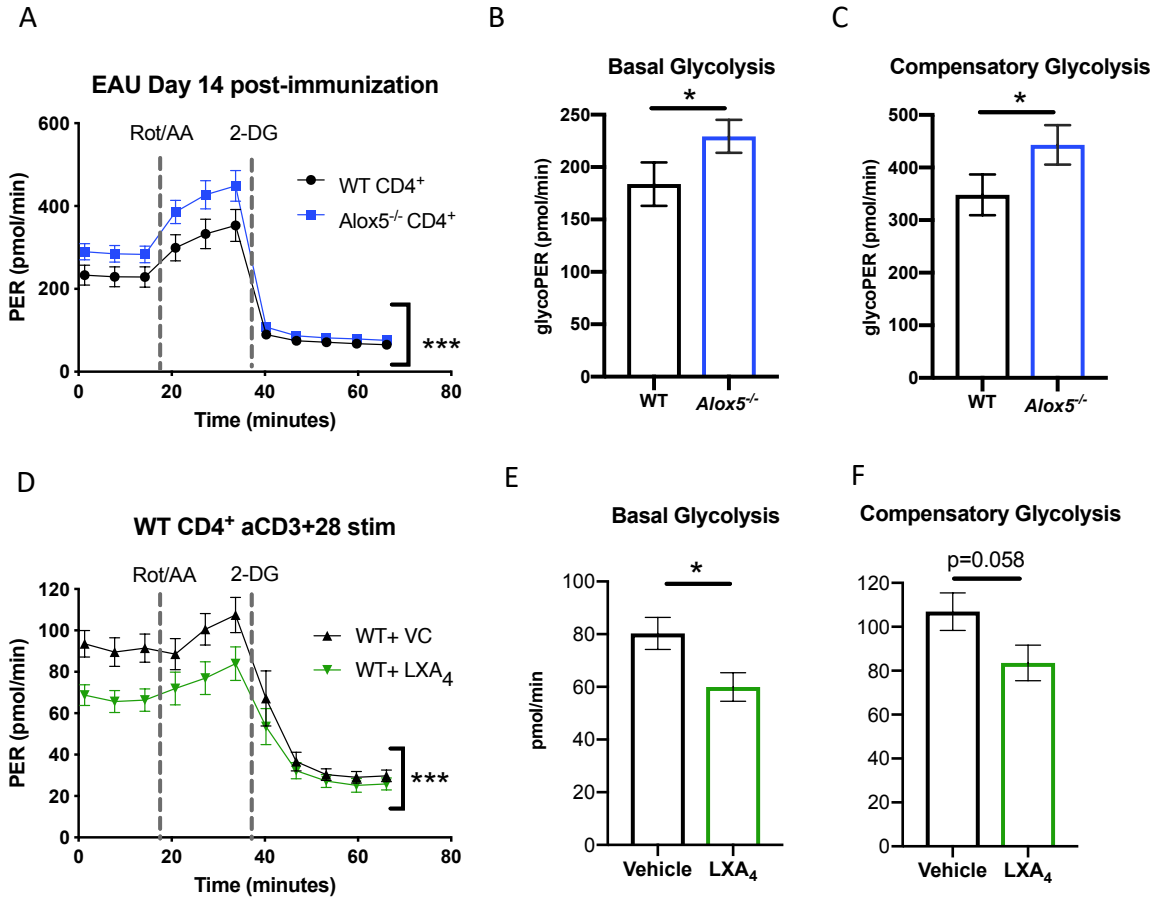


Figure 3.17. The LXA_4 circuit regulates effector function and metabolism of $CD4^+$ T cells. Inguinal lymph nodes and spleens were harvested from EAU-immunized WT and *Alox5*$^{-/-}$ mice on day 13 post-immunization. A) $CD4^+$ T cells isolated from immunized WT and *Alox5*$^{-/-}$ mice were stimulated with anti-CD3 and anti-CD28 antibodies for 18 hours then subjected to glycolytic rate assay by Seahorse analyzer, n=12 mice per group. Bar graphs of B) basal glycolysis and C) compensatory glycolysis from the glycolytic rate assay in A). D) WT $CD4^+$ T cells isolated from immunized WT and *Alox5*$^{-/-}$ mice were treated with vehicle control or LXA_4 *in vitro* and stimulated with anti-CD3 and anti-CD28 antibodies for 18 hours then subjected to glycolytic rate assay by Seahorse analyzer, n=4 mice per group. Bar graphs of E) basal glycolysis and F) compensatory glycolysis from the glycolytic rate assay in D). * $p < 0.05$, *** $p < 0.001$, Wilcoxon matched-pairs signed rank test and Mann-Whitney test.

3.4. Discussion

In this study, we demonstrated that the tissue resident LXA_4 circuit is essential for regulating T effector cell functions during EAU pathogenesis. The temporal expression of the LXA_4 circuit was mapped through the course of EAU development and revealed disease- and tissue-dependent modulation of the pathway. Deletion of LXA_4 in mice led

to exacerbated EAU pathogenesis and intensified T cell effector functions by facilitating more $CD4^+$ and $CD4^+$ IFN-γ producing T cell infiltration into the eye, while treatment with LXA_4 during EAU mitigated disease development. The therapeutic effects of LXA_4 limited pathogenic T cell infiltration into the eye by upregulating the expression of *Ccr7* and downregulating the expression of *S1pr1* on $CD4^+$ T cells in the inguinal lymph nodes, thereby limiting T cell egress. T effector cell function appeared to be sustained by metabolic reprogramming toward glycolysis in the absence of LXA_4 signaling. These findings indicate that the LXA_4 circuit regulates effector function and migration of autoreactive T cells in a T cell-driven autoimmune disease.

This is the first report that identifies the molecular mechanism of LXA_4 regulating T cell function and limiting T cell egress out of peripheral lymph nodes in the setting of autoimmune uveitis involving the adaptive immune mechanisms. We employed *Alox5*$^{-/-}$ mice to establish that disruption of the LXA_4 circuit leads to more severe disease in EAU. In the T cell transfer model into *TCR*$\beta^{-/-}$ hosts, we demonstrate that LXA_4 deficiency leads to pathogenic and dysregulated T effector cell function. In addition to its widely recognized role in regulating innate immune cell function, this study further establishes the importance of the LXA_4 tissue resident circuit in maintaining homeostasis under the healthy conditions, and the new paradigm of LXA_4 regulation of T lymphocyte effector function.

CCR7 is the key molecule that direct T cell homing into secondary lymphoid organs toward its sole ligands CCL19 and CCL21, with CCL21 being the primary ligand expressed in secondary lymphoid organs. Once T cells are in the lymph nodes, the CCR7-CCL19/CCL21 axis facilitates the interaction between T cells and dendritic cells (DC) to induce peripheral tolerance or to elicit an adaptive immune response (Comerford et al., 2013). Our nanoString data support previous findings that showed during neuroinflammation CCL19 expression becomes upregulated (Krumbholz et al., 2007), with increase of CCR7 expression on the infiltrating immune cells (Kivisakk et al., 2004) in CNS lesions of multiple sclerosis patients. While we did not investigate the source of CCL19 ligand expression, endothelia, macrophages, microglia, astrocytes and $CD3^+$ T cells were shown to contribute to increase CCL19 and CCL21 ligand expression in the lesions of the experimental autoimmune encephalymyelitis (EAE) model (Alt et al., 2002; Bielecki et al., 2015). In several studies, the pleiotropic lipid mediator prostaglandin E_2 (PGE_2) was shown to induce CCR7 expression on mature DC along with reduced CCL19 expression, affecting their migratory potential (Muthuswamy et al., 2010; Scandella et al., 2002). In the present study, we show that LXA_4 treatment can induce CCR7 expression in inguinal lymph nodes that drain the site of immunization, and that LXA_4 deletion *in vivo* results in downregulated CCR7 expression on T cells. As CCR7 is also essential in regulating DC migration and function, it will be important to assess whether LXA_4 modulates CCR7 expression on DC and how LXA_4 affects DC priming of T cells. Collectively, these findings provide evidence that lipid mediators can direct T cell migration through modulation of the CCR7-CCL19/CCL21 axis, and offer insights to the undetermined mechanisms of how CCR7 expression is regulated.

T cell metabolism is vital in regulating T effector cell function. Metabolic switch from oxidative phosphorylation to glycolysis is required to sustain T effector function and cytokine production (Coe et al., 2014; Salmond, 2018). IFN-γ production by activated $CD4^+$ T cells is dramatically reduced when blocked from engaging in glycolysis, implicating glycolysis could be a potential target for controlling T effector responses (Chang et al., 2013). It has previously been shown that T cells of systemic lupus erythematosus patients have elevated glycolytic metabolism in comparison to non-autoimmune subjects, and that inhibition of $CD4^+$ T cell glycolysis reduced IFN-γ production and disease biomarkers (Yin et al., 2015). Our data show that the absence of tissue-resident LXA_4 pathway increases glycolytic responses of $CD4^+$ T cells, which in turn upregulates downstream IFN-γ production by $CD4^+$ T effector cells during EAU. Downregulation of activated $CD4^+$ T cell glycolysis though LXA_4 treatment suggests that the LXA_4 circuit can "de-license" T effector cells by diminishing their ability to engage in glycolysis, leading to reduction in pathogenic cytokine production and magnitude of inflammation.

Sphingosine-1 phosphate (S1P) is a lipid mediator that directs lymphocyte recirculation through G protein-coupled S1P receptors. Modulation of *S1pr1* by FYT720 prevents trafficking of autoreactive T cells into the central nervous system (CNS) and is an FDA-approved treatment for multiple sclerosis (Garris et al., 2014). Similarly, in the active immunization model of EAU, LXA_4 appears to modulate gene expression of *S1pr1* on T cells from secondary lymphoid organs. It would be of great importance to connect the effects of LXA_4 on T cell egress with protein expression and function of *S1pr1* directly. However, current technology of S1P and S1pr1 staining by flow cytometry is limited and unreliable, therefore most studies on the function of S1P signaling and lymphocyte biology relies on manipulation of the signaling pathway through S1pr1 receptor antagonist FYT720.

Although the LXA_4 cognate receptor ALX/FPR2 is expressed on human and mouse T cells, G protein-coupled receptor 32 (GPR32), previously known as the receptor for SPM resolvin D1, has recently been identified as a second receptor for LXA_4 in humans (Dalli and Serhan, 2018). LXA_4 receptor knockout *Fpr2*$^{-/-}$ mice exhibit enhanced inflammatory phenotype in some diseases, however, unlike *Alox5*$^{-/-}$ mice, *Fpr2*$^{-/-}$ mice did not develop more severe EAU than WT controls after $IRBP_{651-670}$ immunization. The newly identified receptor in the human system could provide an explanation for the inconsistencies that we observed in EAU-immunized *Fpr2*$^{-/-}$ mice, since it is likely that there could be additional LXA_4 receptors in mice. The ALX/FPR2 receptor is promiscuous in the sense that it can bind to multiple ligands, despite binding to LXA_4 with high affinity (Chiang et al., 2006). Annexin A1, a calcium-dependent phospholipid binding protein that also is a ligand for ALX/FPR2, can counter-regulate inflammatory innate immune cells but activate T cells (Perretti and D'Acquisto, 2009). Thus ALX/FPR2 could mediate multiple immune regulatory signals depending on which ligand is available. Interestingly, however, EAU-immunized *TCRβ*$^{-/-}$ recipients of *Fpr2*$^{-/-}$ $CD3^+$ T cells did develop significantly augmented disease than that of WT T cells. The *Fpr2*$^{-/-}$ donor T cell phenotype in retinal infiltrates were not statistically different from the WT controls. The retinal inflammation in the recipients of *Fpr2*$^{-/-}$ T cells appeared to be

mediated through host macrophages, with higher number of macrophage infiltrates than recipients of WT T cells (data not shown), suggesting that EAU pathogenesis in recipients of *Fpr2*$^{-/-}$ T cells is mediated through distinct cell recruitment program.

Taken together, we propose that T cells that develop and mature in an environment without receiving LXA_4 signals are more susceptible to effector-like programming. Our findings could be further validated using more refined transgenic mice with cell type specific deletion of *Alox5*, as well as identifying which cell types in the inguinal lymph node express 5-LOX and the cellular source of LXA_4 to further dissect regulation of T cell responses.

While most research focuses on understanding the roles of cytokines in autoimmune diseases, little is known about the role of lipid mediators in autoimmunity. Systemic immune suppression and cytokine inhibition are currently the standard treatment regimen, but these sledgehammer approaches often lead to opportunistic infections and adverse side effects. However, a more targeted approach that maintains the basal tone of the endogenous LXA_4 circuit could potentially prevent dysregulation and fine-tune adaptive immune responses. Therapies targeting these predominantly T cell-driven pathologies have sparked efforts in understanding the disease pathogenesis and discovering potential drug targets. Since ocular tissues in the posterior segment do not possess regenerative abilities, it is of great interest to understand how to maintain homeostasis of the retina and the retinal barrier function in order to prevent extensive tissue damage that results in blindness.

Chapter Four: Lipoxins A_4 and B_4 Promote Direct Neuroprotection from Acute and Chronic Retinal Injury

4.1. Introduction

In this chapter, we discovered a novel bioaction of lipoxins in acute and chronic models of neurodegeneration. DHA-derived lipid mediator neuroprotectin D1 (NPD1) has been shown to limit DNA damage, downregulate inflammatory gene expression, and reduce oxidative stress of retinal pigment epithelial (RPE) cells in ischemia (Bazan, 2005). However, eicosanoids had not been linked to neuroprotection until now.

Glaucoma is a leading cause of blindness along with diabetic retinopathy, age-related macular degeneration and cataract. It is characterized by slow, progressive degeneration of retinal ganglion cells (RGC) that leads to irreversible cell death, and the damage is often correlated with elevated intraocular pressure (IOP) and inefficient aqueous humor drainage (Weinreb and Khaw, 2004). The neural retina provides a gateway for neurodegeneration studies due to the ease of fundus visualization and tissue access as opposed to the central nervous system (CNS).

Astrocytes are the most abundant glia cell type in the CNS and are essential for maintaining homeostasis. Originally thought as “brain glue” that passively provides structural support and nutrients to neighboring cells, studies have now shown immune-competent astrocytes actively participating in immune sensing, cytokine secretion, and immune cell trafficking and activation (Colombo and Farina, 2016). Previous studies showed that reactive astrocytes in inflammatory conditions can secrete TNF-α and cause RGC damage *in vitro* (Lebrun-Julien et al., 2010; Tezel et al., 2001; Tezel and Wax, 2000; Yuan and Neufeld, 2000). This neurotoxic effect can be reverse by TNF-α inhibition, but astrocyte reactivity alone was not sufficient to induce RGC death (Livne-Bar et al., 2016). Therefore our collaborators sought to address whether a paracrine protective signal is involved in supporting neuronal survival.

This project was performed in collaboration with Jeremy Sivak’s lab at University of Toronto and John Flanagan’s lab at UC Berkeley, where Izzy my fellow co-first author, had previously discovered that astrocyte-condition media (ACM) rescued retinal ganglion cell survival from acute retinal injury induced by oxidative stress. However, there was no positive hits in the identification of neurotrophic factors in proteomic screens. Upon seeing this result, I suggested to perform a comprehensive lipid mediator screen, with the hypothesis that we may see upregulation in NPD1 levels in ACM in comparison to cell-free media control. Unexpectedly, we detected highly enriched levels of lipoxins A_4 and B_4 in ACM, which launched the collaboration that discovered a novel function of lipoxins. This project lead to a publication in the *Journal of Clinical Investigation* in which I share co-first authorship (Livne-Bar et al., 2017).

4.2. Materials and Methods

Mouse acute retinal insult model: Male C57BL/6 mice, 4-6 weeks of age, were purchased from Taconic or Jackson Laboratory. All experimental protocols were approved by the UHN and UC Berkeley ACUC in accordance with applicable regulations. Mice were anesthetized by intraperitoneal injection of ketamine/xylazine. Intravitreal injections with 10 mM kainic acid (KA) were performed as previously described (Guo et al., 2014). Briefly, a 30-gauge needle was inserted tangentially into the vitreous and replaced with a Hamilton syringe to inject a volume of 2 µL, followed by application of ophthalmic antibiotic ointment (BNP, Vetoquinol). In some experiments, 10x concentrated ACM or test compounds were injected intravitreally 24 hr or 1 hr prior to KA injection in the following concentrations: LXA_4 and LXB_4 at 10 µM, zileuton at 2 µg/ul, and WRW4 at 15 µM, all dissolved in PBS. Mice were euthanized by CO_2 asphyxiation 18 hrs post KA treatment and the eyes were fixed in 4% paraformaldehyde. In all experiments *n* refers to the number of animals tested.

Astrocyte cultures: Primary retinal astrocytes (RA) were isolated and cultured as previously described (Rogers et al., 2012). Briefly, eyes were dissected out of adult Wistar rats and placed in ice-cold MEM-H17 media. Isolated retinas were digested by shaking in MEM-H17 containing papain and DNAse, followed by trituration to disperse cell aggregates. When cultures reached confluence, the cells were placed on a rotating shaker for 6-8 hours to remove microglia and then re-plated. An astrocyte expression profile was confirmed by probing the cultures with a panel of glial and neuronal markers (Nahirnyj et al., 2013). Note that efforts to generate a serum-free defined media resulted in cellular stress that compromised the protective activity. Therefore, conditioned media experiments contained serum, although control media exhibited no activity. RA conditioned media (ACM) or cell-free control media were harvested after 24 hours incubation and stored at -80°C.

Neuronal cultures: HT22 cells were cultured in MEM media with 10% FBS. For neuroprotection experiments injury was induced by incubation with 5mM glutamate. Cell viability was assessed by XTT assay and absorbance was measured at 490 nm, according to the manufacturer's direction (FroggaBio). RGCs were purified using magnetic MicroBeads (Miltenyi Biotec, Germany) according to the manufacturer's protocol. Briefly; retinal cell suspensions were prepared from 8-10 day old rats, incubated with CD90.1 microbeads and washed with DPBS/BSA buffer and gently centrifuged. Cells were resuspended and incubated with biotinylated depletion antibodies against microglia and endothelial cells, and sorted with magnetic Anti-Biotin MACSiBeads™. This pre-enriched population was centrifuged, and resuspended in fresh buffer and applied to a MACs MS separation column to enrich for CD90.1-bound RGCs. RGCs were plated in poly-D-lysine coated wells and cultured in Neurobasal-A media (Gibco), supplemented with 2% calf serum, B27, L-glutamine, BDNF, CNTF, forskolin and pen/strep. To assess RGC survival, cultures were challenged with serum deprivation or 30 uM paraquat (PQ), fixed, probed with tubulin-β3 antibody and imaged with a Nikon confocal microscope. The number of cell bodies and ratio of neurites/cell

was established for each acquired frame, with 32 frames scored from each experimental group. Data represents three independent experiments for each treatment.

LC-MS/MS: Eicosanoids and PUFA in the conditioned media were quantified via LC-MS/MS according to our published protocol (Hassan and Gronert, 2009; Murphy et al., 2005; Sapieha et al., 2011; Serhan et al., 2007). Briefly; class specific deuterated internal standards (PGE_2-d4, LTB4-d4, 15-HETE-d8, LXA_4-d5, DHA-d5, AA-d8) were used to calculate extraction recovery on an LC-MS/MS system consisting of an Agilent 1200 Series HPLC, Kinetex C18 minibore column (Phenomenex), and AB Sciex QTRAP 4500 system. Analysis was carried out in negative ion mode, and eicosanoids and PUFA were quantitated using scheduled multiple reaction monitoring (MRM) using 4-6 specific transition ions for each analyte. Calibration curves were established with synthetic standards (Cayman Chemicals).

Quantitative RT-PCR: Mouse retinal mRNA was isolated using RNeasy isolation kit (Qiagen), quantified using NanoDrop, and mRNA reverse transcribed with High Capacity cDNA kit (Applied Biosystems). q-PCR was performed with SYBR Green Master Mix (Applied Biosystems) using the ΔΔcT method in a Step One Plus qPCR system (Applied Biosystems). β-actin was used as the reference gene. The primers used were as follows: β-Actin: F=ACGGCCAGGTCATCACTATTG, R=AGGGGCCGGACTCATCGTA, 5-LOX: F=ACTACATCTACCTCAGCCTCATT, R=GGTGACATCGTAGGAGTCCAC, 12/15-LOX: F=GCGACGCTGCCCAATCCTAATC, R=ATATGGCCACGCTGTTTTCTACC, FPR2 (ALX): F=GCCAGG ACTTTCGTGGAGAGAT, R=GATGAACTGGTGCTTGAATCACT.

Immunofluorescence microscopy: Eyes were fixed in 4% PFA and equilibrated in 30% Sucrose for 12 hours, embedded in OCT for sectioning. Tissue sections were blocked and probed with primary antibodies to GFAP (Sigma), CD68, GR-1 and F4/80 (Biolegend), FPR2 (Abnova), RBPMS (Phosphosolutions), BRN3a (Santa Cruz), 5-LOX (Millipore), according to standard protocols. Following PBS-t washes, sections were incubated with fluorescent-conjugated secondary antibodies (Molecular Probes) and mounted with glycerol-based medium containing DAPI. TUNEL staining was performed according to the manufacturer's instructions (DeadEnd; Promega). Briefly, sections were fixed with 4% PFA for 5 minutes and washed in PBS. Equilibration buffer was added, and rTdT reaction mix was applied to each slide and incubated at 37 °C for 60 min. Slides were immersed in 2xSSC and then washed with PBS. This was followed by blocking with 5% goat serum and overnight incubation with primary antibodies at 4°C. Immunofluorescent images were acquired with a Zeiss AxioImager microscope and confocal images were acquired with Nikon Eclipse-Ti confocal microscope.

Quantification of RGC survival: For *in vivo* experiments: RBPMS-positive cells in the GCL were counted and expressed as a fraction of total ganglion cell layer (GCL) nuclei. For each eye, at least five central retinal sections were analyzed at the level of the optic nerve to the periphery, and the results averaged, as previously described (Livne-Bar et al., 2016). In parallel, TUNEL-positive GCL nuclei were counted and expressed it as a fraction of the total GCL nuclei (Chen et al., 2009; Guo et al., 2014; Harada et al., 2010;

Nishijima et al., 2007; Riesenberg et al., 2009) . For *in vitro* experiments: RGCs were isolated and treated as above and probed with an antibody to tubulin-β3. A Nikon confocal microscope was used to image immunopositive cells. A total of 32 frames from each experimental group were scored for the number of cell bodies and intact neurites. The ratio of neurites to RGC cell bodies was established for each acquired frame.

Rat chronic IOP model: A method for reliably inducing sustained IOPs in the rodent eye was recently developed by Liu et al (Liu et al., 2015; Liu and Flanagan, 2017). In this minimally invasive approach, a circumlimbal suture is placed to induce chronic ocular hypertension for up to 15 weeks (Liu et al., 2015). Circumlimbal sutures were placed in the randomized right or left eyes of anesthetized rats using a sterile 8-0 nylon suture. IOP was measured twice a week, and only animals showing sustained elevated IOP over 21 mmHg for the duration of the experiment were included in the analyses. The delivery and dosage of LXB_4 was based on previous publications (Biteman et al., 2007; Borgeson et al., 2015; Dunn et al., 2015). Briefly, LXB_4 or PBS vehicle were administered starting from week 8 on alternate days, 3 times a week. Due to the unstable nature of lipoxins, a hybrid approach was taken to ensure consistent delivery, including both IP delivery (1 µg) and topically to each eye (100 ng). ERG was measured monthly to assess RGC functional changes using the positive scotopic threshold response (pSTR), as we have previously used to measure the impact of elevated chronic IOP (Fortune et al., 2004; Liu et al., 2015). In addition, RNFL and total retinal thickness were quantified once a month using optical coherence tomography (OCT). After 15 weeks, animals were euthanized and RGC numbers were counted on central and peripheral regions of retinal flat mounts following immunofluorescent staining for BRN3a as above.

Statistical analyses: For all experiments n refers to the number of animals or biological replicates. For TUNEL staining and RGC counts, statistical analyses were performed by t-test or one-way ANOVA with TUKEY post-hoc analyses. ERG and OCT results were analyzed by two-way ANOVA with Bonferroni post-hoc test.

4.3. Results

4.3.1. Retinal astrocyte neuroprotection is mediated through lipoxins A_4 and B_4

To distinguish whether the protective activities of astrocytes originate from secreted factors, ACM was collected and tested for its effects *in vivo*. Concentrated ACM or cell-free control media incubated under identical conditions was injected intravitreally into C57BL/6 mice 24 hours prior to KA insult. Survival of RGC was assessed by staining with the marker RBPMS, along with TUNEL staining for apoptosis. Extensive RGC loss was observed in KA-challenged eyes treated with control media, while significant rescue of RGC was observed in eyes injected with ACM (Fig. 4.1A, B). TUNEL staining showed reduced RGC apoptosis in eyes injected with ACM (Fig. 4.1C, D).

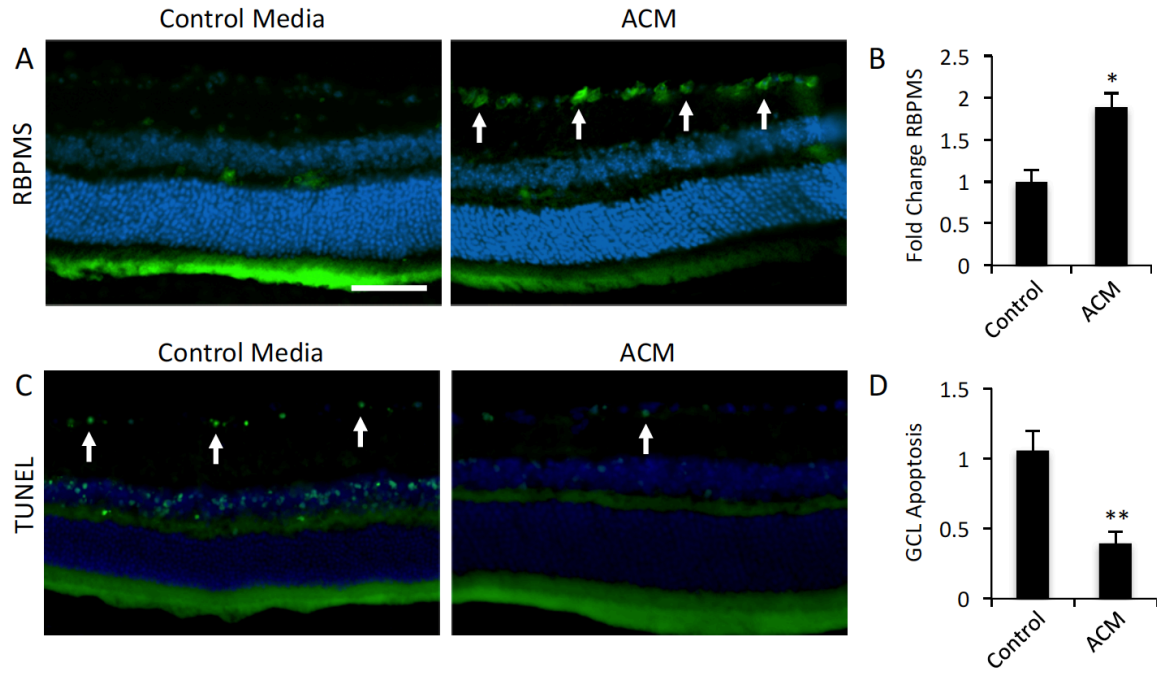


Figure 4.1. Astrocyte conditioned media promotes neuronal survival. ACM or cell-free control media were injected intravitreally, one day prior to KA challenge. A) ACM treatment reduced KA-induced RGC loss compared to control media, as detected by RBPMS (green, arrows), scale bar = 50μm. B) Quantification of the RBPMS results in A) showing significant increase in RGC survival with ACM injection, n=5 mice per group. C) TUNEL staining showing ACM mediated reduction in apoptotic cells (green, arrows) compared to control media. D) Quantification of TUNEL staining in C) showing significant decrease in RGC apoptosis from ACM media, n=5 mice per group. (Livne-Bar et al., 2017).

An *in vitro* assay was designed to recapitulate key aspects of the acute KA model. ACM was applied to the glutamate-sensitive neuronal cell line HT22 (Lee et al., 2007; Stanciu et al., 2000) to induce 60-80% cell death. Consistent with results in Fig. 3.1A-D, ACM treatment conferred significant protection of HT22 cells (Fig. 4.2A). Preliminary fractionation experiments showed that the protective action of ACM likely was not of peptide or protein origin since the protective activity was contained in filtrate smaller than 3kDa (Fig. 4.2B). Lipid mediators are small molecules with molecular weights less than 400g/mol. To identify the protective factors enriched in ACM, a comprehensive lipidomic analysis was performed using LC-MS/MS. Pathway markers for DHA-derived resolvins and protectins were detected (DHA, 17-HDHA) but formation of SPMs was not quantifiable. In contrast, lipoxins LXA_4 and LXB_4 were highly enriched in ACM compared to control media (Fig. 4.2C).

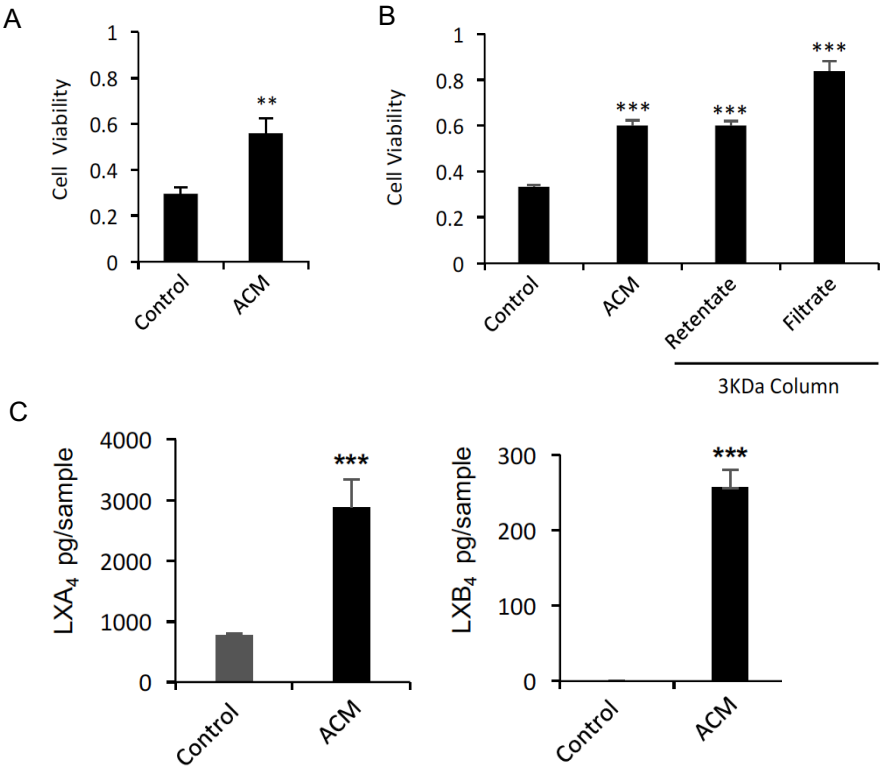


Figure 4.2. Astrocyte neuroprotection is mediated by lipoxins. A) ACM shows protective effect *in vitro* with HT22 neuronal cells challenged with 5mM glutamate, n=3 samples. B) Protective activity of ACM is contained in a 3kDa filtrate, n=3 samples. C) LXA_4 and LXB_4 were detected in ACM at high levels compared to control media by LC-MS/MS n=3 samples. * p <0.05, ** p <0.01, *** p <0.001. (Livne-Bar et al., 2017).

4.3.2. LXA_4 and LXB_4 synthesis and signaling are regulated in the inner retina

We next assessed the expression of 5-LOX, 12/15-LOX and FPR2 in the mouse retina by RT-PCR. Expression levels of *Alox5* (5-LOX) and *Fpr2*, but not *Alox15* (12/15-LOX), were significantly reduced following retinal insult (Figure 4.3A). Due to the retinal tissue matrix and size, endogenous lipoxins were difficult to detect in a single retina. However, by pooling 10 retinas aggregate concentrations of LXA_4 and LXB_4 could be quantified in control eyes, and six hours after KA challenge. LXA_4 levels were low in both control and KA-challenged samples, however, LXB_4 levels were reduced by nearly 50% following injury (Fig. 4.3B). We also assessed lipoxin levels in pooled optic nerves (ON) where LXA_4 levels were significantly reduced six hours after KA challenge, and a slight reduction of LXB_4 levels in KA-challenged samples (Fig. 4.3B).

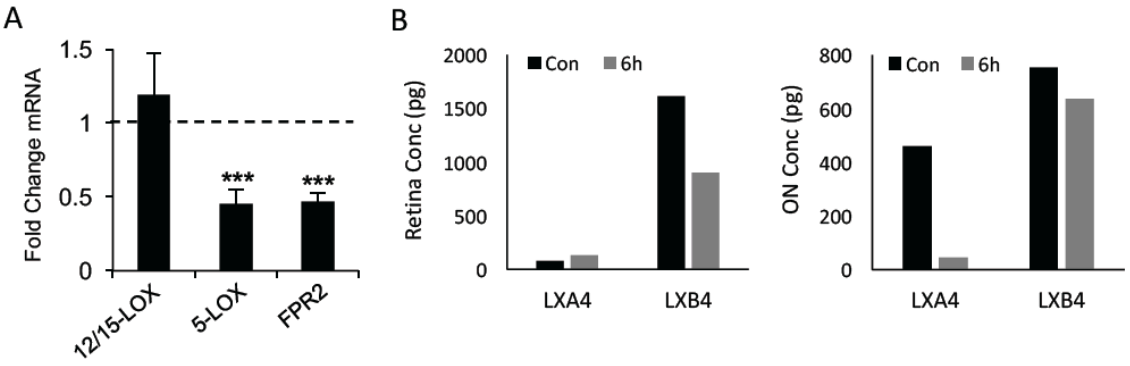
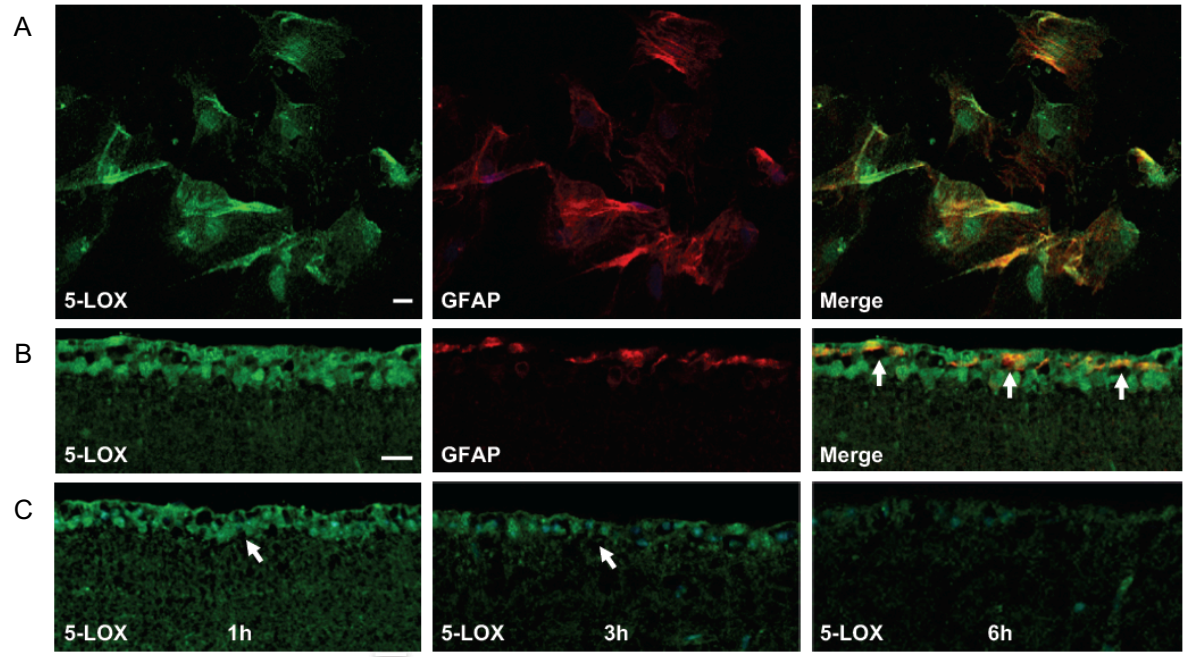


Figure 4.3. *In vivo* formation of lipoxins is reduced after acute injury. A) RT-PCR shows significantly reduced expression of 5-LOX, and *Fpr2* in the retina two hours after KA-induced injury. No change in 12/15-LOX expression was observed, n=3 mice per group. B) LXB_4 concentration in pooled retina sample was reduced six hours following injury, while LXA_4 concentration was reduced in the ON after injury, n=10 pooled retina per group. *** p <0.005. (Livne-Bar et al., 2017).

Since 5-LOX is the rate limiting enzyme for lipoxin formation, we confirmed 5-LOX expression in cultured astrocytes (Fig. 4.4A). 5-LOX expression was also present in astrocytes and RGC in the inner retina (Fig. 4.4B). Consistent with the RT-PCR results, retinal insult reduced 5-LOX signal after three and six hours post injury (Fig. 4.4C). FPR2 receptor expression was detected in RGC *in vitro* (Fig. 4.4D) and *in vivo* (Fig. 4.4E). These data suggest that the lipoxin circuit is present in the inner retina, and its endogenous formation is reduced in response to acute injury.



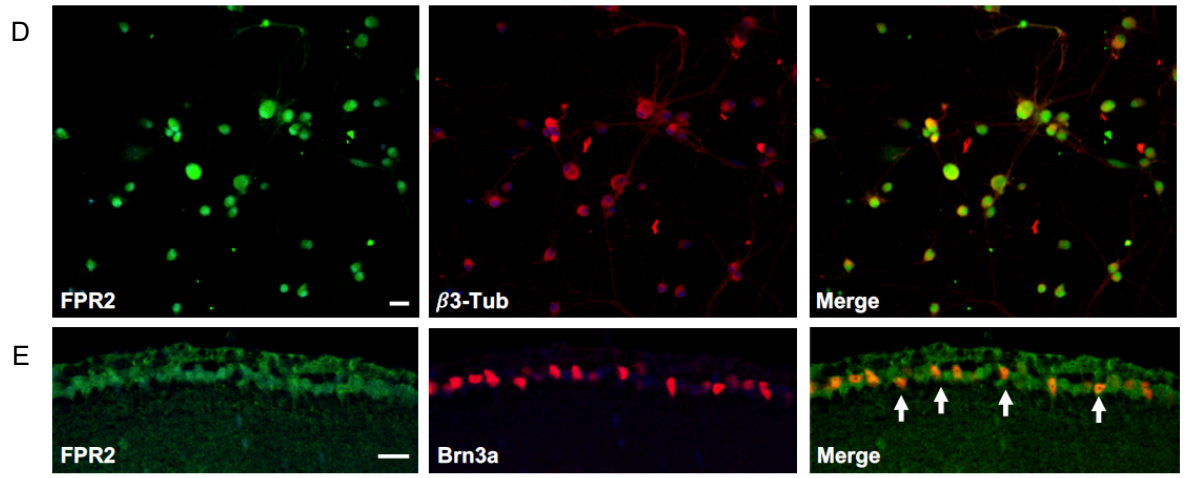


Figure 4.4. 5-LOX and Fpr2 are expressed on astrocytes and retinal ganglion cells respectively. A) Confocal microscopy of 5-LOX immunostaining in primary retinal astrocytes, scale bar= 20μm. B) Confocal microscopy of 5-LOX immunostaining (green) shows expression in the ganglion cell layer and nerve fiber layer, with partial colocalization (yellow, arrows) with astrocytes (GFAP; red), scale bar= 10μm. C) 5-LOX expression in the inner retina is reduced at three and six hours after injury (arrows). D) FPR2 immunostaining (green) is prominent in cultured primary RGC stained with neuron-specific β3-tubulin (red), scale bar= 20μm. E) FPR2 staining (green) is specific to the ganglion cell layer and co-localizes (yellow, arrows) with RGC (Brn3a; red), scale bar= 10μm. (Livne-Bar et al., 2017).

4.3.3. LXA_4 and LXB_4 promote RGC survival following acute insult

We next investigated whether LXA_4 or LXB_4 treatment *in vivo* can promote RGC survival. 10μM of LXA_4 or LXB_4 was injected intravitreally, one hour prior to retinal KA challenge. Both LXA_4 and LXB_4 treatments significantly increased RGC survival by 57% (±3.8)) and 99% (±4.7), respectively, compared to vehicle control (Fig. 4.5A, B). Interestingly, LXB_4 was the more potent molecule compared to LXA_4, despite being the less studied lipoxin molecule.

To confirm the role of lipoxin signaling in the inner retina, zileuton, a 5-LOX inhibitor was administered (Gregor et al., 2005; Sapieha et al., 2011; Smith et al., 2015). Intravitreal injection of zileuton significantly exacerbated RGC loss after KA challenge by 60% (±1.2) (Fig. 4.5C, D). Intravitreal FPR2 receptor antagonist WRW4 injection increased RGC loss by 67% (±1.8) compared to vehicle injected eyes (Fig. 4.5E, F). Taken together, the data demonstrate the critical role of the lipoxin circuit in mediating neuroprotection during acute retinal injury, and that disruption of the LXA_4 pathway through inhibition of FPR2 exacerbates RGC loss.

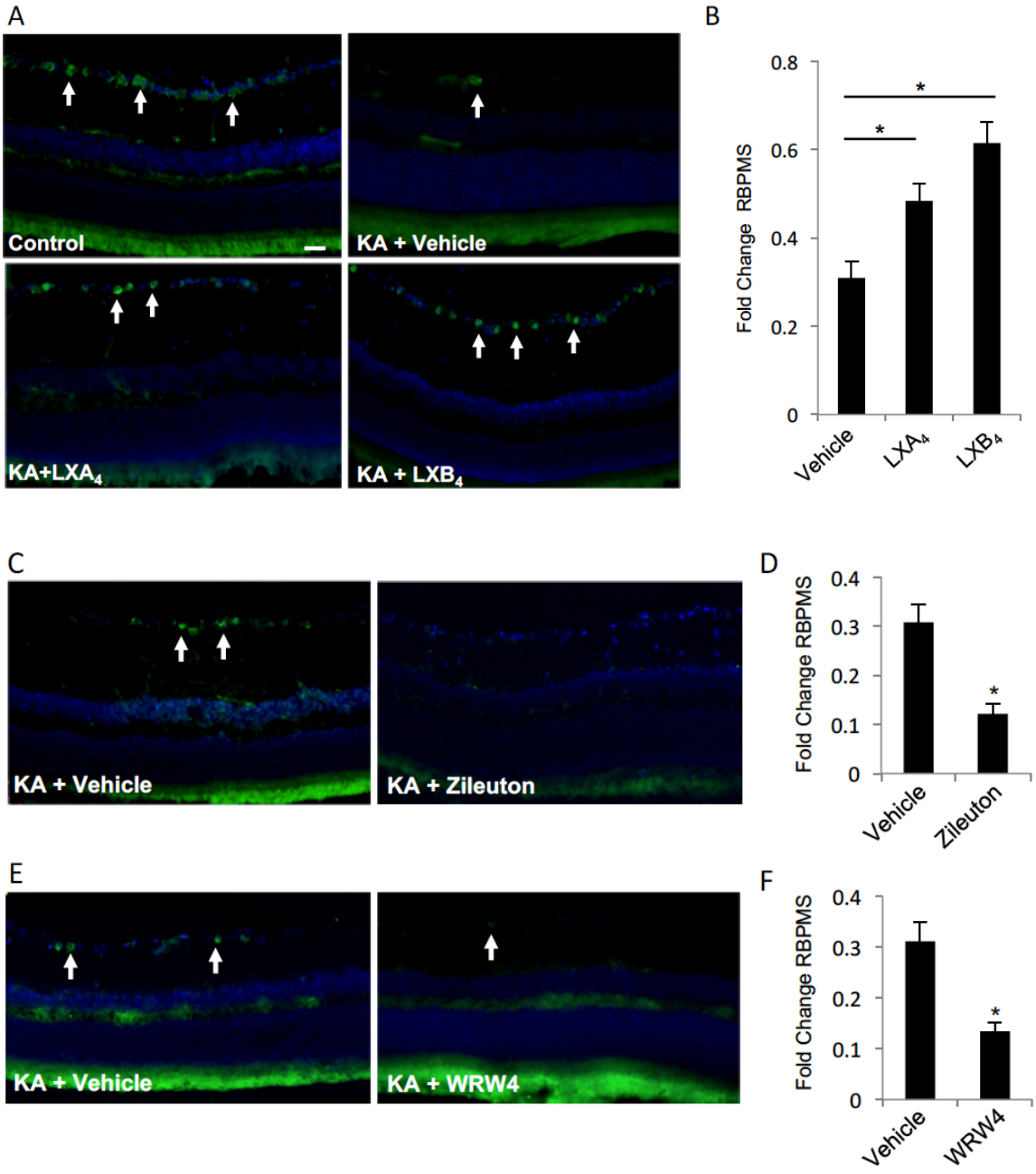


Figure 4.5. LXA_4 and LXB_4 promote RGC survival following acute injury. A) 10μM LXA_4 or LXB_4 was injected intravitreally prior to KA-induced insult. Lipoxin treatment increased RGC survival compared to PBS treated control, shown by RBPMS staining (green, arrows). B) Quantification of RGC shows significant increase in survival with LXA_4 or LXB_4 treatment compared to control, n=8. C-D) intravitreal injection of 10μM zileuton significantly reduced RGC survival following acute stress compared to vehicle injected control, n=5. E-F) Intravitreal injection of 15μM WRW4 significantly reduced RGC survival following acute stress compared to vehicle injected control, n=5. *p < 0.05. (Livne-Bar et al., 2017).

4.3.4. LXA_4 and LXB_4 provide direct neuroprotection

Anti-inflammatory actions of lipoxins have been reported in the CNS (Czapski et al., 2016; Martini et al., 2014; Tassoni et al., 2008; Wang et al., 2015b), but not neuroprotective functions. To assess whether the neuroprotective activity observed was direct or indirect, LXA_4 and LXB_4 were added to neuron cultures *in vitro*. LXA_4 and LXB_4 dose-dependently increased the viability of glutamate-challenged HT22 cells, demonstrating a direct protective action (Fig. 4.6A). Consistent with data in Fig. 3.3, LXB_4 showed more potent protective activity than LXA_4, at concentrations as low as 50nM as opposed to 500nM (Fig. 4.6A). In contrast, 15-HETE and the DHA-derived LXB_4 analogue resolvin D2 (RvD2) did not exhibit protective activity (Fig. 4.6B).

LXB_4 and LXA_4 are both generated by the double oxygenation of arachidonic acid by 5-LOX and 12/15-LOX, but do not mediate their action through the same receptor (Romano et al., 1996; Ryan and Godson, 2010). The LXB_4 receptor has yet to be identified, however, LXB_4 shares structural homology with LXA_4 and RvD2 which mediate their actions via the FPR2 and GPR18 receptors (Chiang et al., 2015), respectively. Therefore, we asked whether administering receptor antagonists for FPR2 or GPR18 could inhibit LXB_4-mediated protection. Increasing concentrations of the FPR2 antagonist WRW4 or GPR18 antagonist O-1918 did not inhibit LXB_4 mediated protection in HT22 cells (Fig. 4.6C). These data suggest that LXB_4 neuroprotection is mediated through an LXA_4,-and RvD2- receptor independent mechanism.

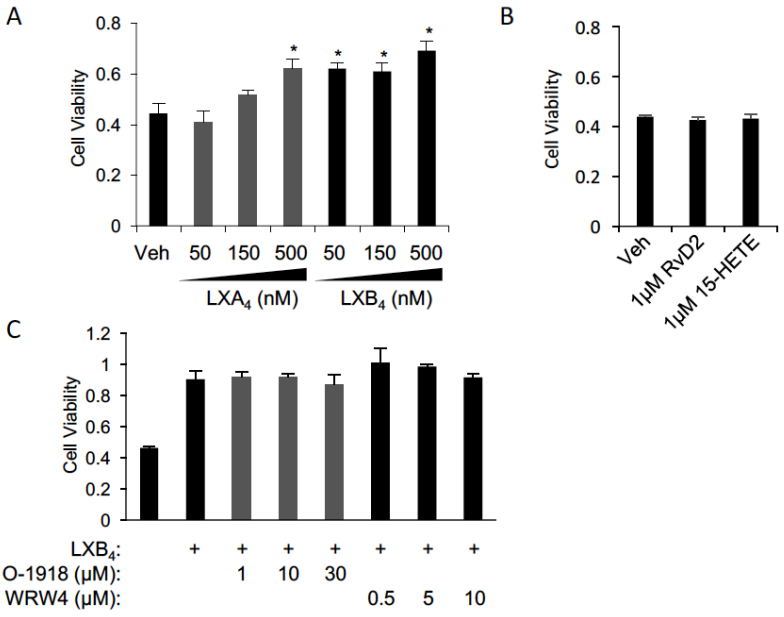


Figure 4.6. LXB_4 exerts more potent neuroprotective effects in a LXA_4 receptor independent manner. A) Treatment of HT22 neuronal cells with LXA_4 or LXB_4 significantly increased cell viability compared to vehicle, n=3. B) 15-HETE and RvD2 did not have neuroprotective actions, n=3. C) LXB_4 mediated neuroprotection was not inhibited by WRW4 or O-1918, n=3. (Livne-Bar et al., 2017).

To confirm direct protection of neurons, we treated primary RGC with LXA_4 or LXB_4, then challenged RGC with 30μM paraquat (PQ) to induce acute oxidative stress (Guo et al., 2014; Nahirnyj et al., 2013). Quantitation of cell and neurite survival were from fluorescence stained β3-tubulin. In normal culture conditions, healthy RGC exhibited an extensive network of neurites (Fig. 4.7A). However, both neurite and cell numbers were reduced after 24 hours of PQ exposure (Fig. 4.7A, B). Treatment with 1μM of LXA_4 or LXB_4 significantly increased RGC numbers (Fig. 4.7B), although only LXB_4 significantly reduced neurite disintegration (Fig. 4.7C). In comparison, RvD2 did not exert any protective activity (Fig. 4.7A-C). These data provide further evidence that LXB_4 and LXA_4 have direct and distinct protective actions on neurons.

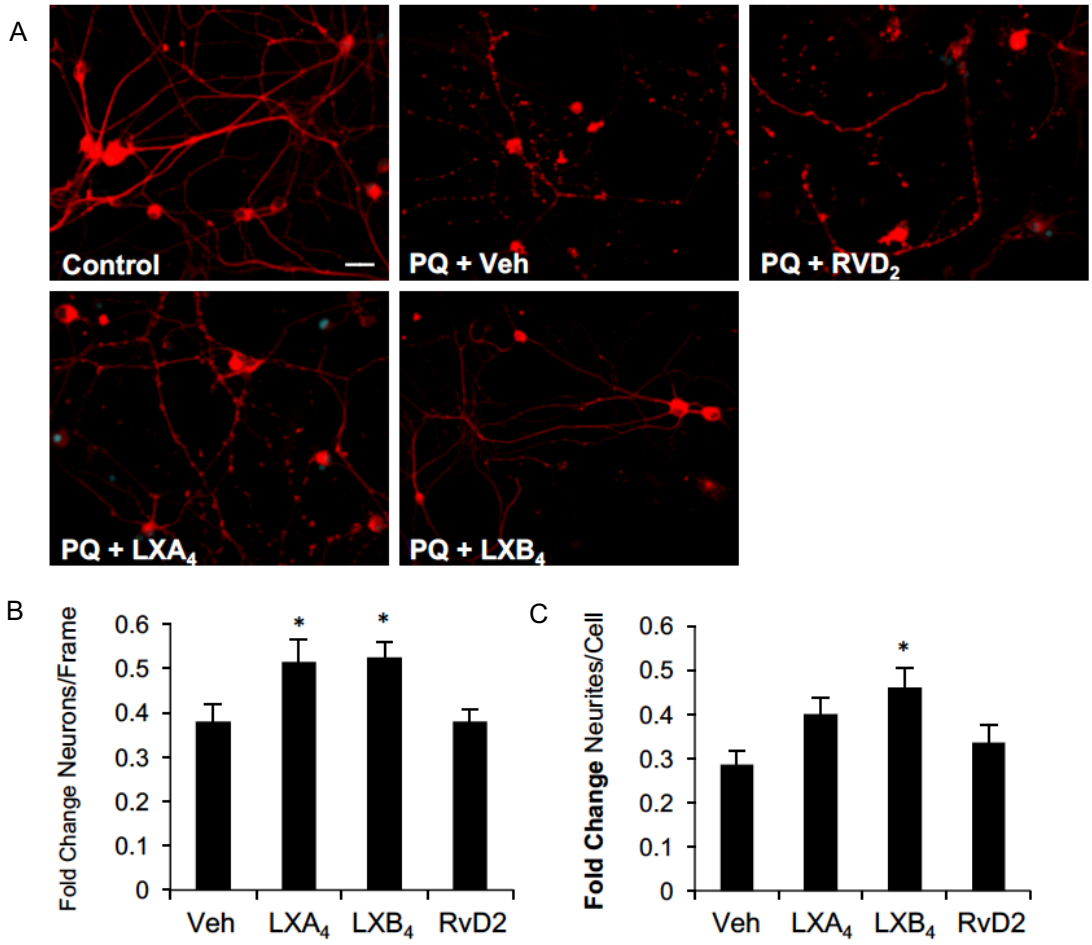
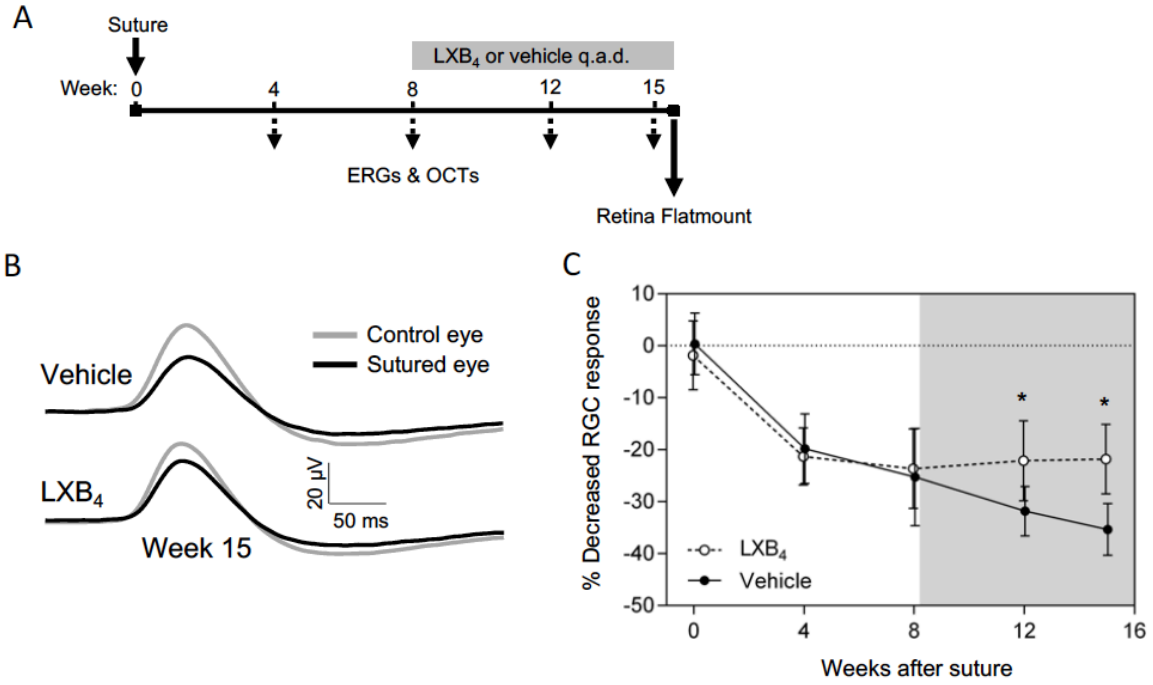


Figure 4.7. LXA_4 and LXB_4 exert direct protection on neurons. A) Treatment with 1μM LXB_4, but not LXA_4 or RvD2, maintained intact neurites, scale bar=20μm. B) Quantification of neuron survival following oxidative stress. LXA_4 and LXB_4 treatment rescued RGC survival, n=3. C) Quantification of neurite network. Following PQ challenge, neurites was significantly maintained with LXB_4 treatment, but not LXA_4 or RvD2, n=3. *p <0.05. (Livne-Bar et al., 2017).

4.3.5. LXB_4 treatment rescues RGC function in a chronic elevated IOP glaucoma model

To assess whether lipoxin neuroprotection extends beyond acute stress models, we employed a chronic elevated IOP model that better recapitulates chronic neurodegeneration in glaucoma. The rat perilimbal suture model of glaucoma maintains moderately elevated IOP over 15 weeks (Liu et al., 2015; Liu and Flanagan, 2017), and results in compromised RGC function, reduced retinal nerve fiber layer (RNFL) thickness, and RGC death by week 15 (Liu et al., 2015). Elevated IOP and RGC functional deficits are reversible with suture removal at 8 weeks post suture placement (Liu and Flanagan, 2017). We therefore started treatment of rats with elevated IOP at week 8, followed by assessment of RGC function and survival. For this *in vivo* study, we focused on LXB_4 due to its higher potency from the *in vitro* and *in vivo* results.

LXB_4 or vehicle treatment was administered after 8 weeks of elevated IOP every other day until week 15. Increased IOP was induced unilaterally, with the contralateral eye serving as control in each rat. IOP was monitored twice weekly to ensure the chronic induction of neurodegeneration. Electroretinograms (ERG) and optical coherence tomography (OCT) were measured at week 0 before placing the suture, and on weeks 4, 8, and 12, and 15 of elevated IOP to monitor pathology and function of the retina (Fig. 4.8A). IOP levels in the LXB_4 treated group did not differ from the vehicle treated group up to week 8, eliminating a potential indirect effect. However by week 12, the positive scotopic threshold response (pSTR) that serves as RGC functional readout, showed significant recovery in the LXB_4 treated group compared to vehicle (Fig. 4.8B, C). OCT measurement of retinal nerve fiber layer thickness, an indicator or RGC axonal loss, showed increasing thinning in the vehicle treated group. LXB_4 treatment group exhibited significantly less retinal thinning in comparison to control by week 15 (Fig. 4.8D, E).



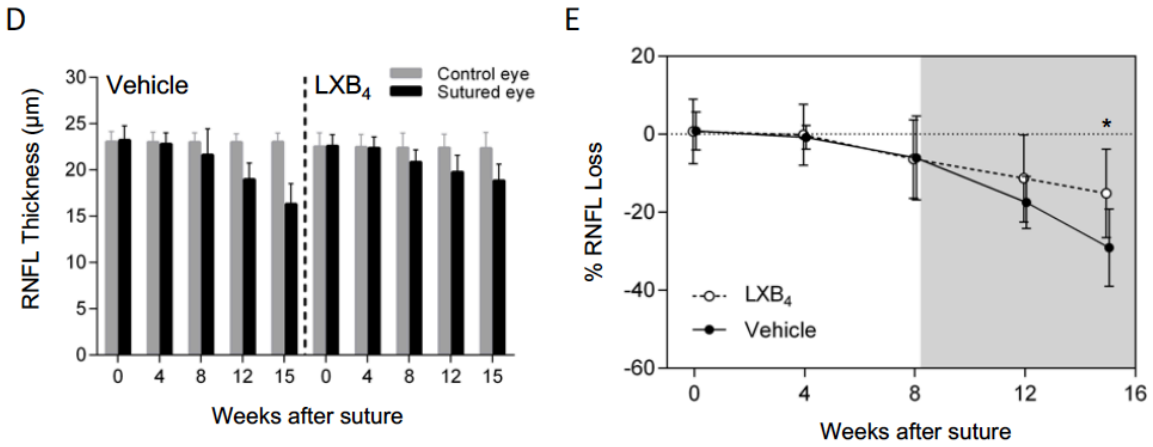


Figure 4.8. LXB_4 treatment protects RGC function following chronic IOP injury. A) Schematic of the experimental design showing ERG and OCT readings every 4 weeks following suture-induced IOP elevation. LXB_4 administration started at week 8, and RGC quantification of enucleated eyes was performed at week 15. B) Average waveforms for RGC (pSTR) responses at week 15 for LXB_4 and vehicle treated groups, and C) relative changes in RGC function across 15 weeks. Starting at week 12, there was a significant increasing rescue of RGC response in LXB_4 treated eyes compared to vehicle, n=8 per group, error bars are S.D., the shaded area indicates the treatment duration. D) RNFL thickness was monitored by OCT across 15 weeks, comparing sutured to control eyes in LXB_4 and vehicle treated groups. E) Percent RNFL loss of LXB_4 and vehicle treated groups shows a significant reduction in RNFL loss in the LXB_4 group at week 15, n=8, error bars are S.D., the shaded area indicates the treatment duration. * $p < 0.05$. (Livne-Bar et al., 2017).

To correlate these functional readouts with pathology, rats were euthanized at week 15 and the retinas stained for BRN3a. In vehicle treated eyes, RGC were reduced by 35% and 30% in sutured eyes compared to controls in the peripheral and central retinas, respectively (Fig. 4.9A, B). However, consistent with the ERG and OCT findings, LXB_4 treated retinas presented a significant rescue of RGC compared to vehicle treated retinas (Fig. 4.9A, B). Together, these data demonstrate substantial recovery of RGC function and increased survival following therapeutic administration of LXB_4 in a model of chronic elevated IOP glaucomatous retinopathy.

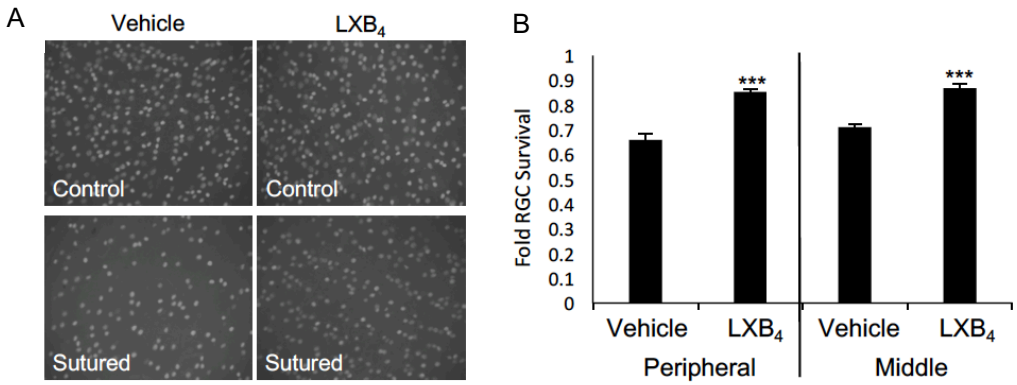


Figure 4.9. LXB_4 treatment promotes RGC survival following chronic IOP injury. A) Representative frames from retinal flatmounts stained BRN3a after 15 weeks of elevated IOP compared to contralateral control eyes, from LXB_4 or vehicle treated groups. B) Quantification of RGC density revealed the IOP-induced loss was significantly rescued by LXB_4 treatment compared to vehicle in both the central and peripheral retinas, n=8, error bars are S.E.M. *** $p < 0.001$. (Livne-Bar et al., 2017).

4.4. Discussion

This project presents a novel protective function of lipoxins in neurodegeneration. We showed that astrocytes produce LXA_4 and LXB_4 in the inner retina to promote RGC survival. Proteins such as neurotrophins have established neuroprotective roles in the CNS, and NPD1 was the only lipid mediator that showed direct neuroprotection (Bazan, 2005; Calandria et al., 2015a; Calandria et al., 2015b; Mukherjee et al., 2004) up until now. Deficiency of lipoxin enzyme precursors 5-LOX and 12/15-LOX has been linked to increased CNS pathology in mice (Emerson and LeVine, 2004; He et al., 2017), and dysregulation of SPMs and anti-inflammatory bioactions of LXA_4 have been implicated in neuroinflammation models such as Alzheimers disease, stroke, and age related macular degeneration (Gordon and Bazan, 2013; Martini et al., 2014; Serhan et al., 2015, Czapski, 2016 #1155; Tassoni et al., 2008; Wang et al., 2015b). However, direct neuroprotection through endogenous generation of lipoxins by astrocytes has not been reported.

Our data showed that LXB_4 was more potent in mediating neuroprotective effects than LXA_4, sparking a renewed interest in this widely overlooked molecule. LXB_4 rescued neurite survival and maintained neuronal cell viability at lower concentrations in comparison to LXA_4. Because the LXB_4 receptor has yet to be identified, mechanistic studies on LXB_4 signaling prove to be difficult. We demonstrated that inhibition of LXA_4 and RvD2 receptors did not abrogate the therapeutic effects of LXB_4, indicating a completely independent signaling pathway. Further studies on identification of the LXB_4 receptor to dissect exact mechanisms of actions are warranted.

The protective functions of astrocyte-derived lipoxins in neurodegeneration fall in line with the role of tissue resident LXA_4 that maintains homeostasis in models of

inflammation. Constitutive lipoxins levels in the neuroretina were reduced after acute retinal injury, in parallel to data from Chapter Two showing that after autoimmune uveitis induction, LXA_4 levels were reduced in the inguinal lymph nodes where T cell priming takes effect. Astrocytes can take on neurotoxic A1 or neuroprotective A2 phenotypes during injury or neurodegeneration, which subsequently dictates the inflammatory status within the microenvironment (Liddelow et al., 2017). Our data indicates that astrocytes constitutively secrete lipoxins to maintain neuronal health, in line with their essential role of supporting CNS homeostasis. Lipoxins in the context of neurodegeneration confer neuroprotection and also counter regulate reactive astrocytes or other inflammatory cell types by serving as a gatekeeper of the threshold to activate inflammatory responses in the immune privileged CNS.

Conventional treatment of glaucoma involves lowering IOP through surgical interventions or latanoprost treatment that targets the prostaglandin F_{2a} receptor to increase aqueous humor outflow. However, these treatments are simply reducing the symptoms that lead to structural damage in the optic nerve and not targeting the root cause of RGC death, since patients treated with IOP lowering methods still have progressive neurodegeneration (Anderson et al., 2001; Heijl et al., 2002; Quigley, 2005). LXB_4 was able to promote RGC function and survival *in vivo* in the chronic elevated IOP model, showing great therapeutic potential for attenuating irreversible neuronal damage.

Chapter Five: Concluding Remarks

The main goal of my thesis project presented in Chapter Three was to delineate whether LXA_4 can regulate T cell responses. We approached this question using the experimental autoimmune uveitis model because it is a pathogenic T cell driven ocular disease. The role of lipoxins in posterior autoimmune uveitis was not previously investigated, thus we first established that upon disease induction LXA_4 formation was increased locally in the eye and reduced in the peripheral lymph node where T cell priming takes place. We next showed that mice deficient of LXA_4 developed more severe uveitis with increased ocular infiltrates and treatment with LXA_4 limited disease progression. The LXA_4 circuit regulated pathogenic $CD4^+$ T cell IFN-γ production and migration by metabolic reprogramming and modulating expression of T cell trafficking marker *Ccr7*. To our knowledge, this was the first characterization of LXA_4 bioactions *in vivo* in autoimmunity, and the first report of antigen-specific T cell migration regulated by the LXA_4 circuit.

In Chapter Four, we identified a new bioaction for lipoxins A_4 and B_4. Lipoxins are conventionally linked to anti-inflammatory functions, however in neurodegeneration models, we showed astrocytes produce lipoxins in the inner retina to provide neuroprotection to RGC during acute and chronic retinal injury. Using receptor antagonists, we demonstrated that LXA_4 and LXB_4 exert neuroprotective functions through distinct signaling pathways. Although LXB_4 conferred more potent neuroprotection than LXA_4, mechanistic studies were limited in scope because the LXB_4 receptor is currently unidentified. Nonetheless, we demonstrated that LXB_4 treatment in a glaucoma model rescued RGC function and survival *in vivo*, providing a promising therapeutic target for neurodegenerative diseases.

Identification of unknown receptors, characterization of lipoxygenase orthologs in mouse and human, and carefully designed transgenic mouse models would further support studies of lipoxin bioactions *in vivo*. Knocking out the 5-LOX enzyme in mice abrogates the formation of lipoxins A_4 and B_4, as well as leukotrienes. 12/15-LOX deletion in mice only partially abrogates lipoxin formation, and *in vivo* studies from these mice are difficult to interpret since the mouse and human enzyme orthologs exert disparate activities. Currently there are no tools available to delete one specific lipid mediator metabolized by lipoxygenases, since there is a series of intermediate metabolizing steps before the final lipid mediator formation. Lipoxygenases are involved in the formation of various lipid mediators, 5-LOX metabolizes leukotrienes and lipoxins and 12/15-LOX metabolizes lipoxins as well as SPMs derived from the DHA biochemical pathway. To circumnavigate this, efforts in identification of lipoxin receptors would be invaluable for establishing pathway specific effects of lipoxin A_4 and B_4. Cell-type specific deletion of lipoxygenase could also further strengthen the role of astrocyte- or other cell type- derived lipoxin in various models. Optimization and specificity improvement of flow cytometry antibodies for lipoxin receptors and immunohistochemistry antibodies for lipoxygenases can strengthen studies that aim to delineate the cellular source of lipoxin formation and the cell types that respond to the lipoxin signaling.

Together, these projects demonstrate key roles of lipoxins in counter regulating inflammatory responses of different cell types and diseases, calibrating and stabilizing the immune balance in local tissue environment and the importance of understanding lipid mediator functions in a context- dependent manner. From the acute inflammation and chronic disease models, LXA_4 proves to be an attractive therapeutic target to circumvent chronic pathology that results in irreversible tissue damage by controlling dysregulated innate and adaptive immune responses. In neurodegeneration, lipoxins A_4 and B_4 can potentially deliver a significant therapeutic outcome in treating the root cause of disease by preventing neuronal cell death. Overall, permanent loss of tissue function can be abated by amplifying the endogenous lipoxin circuit. Further elucidation of the mechanisms in which lipoxins mediate disease and interact with other biological pathways will provide avenues to fine-tune the immune system and restore homeostasis, and ultimately lead to more effective and targeted therapeutics.

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