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Role of Macrophages in Ocular Surface Fibrosis

A Thesis by

Alyanna Beatrice Corpuz

Chapman University

Irvine, CA

School of Pharmacy

Submitted in partial fulfillment of the requirements for the degree of

Master of Science in Pharmaceutical Sciences

December 2022

Committee in charge:

Dr. Ajay Sharma (Chair)

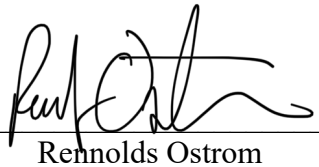
Dr. Jennifer Totonchy

Dr. Rennolds Ostrom

The thesis of Alyanna Beatrice Corpuz is approved.

Ajay Sharma (Chair)

Jennifer Totonchy



Rennolds Ostrom

September 2022

Role of Macrophages in Ocular Surface Fibrosis

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ABSTRACT

Role of Macrophages in Ocular Surface Fibrosis

by Alyanna Beatrice Corpuz

The present study is designed to investigate whether irradiation as used for conditioning regimen to ablate recipient's diseased bone marrow prior to transplantation could trigger macrophage activation and polarization and whether macrophages could cause the transdifferentiation of ocular surface fibroblasts into myofibroblasts, thus contributing to oGVHD-associated fibrosis.

Bone marrow cells were cultured in M-CSF to obtain M0 macrophages, which were subsequently polarized into M1 and M2 phenotypes using IFN-γ + LPS and IL-4, respectively. The macrophages were exposed to 7 Gy radiation. The effect of irradiation on macrophage activation markers, phagocytosis, and apoptosis was assessed using real-time PCR, confocal microscopy, and flow cytometry. Ocular surface fibroblasts were co-cultured using membrane inserts placed on top of cultured M0, M1, and M2 macrophages for 3 days. Myofibroblast formation was assessed using α-SMA immunostaining and gene expression. The effect of macrophages on profibrotic mediators in the fibroblasts was quantified using real-time PCR. Finally, chemokine release from macrophages was analyzed using real-time PCR and bead-based immunoassay.

Our data demonstrates successful generation of M0 macrophages and their polarization into M1 and M2 phenotypes as confirmed by gene quantification and flow cytometry for macrophage markers CD11b, F4/80, CD86, CD206, iNOS, and arginase-1. Our data demonstrates that irradiation caused an increase in macrophage pro-inflammatory cytokines IL-1β, TNF-α, and IL-6 and chemokine CCL2. While irradiation did not cause increase in M1

markers, there was a robust increase in M2 markers. Furthermore, irradiation significantly increased macrophage phagocytosis without compromising their viability. Our data also demonstrates that M0 and M2 macrophages induced significant increase of α-SMA expression in ocular surface fibroblasts and a concomitant notable increase in RAS components and TGF-β1 receptor, but no change in PDGF and M-CSF expression was noted. Finally, our data demonstrates that both M1 and M2 macrophages showed increased gene expression and secretion of chemokines CCL17 and CCL22.

In conclusion, our data demonstrates that macrophages could play a key role in the pathology of oGVHD-associated fibrosis due to their likely activation in response to irradiation and cross-talk with ocular surface fibroblasts, resulting in their transdifferentiation to myofibroblasts.

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CHAPTER 1

INTRODUCTION

The cornea and conjunctiva collectively constitute the ocular surface. Healthy ocular surface is vital for normal vision. Noxious stimuli such as laser surgery, chemical or thermal burns, physical or mechanical injury, radiation, or systemic diseases can cause injury to the ocular surface leading to excessive wound healing response. Aberrant wound healing response can result in corneal and conjunctival fibrosis. Consequent fibrotic changes in the cornea and conjunctiva can severely impact vision, leading to significant morbidity and reduced quality of life (Inamoto et al., 2019).

Fibrosis is marked by the accumulation of extracellular matrix (ECM) components. Myofibroblasts, which transdifferentiate from activated fibroblasts, are responsible for the deposition of ECM and dysregulated ECM production could lead to excessive tissue remodeling and permanent scarring due to fibrosis (Distler et al., 2019). Fibrosis is highly prevalent in chronic conditions associated with inflammation including scleroderma, Crohn's disease, systemic lupus erythematosus, and graft-versus-host disease (Wynn and Ramalingam, 2012).

Graft-versus-host disease (GVHD) is one of the main causes of morbidity and mortality following allogeneic bone marrow transplantation in which donor marrow (graft) attacks the host's tissues. Prior to receiving a bone marrow transplant, patients must undergo a conditioning regimen to ablate their diseased marrow using either total body irradiation or chemotherapy drugs which can cause tissue injury and initiate wound healing response (Gyurkocza and Sandmaier, 2014). GVHD is classified as either acute or chronic. In acute GVHD, the skin, gastrointestinal tract, and the liver are severely impacted. In chronic GVHD, the clinical manifestations can spread further into the mucosal surfaces in addition to the skin such as the mouth, lungs, and eyes (Hill et al., 2021). Clinical reports have indicated that damage to the ocular surface is highly prevalent in chronic GVHD, wherein particularly the conjunctiva is

severely inflicted with fibrotic scarring (Kusne et al., 2017; Kheirkhah et al., 2018). Ocular GVHD occurs in more than 60% of patients with chronic GVHD (Herretes et al., 2015).

Like all mucosal surfaces, the ocular surface also contains an innate immune system that protects the eye from invading pathogens and maintains homeostasis. Both the cornea and conjunctiva are endowed with macrophage and fibroblast populations. Macrophages are sentinel, phagocytic cells of the innate immune system that are prominently involved in clearance of cellular debris, dead cells, and pathogens. Macrophages also act as antigen-presenting cells. Macrophages exhibit remarkable phenotypic plasticity and are known to release an array of pro-inflammatory or profibrotic cytokines (Mosser and Edwards, 2008). Macrophages can be classified under two main phenotypes: M1 classically-activated, pro-inflammatory and M2 alternatively-activated, anti-inflammatory or profibrotic. M1 macrophages perform the characteristic microbicidal host defense by producing pro-inflammatory mediators to induce an inflammatory response. In contrast, M2 macrophages resolve inflammation and initiate wound healing through secretion of anti-inflammatory and profibrotic mediators (Klopfleisch, 2016). Macrophage activation has been implicated in several pathological conditions such as chronic inflammatory and autoimmune diseases, cancer, and fibrosis (Wynn et al., 2013).

Studies have demonstrated that polarized macrophages contribute to the pathogenesis of fibrosis. Unresolved inflammation could lead to exacerbation of tissue damage at the site of injury as well as neighboring tissues. Consequently, chronic inflammation is accompanied by excessive tissue restoration, which could be a result of activation of profibrotic signaling pathways. Macrophages are sources of profibrotic mediators including TGF-β, components of the renin-angiotensin system, platelet derived growth factor (PDGF) and connective tissue growth factor (CTGF) (Wang et al., 2021). Thus, macrophage activation, phenotype change and

the milieu of profibrotic cytokines and mediators can play a critical role in ocular surface inflammation, wound healing, and fibrosis. Studies have also demonstrated that macrophages could become activated and able to polarize preferentially toward either the pro-inflammatory or anti-inflammatory phenotype upon radiation exposure. Additionally, it has been observed that macrophages may show resistance toward radiation and may not undergo cellular death. (Teresa Pinto et al., 2016; Wu et al., 2017).

Currently, there are no studies that investigate how noxious stimuli such as irradiation can affect macrophage activation and phenotype changes, which in turn can modulate ocular surface fibroblasts to contribute to ocular surface fibrosis in the context of ocular GVHD. Thus, the present study is designed to investigate whether irradiation as used as a conditioning regimen for bone marrow ablation prior to transplantation could trigger macrophage activation and polarization. Further, this study also investigates whether macrophage activation, polarization, and associated upregulation of profibrotic pathways could potentially cause the transdifferentiation of ocular surface fibroblasts into myofibroblasts, thus contributing to ocular surface fibrosis. We will test this hypothesis using the following aims:

Aim 1: To determine whether exposing macrophages to radiation affects macrophage activation, polarization, and phagocytosis.

Aim 2: To identify whether polarized macrophages can cause ocular surface fibroblast transdifferentiation to myofibroblasts and can modulate profibrotic pathways in these fibroblasts.

CHAPTER 2

REVIEW OF LITERATURE

Ocular Surface Injury and Fibrosis

i. Tissue Injury & Fibrosis

Tissue damage could arise from a variety of causes including mechanical or chemical injury, infections, autoimmune reactions, radiation and systemic diseases-associated with chronic inflammation. Tissue injury initiates an inflammatory response that activates innate immunity, which involves the infiltration, clearance of tissue debris, and release of pro-inflammatory mediators by leukocytes such as macrophages and neutrophils. These immune cells also release profibrotic mediators that could activate and induce the transdifferentiation of fibroblasts into myofibroblasts leading to fibrosis.

Fibrosis can occur in virtually every tissue and organ and is also prevalent in chronic systemic diseases causing tissue injury due to inflammation including atherosclerosis, kidney disease, chronic obstructive pulmonary disease, and graft-versus-host disease. Wound healing involves two phases: regeneration and fibroplasia (Wynn, 2008). Formation of fibrous tissue occurs when myofibroblasts generate and deposit extracellular matrix (ECM) components such as collagen and fibronectin at the site of injury. In normal wound healing, inflammation as well as the deposition of ECM should cease after tissue damage has been resolved. Chronic inflammation and accumulation of ECM components could lead to prolonged activation of wound repair processes that could further exacerbate damaged tissues. Consequently, this dysregulation could result in permanent fibrotic scarring known as fibrosis (Distler et al., 2019). There are several causes of fibrosis at the ocular surface that could lead to severe visual impairment. For example, fibrosis of the cornea can occur due to viral infections, dysfunctional metabolic processes, or physical trauma or chemical contact to the eye. Conjunctival fibrosis

could arise following immune-mediated conditions including ocular mucous membrane pemphigoid, Stevens-Johnson syndrome, and graft-versus-host disease (Mallone et al., 2021).

ii. Graft-Versus-Host Disease (GVHD)

Globally, more than 30,000 patients each year undergo allogeneic hematopoietic stem cell transplantation (HSCT) in order to treat life-threatening malignancies including leukemia, lymphoma, sickle cell anemia, and immunodeficiency diseases (Hill et al., 2021). Graft-versus-host disease (GVHD) is a severe complication of allogeneic HSCT. GVHD is an autoimmune condition in which donor marrow (graft) attacks the recipient's (host) tissues as foreign. Prior to receiving a bone marrow transplant, patients undergo either total body irradiation or chemotherapy to ablate their marrow (Gehlsen et al., 2021). HLA mismatch between the donor and the recipient, female donor to male recipient, patient age, and intensity of conditioning regimen such as irradiation are a few of the risk factors that have been consistently reported for GVHD (Flowers et al., 2011). There are two forms of GVHD: acute and chronic. The clinical manifestations of acute GVHD primarily occur in the skin, liver, and the gastrointestinal tract. Chronic GVHD impacts the skin and the mucosal surfaces including those found in the mouth, lungs, gastrointestinal tract as well as the eyes (Ferrara et al., 2009).

iii. Ocular GVHD-Associated Fibrosis

Ocular involvement has been reported in patients suffering from acute or chronic GVHD. More than 40% of patients who have undergone allogeneic HSCT develop ocular GVHD (oGVHD). Moreover, oGVHD is mainly associated with chronic GVHD and develops in 40-60% of patient cases (Nassiri et al. 2013). In acute oGVHD, the clinical manifestations commonly include conjunctivitis, photophobia, corneal epithelial keratitis, corneal ulceration, and lagophthalmos. Chronic oGVHD is primarily characterized by severe ocular damage due to

fibrotic changes in the ocular surface and especially to the ocular mucosa. The clinical manifestations of chronic oGVHD include dry eye, keratoconjunctivitis sicca, keratinization, epithelial thinning, and meibomian gland atrophy (Nassar et al., 2013; Nair et al., 2021).

The ocular surface includes the cornea, conjunctiva, and lacrimal glands (Figure 1). The conjunctiva is severely affected in oGVHD and the manifestations include conjunctival fibrosis, symblepharon, and fornix shortening. Clinical reports have shown the presence of fibrotic scars and damage to the cellular structure and function of the conjunctiva in patients with chronic GVHD (Kusne et al., 2017; Kheirkhah et al., 2018). Our lab has also previously shown the presence of conjunctival fibrosis in a mouse model of major MHC-matched and minor MHC-mismatched ocular GVHD (Shamloo et al., 2021). In addition to conjunctival fibrosis, fibrosis of the lacrimal glands has been demonstrated in ocular GVHD (Yamane et al., 2018; Ogawa et al., 2021).

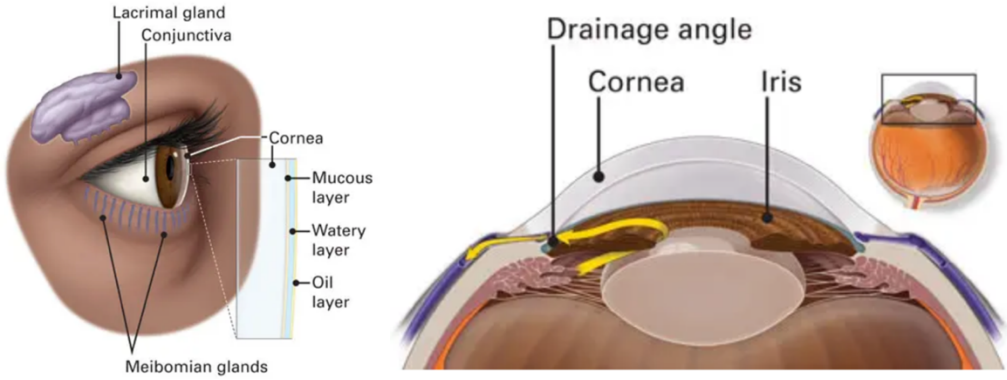

***Figure 1**: Diagram showing anatomical parts of ocular surface and tear film.*
(American Academy of Ophthalmology; https://www.aao.org/eye-health/anatomy/parts-of-eye)

Macrophage Biology

i. Macrophage Origins

The ocular surface is endowed with heterogenous populations of innate immune cells including macrophages, dendritic cells, neutrophils, mast cells, natural killer cells, and

Langerhans cells. Macrophages are sentinel phagocytic cells of the innate immune system. These large cells protect the host against microbes and clear dead and senescent cells as well as debris by phagocytosis. They also act as antigen-presenting cells, thus playing a role in both innate and adaptive immunity (Iwasaki and Medzhitov, 2015).

Based on their hematopoietic origin, there are two main macrophage populations: tissue-resident and bone marrow-derived (Figure 2). Recent studies have identified that tissue-resident macrophages primarily derive from the yolk sac during embryogenesis and populate into the tissues prior to birth (Davies and Taylor, 2015). Moreover, there are two subpopulations of yolk-sac derived tissue-resident macrophages: erythromyeloid progenitor cell-derived and fetal liver precursor cell-derived. These yolk sac-origin erythroid and liver precursor cell-derived macrophages represent long-lived tissue resident populations. These macrophages undergo local self-renewal and self-proliferation after birth (Liu and Li, 2021). After establishing residence in the tissues, these macrophages can maintain their presence into adulthood independently of contributions from bone marrow-derived precursors. Finally, tissue-resident macrophages perform the characteristic localized clearance by phagocytosis and immune regulation (Ginhoux and Guilliams, 2016). The second set of macrophage population originates from monocytes derived from hematopoietic stem cells (Lu et al., 2020). These monocyte-derived macrophages represent bone marrow-derived populations that circulate in the bloodstream and undergo chemotaxis to enter the tissues during immune response. These infiltrating macrophages perform the characteristic inflammatory response to invading pathogens as well as tissue repair and inflammation resolution. Upon depletion, these bone marrow-derived macrophages are replenished by circulating monocytes (Bailey et al., 2020; Liu and Li, 2021).

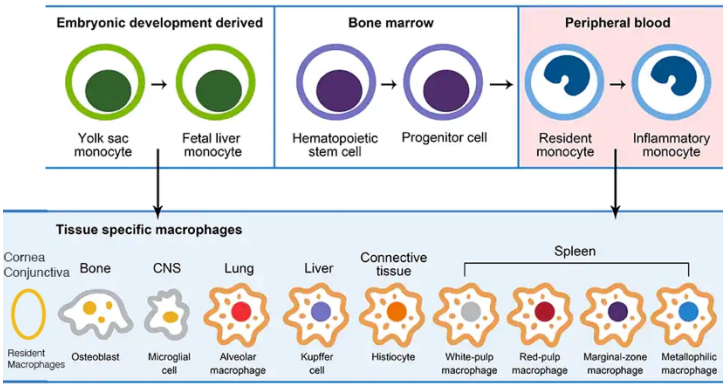

***Figure 2**: Origin of Tissue-Resident and Bone Marrow-Derived Macrophages.*
(Thermo Fisher Scientific: https://www.thermofisher.com/us/en/home/life-science/cell-analysis/cell-analysis-learning-center/immunology-at-work/macrophage-cell-overview.html)

ii. Macrophage Activation & Polarization

a) Macrophage Phenotypes: Inflammatory & Profibrotic

In addition to hematopoietic diversity, macrophages are heterogeneous cells that display a spectrum of plasticity in response to a variety of stimuli (Figure 3). These macrophage phenotypes are prominently involved in initiating and resolving inflammation as well as tissue restoration. Based on their phenotypic plasticity, macrophages can be classified into two main functional phenotypes: M1 classically-activated pro-inflammatory and M2 alternatively-activated anti-inflammatory or profibrotic (Funes et al., 2018).

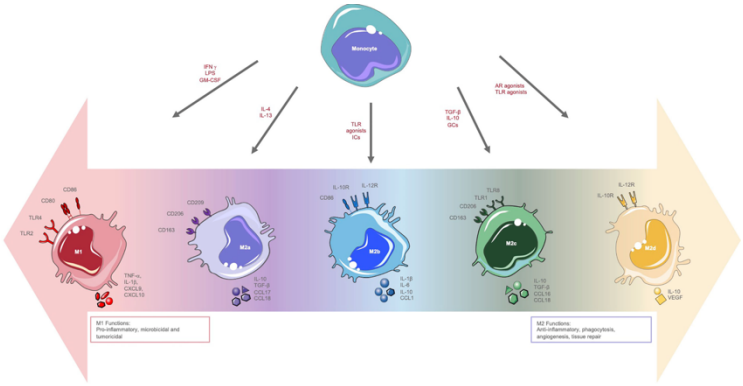

***Figure 3: Macrophage Plasticity**.*
Macrophages display a diverse spectrum of plasticity and can polarize into two main phenotypes: M1 classically-activated and M2 alternative-activated. (Chambers et al., 2021)

Non-activated M0 macrophages can differentiate into the M1 phenotype in response to pro-inflammatory stimulation such as IFN-γ and TNF-α, pathogen-associated molecular patterns including lipopolysaccharide (LPS), growth factors including GM-CSF, and endogenous noxious signals. M1 macrophages are $CD80^{+}CD86^{+}$ that promote acute and chronic inflammation by expressing inflammatory mediators including inducible nitric oxide synthase (iNOS), reactive oxygen species, IL-1β, IL-6, TNF-α, CCL2, and COX2. M1 macrophages induce an increase in nitric oxide production through iNOS activity, which promotes the characteristic inflammatory response during cell-mediated immune reactions (Klopfleisch, 2016). M1 macrophages possess enhanced cytotoxic mechanisms, microbicidal and tumoricidal capabilities, and anti-proliferative effects that are important components of host defense against invading pathogens (Mosser and Edwards, 2008). However, unresolved inflammation may exacerbate tissue injury especially by damaging neighboring tissue, which can further impede the wound healing process (Figure 4).

In contrast, M0 macrophages can differentiate into the M2 phenotype in response to stimulation by IL-4 and IL-13, immune complexes, adenosine, and glucocorticoids. M2 macrophages can be further classified as M2a, M2b, M2c, and M2d (Figure 3). Particularly, M2a macrophages are $CD163^{+}CD206^{+}$ that can attenuate inflammatory responses by expressing anti-inflammatory and profibrotic mediators including arginase-1, IL-10, and TGF-β. M2 macrophages can also express angiogenesis mediators such as vascular endothelial growth factors (VEGFs) and epidermal growth factors (EGFs). The M2 phenotype can oppose M1 pro-inflammatory activity to promote tissue regeneration (Figure 4). M2 macrophages show higher levels of urea and ornithine production through arginase-1 activity, further leading to promoting the production of ECM components such as collagen during wound healing (Klopfleisch, 2016; Funes et al., 2018). However, M2 macrophages have also been associated with uncontrolled

activation of fibrotic pathways and pathological tissue remodeling due to overproduction of extracellular matrix that is characteristic of fibrosis (Mosser and Edwards, 2008).

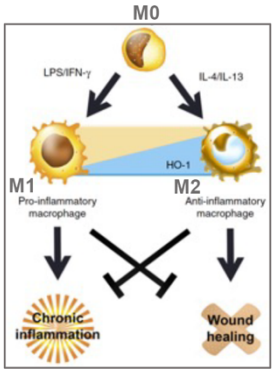

Figure 4*: Macrophage Phenotypes having Pro-inflammatory and Anti-inflammatory Functions to initiate and resolve inflammation, respectively. (Funes et al., 2018)*

b) Transcriptional Mechanisms Underlying Macrophage Polarization

There are several regulatory pathways that impact macrophage polarization (Figure 5). IFN-γ is one of the major stimuli that activates transcription factor STAT1. LPS stimulation leads to activation of toll-like receptor 4, which further activates nuclear factor kappa B (NF-κB). These key transcription factors together help drive non-activated macrophages to preferentially polarize into the M1 classically-activated phenotype that exhibits the characteristic pro-inflammatory and cytotoxic profile (Murray et al., 2014; Wang et al., 2014).

In contrast, macrophages can also be preferentially driven toward the M2 anti-inflammatory phenotype by stimulation with cytokines such as IL-4, IL-13, and IL-10. Stimulation by IL-4 and IL-13 leads to the activation of transcription factor STAT6, while IL-10 stimulation activates STAT3. Together these factors can direct macrophages to polarize into the M2 phenotype associated with inflammatory resolution and wound healing processes (Murray et al., 2014; Wang et al., 2014).

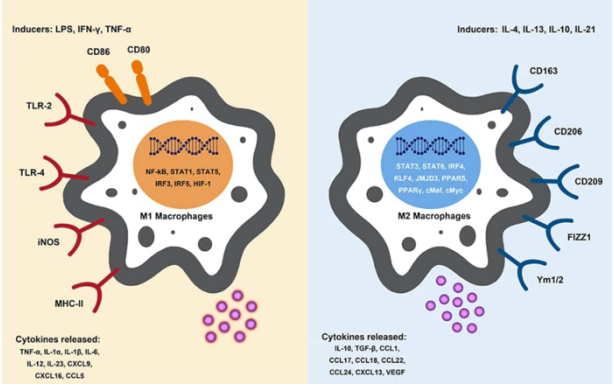

Figure 5*: Stimuli and Transcription Factors Mediating Macrophage Polarization. (Thermo Fisher Scientific: https://www.thermofisher.com/us/en/home/life-science/cell-analysis/cell-analysis-learning-center/immunology-at-work/macrophage-cell-overview.html)*

c) Metabolic Mechanisms Underlying Macrophage Polarization

One of the most critical metabolic pathways that regulates macrophage phenotype is arginine metabolism (Figure 6). Although classified as a non-essential amino acid, arginine plays a role in regulating both innate and adaptive immunity. The major enzymes that compete for arginine metabolism are nitric oxide synthase (NOS) and arginase, where metabolic activity is dictated by the presence of pro- and anti-inflammatory cytokines (Yang and Ming, 2014; Rodriguez et al., 2017). Pro-inflammatory stimulation can activate transcription factors NF-κB and STAT1, leading to classically-activated M1 macrophages that express inducible nitric oxide synthase (iNOS). In contrast, stimulation by anti-inflammatory cytokines leads to activation of STAT3 and STAT6, resulting in alternatively-activated M2 macrophages that express arginase-1 (Murray et. al, 2014).

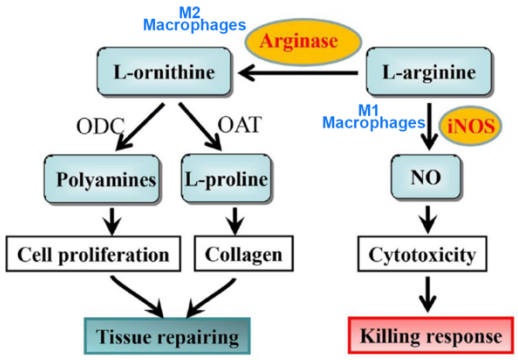

***Figure 6**: Arginine Metabolism and Polarization of Macrophages. (Yang and Ming, 2014).*

Inducible NOS (known as NOS2) is regulated by pro-inflammatory cytokines including IFN-γ, TNF-α, and IL-1β as well as bacterial LPS. Upon stimulation, iNOS metabolizes arginine to ultimately produce nitric oxide (NO) and citrulline, which promote cytotoxic reactions and the characteristic killing mechanisms by classically-activated macrophages (Rodriguez et al., 2017). NO production by M1 macrophages also leads to reduced cell proliferation. Therefore, production of significant quantities of NO is critical for innate immunity, where iNOS acts as an important mediator during inflammation and defense against invading pathogens (Gerber et al., 2019). However, overexpression of iNOS can lead to dysregulated production of NO, resulting in toxic effects and tissue damage implicated in diseases associated with chronic inflammation, sepsis, diabetes, and cancer (Cinelli et al., 2020).

Arginase-1 (Arg-1) is regulated by anti-inflammatory cytokines such as IL-4 and IL-13. Arg-1 metabolizes arginine to produce ornithine as the precursor for polyamines and proline, which promote cell proliferation and wound healing processes, respectively, associated with the M2 macrophage phenotype. Production of ornithine ultimately contributes to the production of extracellular matrix, which is a critical component of the wound healing process. Arg-1 also metabolizes arginine to produce urea, which plays a major role in detoxification of amino acid metabolism and protein degradation by reducing nitrogen levels from the body. Arg-1 acts as an

anti-inflammatory mediator by limiting the local availability of arginine, leading to reduced nitric oxide levels and iNOS activity. Thus, directing arginine metabolism toward Arg-1 activity is critical in resolving inflammation and promoting tissue repair (Yang and Ming, 2014; Rodriguez et al., 2017). However, M2 macrophages have also been shown to induce tissue fibrosis. In recent years, the M2 phenotype has been also linked to possessing tumorigenic characteristics, where immunosuppressive tumor-associated macrophages have been shown to express Arg-1 (Arlauckas et al., 2018).

Ocular Surface Macrophages and Their Physiological Functions

Due to its surface location, the ocular surface can be inflicted by potential abrasion as well as encounter harmful agents such as toxic substances and microbes. Consequently, the ocular surface possesses a unique innate immune system to protect it. Macrophages are present in both major components of ocular tissues i.e., the conjunctiva and the cornea. As in other tissues, ocular surface macrophages perform their characteristic functions essential for host defense and homeostasis including phagocytosis, initiating and resolving inflammation, tissue repair, and antigen presentation (Akpek and Gottsch, 2003; Hadrian et al., 2021). For example, macrophages in the subepithelial tissue of the conjunctiva are one of the primary cells that exert an initial acute immune response against invading pathogens (Bauer et al., 2002). In addition, studies have identified two main corneal macrophage populations using macrophage marker CD64. These populations can be categorized as $CD64^{+}CCR2^{+}$ and $CD64^{+}CCR2^{-}$ macrophages. $CD64^{+}CCR2^{+}$ corneal macrophages are mainly replenished by circulating monocytes, whereas $CD64^{+}CCR2^{-}$ corneal macrophages have been noted to perform local self-maintenance and self-proliferation. Studies have also shown that $CD64^{+}CCR2^{+}$ corneal macrophages express classical pro-inflammatory genes, such as IL-1β and TNF-α, characteristic of the M1 phenotype to

promote an inflammatory response upon corneal injury. In contrast, $CD64^{+}CCR2^{-}$ corneal macrophages express anti-inflammatory genes, such as IL-10 and Arg-1, characteristic of the M2 alternatively-activated phenotype that resolve inflammation. $CD64^{+}CCR2^{-}$ corneal macrophages have also shown to contribute to the morphological features of the cornea and play a critical role in corneal wound repair. Overall, these two corneal macrophage populations play a crucial role during corneal wound healing. Dysregulation of these macrophages could lead to severe impairment of the corneal wound healing, compromise of corneal transparency and ultimately vision (Liu and Li, 2021).

Macrophage-Mediated Profibrotic Pathways

Macrophages play a key role in promoting profibrotic signaling pathways involving the activation of fibroblasts. A variety of profibrotic cytokines and mediators released by macrophages can affect fibroblast transdifferentiation into myofibroblasts (Figure 7). These profibrotic mediators include transforming growth factor-β (TGF-β), components of the renin-angiotensin system (RAS), macrophage colony-stimulating factor (M-CSF), and platelet-derived growth factor (PDGF). Particularly, it has been demonstrated that alternatively-activated M2 macrophages are involved with wound healing as well as tissue fibrosis. Fibrosis studies in a variety of tissues such as the kidneys, lungs, and heart have shown that the M2 phenotype promotes the progressive deposition of ECM and tissue scarring by secreting significant quantities of profibrotic mediators that affect fibroblast activation and transdifferentiation (Wynn and Vannella, 2016; Wang et al., 2021).

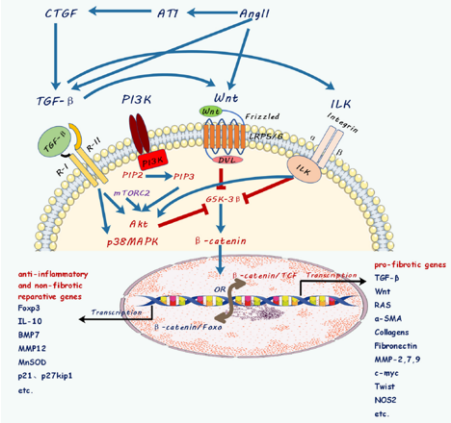

***Figure** 7: Schematic Diagram of Various Fibrotic Signaling Pathways. (Wang et al., 2021)*

i. Transforming Growth Factor-β1 (TGF-β1)

The TGF-β superfamily consists of potent, pleiotropic cytokines that play critical roles in tissue homeostasis, angiogenesis, wound healing, immunoregulation, and apoptosis. There are three known isoforms of TGF-β that are identified as TGF-β1, 2, and 3, where all three isoforms are expressed in fibrotic tissues. However, tissue fibrosis is prominently associated with TGF-β1. Signaling for members of the TGF-β family consists of two major pathways: Smad-dependent (canonical signaling) and Smad-independent (non-canonical signaling). Moreover, studies have suggested that the canonical ALK5/Smad3 pathway largely contributes to the pathogenesis of fibrosis in various tissues (Biernacka et al., 2011). While TGF-βs are essential for the growth and differentiation of various cells including fibroblasts and myofibroblasts, it has been widely studied in renal, cardiac, and ocular tissues that excessive secretion of TGF-β1 is highly implicated in fibrogenesis (Gupta et al., 2020; Wang et al., 2021). One of the most important functions of TGF-β is the upregulation of ECM components during wound repair, including collagens, proteoglycans, and glycoproteins. TGF-β also inhibits metalloproteinase activity, thereby enhancing ECM production (Isaka, 2018). Excessive secretion of TGF-β leads to significant fibrotic changes and increased levels of α-smooth muscle actin (α-SMA), which is a

myofibroblast biomarker of fibrosis (Figure 8). In addition, uncontrolled TGF-β signaling could result in overproduction of ECM components by fibroblasts that contribute to the progressive formation of scar tissue (Ueshima et al., 2019).

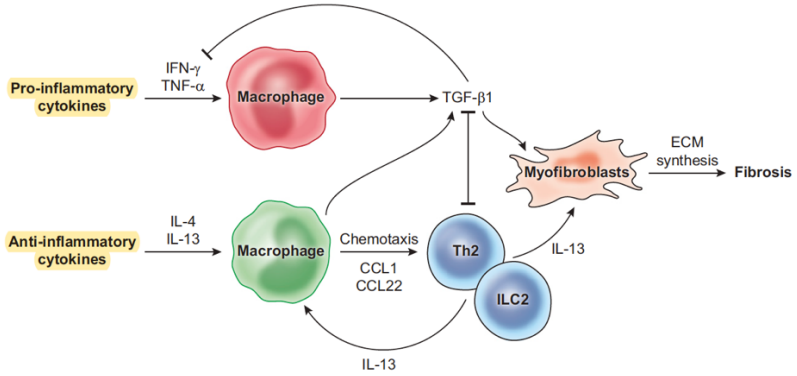

***Figure 8**: Macrophages, TGF-β and Fibrosis. (Wynn and Vannella, 2016)*

ii. Wnt/β-Catenin Signaling Pathway

Wnt/β-catenin signaling is a conserved pathway. The Wnt ligand family consists of a variety of secreted glycolipoproteins essential for cell proliferation, cell polarity, embryonic development, and tissue homeostasis. Canonical Wnt signaling involves the regulation of transcriptional co-activator β-catenin, where stabilization of cytosolic β-catenin activity is critical for Wnt signaling transduction, intercellular adhesion, and developmental processes (MacDonald et al., 2009). Mutations in the Wnt pathway have been associated with birth defects, hereditary disorders, and cancer. In renal studies, the Wnt/β-catenin signaling pathway is activated in several kidney diseases. While the Wnt/β-catenin pathway promotes tissue repair during acute kidney injury, prolonged and dysregulated activation may lead to fibrosis. Macrophages are sources of Wnt components that can direct tissue repair. In parallel, Wnt components can promote macrophage proliferation and activation, which could contribute to a cycle of activation of fibrotic pathways. Studies in other tissues have also demonstrated

macrophage involvement in inducing the expression of components of the Wnt/β-catenin pathway that are implicated in fibrogenesis (Wang et al., 2021).

iii. Renin-Angiotensin System

The renin-angiotensin system (RAS) is best known for its critical role in regulating blood volume, blood pressure, and sodium homeostasis. However, activation of components of RAS have been shown to contribute to the pathogenesis of several conditions including hypertension, cardiac diseases, and fibrosis. RAS consists of two pathways: classical and alternative. In the classical pathway, renin converts angiotensinogen into angiotensin I, which further converts into angiotensin II by angiotensin-converting enzyme (ACE). It is noteworthy that the classical pathway is involved with potentiating profibrotic signaling. In contrast, the alternative pathway reduces the activity of the classical pathway by promoting anti-inflammatory and anti-fibrotic mechanisms. In addition to the heart, activation of RAS has been consistently demonstrated in fibrosis of other tissue such as the skin, liver, and kidneys after a diverse array of injury (Kong et al., 2014; AlQudah et al., 2020). Our lab has also demonstrated for the first time that RAS activation plays a role in GVHD-associated conjunctival fibrosis, where there was a significant increase in the gene expression of angiotensinogen and ACE in the conjunctival tissue of mice with GVHD (Shamloo et al., 2021).

iv. Macrophage Colony-Stimulating Factor (M-CSF)

M-CSF is a potent cytokine that regulates and simulates the proliferation, differentiation, and activation of monocytes and macrophages. While macrophages are sources of M-CSF, this signaling has been found to potentiate fibrotic pathways. A pulmonary fibrosis study has demonstrated that inhibition of M-CSF/M-CSFR signaling led to selective loss of monocyte-derived alveolar macrophages. Moreover, depletion of these macrophages had resulted in

reducing the severity of fibrosis in the lungs (Joshi et al., 2020). It is worthwhile to mention that fibroblasts can also modulate macrophage biology through the secretion of M-CSF. Therefore, fibrosis is associated with elevated levels of M-CSF, which could result in further proliferation and activation of macrophages and setting up a vicious cycle of fibrosis (Borrello and Phipps, 1999).

v. Platelet-Derived Growth Factor (PDGF) & Connective Tissue Growth Factor (CTGF)

Platelet-derived growth factor is one of the initial growth factors deposited in damaged tissues by degranulating platelets. PDGF is essential for fibroblast proliferation and migration into the site of injury. In addition, increased levels of PDGF may lead to enhanced fibroblast activity as well as indirectly increasing ECM synthesis and wound tensile strength. However, uncontrolled PDGF signaling in injured connective tissue has been implicated in fibrotic disorders such as systemic sclerosis and dermal scarring (Rajkumar et al., 2006). Macrophages can be sources of PDGFs, where these growth factors serve as chemotactic agents for myofibroblasts and promote myofibroblast proliferation and production of ECM components such as collagen and fibronectin (Chegini, 2010). Another profibrotic mediator produced by macrophages is connective tissue growth factor, which is one of the key regulators of cell proliferation, angiogenesis, and connective tissue repair mechanisms and wound healing. While CTGF is critical for embryonic development and the physiological function of various organs, overexpression of CTGF has been implicated in several adulthood diseases including tissue fibrosis (Chen et al., 2020).

vi. Other Profibrotic Mediators

Epidermal growth factors such as EGF, TGF-α, and heparin-binding EGF act as mitogenic factors and regulate differentiation of a variety of cells including fibroblasts. Expression of EGFs

plays a role in wound repair processes. However, these growth factors have also been indicated in tissue fibrosis and could promote fibroblasts and myofibroblasts to overproduce extracellular matrix. EGFs may also interact with other growth factors such as PDGFs that could contribute to the progression of fibrosis. Insulin-like growth factors (IGFs) are involved in growth and metabolism. IGFs in tandem with EGFs and PDGFs can induce fibroblast proliferation that could further give rise to fibrogenesis. VEGFs and fibroblast growth factors play important roles in angiogenesis and wound healing by stimulating the proliferation and activation of fibroblasts. However, these factors can also be involved in the pathogenesis of tissue fibrosis (Chegini, 2010).

Effects of Radiation on Macrophage Activation

Radiation therapy remains as one of the widely used treatment options for life-threatening conditions including many cancers. For bone marrow transplant, patients must undergo a conditioning regimen such as total body irradiation to ablate their diseased bone marrow. Although irradiation impacts the biological function and viability of immune cells, studies have indicated that radiation exposure may induce macrophage activation status. For example, ionizing radiation has been reported to affect macrophage activation by modulating the expression of classical and alternative macrophage markers (Wu et al., 2017). A variable trend of pro-inflammatory markers, such as CCL2, IL-6, and CD80, have been observed upon radiation exposure. Studies have also shown a variable trend in the expression of anti-inflammatory markers including CD163, CD206, and IL-10 (Teresa Pinto et al., 2016; Mikhalkevich et al., 2021).

Macrophages exhibit resistance to the effects of ionizing radiation. Although DNA damage can be induced by irradiation in macrophages, apoptosis may or may not occur

depending on the level of exposure. It has been shown that macrophages are viable and metabolically active even after radiation exposure of 10 Gy, suggesting that irradiation does not significantly induce apoptosis (Teresa Pinto et al., 2016). Moreover, it is also worthy to note that M2 anti-inflammatory macrophages have been shown to be more radioresistant in comparison to M0 and M1 pro-inflammatory macrophages (Leblond et al., 2017).

Macrophage-Fibroblast Cross-Talk in Ocular GVHD-Associated Fibrosis

The outermost layer of the ocular surface is composed of corneal epithelium and palpebral and bulbar conjunctiva that represent features of connective tissue and the mucosa. The ocular surface contains resident macrophages and fibroblasts (Palomar et al., 2019; Alfuraih et al., 2020). Insults on the ocular surface such as mechanical injury or radiation could lead to the activation of macrophages and release of profibrotic mediators that could induce the transdifferentiation of fibroblasts into myofibroblasts. Consequently, this unregulated activation could result in ocular surface fibrosis. The underlying mechanisms of ocular surface fibrosis in the context of GVHD are still being understood. Currently, there are no studies that have investigated the effect of radiation on macrophage activation and how it affects the crosstalk between macrophages and fibroblasts in the context of GVHD-associated ocular surface fibrosis.

CHAPTER 3

METHODOLOGY

Murine Macrophage Culture & Polarization

To obtain murine macrophages, hematopoietic stem cells from the bone marrow of femurs and tibias isolated from BALB/c mice. The mice were euthanized using CO_2 and marrow was flushed out using 1X solution of Ca^{2+}/Mg^{2+}-free PBS with 2% FBS. Red blood cells were lysed by incubating with a commercially available lysis buffer for 3 minutes (BD Biosciences, Frankline Lakes, NJ, USA). The cells were centrifuged at 1500 rpm for 5 minutes and resuspended in 18 ml of macrophage growth medium (Table 1). The cells were plated in three 6-well plates (USA Scientific, Inc, Ocala, FL, USA) and cultured in macrophage medium containing M-CSF at 37°C with 5% CO_2 and 95% humidity in a cell culture incubator (Forma series II water jacket, Thermo Fisher Scientific, Marietta, OH, USA). The media was replaced three times during a 7-day period to obtain M0 macrophages. The M0 macrophages thus obtained were further polarized into M1 and M2 macrophages (Ying et al., 2013).

M1 macrophage polarization was initiated by culturing the M0 macrophages in medium supplemented with IFN-γ + LPS (Table 2). These cells were exposed to M1 macrophage medium for 48 hours. M2 macrophage polarization was initiated by culturing the M0 macrophages in medium supplemented with IL-4 (Table 3). These cells were exposed to M2 macrophage medium for 72 hours. Representative images of the polarized macrophages were obtained using EVOS microscope (Thermo Fisher Scientific, Marietta, OH, USA). The macrophages were stained for M0, M1, and M2 markers (Table 4) and analyzed using flow cytometry to confirm polarization (FACSVerse Flow Cytometer, BD Biosciences, Franklin Lakes, NJ, USA). Real-time PCR was used to quantify gene expression of M1 and M2 markers iNOS and arginase-1, respectively. Real-time PCR and antibody-coated bead-mediated flow cytometry protein quantification were used to quantify macrophage chemokines CCL17 and CCL22.

Table 1: Composition of Macrophage Growth Medium

Components	Concentration
Iscove's Modified Dulbecco's Medium	
Heat-Inactivated Fetal Bovine Serum	10%
Penicillin/Streptomycin	100 U/mL
M-CSF	10 ng/μL

Table 2: Composition of M1 Macrophage Medium

Components	Concentration
Macrophage Growth Medium	
IFN-γ	50 ng/μL
LPS	100 ng/mL

Table 3: Composition of M2 Macrophage Medium

Components	Concentration
Macrophage Growth Medium	
IL-4	20 ng/μL

Table 4: Macrophage Markers

Macrophage Phenotype	Anti-Mouse Marker	Fluorochrome	Detection
M0	CD11b	Alexa 647	Red (633 nm)
M0	F4/80	BV510	Violet (405 nm)
M1	CD86	APC/Cy7	Red (633 nm)
M2	CD206	PE	Blue (488 nm)

Murine Corneal Fibroblast Culture

To test the effect of macrophage polarization on fibroblast transdifferentiation, murine corneal fibroblasts were used (Mittal et al., 2016; Lin et al., 2007). These murine corneal fibroblasts are human telomerase reverse transcriptase (hTERT) immortalized cells and do not show any sign of senescence until 100 passages. These murine fibroblasts are termed as MK/T-1

cells (Gendron et al., 2001). The cells were plated at a density of 1 x 10^6 cells/mL in a T-25 flask (CytoOne® USA Scientific, Inc, Ocala, FL, USA). The cells were cultured in fibroblast growth medium containing 2% FBS at 37°C with 5% CO_2 and 95% humidity (Table 5). The cells were maintained until at least 80% confluence was reached. The cells were then trypsinized and plated in 12-well inserts (SABEU GmbH & Co. KG, Northeim, Germany) for a 12-well plate (CytoOne® USA Scientific, Inc, Ocala, FL, USA) at a density of 1x10^4 cells/mL. For immunostaining experiments, the cells were plated in tissue cultured treated 4-chambered glass slides (Corning Inc., Big Flats, NY, USA) at a density of 8 x 10^4 cells/mL. The corneal fibroblasts were co-cultured in inserts placed on top of M0, M1, and M2 macrophages.

Table 5: Composition of Fibroblast Growth Medium

Components	Concentration
Dulbecco's Modified Eagle Medium	
L-Glutamine	200 mM
Heat-Inactivated Fetal Bovine Serum	2%
Penicillin/Streptomycin	100 U/mL
Sodium Pyruvate	100 mM

Macrophage Polarization and Fibroblast Transdifferentiation

To test the effect of macrophage polarization on fibroblast differentiation, ocular surface fibroblasts were cultured on clear 0.4 μm PET-membrane cell culture inserts (SABEU GmbH & Co. KG, Northeim, Germany). The inserts were then placed in 6 or 12 well plates containing cultures of M0, M1 or M2 macrophages on the bottom well of these plates as shown below in Table 6. The fibroblasts and macrophages were co-cultured for 3 days. The experiments were conducted in duplicate. Immunostaining and gene expression quantification for α-SMA was used as a marker for myofibroblast formation.

Table 6: Experimental Design of Fibroblast Treatment

Treatment				
Inserts	Fibroblasts	Fibroblasts	Fibroblasts	Fibroblasts
Bottom Wells	Media only	M0 macrophages	M1 macrophages	M2 macrophages

α-SMA Immunostaining

The cells were rinsed with 1x PBS and fixed in 4% paraformaldehyde for 15 minutes followed by rinsing with 1x PBS. The cell membranes were permeabilized with a 1x PBS solution containing 0.25% Tween20 for 15 minutes. The cells were rinsed again with 1x PBS and non-specific binding was blocked with a 1x PBS solution containing 2% BSA for 30 minutes. The cells were incubated with the murine primary antibody for α-SMA at a dilution of 1:100 for 90 minutes (Invitrogen, Waltham, MA, USA). The cells were washed with 1x PBS and then incubated with Alexa Fluor 488 goat anti-mouse secondary antibody at a dilution of 1:250 for 60 minutes (Invitrogen, Waltham, MA, USA). The nuclei were stained with DAPI. The immunostained cells were imaged using confocal microscopy (Nikon, Melville, NY, USA). Image J Software (National Institutes of Health, MD, USA) was used to quantify the number of nuclei stained for α-SMA.

Isolation of mRNA and Preparation of cDNA

To test the effect of polarized macrophages on the expression of profibrotic gene in the ocular surface fibroblasts, RNA was isolated, reversed transcribed to cDNA and gene expression was quantified using real-time PCR.

A commercially available kit (QIAGEN's RNeasy Mini Kit) was used to isolate the mRNA. The media was aspirated from each well and 350 μL of RNase inhibitor containing RLT buffer was added to each well to induce cell lysis. The lysate was then transferred to the QIA

shredder spin column and centrifuged to further facilitate shredding. The flow-through was collected and an equal volume of 70% molecular biology grade ethanol was added to the lysate to precipitate the DNA. The lysate was transferred to the RNeasy spin column which binds RNA. The column was centrifuged at 10,000 rpm for 30 seconds. The column was then washed with RW1 buffer and RPE buffer. Finally, 30 μL of RNase-free water was added to the column membrane and incubated for 5 minutes at room temperature followed by centrifugation for 1 minute to elute the RNA. The isolated RNA was immediately used for cDNA synthesis.

The RNA was reversed transcribed to cDNA using a commercially available kit (SuperScript® III First-Strand, Invitrogen, CA, USA). For each RNA sample, 8 μL of isolated RNA sample was combined with 1 μL of 50 μM oligo$(dT)_{20}$ primer and 1 μL of 10 mM dNTP mix. The mixture was incubated at 65°C for 5 minutes using MiniAmp Thermocycler followed by incubation on ice for 1 minute (Applied Biosystems, Thermo Fisher Scientific, Singapore). A 10 μL of cDNA synthesis mixture was added to the reaction mixture containing the RNA sample (Table 7). The mixture was incubated at 50°C for 50 minutes followed by 85°C for 5 minutes. The samples were incubated on ice for 1 minute followed by the addition of 1 μL of RNase H. The mixture was incubated at 37°C for 20 minutes. Lastly, 40 μL of DEPC water was added to the reaction mixture to complete cDNA synthesis.

Table 7: Composition of cDNA Synthesis Mixture

Components	1 Sample
10X RT Buffer	2 µL
25 mM $MgCl_2$	4 µL
0.1 M DTT	2 µL
RNaseOut™ (40 U/ µL)	1 µL
Superscript III RT (200 U/ µL)	1 µL

Real-Time PCR

The gene expression of the following genes was quantified using real-time PCR: α-SMA, TGF-β1, angiotensinogen, ACE, M-CSF, and PDGF. For each gene, a PCR master mixture containing SYBR green PCR master mix (Applied Biosystems, Thermo Fisher Scientific, Waltham, MA) was prepared as shown in Table 8 and using the primers shown in Table 9. A total of 20 µL was used per reaction (2 µL cDNA + 18 µL master mix). Beta actin was used as the housekeeping gene. QuantStudio™ 3 Real-Time Thermocycler was used to run the PCR reactions (Applied biosystems, Thermo Fisher Scientific, Singapore). The thermocycler settings for PCR: a) Step 1 (Activation): 50°C for 2 minutes then 95°C for 2 minutes for thermal activation of AmpliTaq Gold DNA Polymerase; b) Step 2 (PCR): 40 cycles of 95°C for 15 seconds to cause denaturation and 60°C for 1 minute for annealing and extension; c) Step 3 (Melt Curve): 95°C for 15 seconds, 60°C for 1 minute, and then 95°C for 15 seconds.

Table 8: Composition of Real-Time PCR Master Mix

COMPONENTS	VOLUME PER REACTION
Master Mix (SYBR Green)	10 µl
F primer for target gene	2 µl
R primer for target gene	2 µl
DEPC water	4 µl
Total Volume	18 µl

Table 9: List of Murine Genes and Forward and Reverse Primers

GENE	FORWARD PRIMER	REVERSE PRIMER	NM ACCESSION NUMBER
α-SMA	CGA TCA TGC GTC TGG ACT T	GGC AGT AGT CAC GAA GGA ATA	NM_007392.3
TGF-βR1	GGG CTT AGT GTT CTG GGA AA	CCG ATG GAT CAG AAG GTA CAA G	NM_009370.3
Angiotensinogen	TCC CAG GCT CTC TGG ATT TA	CAA GTT CAT CTT CCA CCC TGT	NM_007428.4
ACE	GAC AGG TTC GTG GAA GAG TAT	TTG CTG CCC TCT ATG GTA ATG	NM_207624.6
M-CSF	GAC AGA TGA GAA GGA GCA GAA G	GCT GTC CCA CCC TTT GAA TA	NM_007778.4
PDGF	GTC CAT ACG GGA AGA GCT AAA G	GGA GAC AAC AGG CAC AAT TTC	NM_019971.3
CD80	TCG GCG CAG TAA TAA CAG TC	GTT TCT CTG CTT GCC TCA TTT C	NM_009855.2
iNOS	GCC TGT GAG ACC TTT GAT GT	TGG ATG AGC CTA TAT TGC TGT G	NM_001313922.1
CCL2	GAA GGA ATG GGT CCA GAC ATA C	CAC ATT CAA AGG TGC TGA AGA C	NM_011333.3
IL-1β	CCA CCT CAA TGG ACA GAA TAT CA	CCC AAG GCC ACA GGT ATT T	NM_008361.4
TNF-α	TTG CTC TGT GAA GGG AAT GG	GGC TCT GAG GAG TAG ACA ATA AAG	NM_013693.3
IL-6	TTT CCT CTG GTC TTC TGG AGT A	CTC TGA AGG ACT CTG GCT TTG	NM_031168.2
CD206	CAG GTG GCT TAT GGG ATG TT	CAT TTG GGT TCA GGA GTT GTT G	NM_008625.2
Arginase-1	GGG CTC CTT TCA GGA CTA GAT A	CGA AGC AAG CCA AGG TTA AAG	NM_007482.3
IL-10	TTG AAT TCC CTG GGT GAG AAG	TCC ACT GCC TTG CTC TTA TTT	NM_010548.2
CCL17	GGA AGT TGG TGA GCT GGT ATA A	GAT GGC CTT CTT CAC ATG TTT G	NM_011332.3
CCL22	ACA CAC CTC CCA AGT TTC TAT C	CAG CCT GAA ACT CCC AGA AT	NM_009137.2
β-Actin	CTC CCT GGA GAA GAG CTA TGA	CCA AGA AGG AAG GCT GGA AA	NM_007393.5

Effect of Irradiation on Macrophage Activation and Polarization

Bone marrow-derived M0 macrophages were exposed to 7 Gy radiation using an x-ray irradiator shown in Figure 9 for 3 minutes and 26 seconds (RS 2000 X-ray Biological Irradiator: Rad Source Technologies, GA, USA). Macrophages were incubated at 37°C with 5% CO_2 and 95% humidity to allow for radiation effects.

Figure 9: X-Ray Irradiator. *RS 2000 X-ray Biological Irradiator was used to irradiate macrophages. The following parameters were set: 160 kV, 25.0 mA, Configuration: Shelf Only, Shelf Level: 3, Radiation: 7 Gy, Exposure Time: 3 mins 36 secs.*

Effect of irradiation on macrophage phagocytosis

M0 macrophages were plated at a density of 5 x 10^4 cells/mL in tissue cultured treated 4-chambered glass slides and exposed to irradiation as described above. Two hours after irradiation, macrophages were exposed to Alexa Fluor™ 594 conjugated zymosan A (*S. cerevisiae*) particles (Invitrogen, Waltham, MA, USA) at a dilution of 1:50 and incubated for 15 minutes at 37°C with 5% CO_2 and 95% humidity followed by washing twice with 1x PBS. The cells were fixed with 4% paraformaldehyde for 15 minutes followed by washing with 1x PBS. The cells were permeabilized using 0.25% Tween20 for 15 minutes followed by washing with 1x PBS. Non-specific binding was blocked using 2% BSA for 15 minutes. The cell outlines were visualized by f-actin staining using Alexa Fluor 488 conjugated phalloidin (Invitrogen, Waltham, MA, USA) at a concentration of 2 drops/mL and incubated for 30 minutes followed by washing with 1x PBS. The nuclei were stained using DAPI. Phagocytic activity was assessed using confocal microscopy and the number of macrophages containing zymosan particles were quantified using Image J Software (Ragsdale and Grasso, 1989).

Effect of irradiation on macrophage apoptosis/necrosis

Six hours after irradiation, macrophages were stained using an Annexin V APC Conjugated Apoptosis Assay Kit (Cayman Chemical, Ann Arbor, MI, USA). Unstained macrophages were used as the negative control. A 1x Annexin V binding buffer was prepared using a cell-based assay Annexin V binding buffer (10x) at a dilution of 1:10 with DI H_20. The cells were trypsinized and collected from 6-well plates followed by centrifugation at 400 x g for 5 minutes. The cells were resuspended in Annexin V APC/DAPI staining solution (Table 10) using an Annexin V APC assay reagent and DAPI viability dye and incubated in the dark at

room temperature for 10 minutes. The cells were centrifuged at 400 x g for 5 minutes and resuspended in 1x PBS. Apoptosis was analyzed using FACS flow cytometry.

Table 10: Composition of Annexin V APC/DAPI Staining Solution

Components	Quantity
Annexin V APC Assay Reagent	20 µL
DAPI Viability Dye	20 µL
1x Annexin V Binding Buffer	2 mL

Effect of irradiation on macrophage activation and polarization

M0 macrophages were plated at a density of 1.6 x 10^5 cells/well in 6-well plates. Twenty-four hours after irradiation, the mRNA and cDNA were isolated using the methods described earlier. Real-time PCR was used to quantify the cytokines and markers of classical and alternative macrophage activation status CD80, iNOS, CCL2, IL-1β, TNF-α, IL-6, CD206, Arg-1, and IL-10 using primers listed in Table 9 (Teresa Pinto et al., 2016; Leblond et al., 2017; Mikhalkevich et al., 2021).

Statistical Analysis

All data are presented as mean ± standard error of the mean. Fold changes of mRNA were calculated using the $^{\Delta\Delta}$Ct method. Statistical analysis was performed using GraphPad Prism software (Version 9, San Diego, CA, USA). t test was used for analysis of the data for the macrophages exposed to irradiation compared to macrophages not exposed to irradiation. One way ANOVA followed by Tukey's test was used to perform statistical analysis for comparisons between M0, M1 and M2 macrophages.

CHAPTER 4

RESULTS

Polarized Macrophage Culture and Characterization

M0 macrophages were successfully generated from hematopoietic stem cells isolated from murine bone marrow by culturing them in M-CSF (Figure 10). Flow cytometry data shows that an average of 70% of these cells expressed Pan macrophage markers CD11b and F4/80 after culturing them in M-CSF for one week, thus confirming the successful differentiation of M0 macrophages from hematopoietic stem cells (Figure 11). Further polarization of M0 macrophages to M1 and M2 phenotypes was obtained by culturing these cells in M-CSF and IFN-γ + LPS or M-CSF + IL-4, respectively (Figure 10). Flow cytometry data confirmed that an average of 80% of the macrophages expressed the M1 marker CD86 after culturing them in M-CSF and IFN-γ + LPS for 48 hours (Figure 11). An average of about 40% of the macrophages expressed the M2 marker CD206 after culturing them for 72 hours in M-CSF + IL-4 (Figure 11). Figure 10 shows there is a notable difference in the morphology and confluency of M1 macrophages compared to M0 and M2 macrophages since M1 macrophages undergo significant levels of apoptosis.

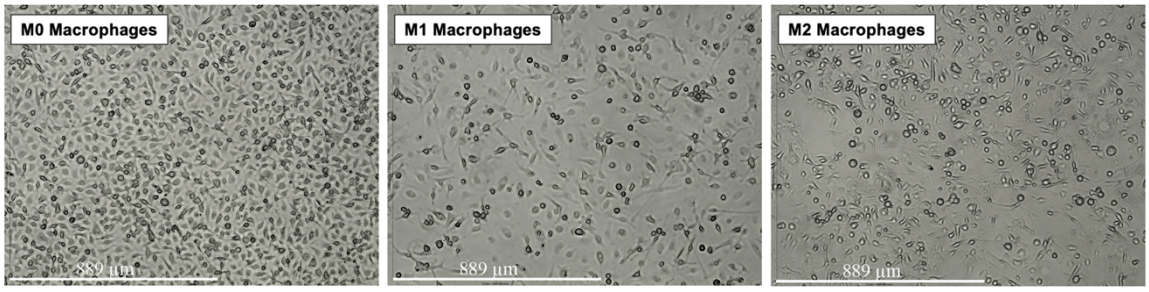

Figure 10*: Representative phase contrast microscopy images of cultured and polarized murine bone marrow-derived macrophages.*

We further confirmed the M1 and M2 macrophage phenotypes by gene expression analysis (Figure 12). In congruence with the flow cytometry data, our gene expression data confirmed that M1 macrophages expressed high levels of iNOS, a key M1 marker. Similarly, M2

macrophages showed a very high expression of arginase-1, which is specific for the M2 phenotype (Figure 12).

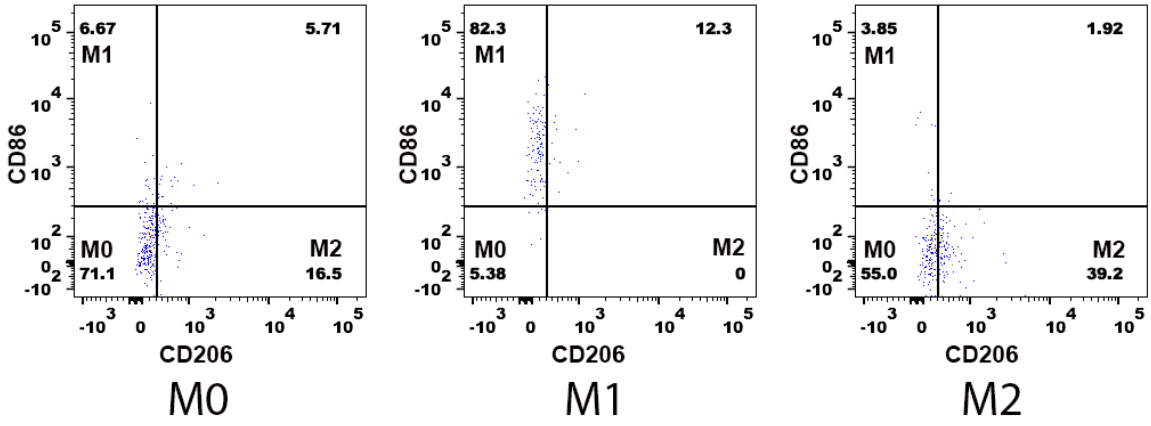

***Figure 11**: Flow cytometry data showing the anticipated expression of Pan (CD11b, F4/80), M1 (CD86) and M2 cell surface (CD206) markers confirming the successful generation of macrophage phenotypes. Full gating scheme for CD11b and F4/80 plots shown in Figure 13.*

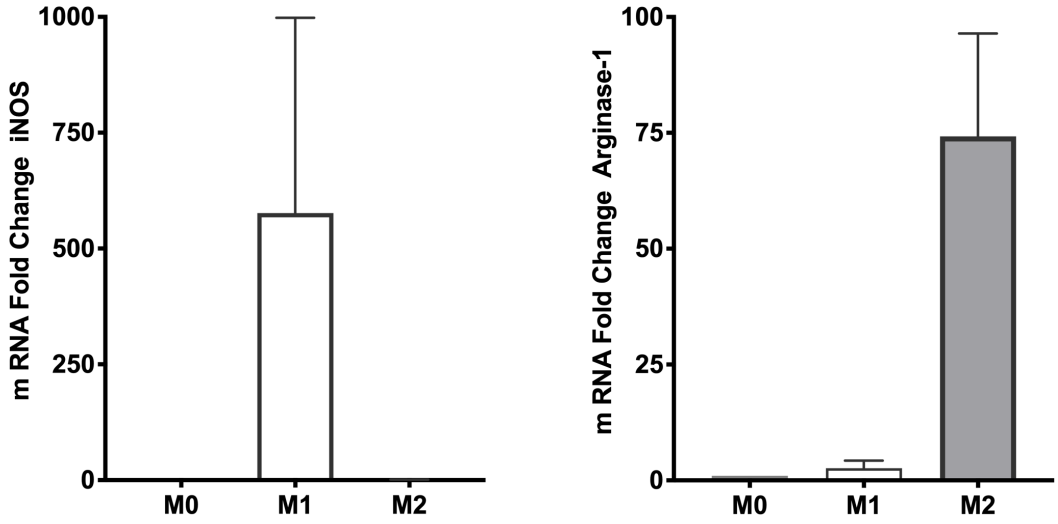

***Figure 12**: Real-time PCR quantification showing the anticipated high expression of iNOS genes in M1 macrophages and arginase-1 genes in M2 macrophages. (The graph shows mean ± SEM data from N = 3 replicates)*

Lastly, we confirmed that we were able to successfully identify macrophage phenotypes in a heterogenous macrophage population. To achieve this goal, we pooled a mixture of M0, M1 and M2 macrophage and characterized them by flow cytometry. Our flow cytometry data shows

that about 91% of the pooled macrophages expressed CD11b, and 77% of those $CD11b^+$ cells were also positive for F4/80 (Figure 13). In the mixture, 50% cells were M0 ($CD11b^+F4/80^+CD86^-CD206^-$) and as anticipated based on our mixture of cells, about 17% of the cells were positive for M1 surface marker CD86, while 23% of the cells were positive for M2 surface marker CD206 (Figure 13). Thus, our data shows that we were able to successfully characterize M0, M1 and M2 phenotypes in a heterogeneous mixture of macrophages.

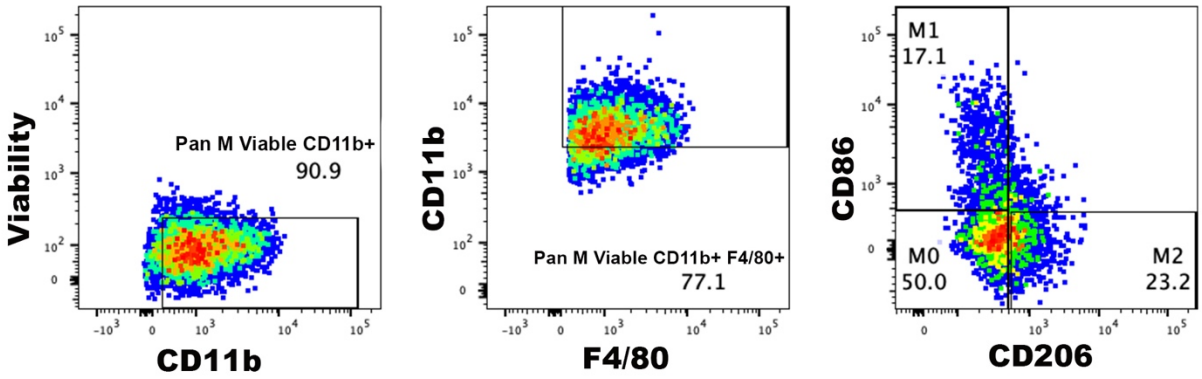

***Figure 13**: Flow cytometry characterization of macrophage phenotypes using Pan (CD11b, F4/80), M1 (CD86) and M2 cell surface (CD206) markers.*

Effect of Irradiation on Macrophage Activation

After generating and charactering M0, M1 and M2 macrophages, we wanted to test the effect of irradiation on these macrophages. Irradiation is used to ablate diseased bone marrow prior to the transplantation. Clinical data shows that irradiation is a major risk factor for ocular GVHD-associated fibrosis. Activated macrophages, especially the M2 phenotype, can release profibrotic mediators, thus we hypothesized that irradiation-activated generation of M2 macrophages can orchestrate GVHD-associated fibrosis. Therefore, we assessed whether irradiation causes macrophage activation and polarization. Our data demonstrates that irradiation caused an increase in gene expression of pro-inflammatory cytokines in M0 macrophages (Figure 14). After radiation exposure, there was a 2-fold ± 0.29 increase in IL-1β, a 4.2-fold ±

1.21 increase in TNF-α, and a 2-fold ± 0.99 increase in IL-6. Further, a 2.4-fold ± 0.77 increase in chemokine CCL2 was also noted (Figure 14). CCL2 is known to cause the recruitment of monocytes, dendritic cells, and T cells. However, irradiation did not cause any upregulation of M1 macrophage markers i.e., CD80 and iNOS. In contrast, our data demonstrates that irradiation caused a robust increase in gene expression of M2 phenotype markers i.e., a 2.7-fold ± 1.5 increase in CD206, a 16.4-fold ± 4.1 increase in arginase-1, and a 4-fold ± 0.4 increase in IL-10 (Figure 14).

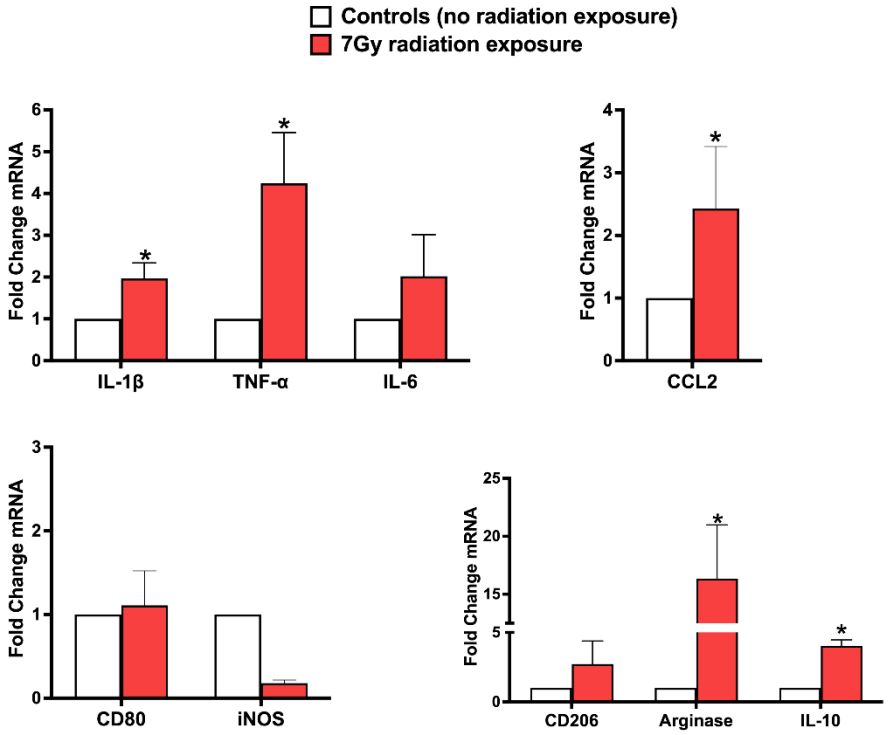

***Figure 14**: Real-time PCR quantification of changes in expression of pro-inflammatory cytokines, chemokines, M1 and M2 markers twenty-four hours after exposing M0 macrophages to 7 Gy radiation. (* p< 0.05 compared to control M0 macrophages that were not exposed to irradiation. The graphs show mean ± SEM data from N = 5 replicates)*

Effect of Irradiation on Macrophage Phagocytic Activity

Our data demonstrates that exposing M0 macrophages to irradiation caused a notable increase in the uptake of zymosan particles compared to control macrophages that were not

exposed to irradiation (Figure 15A). Quantification from 54 images captured at 20X magnification showed that without irradiation, 41± 2 macrophages engulfed zymosan particles whereas after irradiation 52±1 macrophages showed engulfed zymosan particles (Figure 15B). Overall, these results suggest that irradiation potentially promotes macrophage phagocytic activity.

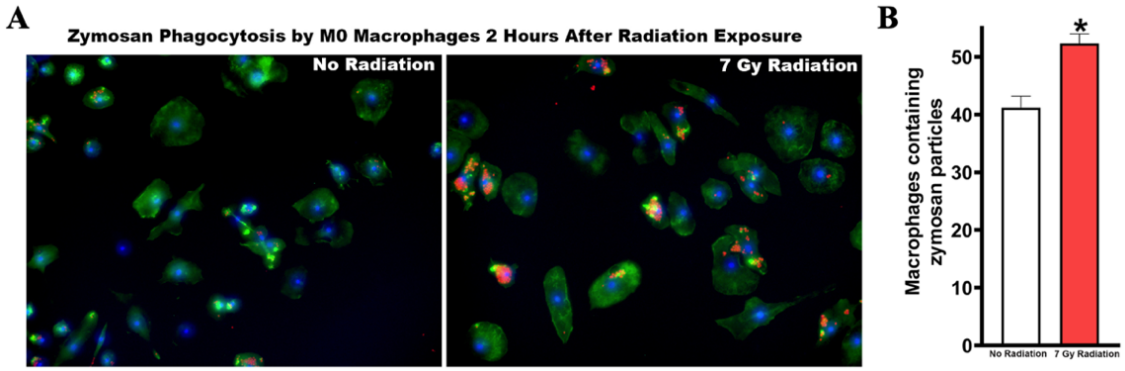

***Figure 15**: (A) Representative immunofluorescent confocal microscopy images showing the effect of irradiation on macrophage phagocytic activity two hours after 7 Gy radiation exposure, and (B) quantification macrophage zymosan uptake counted from 54 images. (* $p < 0.05$ compared to control macrophages that were not exposed to irradiation. The graph shows mean ± SEM data from N= 4 replicates)*

Effect of Irradiation on Macrophage Cell Death

Finally, we assessed whether irradiation could cause macrophage cell death. As shown in Figure 16, our data demonstrates that control macrophages not exposed to irradiation had 85.6± 4.5% viable cells and had 0.22±0.03% necrotic and 14.1±4.5% apoptotic cells. Irradiation did not alter macrophage viability since irradiated macrophages showed 85.7±3% viability and had 0.11± 0.04% necrotic and 19.8±5.8% apoptotic cells. Therefore, the data suggest that while irradiation could potentially induce macrophage activation and phagocytic activity, radiation exposure has no effect on macrophage apoptosis and necrosis and cell viability.

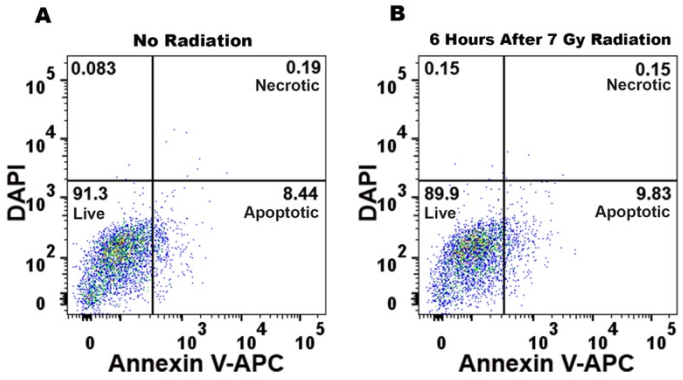

***Figure 16**: Representative flow cytometry data assessing macrophage apoptosis/necrosis six hours after irradiation exposure. The cell population in the lower left quadrant which is not stained by DAPI and annexin is viable. The cells in the lower right quadrant which are stained by annexin are apoptotic and cells in upper right quadrant which are DAPI and annexin stained are necrotic. (The results were mean ± SEM data from N= 3 replicates)*

Effect of Polarized Macrophages on Ocular Surface Fibroblast Transdifferentiation

Next, we tested whether macrophages could cause transdifferentiation of ocular surface fibroblasts to myofibroblasts through the release of profibrotic mediators in a paracrine manner. To test the transdifferentiation of fibroblasts to myofibroblasts, we used α-SMA as a marker for myofibroblast formation. Our data demonstrates that exposing the fibroblasts to M0 and M2 macrophages was able to induce a noticeable increase in α-SMA expression as shown in Figure 17A. This observation is further supported by the quantification of gene expression of α-SMA, which shows that both M0 and M2 macrophages were able to induce a significant increase in α-SMA gene expression in fibroblasts (Figure 17B). In contrast, M1 macrophages did not induce α-SMA expression. In summary, our data demonstrates that M0 and M2 macrophages could potentially induce the transdifferentiation of ocular surface fibroblasts to myofibroblasts.

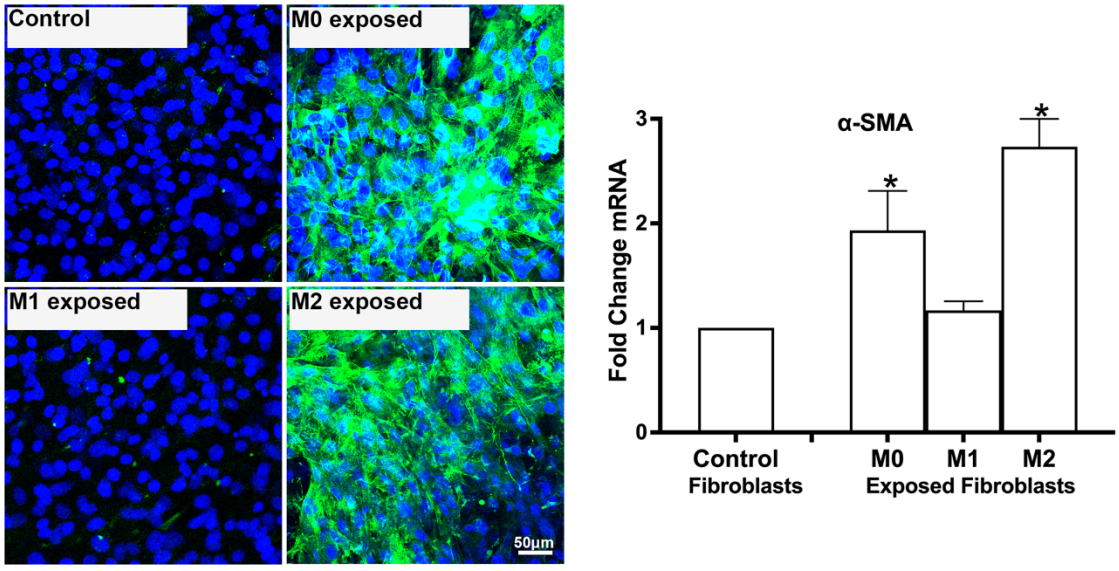

***Figure 17**: (A) Representative immunofluorescent microscopy images and (B) real-time PCR data showing α-SMA protein and gene expression in cultured corneal fibroblasts that were exposed to M0, M1 and M2 macrophages. (*p < 0.05 compared to control fibroblasts that were not exposed to macrophages. The graph shows mean ± SEM data from N=4 replicates)*

Effect of Polarized Macrophage Exposure on Profibrotic Mediators Expression in Ocular Surface Fibroblasts

It is already well known that macrophages, especially the M2 phenotype, release a multitude of profibrotic mediators that can cause transdifferentiation of fibroblasts to myofibroblasts as was observed in our experiments. We further wanted to test whether these profibrotic mediators released from macrophages could cause an increase in the expression of profibrotic mediators in the ocular surface fibroblasts. Our data demonstrates that co-culturing corneal fibroblasts in inserts on the top of M2 macrophages caused a notable increase in components of renin angiotensin system i.e., angiotensinogen and angiotensin-converting enzyme (Figure 18). An increase in TGF-β1 receptor expression was also noted in fibroblasts upon exposure to both M1 and M2 phenotypes. Lastly, co-culturing corneal fibroblasts in inserts on the top of macrophages caused a notable decrease in expression of PDGF and no change was observed in M-CSF gene expression (Figure 18).

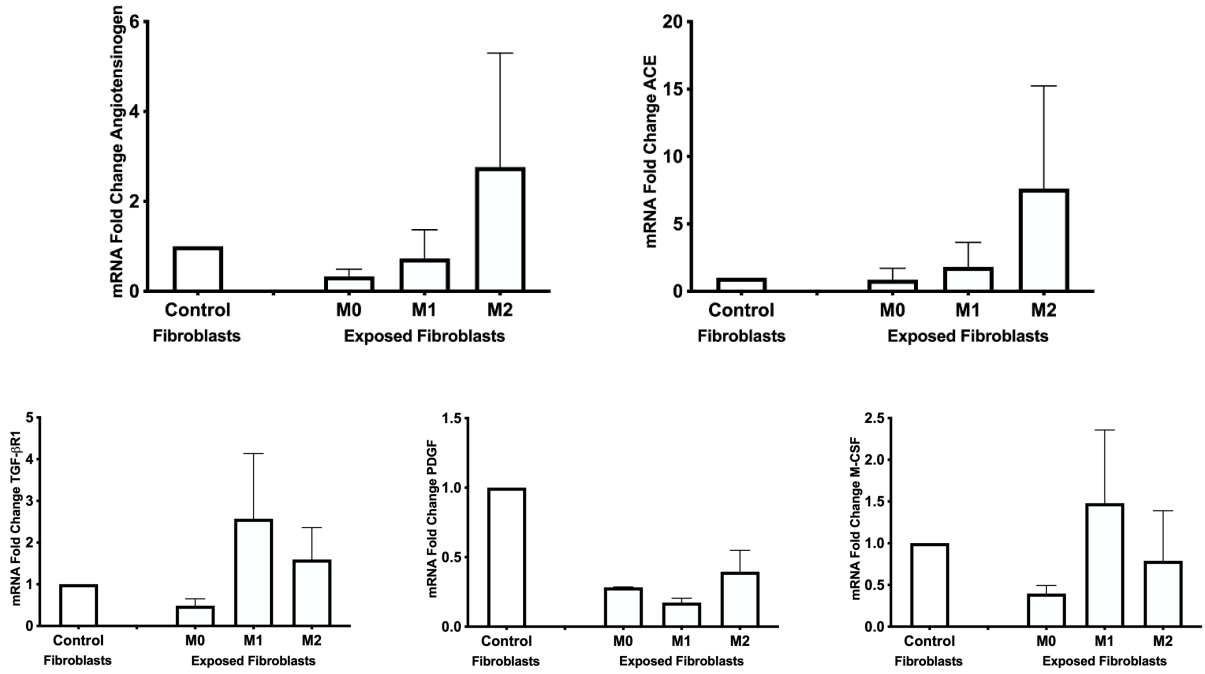

Figure 18*: Real-time PCR gene quantification of profibrotic mediators in corneal fibroblasts exposed to M0, M1, and M2 macrophages. (The graph shows mean ± SEM data from N=2 replicates)*

Macrophage Phenotypes and T-Helper Attractant Chemokines

Pathological features of ocular GVHD can be divided into three phases and infiltration of T cells is a key feature towards the end of the first phase extending into the second phase of the disease pathology. CCL17 and CCL22 are two key chemokines involved in CD4+ T cell chemotaxis. Thus, we wanted to test whether macrophage polarization results in differential secretion of these two chemokines. Our data shows that M1 phenotype macrophages have high gene expression of CCL22 and secrete statistically significantly large quantities of this chemokine compared to both M0 and M2 phenotypes (Figure 19). M2 macrophages also show a notably higher expression and secretion of CCL22 compared to M0 phenotypes but not nearly as high as M1 phenotype. Lastly, both M1 and M2 macrophages secrete CCL17 in notably higher quantities compared to M0 macrophages (Figure 19).

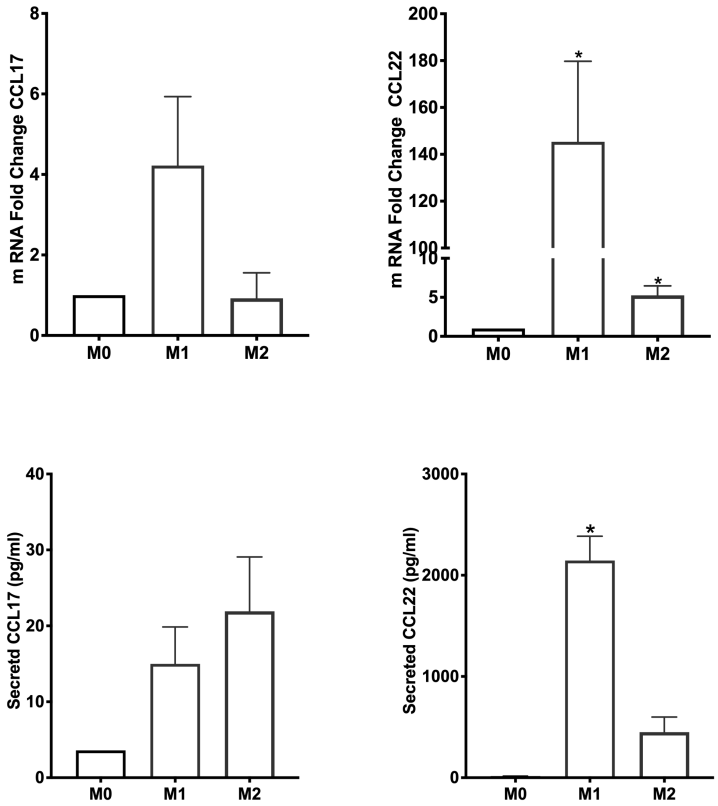

Figure 19*: Real-time PCR gene quantification and antibody-coated bead-mediated flow cytometry protein quantification of CCL17 and CCL22 in M0, M1, and M2 macrophages. (*p<0.05 compared to M0 and M2. The graphs show mean ± SEM data from N=3 replicates)*

CHAPTER 5

DISCUSSION

The ocular surface consists of the cornea and conjunctiva, which contain rich populations of macrophages and fibroblasts. Damage to the ocular surface can occur due to various noxious stimuli including mechanical injury, microbial infection, and chronic systemic diseases. As in all tissues, local response to ocular surface injury involves the initiation of an inflammatory response by innate immune cells, such as macrophages, followed by wound repair. However, excessive wound healing can lead to fibrotic alterations to the cornea and conjunctiva, which can severely impact normal vision (Friedlander, 2007). Damage to the ocular surface has been implicated in chronic graft-versus-host disease (GVHD), where at least 60% of patients with GVHD have reported ocular complications following bone marrow transplantation (Herretes et al., 2015). Moreover, clinical observations have noted that the conjunctiva is significantly affected by fibrotic scarring in patients with GVHD (Kusne et al., 2017; Kheirkhah et al., 2018). Innate immunity plays a role in inducing fibrogenesis in ocular GVHD (oGVHD). Our lab has previously demonstrated that there is a concomitant influx and activation of macrophages preceding and alongside the onset of fibrosis in the conjunctiva of mice with GVHD (Shamloo et al., 2021). In addition, studies have demonstrated that M2 phenotype macrophages are involved in initiating and progressing the pathology of fibrosis by releasing profibrotic mediators (Wang et al., 2021). However, there are currently no studies that explore whether irradiation prior to bone marrow transplant induces an effect on macrophage activation and phenotypic plasticity in the context of ocular GVHD. The present study demonstrates that irradiation can trigger macrophage activation and polarization, and macrophages can cause the transdifferentiation of ocular surface fibroblasts to myofibroblasts, thus contributing to ocular surface fibrosis.

In order to study the role of macrophages in ocular surface fibrosis, M0 macrophages were first obtained by isolating hematopoietic stem cells from murine bone marrow and culturing

these cells in M-CSF. Macrophages display a spectrum of plasticity in response to a variety of stimuli and can polarize into two main phenotypes: M1 classically-activated pro-inflammatory and M2 alternatively-activated anti-inflammatory or profibrotic. Our data demonstrates that we have successfully obtained the three macrophage phenotypes M0, M1, and M2 using published methods (Ying et al., 2013) as demonstrated by anticipated expression of Pan, M1 and M2 macrophage markers including CD11b, F4/80, CD86, CD206, iNOS and arginase-1, (Klopfleisch, 2016; Rodriguez et al., 2017). It is worth noting that our data also demonstrates a noticeable increase in the expression of the chemoattractants CCL17 and CCL22 in polarized macrophages, further suggesting that successful macrophage activation was achieved using published methods.

In addition to biological stimuli, studies have demonstrated that irradiation could induce macrophage activation and phenotypic changes toward either pro-inflammatory or anti-inflammatory (Wu et al., 2017). Several studies have also shown that irradiation as used for conditioning regimen prior to bone marrow transplantation is a key stimulus to trigger GVHD including oGVHD (Corvò et al., 1999; Flowers et al., 2011). Moreover, intensity of radiation dose can affect the severity of oGVHD, wherein injury by irradiation and high autoantigen load at the ocular surface could potentially activate host tissue-resident macrophages (Perez et al., 2016). Mikhalkevich et al., 2021 have demonstrated that radiation triggers the expression of pro- and anti-inflammatory genes in monocytes and macrophages. In line with published literature, our data also demonstrates that radiation exposure increases the gene expression of pro-inflammatory cytokines in macrophages and also increases the expression of M2 markers. While the results indicate that irradiation may trigger the release of pro-inflammatory cytokines from M0 macrophages, our data suggests that radiation can lead to differentiation toward the M2

phenotype as shown by the robust increase in gene expression of M2 markers. Our data also demonstrates that macrophages exhibit increased phagocytic activity and remain largely viable after radiation exposure, which is complemented by published observations that irradiation does not affect macrophage metabolic activity and viability (Teresa Pinto et al., 2016). Moreover, studies have shown that the M2 macrophage phenotype exhibits increased radioresistance and share many of the characteristic expression patterns as tumor-associated macrophages (Leblond et al., 2017). Therefore, while radiation-induced injury can also cause macrophage activation, our data further demonstrates that radiation exposure could direct macrophages toward the M2 phenotype, which could potentially remain viable and initiate and promote aberrant tissue remodeling in response tissue injury leading to fibrosis as noted in oGVHD (Barker et al., 2015; Shi and Shiao, 2018).

Clinical studies have shown that patients with oGVHD exhibit features of subtarsal conjunctival fibrosis and lacrimal gland fibrosis (Ogawa et al., 2010; Kusne et al., 2017; Kheirkhah et al., 2018). As mentioned, our lab has published data showing the presence of fibrosis in the palpebral conjunctiva of mice with GVHD. Our lab has also previously demonstrated that conjunctival fibrosis coincides with an increased influx and activation of macrophages (Shamloo et al., 2021). Macrophages are sources of a variety of profibrotic mediators and particularly the M2 phenotype is involved in activating wound healing processes and present in fibrotic tissues (Arango Duque and Descoteaux, 2014; Braga et al., 2015). As anticipated, our data demonstrates that M2 macrophages induced transdifferentiation of ocular surface fibroblasts to myofibroblasts, as indicated by prominent α-SMA immunostaining accompanied by increased α-SMA gene expression. Interestingly, M0 macrophages had also induced transdifferentiation of ocular surface fibroblasts to myofibroblasts and caused increased

α-SMA gene expression. In addition, our data demonstrates that polarized macrophages increased fibroblast gene expression of profibrotic mediators. Interestingly, our data shows that M2 macrophages induced a notable increase in gene expression of RAS components, which is in tandem with our published data that fibrosis in the conjunctiva is associated with RAS activation (Shamloo et al., 2021). Chronic GVHD including oGVHD is divided into three phases: 1) early inflammation and tissue injury; 2) chronic inflammation and dysregulated immune response; and 3) aberrant tissue repair leading to fibrosis (Cooke et al., 2017; Zeiser and Blazar, 2017). Activation of macrophages, especially the M1 phenotype, during phase 1 inflammatory response may lead to influx of $CD4^+$ T cells. Earlier studies have demonstrated that macrophages secrete CCL22, a key chemokine to cause $CD4^+$ T cell influx into the ocular surface in Sjögren's disease-associated ocular surface inflammation (Ushio et al., 2018). Studies have also demonstrated that tumor-infiltrating macrophages can secrete CCL22 to recruit regulatory T cells, which could lead to suppression of anti-tumor immunity (Martinenaite et al., 2016). Our data demonstrates that M1 polarized macrophages can secrete large amounts of CCL22, which could potentially act as a stimulus for T cell infiltration during phase 2 of oGVHD. In fibrotic conditions, naïve $CD4^+$ T cells have shown to polarize toward the Th2 phenotype, and Th2 cells secrete cytokines IL-4 and IL-13 that could induce the macrophage milieu to polarize toward the M2 phenotype. Thus, this interaction between Th2 cells and macrophages could potentially create a progression from the inflammatory phase to the profibrotic phase in oGVHD (Distler et al., 2019). Taken together our data provides evidence for the potential role of irradiation-mediated activation of macrophages, their phenotypic changes, and their cross talk with other cells as an underlying mechanism for oGVHD-associated fibrosis.

CHAPTER 6

CONCLUSION

Based on the data from the present study in parallel with published literature in non-ocular tissues, macrophages could play a key role in the pathology of ocular surface fibrosis especially in the context of oGVHD. Our data demonstrates that macrophages are activated and remain viable upon radiation exposure. In addition, our data shows that activated and polarized macrophages are able to induce profibrotic gene expression in ocular surface fibroblasts and cause their transdifferentiation to myofibroblasts, therefore serving as a potential mechanism of oGVHD-associated ocular surface fibrosis.

APPENDIX

MATERIALS	SUPPLIER
BALB/c Mice	Chapman School of Pharmacy Vivarium
Lysis Buffer	BD Biosciences
Iscove's Modified Dulbecco's Medium	Gibco
Antibodies for CD11b, F4/80, CD86, CD206	Thermo Fisher Scientific
Dulbecco's Modified Eagle Medium	Gibco
Antibodies for α-SMA (primary), Alexa Fluor 488 (secondary)	Invitrogen
FACSVerse Flow Cytometer	BD Biosciences
Confocal Microscopy	Nikon
RNeasy Mini Kit	Qiagen
SuperScript® III First-Strand Synthesis Kit	Invitrogen
Thermocycler	Applied Biosystems
Forward/Reverse Primers of α-SMA, TGF-β1, Angiotensinogen, ACE, M-CSF, PDGF, CD80, iNOS, CCL2, IL-1β, CD206, Arginase-1, IL-10, β-actin	Integrated DNA Technologies
Quant3Studio	Applied Biosystems
RS 2000 X-Ray Biological Irradiator	Rad Sources Technologies
Zymosan A Particles	Invitrogen
Alexa Fluor 488-Conjugated Phalloidin	Invitrogen
Annexin V APC Conjugated Apoptosis Assay Kit	Cayman Chemical

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