MOLECULAR MECHANISMS REGULATING WOUND HEALING AND FIBROSIS

A Dissertation

by

KAREN MELISSA DOERSCH

Submitted to the Office of Graduate and Professional Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

DOCTOR OF MEDICINE

Chair of Committee,	Martha Karen Newell-Rogers
Committee Members,	Brett M. Mitchell
	Cynthia J. Meininger
	Shaodong Guo
Head of Program,	Warren Zimmer

May 2018

Major Subject: Medicine

ABSTRACT

Impaired wound healing and excess scarring are substantial clinical problems that affect millions of Americans annually and cost billions of dollars to treat. Currently there are insufficient therapies available to improve wound healing. Many patients without underlying comorbidities experience problematic wound healing every year. Additionally, certain diseases predispose individuals to excess scarring and poor healing. One such disease, Systemic Lupus Erythematosus (SLE), affects roughly one million Americans and leads to numerous skin pathologies, including excess scarring and wound healing delays. Interleukin-2 (IL-2), a cytokine that impacts the signaling of immune cells and may contribute to wound healing, is dysregulated in SLE. We hypothesized that IL-2 signaling acts as a growth factor for cutaneous wound healing but leads to scarring in SLE. We employed wound healing models in both wild-type (WT) and SLE-model mice and a fibroblast cell culture to study wound healing. Because fibrosis is a key contributor to impaired wound healing, especially in SLE, we employed an anti-fibrotic agent, quercetin, to determine whether blockade of fibrosis improves the quality of healed wounds in healthy and SLE-mode mice. We used Trichrome staining and flow cytometric analysis to understand quercetin's impact on wounds. Additionally, immunohistochemistry (IHC) for IL-2 and IL-2-receptor-α (IL-2Rα) in wounds and flow cytometry of immune cells revealed how IL-2 signaling impacts wound healing in the SLE-model mice. Our findings indicate that the mechanism by which quercetin reduces fibrosis at the wound site may involve reduced expression of β1 integrin and increased αV integrin. However, a comparative analysis between the WT and the SLE-model mice

demonstrated that quercetin impairs wound healing only in the SLE model. In the wounds of quercetin-treated SLE-model mice, there is also evidence of impaired IL-2 signaling. Paradoxically, we show that in cultured fibroblasts, combination treatment with quercetin and IL-2 increases IL-2R expression. To better understand the difference between the *in vitro* and *in vivo* studies, we demonstrate that quercetin alters immune cell signaling in the SLE-model mice, and that this reduces IL-2Rα at the wound site. Thus, by using quercetin to prevent fibrosis, we block an increase in IL-2 signaling that may be necessary for wound healing in SLE. More work is needed to better understand how IL-2 impacts wound healing and scar formation in patients with wounds with and without SLE.

ACKNOWLEDGEMENTS

I thank my friends, colleagues and the Texas A&M University Health Science Center faculty and staff for their support. I am also especially grateful to my family, including my mother, Candace Doersch, and my father, Robert Doersch, for being there for me throughout this project. I am especially grateful to my brother, Carl Doersch, for his unique understanding of my graduate studies.

CONTRIBUTORS AND FUNDING SOURCES

This work was supervised by the members of my dissertation committee: my committee chair, Dr. M Karen Newell-Rogers, and my committee members, Dr. Brett Mitchell, Dr. Cynthia Meininger, and Dr. Shaodong Guo. I would also like to thank my substitute committee member, Dr. Travis Hein.

I would like to thank the members of the Chatterjee lab, specifically Dr. Piyali Chatterjee, Valorie Chiasson, and Kelsey Bounds, for the use of immunohistochemical and western blotting supplies and for assistance with these methods. I would like to thank the Zawieja lab and the Integrated Imaging and Microscopy Laboratory for the use of microscopy and immunohistochemical equipment. I would like to thank Dr. Vincent VanBuren and Dr. Carl Tong for the use of microscopy equipment in their laboratories. I also appreciate Dr. Vincent VanBuren's advice on the statistical methods used in this dissertation. I would like to acknowledge training I received from Dr. Giuseppina Dusio, Dr. Walter Cromer, and Dr. Richard Tobin on the techniques utilized in this dissertation. Finally, Dr. Robinna Lorenz, who served as an extramural faculty advisor, has also been extremely helpful.

Funding sources included the Sigma Xi the Grant-in-Aid (Grant ID #201603152030013). Additional support was provided by funding from the Texas A&M University Health Science Center and Baylor Scott and White to Dr. M. Karen Newell-Rogers.

NOMENCLATURE

AGE	Advanced Glycation End Product
AO/PI	Acridine Orange/Propidium Iodide
APC	Allophycocyanin
CD	Cluster of Differentiation
CLE	Cutaneous Lupus Erythematosus
CTL	Cytotoxic T-Lymphocyte
CTLA	Cytotoxic T-Lymphocyte Antigen
DM	Diabetes Mellitus
DMSO	Dimethylsulfoxide
DN	Double Negative
ECM	Extracellular Matrix
ERK	Extracellular Signal-Related Kinase
FAK	Focal Adhesion Kinase
FGF	Fibroblast Growth Factor
FITC	Fluorescein
GPCR	G Protein-Coupled Receptor
GWAS	Genome-Wide Association Studies
HER	Human Epidermal Growth Factor Receptor
HLA	Human Leukocyte Antigen
ICAM-1	Intracellular Adhesion Molecule-1
IFN	Interferon

IgG	Immunoglobulin G
IL-1	Interleukin-1
IL-2	Interleukin-1
IL-2R	Interleukin-2 Receptor
Jak	Janus Kinase
LN	Lymph Node
lpr	The Mouse Model for Systemic Lupus Erythematosus
MCP-1	Monocyte Chemotactic Protein-1
MEK	Mitogen Activated Protein Kinase Kinase
MFI	Mean Fluorescence Intensity
MI	Myocardial Infarction
MRL	Murphy Roths Large
mRNA	Messenger Ribonucleic Acid
mTOR	Mammalian Target of Rapamycin
NK	Natural Killer
PCa	Prostate Cancer
PE	Phycoerythrin
PI3K	Phosphotidylinositol-3-Kinase
PSA	Prostate Specific Antigen
PSMA	Prostate-Specific Membrane Antigen
PTPN2	Tyrosine-Protein Phosphatase Non-Receptor Type-2
RAF	Rapidly Accelerated Fibrosarcoma

SHC	Src Homology Domain-Containing
sIL-2Rα	Soluble Interleukin-2-Receptor-α
SLE	Systemic Lupus Erythematosus
snRNP	Small Nuclear Ribonucleoproteins
STAT	Signal Transducer and Activator of Transcription
T1DM	Type-1 Diabetes Mellitus
T2DM	Type-2 Diabetes Mellitus
TCR	T-Cell Receptor
TGF	Transforming Growth Factor
TH_{17}	T-Helper-17 Cells
THC	T-Helper Cells
TNF-α	Tumor Necrosis Factor-α
T-reg	T-Regulatory Cell
VLA	Very Late Antigen
WT	Wild-Type

TABLE OF CONTENTS

LIST OF FIGURES

Page

1. INTRODUCTION*

1.1 Wound Healing

Wound healing requires orchestration of the cells that close the wound, such as platelets and fibroblasts, with the immune mediators of wound repair. The process of wound healing is divided into multiple phases, which are detailed in Figure 1: the hemostatic phase, the inflammatory phase, the proliferative phase, and the remodeling phase (1-4). During the hemostatic phase a clot, which is composed of fibrin, platelets, and other factors, forms to stop blood loss at the injury site (2, 4). After bleeding has been controlled, the wound site transitions into the inflammatory phase. During the inflammatory phase, immune cells infiltrate the wound to prevent infection and orchestrate the recruitment of other cells to the wound (1, 2). Early in the inflammatory phase the primary immune cells present at the site of injury are neutrophils. These neutrophils are relatively short-lived cells and secrete chemotactic factors that attract macrophages such that, beginning roughly 3 days after injury, macrophages predominate at the injury site. Next is the proliferative phase, in which a layer of keratinocytes cover the wound, over which other epidermal cells migrate and proliferate to create the multiple skin layers (1). Additionally, the proliferative phase is when angiogenesis begins and production of the extracellular matrix (ECM) occurs (1). Finally, during the remodeling phase, the collagen of the ECM matures, a process that is orchestrated by both skin and immune cells, increasing the strength of the closed skin (1, 3).

*Parts of this chapter reprinted with permission from:
"The contribution of interleukin-2 to effective wound healing" by Doersch KM, DelloStritto DJ, Newell-Rogers MK, 2016. *Exp Biol Med*, 242(4), 384-396, Copyright 2016 by the Society for Experimental Biology and Medicine.
"The impact of quercetin on wound healing relates to changes in αV and β1 integrin expression" by Doersch KM, Newell-Rogers MK, 2017. *Exp Biol Med*, 242(14), 1424-1431, Copyright 2017 by the Society for Experimental Biology and Medicine.
"Synergistic immunologic targets for the treatment of prostate cancer" by Doersch KM, Moses KA, Zimmer WE, 2016. *Exp Biol Med*, 241 (17), 1900-1910, Copyright 2016 by the Society for Experimental Biology and Medicine.

Cutaneous wounds can range from insignificant small cuts that heal relatively quickly to substantial wounds that take a long time to heal or fail to heal entirely (5). It is estimated that more than 6.5 million Americans were afflicted with chronic wounds in 2009. However, 40-70% of surgical patients experience hypertrophic scarring, or a large cutaneous defect at the surgical site that results from an overgrowth of skin and connective tissue, which can both impair function and be cosmetically undesirable. Wounds of the skin can be painful and unsightly and may also become infected if they remain open too long. Therefore, it is important for the body to close skin injuries quickly. However, an excessive healing response that leads to scarring is also undesirable. Thus, it is fundamentally important that wounds heal quickly, but not too robustly. Unfortunately, it is unclear how best to promote healing without excess scar formation. Factors that delay wound healing include increasing patient age, obesity, diabetes, tobacco smoking, vascular diseases, and autoimmune diseases such as systemic lupus erythematosus (SLE) (6-8). Wound infections, which occur following roughly 0.3% of surgeries and can also complicate non-surgical injuries, also delay wound healing (7, 9, 10). A wound that reopens following a surgical closure, also known as dehiscence, is often, but not always, caused by a wound infection (6, 9). Thus, a myriad of factors, including infection and debris at the wound site and patient comorbidities, can lead to impairment of wound healing. Wounds that fail to heal can cause significant morbidity for patients and treatment strategies need to be developed to treat and prevent wound healing delays (11).

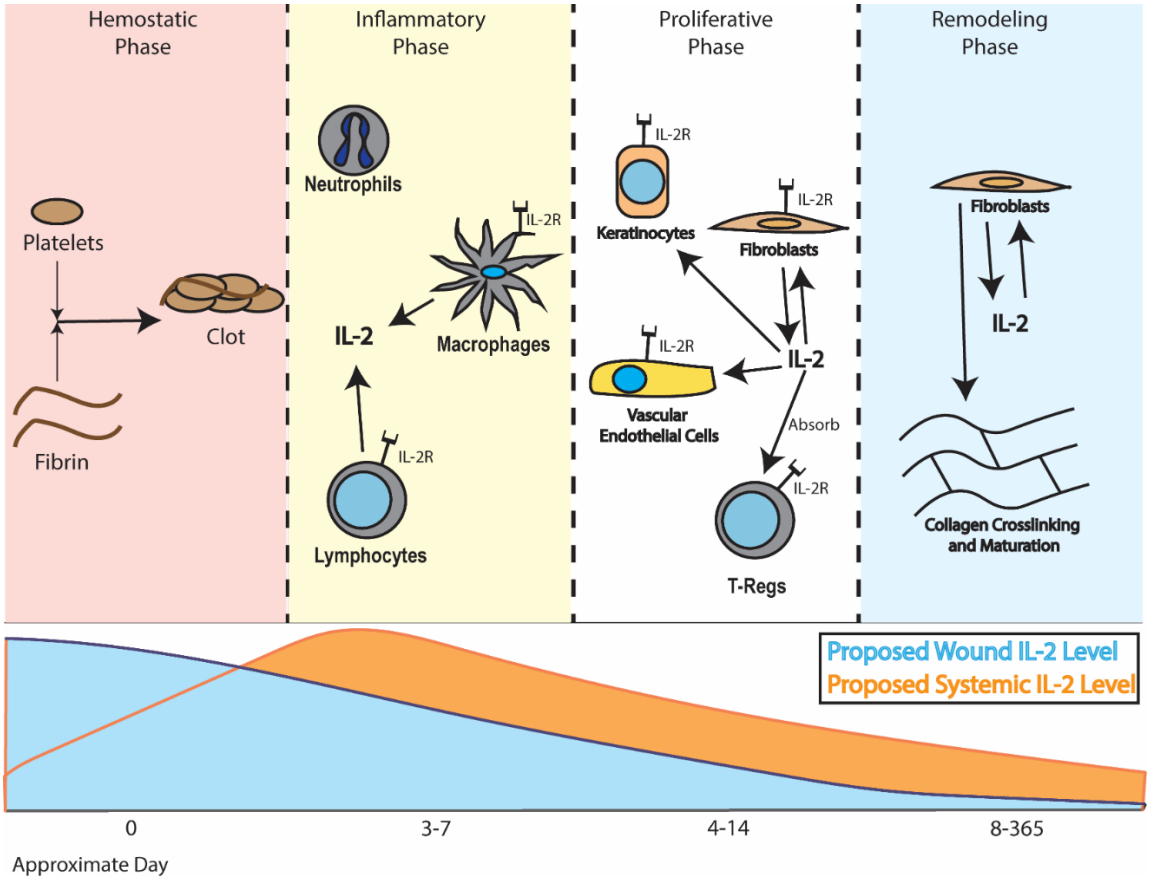

Figure 1: Flow chart describing key players at various times in wound healing. Following an injury, the hemostatic phase occurs, during which platelets, fibrin, and other clotting factors work together to form a clot that serves to block the wound, preventing bleeding and infection. Within the first few days, during the inflammatory phase, first neutrophils and later macrophages enter the wound site, preventing infection and attracting other cells that will help close the wound. During this time, lymphocytes and IL-2 signaling may assist in the wound healing process. During the proliferative phase, the cells that will form the final closure, including keratinocytes, fibroblasts, and vascular endothelial cells, are attracted to the wound site. This phase may also involve IL-2 signaling. Finally, during the remodeling phase, which takes place in the year after the wound is initiated, skin cells such as fibroblasts continue to proliferate and the ECM is remodeled to form the final closure for the wound. Below the figure are shown proposed systemic and wound IL-2 levels. We predict that systemic IL-2 peaks during the inflammatory phase and then is reduced back to normal levels. Wound IL-2 likely is high initially and is slowly reduced over time back to the normal levels that would be found in the skin. Reprinted with permission from (5).

In contrast to delayed closure, wound healing can also be excessively robust, leading to scarring. In scars, excess ECM is laid down, leading to a closure with different qualities from normal skin. Scar types include atrophic scars, hypertrophic scars, and keloids, among others, but this review will focus on hypertrophic scars and keloids (12, 13). Hypertrophic scars typically form immediately after injury or surgery and regress over time (12-14). Risk factors for hypertrophic scars include wound infections, wounds that cross joints or other high-tension skin, and genetics (13, 14). Keloids, on the other hand, can begin growing long after the inciting injury (13, 14). Keloid risk is clearly driven by genetics. Certain ethnicities are more at risk and keloids also run in families. Additionally, certain genetic variations in the antigen presentation system genes, also called human leukocyte antigen (HLA) types, confer a risk for keloids that hints at both a genetic component for this phenomenon and the involvement of the immune system (13, 14). Incidence of keloids seems to decrease with older age and especially following menopause (13, 14). All types of scars have the potential to be itchy or painful, to be cosmetically undesirable, and to cause patient distress, thus warranting treatment (12-14). The limited current strategies to address undesirable scars include corticosteroid injections, pressure treatments for keloids, laser treatment, cryotherapy, radiation, or in severe cases surgical intervention (14). However, these strategies are not universally effective and a better understanding of wound healing may provide novel treatment targets for excess scarring (12-14).

Extracellular Matrix and Wound Healing

Wound healing is a complex process involving the orchestration of immune and skin cells with the structural proteins that compose the ECM (4, 15). Within days after the initial injury, cells at the wound site, including both skin and immune cells, begin to produce ECM components. In the following weeks, cells at the wound site produce, absorb, and reshape the ECM and these processes impact the strength and quality of the final closure (16). The ECM also affects the behavior of cells at the wound site, including skin and immune cells, promoting survival, guiding migration, and altering the metabolism of the cells that go on to heal damaged skin. Over the course of roughly the year after the initial injury, these cells that contribute to the ECM have a great impact on the strength and appearance of the site of injury. However, the factors that determine whether an ideal amount of ECM will be laid down are unclear. Some individuals experience inadequate healing following an injury, while in others, overzealous production of ECM occurs, leaving an excessive scar. Furthermore, it is unclear what factors impact cell growth, such that an ideal cell-to-ECM ratio forms at the wound site.

The Role of the Immune System and Wound Healing

Evidence suggests the immune system plays an active role in wound closure and the balance of proper wound healing and excess scarring (1, 4). The innate immune system, which reacts non-specifically to protect the body, and the adaptive immune system, which promotes specific responses to antigens, both appear to have roles to play in wound closure (17, 18). Innate immune cells, such as neutrophils and macrophages, arrive during the inflammatory phase to phagocytose debris and attract other cells

necessary to the wound healing process (3, 4). Adaptive immune cells, including both B- and T-lymphocytes, are more common during the proliferative phase and may help promote cell proliferation, ECM formation, and wound closure (3). The finding that polymorphisms in the HLA genes, which are involved in antigen presentation, are associated with keloid risk further implicates the adaptive immune system in wound healing (14). Furthermore, keratinocytes and possibly other skin cells are capable of presenting antigens to the adaptive immune system, which may be involved in the wound healing process (19). Skin cells are also capable of producing and responding to innate immune cytokines. Studies have shown that keratinocytes produce immune mediators such as tumor necrosis factor-α (TNF-α) and interleukin-1 (IL-1), which aid in the inflammatory response (15). Furthermore, fibroblasts have been shown to express receptors for some of these factors, including both TNF-α and IL-1. Thus, these cells may not only produce inflammatory mediators but also respond to them, which may mean that the skin cells themselves play a role in the immune response or that these factors have secondary roles as growth factors, drivers of migration, or to prevent over-proliferation of the skin cells healing the wound. Thus, both non-specific and antigen-specific immune responses likely contribute to successful wound closure. The integration of the immune response in the context of wound healing is quite complex, involving many signaling molecules.

1.2 Systemic Lupus Erythematosus

SLE is an autoimmune disease of unclear etiology in which a person's own immune system reacts against self-antigens, causing significant damage to multiple

organ systems, notably the skin (20, 21). While it is a relatively rare disease, it is the most common of the autoimmune diseases and there are roughly one million individuals with SLE in the United States alone (22). SLE patients have numerous immunologic abnormalities, including altered cytokine profiles and the production of antibodies targeting self-antigens, known as autoantibodies, within the body (23). The cause of SLE remains unclear, although numerous genetic variations have been linked with the disease (24). These include HLA genes, genes in the complement system, T-cell receptor (TCR) variability, and multiple ILs. Of note, a percentage of people with SLE possess mutations in their Fas gene or other Fas pathway members, which decreases apoptosis and thus prevents removal of self-reactive immune cells. Regardless of the etiology, patients with SLE have aberrant immune cell function that leads to autoreactivity, including the production of proinflammatory cytokines such as interferons, TNFs, and several IL pathway members. These proinflammatory cytokines contribute to leukocyte recruitment and excess inflammation in multiple organs throughout the body. Additionally, the immune overactivation and excess of self-reactive cells promotes the formation of autoantibodies to antigens from the individual with SLE, especially nuclear antigens, including DNA, RNA, and small nuclear ribonucleoproteins (snRNPs). Together, the autoantibodies, inflammatory cytokines, and autoreactive cells contribute to the breakdown of self-tolerance that is a hallmark of SLE.

Skin Manifestations of and Wound Healing in Systemic Lupus Erythematosus

As stated previously, the skin is impacted by the presence of SLE (23). A majority of SLE patients have at least some skin-related symptoms and SLE has multiple

variants, which include a cutaneous variant (CLE) in which the only apparent symptoms are skin problems. Cutaneous manifestations of SLE include the malar butterfly rash, a rash across the bridge of the nose and cheeks thought to be associated with sun exposure inducing skin cell apoptosis. Additionally, discoid lesions, or excessive scars that form following minor injuries, are present in many SLE patients and skin and mucous membranes are more prone to injury following minor insults. Patients frequently experience scar formation, hyperpigmentation of the skin, and alopecia, or hair loss (25, 26). Wound healing is also impaired in SLE patients. A study of SLE patients undergoing hip arthroplasty demonstrated delayed wound healing in a subset of the patients that was not correlated with the corticosteroids being used to treat their SLE (8). These skin manifestations of SLE have proven very difficult to treat and as yet there is no definitive therapy to prevent or treat skin abnormalities or impaired wound healing in patients with this disease (27).

The mediators of the skin manifestations of SLE have been a topic of intense study (23). Excess interferon (IFN) signaling may be involved, given the risk of SLE and specifically skin manifestations in individuals with IFN mutations. Dendritic cells have been shown to produce IFNs, specifically type I IFNS, in response to sun exposure, especially in SLE patients, promoting cutaneous inflammation. TNFα production by skin cells, notably keratinocytes may also play a role in the skin symptoms of SLE; however, this is less well-understood. Studies have shown that either high levels of TNFα or the administration of a TNFα inhibitor lead to increased cutaneous symptoms in SLE. Thus, it is unclear whether TNFα has an ideal level above or below which skin symptoms

occur, or whether TNFα production is simply a reaction to damage and thus it is actually anti-inflammatory in this context. Additionally, a variety of ILs, including IL-1, IL-6, and IL-18 have been implicated in the pathogenesis of cutaneous SLE manifestations (23). In the context of SLE, these ILs appear to be pro-inflammatory, increasing immune cell recruitment and proliferation and promoting the production of other pro-inflammatory cytokines, such as IFNs and TNFs. Thus, there is a clear role for immune mediators, especially dendritic cells, as well as skin cells, in the cutaneous manifestations of SLE.

1.3 Integrins and Integrin Signaling

Integrins and their ligands play a substantial role in the fibrotic processes associated with wound healing (28). To further understand this role, we must first consider integrin signaling. Integrins are adhesive proteins that mediate the binding of cells to the ECM and to other cells. Integrins consist of two subunits, an α and a β subunit, each of which have many subtypes (29-32). These two subunits associate into dimers and bind to a variety of ECM proteins. The binding of integrin dimers to ECM requires the integrins to be activated, involving a conformational change from a low-affinity structure, which is the default state, to a high-affinity position. This activation can occur in two ways, “inside-out” and “outside-in” activation. Following activation, integrins cluster together, which leads to increased strength of adhesions. Integrin behavior can have a substantial impact on cell motility, adhesion, and shape. There is also evidence that integrins can control proliferation and survival versus apoptosis and senescence (32).

Inside-Out Activation

During inside-out activation, the transition of integrins into their active conformations is dependent on signaling from proteins within the cells. Talin, which binds directly to the β integrin subunit, mediates this process. Talin is activated by a variety of G-protein coupled receptors (GPCR) and tyrosine kinase receptors depending on context and cell type (30). Following its activation, talin binds to both the cytoplasmic tail of β integrin proteins and to proteins that bind actin filaments within the cell, thus mediating interactions between the ECM and a cell's cytoskeleton. Talin binding recruits other proteins that are part of the focal adhesion complex, a group of proteins that mediate activation and grouping of the integrins, and also facilitates signaling downstream of integrin activation. Focal adhesion complex proteins include focal adhesion kinase (FAK) and Src or Fyn, which serve as a scaffold for the complex and also phosphorylate downstream mediators of binding, such as paxillin (32). FAK activation has also been shown to promote a sustained activation of the mitogen activated protein kinase (MAPK) pathway. The MAPK signaling pathway mediates cell growth, differentiation, and diminishes apoptosis. These factors thus promote cell adhesion or migration depending on context.

Outside-In Activation

When an integrin encounters its ligand in its low-affinity state, the integrin is capable of binding to that ligand (30). This binding to the ECM facilitates a conformational change, exposing the talin binding site on the β integrin's cytoplasmic tail and facilitating the formation of the focal adhesion complex mentioned above. The

complex participants then mediate similar downstream signaling as are present during inside-out activation.

1.4 The Role of Integrins in Wound Repair and Fibrosis

As stated previously, the downstream signaling pathways of integrins have the potential to activate pathways involved in cell adhesion and migration as well as pathways that prevent cell death and promote cell proliferation. All of these activities have the potential to play a role in the closure of a wound. Two specific integrin subunits, αV and β1, appear to play a role in fibroblast behavior at wound sites. These two integrins have been shown to activate Fyn, unlike most integrins, which bind but do not activate this adhesion mediator (32, 33). Fyn then recruits Src homologous and collagen (SHC), a protein that activates the Ras-extracellular signal-regulated kinase (ERK) pathway, which is one of the MAPK pathways. Fyn activates the ERK pathway more acutely than the previously mentioned FAK-Src activation, promoting more aggressive cell proliferation. It has been shown that an increase in αV may increase fibroblast migration and improve wound healing (34). Additionally, αV integrin specifically may play a role in fibroblast proliferation at wound sites (35). On the other hand, β1 subunit-containing integrins have been associated with both fibroblast migration and the initiation of fibrosis (36). Both of these integrin subunits are important for fibroblast adhesion to several ECM components, including fibronectin and collagen (28). Thus, it is reasonable to assume that altering the levels of αV and β1 integrin on fibroblasts might be associated with improving the quality of a healed wound by decreasing fibrosis. Finally, integrins also play a role in the recruitment and behavior of

white blood cells, many of which are present at the wound site. Several integrins with β1 as a component, including very late antigen-4 (VLA-4), are involved in the recruitment of monocytes and may play a role in the pro-inflammatory events seen in early wound healing. During the resolution phase, αV-containing integrins are more highly expressed on leukocytes, including macrophages and dendritic cells, and may play a role in the resolution phase of wound healing. Studies have demonstrated that αVβ3 integrin is involved in efferocytosis, the process by which necrotic cells are cleared by phagocytic cells. These data together hint that crosstalk between αV and β1 integrin may mediate the promotion of wound healing and the initiation of fibrosis.

1.5 Quercetin

Quercetin (2-(3,4-dihydroxyphenyl)-3,5,7-trihydroxy-4*H*-chromen-4-one) is a naturally occurring flavonoid found in tea and berries that may have anti-fibrotic properties. In addition to being present in a variety of foods, quercetin supplements are currently available over-the-counter in the United States. Previous studies have shown a healing benefit when quercetin is applied to a wound (37, 38). One study demonstrated accelerated wound healing in rats and mice when quercetin was applied topically to the wound (37). In this study, the effect was shown to be associated with an increase in fibroblast proliferation, a decrease in immune cell infiltration, and changes in signaling in fibrosis-associated signaling pathways. Additionally, other studies have indicated that quercetin decreases fibrosis and scar formation in wound healing, both *in vivo* and *in vitro* (14, 39, 40).

1.6 Interleukin-2

Interleukin-2 (IL-2), a cytokine with a complex signaling cascade, has been extensively reviewed (41-44). This cytokine is secreted in its active form by many cells that participate in wound healing, including dendritic cells T-helper cells (THCs), cytotoxic T-lymphocytes (CTLs), macrophages, and keratinocytes (2, 45-47). The IL-2R subunits, which are described later, are found principally on immune cells but are also present on the surfaces of various skin cell subtypes, including keratinocytes and fibroblasts (41, 43, 48, 49). The behavior of cells in response to IL-2 signaling varies significantly by cell type depending up on the level and subunit complement of the IL-2 receptor (IL-2R) that the cell has on its surface, the external cytokine environment, and the other intracellular signaling molecules present (44).

IL-2 signaling occurs via a receptor with 3 subunits, IL-2R -α, -β, and -γ. The subunits of the IL-2R have different affinities for and responses to IL-2. IL-2Rβ and IL-2Rγ are able to bind other cytokines, but IL-2Rα appears be specific for IL-2 binding (48). Each IL-2R subunit is low-affinity when alone. However, when dimerized, the IL-2Rβ-IL-2Rγ complex achieves a higher affinity. The highest affinity is achieved when all 3 subunits (α, β, and γ) are together (41, 43, 48). IL-2Rs signal via Janus tyrosine kinases (JAK) and signal transducer and activator of transcription (STAT) pathways primarily (Figure 2a). Following IL-2 binding to its receptor, it appears that JAK1 is recruited to IL-2Rβ, while IL-2Rγ recruits JAK3 (44). The recruitment of JAK1 appears to depend upon JAK3 recruitment (50). These JAKs then phosphorylate domains of the IL-2Rβ, which goes on to recruit and phosphorylate STATs, principally STAT5a and

STAT5b, but also STAT1 and STAT3 (50). The phosphorylated STATs dimerizes and this dimer enters the nucleus where it can activate a variety of genes depending on the cell type and other signals present (44). In addition to STATs, other signaling molecules, proteins from the SHC family are also recruited to the IL-2Rβ cytoplasmic tail following its phosphorylation by the JAKs (44). The SHC proteins then go on to activate the phosphatidylinositol-3-kinase (PI3K) and MAPK pathways (41, 43, 48). In both the MAPK pathway and the PI3K pathways, phosphorylation of SHC causes SHC to bind to a complex of several proteins, including Ras (Figure 2b) (50, 51). This complex contributes to rapidly accelerated fibrosarcoma (RAF) activation, which activates mitogen activated protein kinase kinase (MEK); MEK goes on to activate MAPK, which is also known as ERK (50, 51). The ERKs act as transcription factors for a variety of mediators of cell proliferation (50, 51). In the PI3K pathway, Ras activation activates PI3K, which goes on to activate Akt, which activates mammalian target of rapamycin (mTOR) (52). There is a myriad of subtypes of many of the participants in both the MAPK and PI3K pathways that are thus activated by IL-2 signaling. Additionally, many steps of both these cascades are regulated by both feedback mechanisms from within the pathway and signaling events in other pathways. The MAPK and PI3K pathways are both associated with cell proliferation and likely contribute to the expansion of effector T-cells in response to IL-2 (44, 50-52). Thus, it is clear that IL-2 signaling is complicated, dependent upon cell type, extensively regulated, and likely contributes to cell expansion in some cell types.

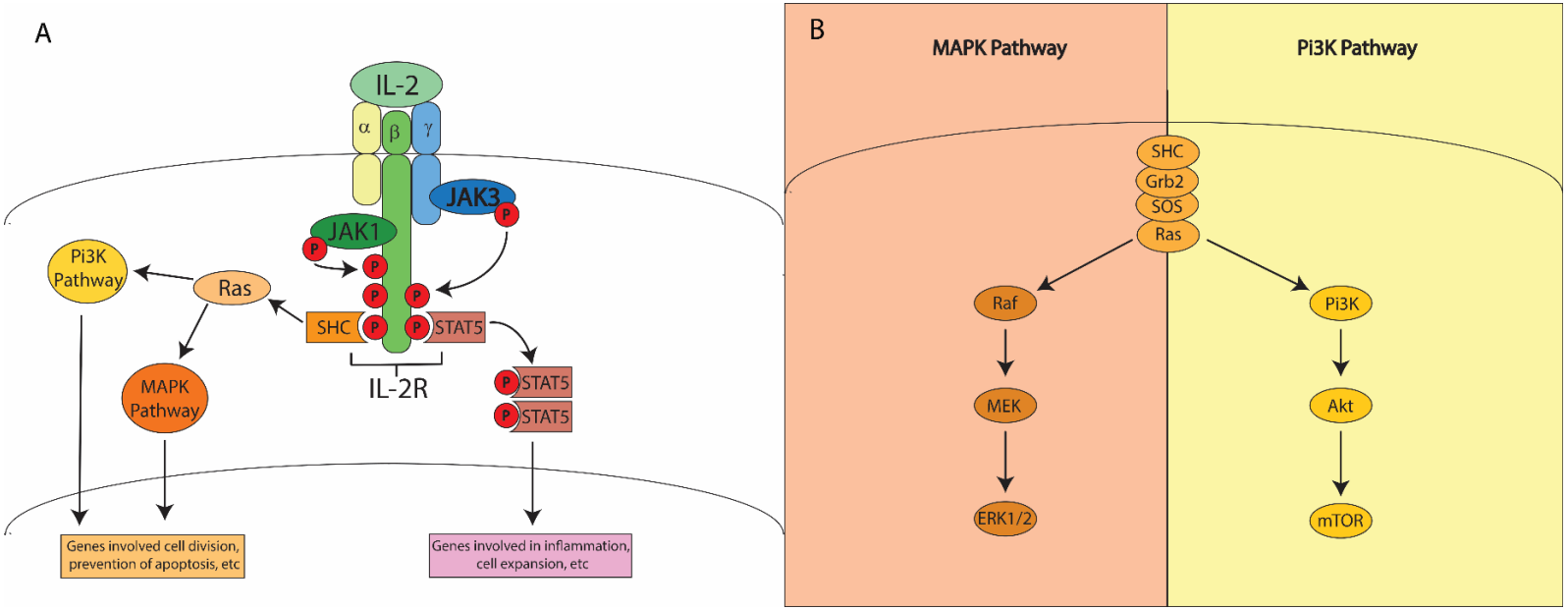

Figure 2: Interleukin-2 Pathway Signaling. a. The IL-2R consists of a trimer of the IL-2-specific α chain and the common β and γ chains. Upon IL-2 binding to its receptor, Jaks are recruited to the β and γ chain of the receptor. The most common Jaks that the receptor signals through are Jak1 and Jak3. Jak3 is recruited to the γ chain and Jak1 is recruited to the β chain. These Jaks phosphorylate the cytoplasmic tail of the β chain, which binds and phosphorylates STATs. The β chain most frequently attracts and phosphorylates STAT5a and b, but can also recruit and phosphorylate STAT1 and STAT3. These phosphorylated STATs then form a dimer and enter the nucleus, where they serve as a transcription factor for genes involved in inflammation and cell proliferation. The β chain cytoplasmic tail also recruits SHC proteins, which signal via Ras activation to activate the MAPK and PI3K pathways. b. Signaling via the SHC pathway downstream of the common β chain of the IL-2 receptor. These pathways lead to activation of Erk1/2 in the MAPK pathway and mTOR downstream of the PI3K pathway. Activation of these pathways increases cell division and decreases apoptosis. Thus, IL-2 signaling acts to enhance cell growth and decrease cell death. Reprinted with permission from (5).

Complex regulation determines the degree to which each of the IL-2 downstream pathways contributes to its action in each separate cell type leading to the variability in cell behavior in response to IL-2 signaling. For example, FoxP3 levels are upregulated in T-regulatory cells (T-regs), but not other T-cells, in response to IL-2 receptor signaling. This effect in T-regs is due to transforming growth factor (TGF)-β cooperation; it has been shown that FoxP3 is upregulated in response to STAT5 signaling and that this effect requires SMADs, which are downstream of TGF-β. Thus, regulation of IL-2's downstream signals involves multiple pathways. IL-2 downstream signaling has also been shown to be indirectly downregulated by protein tyrosine phosphatase non-receptor type II (PTPN2), which appears to decrease STAT5 phosphorylation and activity in response to IL-2R engagement (53). Thus, numerous signaling pathways cooperate with the IL-2R to affect cell fate, proliferation, differentiation, and behavior.

In addition to serving as a cell surface receptor, soluble IL-2Rα (sIL-2Rα) can also be released from cells, primarily THCs. It is unclear whether surface IL-2Rα is released from cell surfaces by proteases or whether it is produced in a secreted form via alternative splicing or altered transcription (43, 54). Both of these mechanisms may contribute to the production of sIL-2Rα. While sIL-2Rα has low affinity for IL-2, it is capable of regulating immune function, although the exact mechanism of this action is not well-understood (55-57). More studies are needed to demonstrate the role of sIL-2Rα in regulating IL-2 signaling, but this molecule may serve as a therapeutic target once its role is better understood.

Interleukin-2 in Immune Cell Development

IL-2 interacts with other cytokines to influence immune cell development and activation (41, 46, 49, 58). IL-2 has been shown to promote the development and activation of both THC subtypes, TH_1 and TH_2, depending upon the presence of other cytokines in the local environment (46, 59, 60). TH_1 cells produce cytokines, including IFN-γ, and appear to be important in the immune response to intracellular pathogens and in autoimmune diseases (46). TH_2 cells, which also produce numerous cytokines, including IL-4, IL-5, and IL-10, mediate defense against helminths and the pathogenesis of allergies (46). IL-2 also influences the development of memory subsets of both CTLs and THCs, which are important for responding to previously-encountered antigens (46, 49, 60). T-regs, which are important for dampening the immune response and promoting tolerance of self-tissues, also require IL-2 for development (46). Additionally, γδ T-cells, a less well-studied immune mediator found in the skin, among other tissues, also respond to IL-2 in a variety of contexts and, given their location in the body, may play a role in cutaneous wound healing (17, 61-63). Thus, numerous cells of the immune system respond to IL-2, implicating this cytokine in processes mediated by the immune system, such as wound healing.

IL-2R subunits are also found principally on immune cells, including CTLs, THCs, macrophages, and natural killer (NK) cells (41, 43, 48, 49). The signaling response upon engagement of various IL-2R configurations may play a role in immunologic reactions, both innate and adaptive.

Clinical Uses of Interleukin-2

Because IL-2 may be a good therapeutic target in wound healing, it is important to consider what IL-2-related treatments are available and the therapeutic and adverse effects of these treatments. IL-2-based products are currently used to treat several types of cancer, including renal cell carcinoma and melanoma (64-66). Unfortunately, when administered systemically at high doses, IL-2 has a narrow therapeutic window, making it difficult to employ as a treatment except when the benefits outweigh its significant risk profile (64-67). IL-2 treatment can lead to hypotension, fever, and nausea, which make its use undesirable in the clinical setting (67, 68). Numerous strategies have been proposed to prevent toxicity, which may be mediated by downstream targets of IL-2 rather than the IL-2 itself (67). Strategies for attenuating IL-2 toxicity have included careful patient selection, appropriate dosing, use with other treatments that decrease the toxicity mediated by downstream targets or engineering of IL-2 treatments in ways that minimize toxicity (67-70). However, it is effective when it is injected intralesionally to treat a melanoma metastasis, indicating that local use is possible (71). Local treatment appears well-tolerated so this strategy might help prevent the toxicity associated with systemic administration of IL-2 (71). Furthermore, if IL-2 is shown to improve wound healing, it could be added to wound dressings, adhesives, and sutures, which would provide options for modes of delivery that could be tailored to skin delivery and to the needs of each individual wound patient.

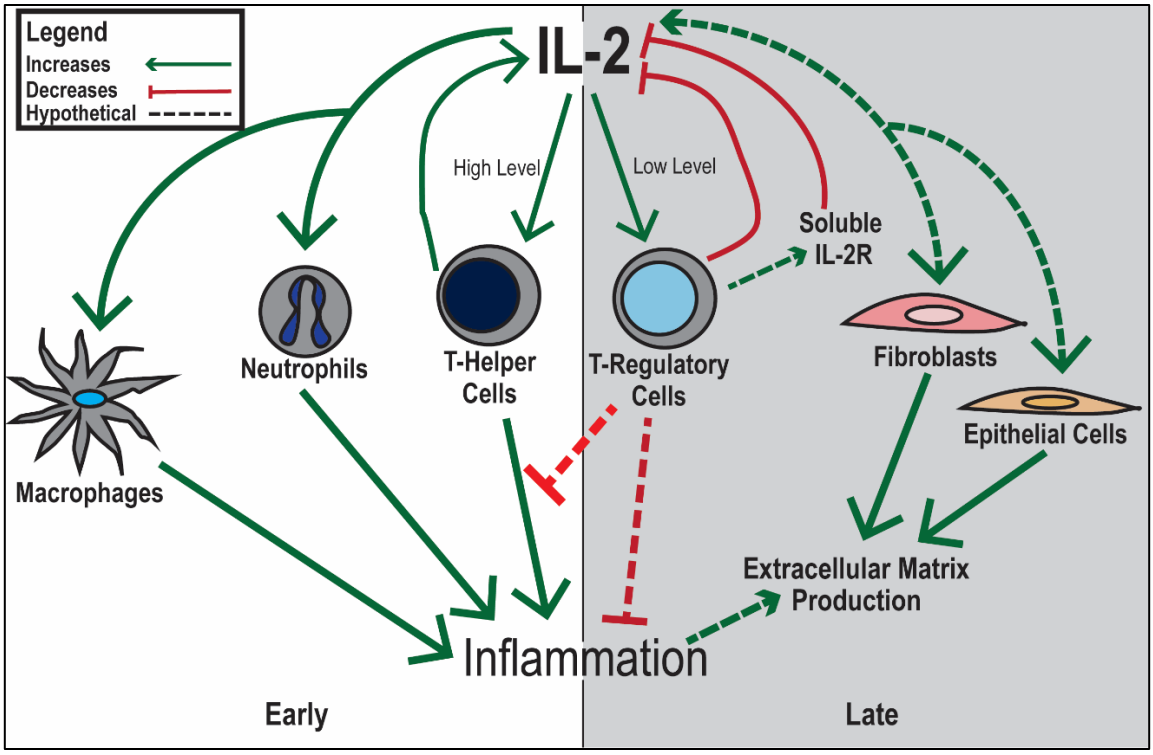

Figure 3: Overview of IL-2 Signaling in Wound Healing. IL-2 appears to play a role in wound healing. High levels of IL-2 in the early wound healing process likely act to increase the action of neutrophils, macrophages, T-cells, and other immune cells at the wound site to promote the inflammation necessary to initiate healing. Later in the wound healing process, IL-2 levels are likely lower, which promotes skin cell replication and production and remodeling of the ECM. This may occur in response to T-reg signaling or high levels of soluble IL-2 receptor, which decreases inflammation, preventing excess fibrosis and promoting cell proliferation. Reprinted with permission from (104).

1.7 Interleukin-2 and Wound Healing

Several wound healing studies hint that IL-2 might play a role in wound healing, although it is unclear whether the role of IL-2 depends on the immune system or reactions intrinsically in the skin cells (Figure 3). In a rat model of wound healing, high doses of IL-2 increased wound breaking strength as tested by a constant speed

tensometer (72). The IL-2-treated wounds had higher levels of hydroxyproline, which is indicative of collagen crosslinking and may elude to increased ECM deposition or scarring in response to IL-2 (72, 73). The authors of this study interpreted these results to mean that lymphocyte activation by IL-2, rather than IL-2 action on skin cells, mediates increased strength following IL-2 treatment, although this study did not directly test that hypothesis (72). Another study demonstrated that T-cells produce IL-2 following stimulation with antibodies to Cluster of Differentiation (CD) 3 along with treatment with fibroblast growth factor (FGF)-1 or -2, which are known to be important for wound healing and angiogenesis and are typically found at wound sites (74). Collectively, these findings may indicate that there is a feed-forward mechanism by which FGF in wounds increases IL-2 production by immune cells, and that both of these signaling molecules act as growth factors at the wound site (74, 75). These results could mean that high-dose IL-2 would increase scar formation but might be appropriate in patients in whom wound healing is delayed.

IL-2 inhibition at the wound site may also be important for adequate wound healing. Wound exudates collected 10 days after wounding contain a specific inhibitor of THC proliferation that can be incompletely overcome by treating with IL-2 (76). In contrast, fibroblasts proliferated *in vitro* in response to the same wound exudates (76). This study does not identify the inhibitor of IL-2-mediated THC proliferation, demonstrate the timing or source of the inhibitor, or determine the exact role of the inhibitor in wound healing (76). However, it is possible that the IL-2 inhibitor helps resolve inflammation, promote T-reg development by favoring a low level of IL-2, or

slow IL-2-mediated collagen fiber crosslinking without preventing cell proliferation, thus improving the quality of wound closure (72, 76-78). The discovery of this inhibitor lends evidence that IL-2, either directly or indirectly, promotes immune activation during the inflammatory phase of wound healing and potentiates skin cell proliferation during the proliferative phase, but can lead to an overly robust closure if it is not inhibited during the proliferative or remodeling phases. Thus, more research is needed to determine the exact role of IL-2 in wound healing before IL-2-related treatments can be designed.

Wounds have been shown to alter body IL-2 levels. One study of adult burn patients demonstrated that blood IL-2 levels are increased on both day 1 and day 5 following burn injury and that the levels of IL-2 correlate positively with the percentage of the body burned (79). This study did not identify the source of IL-2, although it did show that white blood cell counts in the peripheral blood of burn patients are elevated (79). Another study of burns in children demonstrates that IL-2 levels at the wound site are lower than they are in the peripheral blood, which serves as evidence for IL-2 acting systemically rather than locally at the wounds or may indicate that at some points during the healing process low levels of IL-2 are favorable (80). The high levels of IL-2 in the blood and high white blood cell counts may also mean that the role of IL-2 in wound healing involves systemic immune activation. More research is needed to fully understand the timing and location of IL-2's contribution to wound healing.

IL-2 signaling may also contribute directly to the activity of fibroblasts. Several studies have shown that fibroblasts express IL-2R, specifically the β and γ subunits that

are capable of signaling (81, 82). One of these studies, concerning fibroblast-like cells isolated from human joint fluid, demonstrated that IL-2 treatment could induce production of pro-inflammatory cytokines by these cells (82). Another study demonstrated that fibroblast signaling through the IL-2Rγ-STAT3 pathway led to increased production of monocyte chemoattractant factors, including monocyte chemoattractant protein-1 (MCP-1) and intracellular adhesion molecule-1 (ICAM-1). These observations may mean that IL-2 produced in skin cells at a wound may promote the recruitment and activation of immune cells at wound sites (81). IL-2 appears to act as a growth signal in fibroblasts in a mechanism involving autophagy, or the digestion and reallocation of the materials of a cell's organelles (83). Additionally, human skin fibroblasts can secrete IL-2, among other pro- and anti-inflammatory cytokines, upon high-dose exposure to advanced glycation end products (AGEs), which are sugar-conjugated proteins that occur in diabetes mellitus (DM) (84). Blockade of AGE receptors and TGF-β led to decreased IL-2 production, indicating that the fibrosis associated with AGEs may involve IL-2 (84). Fibroblast secretion of IL-2 may be pathologic and lead to poor wound healing or could also be a compensatory response to a lack of IL-2 signal usually provided by other sources (84). Furthermore, because fibroblasts can be induced to make IL-2 by AGEs, they may also be able to produce IL-2 in other contexts, which could have implications for the rate and quality of wound healing (84). T-regs, which are known to respond to IL-2, have been shown to produce TGF-β and may play a role in this mechanism *in vivo* (46).

IL-2 may also contribute to the secretion of, and reaction to, growth-promoting factors and cytokines. IL-2 promotes the release of IFN-γ and the development of IFN-γ-producing TH_1 cells, leading to IL-1 production, which may promote wound healing (46). Additionally, IL-2 appears to synergize with IFN-α to promote endothelial cell proliferation and angiogenesis, which is necessary for revascularization of a wound site (75). Combined treatment with IL-2 and IFN-α increased endothelial cell proliferation and IFN-α alone increased expression and signaling of IL-2R (75). Thus, there may be crosstalk between IL-2 and IFN-α, such that IFN-α increases the capacity for a response to IL-2 and IL-2 promotes endothelial cell proliferation (75). IL-2 and IFN-α together led to increased release of FGF, which likely contributed to the increased cell growth, while FGF blockade decreased cell proliferation in response to IL-2 and IFN-α, thereby demonstrating that IFN-α and IL-2 synergy may be FGF-mediated (75). Growth promotion by IL-2 via FGF, and possibly other growth factors, may also be applicable to other cell types, implying that it could promote both skin cell and blood vessel growth at wound sites (75).

Thus, IL-2 may be an underappreciated mediator of wound healing. Figure 3 contains a diagram of the immune cells that produce and/or react to IL-2 and also play a role in wound healing. The relationship between IL-2 and wound healing and the mediators of this relationship are potential therapeutic targets for wound healing and thus warrant further exploration.

1.8 Interleukin-2 in Systemic Lupus Erythematosus

Studies in lupus-model mice, which have been reported to have IL-2 deficiencies, have yielded heterogeneous results, with studies reporting increased, decreased, or normal healing rates and quality compared to WT mice (85, 86). Interestingly, in studies that have demonstrated improved wound healing in this model, wounds were located on the cartilaginous ear, which may explain the differences in healing (87). Overall, these models provide an avenue to explore causality between SLE, IL-2, and defective wound healing. IL-2 levels in both SLE patients and mouse models of SLE appear to be below that of healthy controls, which may be involved in the pathogenesis of the skin damage and wound healing problems observed in SLE. It has been shown that IL-2 mRNA expression is decreased in skin biopsies from SLE patients in both healthy and lesioned skin compared with controls, which may implicate IL-2 in the cutaneous manifestations of SLE (88). Moreover, IL-2 production by lymphocytes, including cultured cells, and both CD3+ T-cells and peripheral blood mononuclear cells isolated from human patients, is decreased, although the mechanism underlying this decrease remains unclear (89, 90). There have been conflicting reports regarding whether this decreased IL-2 production correlates with disease activity (90, 91). Additionally, one study demonstrated that the Immunoglobulin G (IgG) fraction of SLE patient serum from a subset of study participants was capable of inhibiting IL-2 production by cultured human peripheral blood mononuclear cells, indicating that there may be a factor that inhibits IL-2 production by lymphocytes (92). Furthermore, mice bearing the *lpr* mutation in their Fas receptor gene develop an SLE-like autoimmune disease and have

IL-2 deficiency (93). The IL-2R may also play a role in SLE. Patients experiencing SLE exacerbations have lower levels of IL-2R in their serum, indicating that the downstream mediators of IL-2 may also be playing a role in SLE (91). Blood sIL-2R levels are increased in SLE patients, especially in those experiencing discoid skin lesions (94). Together, these results indicate that a lack of IL-2 production, poor IL-2 signaling, or IL-2 inhibition may play a role in SLE and specifically in the cutaneous manifestations of SLE. If IL-2 deficits are demonstrated to be a significant part of the pathogenesis of wound healing impairment and other skin pathologies in SLE, it would represent an attractive therapeutic target.

1.9 Interleukin-2 in Other Disease States

Sarcoidosis

Another multifactorial inflammatory disease that links IL-2 with wound healing is sarcoidosis. Sarcoidosis is a complex and relatively rare disease that involves immune-mediated damage to multiple organs, including the lungs, kidneys, eyes, and skin (95). Roughly 25-35% of patients experience skin symptoms, including plaque and ulcer formation, which can occur on normal skin or around scars and tattoos (95). The relationship to prior sites of injury is indicative of aberrant wound healing in patients suffering from this disease (95). Furthermore, the lung pathology seen in sarcoidosis includes excessive fibrosis, possibly implicating an overactive scarring response in the disease state, a process which may also impact cutaneous healing. Thus, impaired balance of wound healing versus scarring may warrant additional study in sarcoidosis.

Sarcoidosis involves significant immunopathologies, notably the development of granulomas, or collections of macrophages and lymphocytes (95). The pathogenesis of these granulomas in sarcoidosis involves expansion and activation of T-cell subsets, many of which secrete IL-2 (95). Skin granulomas associated with this disease contain IL-2-secreting T-cell subsets, and these T-cells are more numerous in granulomas of patients with active sarcoidosis compared to those with chronic sarcoidosis (96). Additionally, many of the therapies used for the cutaneous lesions of sarcoidosis decrease IL-2 levels, which may be a part of their mechanism of action (97). IL-2 secretion by T-cells has long been recognized to play a role in the pulmonary fibrosis found in sarcoidosis and patients with active sarcoidosis have lung lymphocytes that spontaneously secrete IL-2 in the absence of activation (98, 99). This aberrant IL-2 secretion in the context of lung fibrosis could be a reaction to the fibrosis or could be involved in its pathogenesis. It is possible that spontaneous IL-2 production by immune cells also occurs in the skin and leads to some of the cutaneous pathologies associated with sarcoidosis. The IL-2 production by T-cells in sarcoidosis can be overcome by immunosuppression using systemic corticosteroids, as demonstrated in a prospective clinical trial, which could be an attractive therapeutic for this disease (100).

The levels of sIL-2Rα is also elevated in sarcoidosis and sIL-2Rα has been extensively studied in the disease (101, 102). Su *et al*. found that sIL-2Rα levels were elevated in sarcoidosis compared with healthy controls, although the sIL-2Rα levels did not correlate with lung disease severity (101). The amount of sIL-2Rα also correlates with eye inflammation in sarcoidosis patients (102). Methotrexate therapy in sarcoidosis

patients is associated with decreased serum sIL-2Rα, and this decrease correlates with improved lung function (103). These studies do not clarify whether sIL-2Rα is pathologic or is produced in response to excess IL-2. Furthermore, it is unclear what role sIL-2Rα may play in the cutaneous lesions accompanying this disease. However, IL-2 and IL-2R signaling likely play a role in the skin damage involved in sarcoidosis and warrant further study in the context of this disease, which may shed light on the role of IL-2 in wound healing in other contexts.

Prostate Cancer

Many of the immune cells implicated in prostate cancer (PCa) produce and respond to IL-2, a cytokine that binds to a multi-subunit receptor and signals via Jak-STAT phosphorylation (77, 104). IL-2 is produced by T-cell subsets, dendritic cells, and other immune cell subsets and many immune and non-immune cells in the body possess its receptor (45, 77). IL-2 has a variety of pro-inflammatory roles in the immune system, including promotion of TH_1 cell and M1 macrophage development, making it a very attractive immunologic candidate for treating cancer (41, 105). Additionally, individuals with a polymorphism in IL-2 are at an increased risk for PCa, although the mechanism for this susceptibility has not been determined (106). This susceptibility due to an IL-2 polymorphism hints that, in the context of PCa, IL-2 may be especially important.

IL-2 has been suggested as a potential PCa therapeutic based on human data, has been studied using both *in vitro* and *in vivo* PCa models, and some IL-2 based therapies have been tested in clinical trials. TG4010 is a recombinant viral vector that expresses IL-2 and Muc1 genes. Muc1 is a factor expressed by prostate tumors and associated with

a poor prognosis, and the role of IL-2 in this therapy is as an adjuvant to increase the immune response (107). TG4010 was associated with a prolonged prostate-specific antigen (PSA) doubling time and improved progression-free survival, although its Phase II clinical trial did not meet its primary endpoint of a 50% reduction in PSA (107). This treatment has gone on to further clinical trials for lung cancer, meeting its endpoints to progress to a phase III trial, and may be commercially available in the future (108). A trial using IL-2 in combination with the γδ T-cell agonist zoledronate improved survival in PCa over zoledronate alone and was associated with increased levels of γδ T-cells in the blood (109). A PCa cell line expressing IFN-γ and recombinant IL-2 has also shown promise in PCa treatment, and, in a phase I/II trial, the vaccine was well-tolerated (110, 111). Median survival was 32 months among patients receiving the vaccine and PSA doubling time slowed in a majority of the patients (110, 111). Other strategies that have been explored are the delivery of IL-2-fusion proteins within a liposome or fused to an antibody (112-114). Leuvectin, a liposomal delivery vector of IL-2, was studied as an intraprostatic injection for the treatment of cancer, with the intent to stimulate immune cells to kill PCa cells (112). The treatment was well-tolerated in a Phase I clinical trial and appeared to cause a drop in PSA in two thirds of patients (112). Two trials of leuvectin in PCa have been terminated without posting results or reasons for termination, so it is unclear whether this treatment remains in development (115). The termination of these studies may indicate that treatment with IL-2 is ineffective in improving outcomes in prostate cancer or may indicate a problem with the liposomal delivery method or with localized treatments in this disease state. Another promising study used an antibody to

prostate specific membrane antigen (PSMA) in combination with low-dose IL-2 treatment and yielded promising results, with tolerable toxicities and PSA stabilization in patients receiving the treatment (115, 116). However, this study ended in 2007 and has not yet led to a commercially available therapeutic. A vaccine containing PSA as well as IL-2 and GM-CSF was studied in a cross-over design with nilutamide, an anti-androgen, as the comparative treatment (117). The combination treatment showed some efficacy in treating patients with hormone-refractory PCa (117, 118). While there were some toxicities attributable to the use of IL-2, patients receiving the three-component treatment first had a trend towards increased survival over the course of the study and many patients experienced a decrease in PSA or a decrease in rate of rise of PSA. Additionally, a phase IA/IB clinical trial is underway to test the use of this vaccine in recurrent hormone naïve and hormone refractory PCa, which will hopefully yield promising results (115). Finally, there are several clinical trials using IL-2 in PCa that have been completed but have not yet reported their results, indicating that this treatment remains an active area of research. Thus, results of trials in human patients are very promising and may yield IL-2-related therapies that are able to improve PCa treatment.

In vitro and *in vivo* models also indicate that IL-2 may be useful in treating PCa and this potential therapeutic aveneu remains an active area of research. Sugimoto *et al*. created a fusion of IL-2 to an anti-PMSA antibody. The addition of IL-2 to this antibody enhanced its cytotoxicity, as demonstrated by coculture of PCa cell lines with human peripheral blood mononuclear cells (113). The anti-PSMA-IL-2 conjugate antibody also caused more tumor regression in an *in vivo* mouse model than the anti-PSMA alone

(113). IL-2 was also used as an adjuvant for *ex vivo* immune cell activation as part of an antibody fused to an IL-15-Receptor-α with IL-2 attached (114). Thus, a variety of engineered anti-tumor treatments utilizing IL-2 are efficacious in PCa, both in patients and in models, demonstrating that its use warrants further study.

PCa can also be treated by activating immune cells *in vitro* or *ex vivo* using IL-2 and other cytokines, which has been demonstrated to be effective both *in vitro* and *in vivo* in animal models (119). This strategy could prevent the excessive inflammation seen in systemic IL-2 treatment if the ideal anti-tumor immune cell subsets are identified, stimulated *ex vivo,* and then used to treat the patient (68). Additionally, treatments that act by increasing IL-2 activity may be another approach to favor immunologic reaction to PCa with fewer off-target IL-2-mediated side effects. For example, use of an agonist of the cell surface marker OX40, a TNF family member that increases IL-2R -α expression on CTLs and promotes tumor-specific lytic activity, led to tumor regression *in vivo* (120). The tumor specificity of OX40 was shown to be dependent on IL-2 (120). Furthermore, a combination of OX40, cytotoxic T-lymphocyte associated protein (CTLA)-4 blockade, and human epidermal growth factor receptor (HER) 2 vaccination improved survival in a mouse PCa model by enhancing CTL recognition of and response to HER2 found in the tumor and increasing their IL-2 production (121). These data indicate that inducing IL-2 signaling in the body's immune system could be used in place of IL-2 itself, especially with more understanding of the IL-2 pathway elements that are most relevant to PCa (120, 121).

However, there are potential problems using IL-2 to treat PCa. One serious concern is the previously mentioned systemic toxicity, which might be alleviated with careful patient selection and dosing or by using the IL-2 locally. Another concern regarding the use of IL-2 in PCa is that one *in vitro* study showed that IL-2 increased proliferation and PSA secretion in androgen sensitive cell lines, although this finding was absent in androgen-insensitive cells (122). These findings indicate that IL-2 may be capable of stimulating PCa cells to grow in some contexts. However, given the success of IL-2 treatment in other models and based on evidence that immune cells are part of the mechanism by which IL-2 could be used to treat cancer, it seems likely that IL-2 treatment is a viable treatment strategy, provided its toxicity can be managed.

Diabetes Mellitus

DM is a growing epidemic within the United States and includes both Type I (T1DM), which is caused by autoimmune destruction of insulin-producing cells, and Type II (T2DM), which is a multifactorial disease involving decreased tissue insulin sensitivity (123). Both T1DM and T2DM lead to, among other pathologies, aberrant wound healing (123). The nature of the impaired wound healing in DM is not entirely characterized, but it appears to involve a combination of neuropathy leading to decreased sensation of injury, vascular insufficiency causing decreased blood delivery to the wound site, and excessive inflammation (124).

In the setting of DM, IL-2 and IL-2R have been extensively studied. In T1DM, genome wide association studies (GWAS) have demonstrated allelic variation in multiple IL-2 signaling cascade participants (53, 125, 126). These include single

nucleotide polymorphisms that occur within the IL-2Rα gene and PTPN2, ultimately conferring susceptibility in acquiring T1DM (53, 125, 126). Moreover, in healthy patients lacking a polymorphism known to decrease T1DM risk, IL-2 signaling was attenuated in several T-cell subsets, including memory T-cells and T-regs (127). Interestingly, decreased IL-2 signaling was observed in diabetic patients as demonstrated by diminished phosphorylation of STAT5 in response to IL-2 treatment and increased expression of PTPN2 in the T-regs of diabetic patients compared with healthy controls (128). Thus, overactive and underactive IL-2 signaling are relevant to DM pathogenesis. Altered IL-2 signaling in some T1DM patients may impair early immune cell engagement at wound sites, which could lead to an increased risk of wound infection and impairment of early inflammatory events necessary to close the wound. Conversely, other patients may experience excessive IL-2 signaling, which may worsen some diabetes-associated pathologies. Thus, in T1DM, more research is needed to clarify the role of IL-2 in diabetes pathogenesis and in its manifestations.

In T2DM, IL-2 signaling is also likely altered, although this association has not been well-studied. One group, Lagman *et al*., demonstrated a robust reduction of IL-2 in the plasma of T2DM patients compared with healthy controls (129). Interestingly, other groups have demonstrated an increase in blood sIL-2R in T2DM patients (130, 131). One of these studies also demonstrated that increased sIL-2R levels were associated with decreased T-regs and increased THC frequencies, and higher percentages of IL-2Rα+ non-T-reg T-cell subsets (130). These findings implicate IL-2Rα-possessing T-cells in the mechanism by which excessive sIL-2Rα is produced in T2DM patients (130).

Overall, the investigations of IL-2 and T2DM generally only measured differences in IL-2 and sIL-2R in peripheral blood and did not relate it back to severity of T2DM-associated pathologies, such as impaired wound healing. The wound healing impairment in T2DM may be due to a decrease in IL-2 signaling early during the skin repair process, which fails to attract inflammatory cells and prevent microbial colonization of the wound site.

Numerous studies in a mouse model of T1DM have demonstrated that IL-2 treatment may prevent or improve T1DM. IL-2 stimulation through a variety of approaches including extrinsic IL-2 treatment, viral vectors containing IL-2, and antibody bound to IL-2 (Ab/IL-2) have been successful in demonstrating that increasing IL-2 signaling is a potential therapeutic avenue that likely acts by expanding T-regs (132-136). However, the protective nature of IL-2 treatment was demonstrated to be dose-dependent. Low doses of IL-2 or IL-2-containing virus resulted in prevention of diabetes, whereas high-dose IL-2 had a deleterious effect on diabetes prevention (132-134). Furthermore, in order to prevent loss of the insulin-producing cells of the pancreas, the β-cells, the treatment must be administered prior to the loss of these β-cells, indicating that IL-2 is attenuating an immune-mediated destruction of the β-cells rather than preventing downstream diabetes complications (135). There have been a few clinical trials assessing IL-2 administration to T1DM patients, and the therapy appears to increase T-reg frequency, although it is associated with an increase in adverse events (137, 138). Rapamycin combined with IL-2 therapy further increased T-reg frequency when compared to other T-cell subsets and improved T-reg signaling via the IL-2

pathway, but had a deleterious effect on insulin production by pancreatic β-cells (137). Thus, in the context of DM, more research is needed to effectively use IL-2 to upregulate T-reg function while minimizing side effects.

Currently there is no direct evidence that IL-2 signaling is disrupted in diabetic wound healing. However, the involvement of the immune system in the wound healing impairment is clear. Thus, the well-established link between diabetes, especially its immunologic derangements, and impaired wound healing, coupled with the role of IL-2 in diabetes pathogenesis, provides an opportunity to explore the role of IL-2 in wound healing.

Myocardial Infarction

Following a myocardial infarction (MI), the healing of the necrotic, apoptotic, and autophagic cellular response is strikingly similar to skin wound repair. In an MI, following the irreversible damage of cardiomyocytes by ischemia, the healing process begins immediately. Part of this process includes recruitment of inflammatory cells (neutrophils, macrophages, and lymphocytes) to the injury site, which strikingly resembles the pattern seen in skin healing (1, 139). The similarity between MI and cutaneous wounds implicates processes similar in the healing of both types of injuries.

There have been a number of studies investigating IL-2 in cardiac remodeling. A majority of these studies utilized rodent models. Overall, there seems to be a consensus that IL-2 treatment (be that preconditioning or following MI induction) attenuates the destructive cardiac response (140-143). These studies propose multiple mechanisms by which IL-2 regulates post-infarction recovery, including modulating T-regs, decreasing

oxygen free radical production, or activating NK cells to promote angiogenesis (140-143). Thus, the mechanism(s) of IL-2 efficacy in limiting cardiac injury appear to be immune-mediated and similar mechanisms might lead to a beneficial immune response during wound healing by using IL-2 treatment.

Interestingly, in contrast to many of these more recent animal studies, clinical trials revealed that high dose treatment of IL-2 (usually for cancer treatment) increased the risk of death from MI and myocarditis (144, 145). The authors of these studies speculated that the observed increase in the ischemic events was due to hypotension caused by the vascular leak associated with IL-2 administration because infarctions occurring in patients sometimes occurred in the absence of atherosclerotic lesions (145). Furthermore, it is also possible that too much IL-2 led to an overaggressive immune response. Either hypotension or excessive inflammation would be detrimental to wound healing, which argues for careful dosing, timing, and patient selection or local administration when using IL-2 to treat a healing wound.

Furthermore, there have been a number of investigations looking at IL-2 signaling in patients with MI. Blum *et al*. found a significantly higher blood sIL-2R lrbrl in patients who experienced a second MI in the week following an initial MI (146). Higher sIL-2R also inversely correlated with ejection fraction and was associated with an increased mortality risk, although it is unclear how the increased sIL-2R is involved in these effects (146). Using a 3-hydroxy-3-methyl-glutaryl-coenzyme A reductase inhibitor, atorvastatin, Zhang *et al*. were able to improve infarct volume in a mechanism involving increased T-regs at the site of infarct (147). While this study does not directly

address IL-2 or IL-2R, they may be involved in the T-reg recruitment in response to atorvastatin. T-reg engagement by atorvastatin and other drugs in its class might also be exploited to improve cutaneous wound healing. Taken together, evidence suggests that supraphysiological doses of IL-2 may not be advantageous for MI, as they might stimulate effector cell expansion and increase the overall inflammatory response. However, low-dose IL-2 treatment aimed at stimulating T-reg or NK cells may be cardioprotective. A similar mechanism may improve wound healing by careful administration of IL-2 following cutaneous injury.

2. HYPOTHESES

The hypothesis tested in our study was that reducing fibrosis could improve wound healing in both healthy mice and those with an SLE-like syndrome. The purpose of this study was to explore the mechanisms by which fibrosis can be prevented and to test them in a model of SLE, a disease in which fibrotic skin lesions are a substantial problem. The first aim was to explore the impact of a naturally occurring anti-fibrotic agent, quercetin, on fibrosis and wound healing in healthy mice. This alteration of fibrosis was connected to quercetin's impact on integrin levels. The hypothesis tested through these experiments was that reducing fibrosis with quercetin would decrease scarring in healed wounds. The second aim was to test this anti-fibrotic agent in a disease model of SLE and explore its impact on wound healing in this model. The effects of anti-fibrotic treatment in this model were connected to IL-2 signaling. These results may help identify possible methods for improving wound healing in both healthy individuals and those with SLE and improving the quality of the wounds during and after this process. The hypothesis tested in this section was that wound healing deficits and scarring seen in SLE are caused by SLE-dependent aberrancies in IL-2 signaling.

3. METHODS*

3.1 Mice

WT C57Bl/6J mice (Jackson Labs) were used as our mouse model of healthy wound healing. The B6.MRL-Fas^{lpr}/J (*lpr*) mouse served as the model of SLE with the C57Bl/6J background strain as the control (Jackson Labs). All procedures were approved by the Baylor Scott & White Institutional Animal Care and Use Committee prior to experimentation.

3.2 Procedure

Animals were anesthetized using isofluorane inhalation and shaved. Wounding was performed on the back with an 8-millimeter biopsy punch (Integra Miltex). Mice were treated daily with 100 uL of 10 uM quercetin solution in sterile saline containing 10% dimethylsulfoxide (DMSO) by volume, a dosing strategy that was based on literature review (148). On post-operative days 0, 1, 3, 7, 10, and 14, mice were anesthetized for photography and measurement of wound sizes. Mice were sacrificed on post-operative day 10 or 14 by isofluorane inhalation followed by cervical dislocation and tissue collection was performed. Day 10 was chosen for further analysis because it was the time point at which the mice with impaired wound healing demonstrated the greatest difference in wound size. Day 14 was chosen for the healthy mouse experiments because that was the day at which the wounds were closed in most of the healthy mice.

---

*Parts of this chapter reprinted with permission from:
"The impact of quercetin on wound healing relates to changes in αV and β1 integrin expression" by Doersch KM, Newell-Rogers MK, 2017. *Exp Biol Med*, 242(14), 1424-1431, Copyright 2017 by the Society for Experimental Biology and Medicine.

3.3 Measurements of Wounds

Wounds were measured using 2 perpendicular measurements with calipers at the horizontal and vertical centers of the wounds. These numbers were then averaged and presented as the average wound diameter. Areas were calculated from these numbers.

3.4 Trichrome Stain of Wounds

On post-operative day 10 or 14, mice were sacrificed under anesthesia. Wound area was cut out, fixed in formalin, and embedded in paraffin. Masson's Trichrome stain of the wound site was performed in accordance with kit instructions (Sigma Aldrich). Photographs were taken using an Olympus BX51 microscope (Olympus).

3.5 Wound Immunohistochemistry (IHC)

Wounds were sectioned in 10 micron sections after fixation and paraffin embedding. Antibodies used were the JES6-5H4 clone of anti-mouse IL-2 (Biolegend) and the PC61.5 clone of anti-mouse IL-2Rα (eBiosciences). Some IHC for IL-2 was performed using the HistoMouse-MAX kit (ThermoFisher). The rest was performed with the appropriate Vectastain Elite ABC HRP Kit and DAB substrate kit (Vector Laboratories).

3.6 Flow Cytometry of Spleens and Lymph Nodes

Spleens and lymph nodes (LNs), including the proper and accessory axillary LNs, which drain the back wound sites, were removed from mice following euthanasia and were crushed through a 40 micron cell strainer. Spleens were then treated with ACK lysing buffer (ThermoFisher Scientific) to remove red blood cells and then isolated cells were counted with an automatic cellometer, using acridine orange and propidium iodide

(AO/PI) (Nexcelom Bioscience). In spleen samples 5 million cells were stained and in LN samples, 1 million cells were stained. To identify live cells, Aqua (ThermoFisher Scientific) was used. Cells were also stained with Pacific Blue-anti-CD3e (Clone 200A2, BD Pharmingen), PerCPCy5.5-anti-CD4 (Clone GK1.5, Biolegend), PeCy7-anti-CD8a (Clone 53-6.7, BD Pharmingen), fluorescein (FITC)-anti-CD11c (Clone HL3, BD Pharmingen), allophycocyanin (APC)-Cy7-anti-CD19 (Clone ID3, BD Biosciences), APC-anti-CD25 (Clone PC61, Biolegend), and phycoerythrin (PE)-anti-FoxP3 (FJK-16s, ThermoFisher Scientific) to identify immune cell subtypes. CD8 and CD19 in this panel were used only to exclude B-cell and CTL cell populations from the analysis. The Foxp3/Transcription Factor Staining Buffer Set (ThermoFisher Scientific) was used to permeabilize the cells for the intracellular FoxP3 stain.

3.7 Cells

L929 fibroblast cells served as the experimental model. Cells were grown in 1640 RPMI (Gibco) with phenol red and supplemented with 10% fetal bovine serum. Where indicated, cells were either plated directly on a plate or plates pre-coated with 0.02% gelatin supplemented with 5 ug/mL fibronectin (Sigma Aldrich) for at least half an hour at 37°C.

3.8 Cell Treatments

Cells were treated with 20 nM quercetin in DMSO or just DMSO vehicle as the control. Quercetin dose was chosen based on a dose-response experiment. Cells treated with IL-2 were treated with 10 μg/mL recombinant mouse IL-2 (Sigma Aldrich) in water or just water as a vehicle control (81, 149).

3.9 Scratch Assay

Six well plates (Corning) were coated with a matrix of 0.02% gelatin supplemented with 5 ug/mL fibronectin (Sigma Aldrich) for at least half an hour at 37°C. These plates were plated with 50,000 cells and grown for 4 days before beginning the experiment. Cells were scratched using a pipet tip, photographed, and then treated. Cells were then grown with treatments as described for 8 hours and photographed using an Olympus IX51 microscope (Olympus). Quantification of the photographs was performed by tracing wound areas using FIJI (150).

3.10 Cell Counts

Cells were exposed for 24 hours to treatments as described in the results section. They were removed from the plate with gentle scraping and stained with trypan blue. Counts were performed using a hemocytometer. Number of cells per well were calculated.

3.11 Flow Cytometry

Cells were grown on 12-well plates (Corning) and were treated with 20 uM quercetin for 24 hours. Cells were scraped off the plate and counted with an automatic cellometer using AO/PI (Nexcelom Bioscience). One million cells were stained with Aqua (ThermoFisher Scientific) to identify live cells and antibodies to αV (using PE-conjugated rat anti-mouse CD51) and β1 integrin (using Pacific Blue-conjugated Armenian hamster anti-mouse CD29) (Biolegend). Cells were analyzed using a BD FACS Canto II (BD Biosciences) and FlowJo (FlowJo). Mean fluorescence intensity (MFI) was calculated with isotype-matched antibodies as the controls for specific stains

(PE-conjugated rat anti-IgG-1ak (BD Pharmingen) as a control for αV and Pacific Blue Armenian hamster IgG as a control for β1 (Biolegend)). For each sample, 500,000 cells were stained with Aqua (ThermoFisher Scientific) to identify live cells and with the PC61 clone of PE-Cy7-conjugated anti-IL-2Rα (BD Pharmingen), the TM-β1 clone of anti-IL-2Rβ conjugated to PerCPCy5.5, and the TUGm2 clone of anti-IL-2Rγ conjugated to APC. After staining, cells were analyzed using a BD FACS Canto II flow cytometer and FlowJo software. MFI was calculated with isotype-matched antibodies as the controls for specific stains (PECy7-conjugated rat IgG1λ (BD Pharmingen), PerCPCy5.5-conjugated rat IgG2bk (Biolegend) for IL-2Rβ and APC-conjugated rat IgG2bk (Biolegend)).

3.12 Human Dataset

Data was analyzed from the NCBI Dataset #GSE52471, which contains human microarray data from healthy controls and SLE patient skin lesions. These data were queried for IL-2 signaling pathway participants including IL-2Rα, IL-2Rβ, IL-2Rγ, Jak1, STAT5a, STAT5b, Jak3, Akt1, Akt3, and MAPK1 and the levels were analyzed (5, 151).

3.13 Statistical Analyses

Statistical analysis was performed with GraphPad (Prism). Graphs present means with error bars representing standard deviations. Student's t-tests were used for comparisons of two groups and analysis of variance (ANOVA) when more than two groups were compared. A Tukey-Kramer post-hoc analysis was then used to compute p values between individual groups. A linear regression with ANOVA was used to

compare measured wound areas with a Holm post-hoc analysis. Statistical significance was indicated by a $p<0.05$. Hierarchical clustering was performed with R (R-Project).

4. RESULTS*

4.1 Fibroblast Reaction to Quercetin

To determine the effect of quercetin on fibroblast growth, we established an *in vitro* system of fibroblast culture and treated the cultures with quercetin at a concentration of 20 nM for 24 hours. The fibroblasts treated with quercetin grow at the same rate as control cells (Figure 4a, b). This indicates that quercetin alone is non-toxic at this concentration and does not impair or improve cell proliferation or survival.

Because quercetin is known to be anti-fibrotic, we sought to explore the expression of integrins in response to quercetin. We specifically chose to examine the response of αV integrin and β1 integrin because of their association with cell migration, skin cell proliferation, and fibrosis (35, 36, 152). After 24 hours of quercetin treatment, we observed changes in the surface expression of both integrin subunits examined, as measured by flow cytometry. Specifically, quercetin-treated fibroblasts have decreased surface β1 integrin (Figure 4c). Given that this integrin is associated with focal adhesions, we suspected that this may indicate a diminished capacity to adhere to the plate. Additionally, quercetin treatment increased surface αV integrin (Figure 4d). Because αV integrin has been associated with promotion of cell migration, this may mean that quercetin may make cells more capable of entering a wound site, even in the absence of substantial ECM deposition. These changes in integrin expression may impact their adhesiveness, ability to migrate, and productivity of ECM and could alter wound closure capabilities by fibroblasts at wound sites. Thus, the alteration of integrin

---

*Parts of this chapter reprinted with permission from:
"The impact of quercetin on wound healing relates to changes in αV and β1 integrin expression" by Doersch KM, Newell-Rogers MK, 2017. *Exp Biol Med*, 242(14), 1424-1431, Copyright 2017 by the Society for Experimental Biology and Medicine.

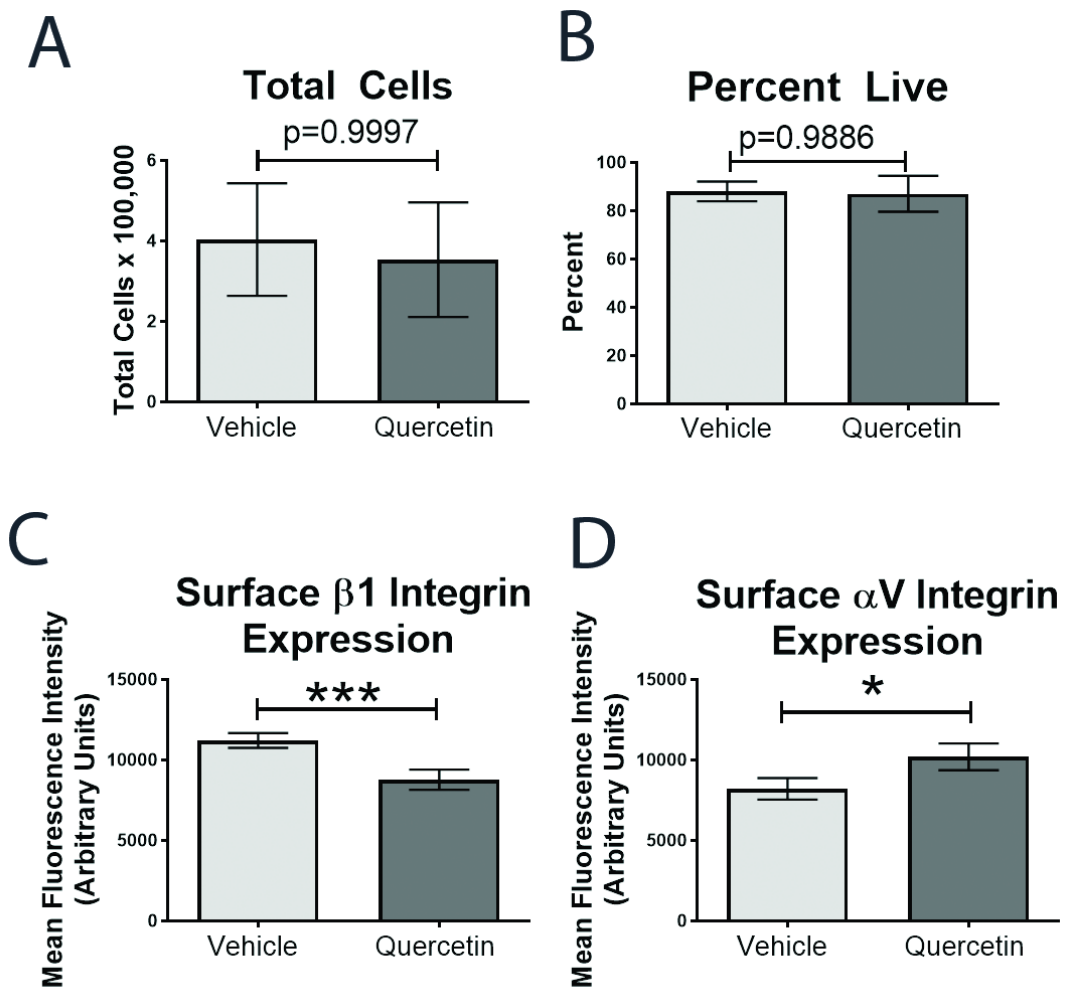

Figure 4: Fibroblast Response to Quercetin. A, B. Quercetin treatment had no effect on total cell number (A) or percent of cells that are alive (B) for L929 fibroblasts. C. Surface β1 integrin expression on L929 fibroblasts is decreased compared with control. D. Surface αV integrin expression on quercetin treated fibroblasts is higher than control. Graph values indicate mean ± standard deviation. *, $p<0.05$; ***, $p<0.001$. Reprinted with permission from (33).

components on the surface of fibroblasts may be part of the mechanism by which quercetin diminishes fibrosis and may improve the quality of healed wounds *in vivo*.

4.2 Quercetin Does Not Impair Wound Healing

Based upon our experience *in vitro*, that quercetin does not impair cell growth or survival, we sought to determine whether quercetin would impact wound healing *in vivo*. To this end, we studied wound healing in C57Bl/6J WT mice. Mice were wounded with an 8-mm biopsy punch and wounds were allowed to heal by second intention. Mice were treated daily with intraperitoneal injections of quercetin. Following the biopsy punch, we observed that wounds in WT mice heal in roughly 14 days. We measured the wounds with calipers to find the average diameter and used these diameters to calculate approximate wound areas. Daily quercetin treatment had no impact on the wound healing rate over the 14-day healing period, based upon these calculated areas ($p=0.55$) (Figure 5a).

Images of typical wounds from the experiments are shown in Figure 5b. The wounds of quercetin-treated mice look grossly normal. Immediately following wounding, which is superficial and does not extend into the underlying adipose layer, the wounds are relatively bloodless and look very similar in both groups. By day 1 post-wounding, wounds stretch to be larger and clots begin to form. On day 3 post-wounding, clots are fully formed and contraction appears to be beginning at the edges of the wounds. By day 7, the unhealed area is substantially smaller and the clot matures in preparation to fall off. On day 10, the wounded area is usually substantially smaller than

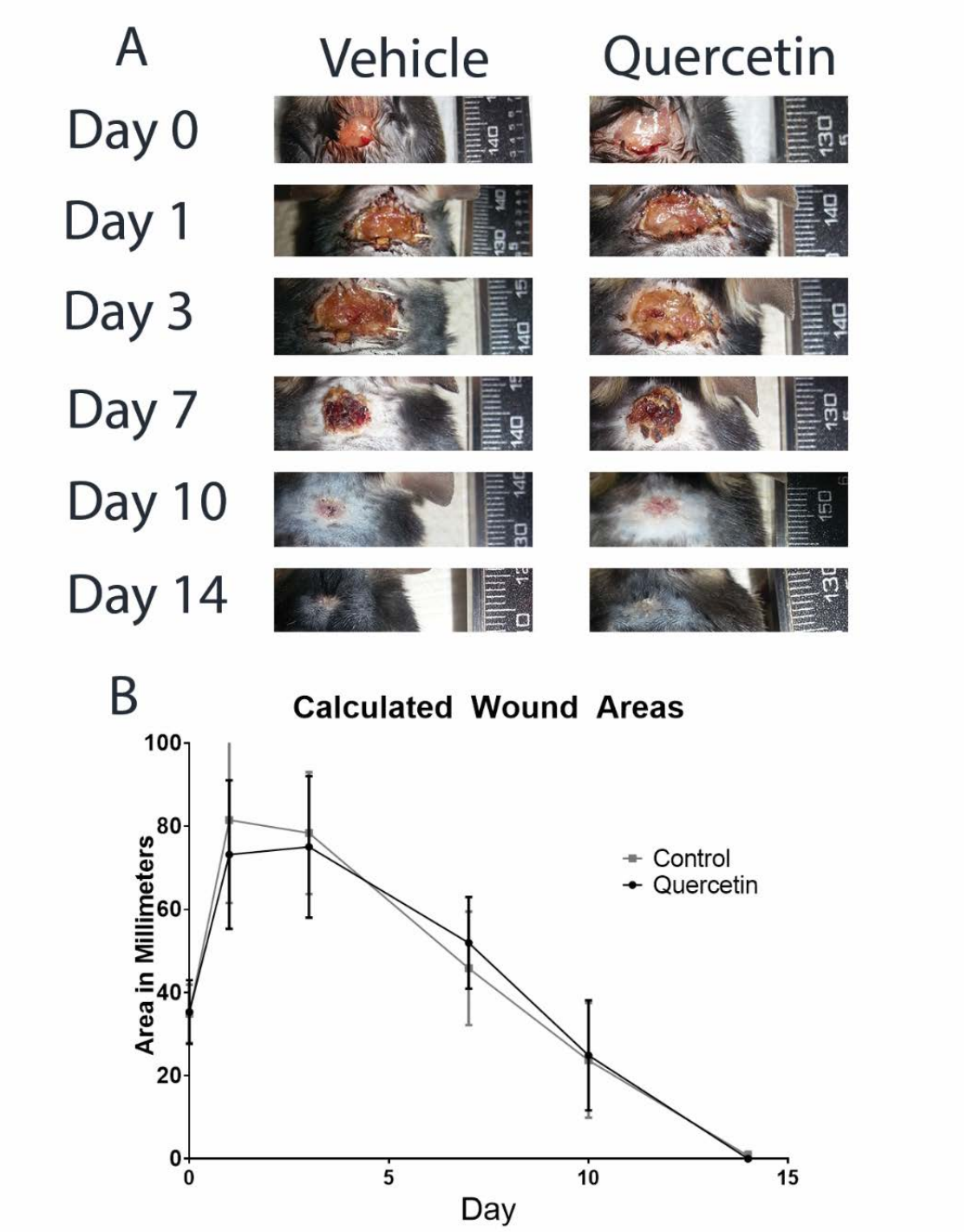

Figure 5: *In Vivo* Wound Healing. A. Gross wound healing for vehicle-treated mice (left column) and quercetin-treated mice (right column) were similar over the course of the 14-day experiment. B. Wild-type mice treated with quercetin heal at the same rate as controls, based on a linear regression analysis of the calculated areas of their wounds that remained open over the course of 14 days (p=0.55). Graph values indicate mean ± standard deviation. Reprinted with permission from (33).

its peak size, returning to roughly the size of the original injury. Additionally, wounds have typically unroofed by day 10. Finally, on day 14, the wound area has continued to contract and the remaining scar appears to be formed. Thus, in the quercetin-treated group, wound healing appears to proceed normally, closing the wound at the same rate and with the same visual quality as in the control mice. Thus, quercetin appears not to impair wound healing in wild-type C57Bl/6J mice.

4.3 Diminished Fibrosis in Healing Wounds

To better understand the impact of quercetin treatment on scar formation in wounds, we sought to characterize the extent of fibrosis using Masson's Trichrome stain. We chose the day 10 and day 14 time points to examine the degree of fibrosis in the wounds that were partially closed at day 10 and wounds that appeared grossly fully closed at day 14. Sections shown are characteristic sections from both day 10 and day 14 post wounding. When compared to controls, we found that quercetin-treated mice have a decrease in fibrosis, as revealed using Masson's Trichrome Stain, shown at both 4X and 10X magnifications. The frequency and density of fibers is substantially decreased at the centers of the wounds in the quercetin-treated mice on day 10 (Figure 6a, b) and day 14 (Figure 6c, d). Furthermore, day 10 collagen fiber density on Masson's Trichrome stain was quantified using ImageJ and represented as particle density. This quantification demonstrated that quercetin-treated WT mice have significantly fewer fibers compared to control mice (Figure 6e). These findings indicate that the high-fibrosis edge of the wound is reduced following quercetin treatment, but that this area is not necessary for the wound to heal at the same rate.

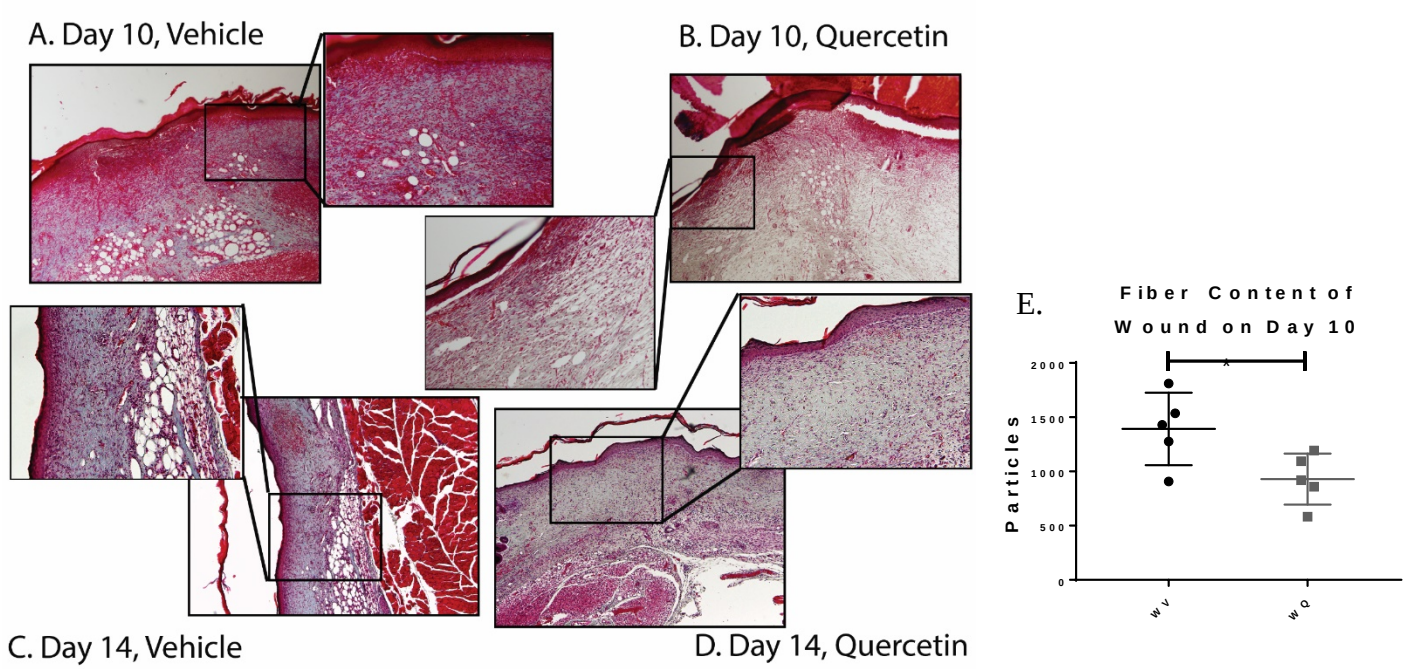

Figure 6: Evidence of Quercetin-Related Wound Healing Advantages *In Vivo:* Masson's Trichrome Stain of Wound Sites. On both day 10 and day 14 post-wounding, sections of wounds from vehicle-treated mice (A, C) had more fibrosis present, characterized by a blue color, than wounds from quercetin-treated mice (B, D). Characteristic sections are shown. E. Quantification of day-10 wound fiber content performed using ImageJ analyzing particles of blue in sections. Quercetin-treated mice demonstrate a statistically significant reduction in particles compared to control. *, $p<0.05$. Reprinted with permission from (33).

Thus, while appearing grossly normal, the wounds of quercetin-treated mice contain fewer ECM fibers, although the presence and cellular appearance of both the dermal and epidermal layers appear unchanged. Thus, quercetin appears to specifically impact ECM deposition, without impairing the ability of skin cells to replicate or migrate. Given that skin cells migrate into the wound over the ECM during the wound healing process, this result is unexpected. However, the fact that wounds were still able to heal with less ECM present may be due to altered integrin expression, allowing cells to migrate more efficiently despite diminished ECM. Furthermore, the cells' ability to migrate adequately may downregulate further ECM production, thus proceeding in a manner that leads to adequate closure of the wound with less ECM. These results may indicate a mechanism by which quercetin treatment diminishes fibrosis, which could decrease scar formation in healed wounds.

4.4 Fibroblast Growth is Impaired When Quercetin is Combined with an Artificial Extracellular Matrix

Following the finding that quercetin did not impact cellular growth *in vitro* or alter wound closure rate *in vivo*, but did diminish *in vivo* ECM production, we sought to test the reaction of quercetin-treated cells to an ECM that is already present on an artificial matrix. We hypothesized that the addition of ECM to the system might impair quercetin-mediated integrin alterations and thereby impact wound healing. To test this hypothesis, we treated fibroblasts with artificial ECM. Fibroblasts were grown on an ECM made of 0.02% bovine gelatin supplemented with 5 uM fibronectin. Fibroblasts grew and survived on this modeled ECM. However, when treated with quercetin in the

context of added ECM, cells demonstrated statistically significant decreases in both total cell number and percent of cells that were alive (Figure 7a, b). Furthermore, fibroblasts were less able to migrate and close a scratch in a scratch assay than in the control groups, a finding that was statistically significant (Figure 7c, d). Thus, it appears that when ECM fibers are already present, quercetin does not provide an advantage. A similar experiment was performed without the artificial ECM to compare the migration across the plate. These experimental results could not be quantified because, while the quercetin-treated fibroblasts remained adherent to the plate and migrated into the scratch, the control group fibroblasts did not adhere to the plate or migrate, but rather peeled off the plate in a sheet. These findings further hint that quercetin makes cells more capable of migrating in their environment and that, following quercetin treatment, cells may require less ECM to migrate. We sought to determine whether αV or β1 integrin levels changes induced by quercetin were impacted by the addition of ECM to the plate. When treated with the artificial ECM, fibroblasts demonstrated the same decrease in β1 integrin expression when treated with quercetin as they had when no ECM was added (Figure 7e). However, the increase in αV integrin levels was statistically significantly attenuated with this treatment (Figure 7f). Thus, when the ECM was already present, quercetin dampened the increase in αV integrin expression without changing the β1 integrin levels, which may mean that the cells are less able to expand and migrate. These results further suggest that quercetin's anti-fibrotic effects may be attenuated by the presence of a pre-constructed ECM, such as is present in certain engineered tissue structures. Finally, the fact that the artificial ECM does not prevent

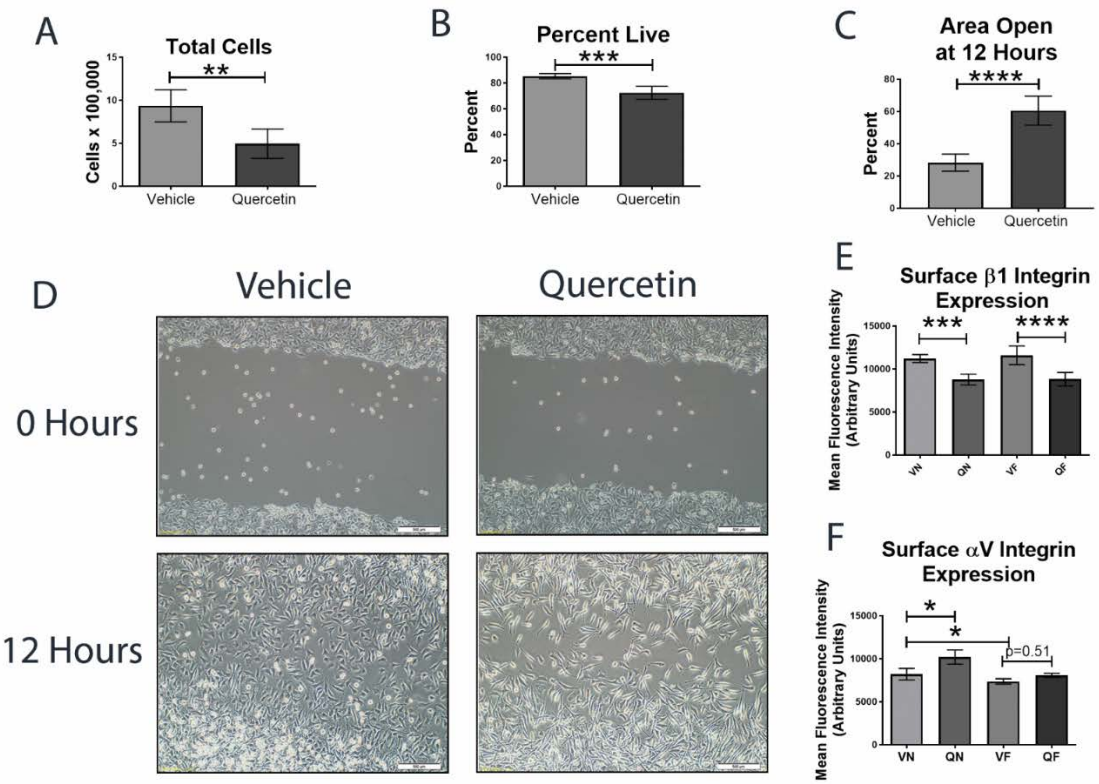

Figure 7: Artificial Extracellular Matrix Alters Response to Quercetin. A. Cell proliferation is diminished when cells are grown on an artificial ECM made of gelatin and fibronectin. B. Cell survival is also diminished on the same ECM. C, D. When grown on gelatin and fibronectin, quercetin diminishes migration into an *in vitro* model of a wound. E. Cells grown on gelatin and fibronectin retain their decreased β1 integrin expression following quercetin treatment. F. When grown on gelatin and fibronectin, cells do not increase their αV levels, unlike the cells grown in the absence of the ECM components. Graph values indicate mean ± standard deviation. *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$; ****, $p<0.0001$. Reprinted with permission from (33).

the decrease in surface β1 integrin expression in response to quercetin indicates that this reaction may be more dependent on “inside-out” signaling rather than “outside-in” signaling. However, changes in αV integrin following quercetin treatment may be more responsive to the outside-in mechanism of integrin signaling due to the fact that changing the ECM of the cells changed the αV integrin expression. These results suggest that changes in integrin expression may play a role in the mechanism by which quercetin and other anti-fibrotic treatments impact the deposition of ECM and thereby the appearance of healed scars.

4.5 Comparison of Wound Healing in a Mouse Model of Systemic Lupus Erythematosus and Wild-Type Mice

We next sought to test the anti-fibrotic treatment in a model of a disease in which wound healing is impaired, specifically SLE. We utilized the *lpr* mouse, which is a well-established SLE model and compared it to its WT background strain. Mouse wound healing, as measured by calculating wound areas from measured diameters, is similar between *lpr* mice and WT (Figure 8a, b). Thus, these mice do not perfectly recapitulate the subtle wound healing delays seen in humans with SLE (8). While quercetin treatment does not impact the wound healing rate in WT mice, *lpr* mice treated with quercetin demonstrated a statistically significant delay in their wound healing, which is most apparent at day 10-post wounding (Figure 8b, c). A histological analysis of the wound sites of these mice at day 10 following wounding reveals that in the WT mice, quercetin reduces the presence of ECM (Figure 8d). Quercetin-treated *lpr* wounds have severely diminished ECM fibers and in these wounds, ECM fibers were disarrayed and severely

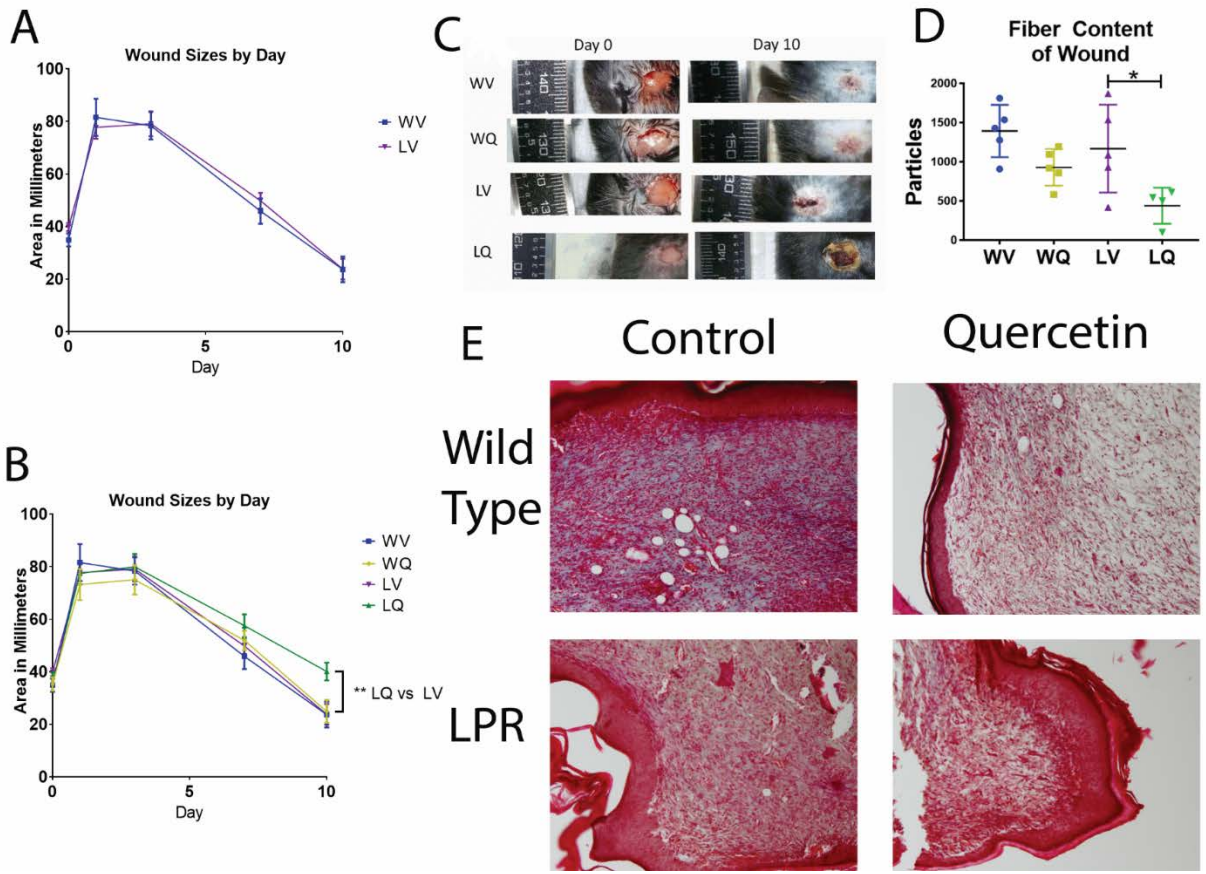

Figure 8: Comparison of Wound Healing in WT and *lpr* mice. A. WT and *lpr* mice heal at the same rate. B. WT and quercetin-treated WT mice heal at the same rate. However, quercetin-treated *lpr* mice heal significantly more slowly than the other groups. C. Photographs of characteristic mouse wounds at days 0 and 10. All mice have roughly equivalent wounds on day 0. However, at day 10 the wounds in the WT (both quercetin-treated and control) and the control *lpr* wounds have shrunk substantially. The wounds in the quercetin-treated *lpr* mice remain larger at the day-10 timepoint. D, E. Quantification of (D) and representation of typical trichrome stains (E). Blue areas represent extracellular matrix and red areas represent cells. WV, WT Control; WQ, WT with quercetin; LV, *lpr* control; LQ, *lpr* with quercetin. *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$; ****, $p<0.0001$.

diminished. Thus, the impact of quercetin on the ECM at the wound site in the SLE model mice led to impaired wound healing.

4.6 Systemic Lupus Erythematosus-Model Mice Have Increased Skin Expression of Interleukin-2

Based upon the evidence that SLE includes aberrant wound healing and the finding that IL-2 may alter the strength of healed wounds we sought to determine whether changes in IL-2 signaling might underlie the altered wound healing in these mice (8, 72). Based on IHC, WT mice had limited IL-2 regardless of quercetin treatment (Figure 9a, b). However, there was substantial IL-2 staining present in both quercetin-treated and control *lpr* mice. These data indicate that, while the serum of *lpr* mice (and SLE patients) is reported to have substantially lower levels of IL-2 compared to WT, there may be a collection of IL-2 in the skin and at wound sites. This IL-2 could play a role in wound healing in the *lpr* mice, perhaps compensating for growth factor pathway signaling that normally plays a role in wound healing. IL-2, which serves as a growth factor in other contexts, specifically for immune cells, could serve as a substitute for as-yet-unidentified growth factor signaling that is a part of healthy wound healing and that is aberrant in these mice and in SLE (44, 60).

4.7 Cultured Fibroblast Response to Interleukin-2

To further understand how IL-2 treatment might impact wound healing we employed the *in vitro* model using cultured L929 fibroblasts. We used these fibroblasts to examine their viability and how effectively the cells migrate into a scratch created in the center of the cell layer on a culture plate. Cells were capable of growing when treated for 24 hours

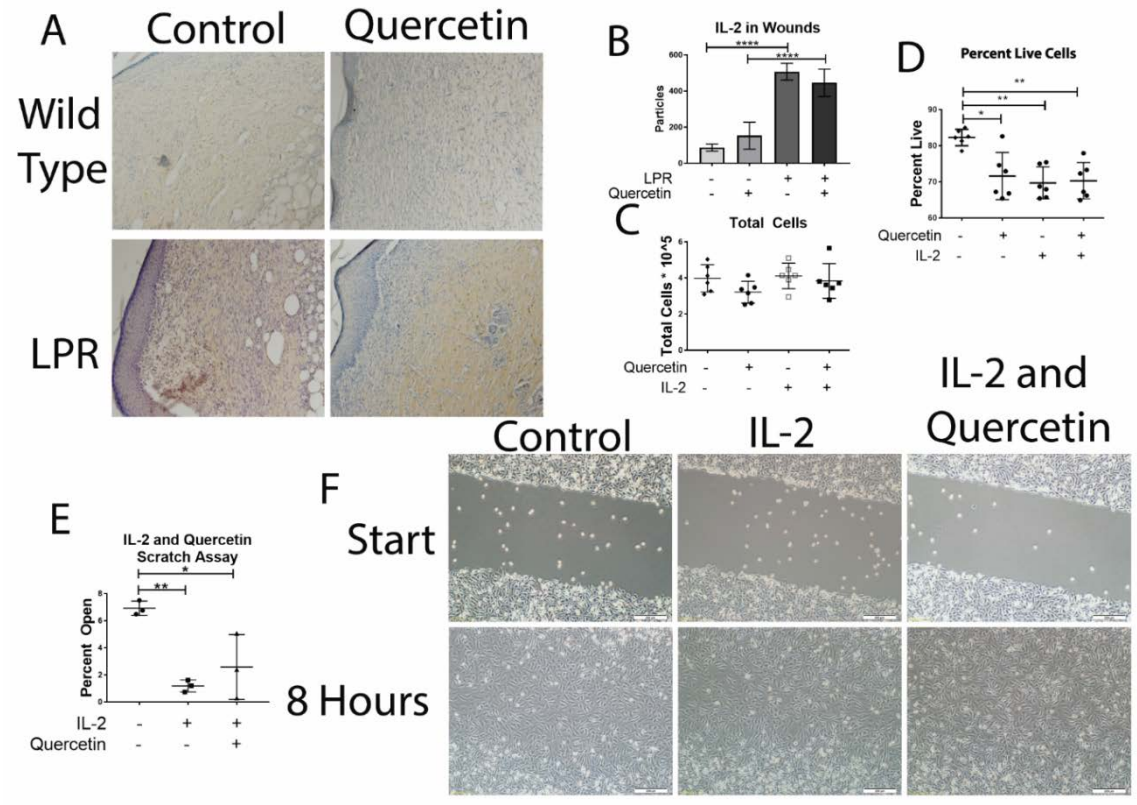

Figure 9: Exploration of IL-2 in Mice and Cells. A, B. Images and quantification (using ImageJ measurement of density of brown particles) of IL-2 in the wounds of mice. IL-2 is apparent in all sections. However, both quercetin-treated and control *lpr* mice had substantially more foci of IL-2 compared to quercetin-treated or control WT. C. L929 cells treated with IL-2, with or without quercetin, did not demonstrate changes in the total number of cells on the plate. D. IL-2 treatment of L929 cells decreased the percentage of cells present in culture that were alive. This effect occurred with or without quercetin treatment. E, F. Despite the decreased viability, cells were more capable of closing scratch assays when treated with IL-2. This IL-2 treatment overcame the previously mentioned impairment of scratch closure seen with quercetin treatment. WV, WT Control; WQ, WT with quercetin; LV, *lpr* control; LQ, *lpr* with quercetin. *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$; ****, $p<0.0001$.

with IL-2 with or without quercetin and these treatments did not impact the total number of cells growing on a plate (Figure 9c). However, IL-2 treatment did significantly decrease the percentage of the cells growing on the plate that were alive (Figure 9d). In contrast to these findings were the scratch assay results with IL-2. In this experiment, IL-2 treatment improved cell migration into the scratched area on a cell culture plate (Figure 9e, f). IL-2 treatment was even sufficient to overcome the scratch closure delay seen with quercetin treatment alone. Stated another way, IL-2 treatment, with or without quercetin, was sufficient to improve wound healing, despite a decrease in the percentage of cells on the plate that were alive. These findings hint that the IL-2 in the wounds of the *lpr* mice may be serving as a growth or migration factor and may assist in the wound closure. However, it does not explain why quercetin treatment diminished the ability of these mice to heal.

To further understand the mechanism underlying our findings, we then sought to determine whether cultured fibroblasts possess surface IL-2R subunits and if expression of those subunits changes in response to IL-2 or quercetin treatment. The results of these experiments are shown in Figure 10. Neither quercetin nor IL-2 alone substantially changed the levels of IL-2Rα, β, or γ. However, a combination of quercetin and IL-2 increased the expression of IL-2Rα and IL-2Rγ compared to quercetin alone only in the presence of the gelatin and fibronectin. This indicates that quercetin sensitizes fibroblast cells to an IL-2-induced upregulation of its receptors, which might mean that IL-2 signaling directly impacts skin cell behavior and indicates that that skin cells and IL-2-

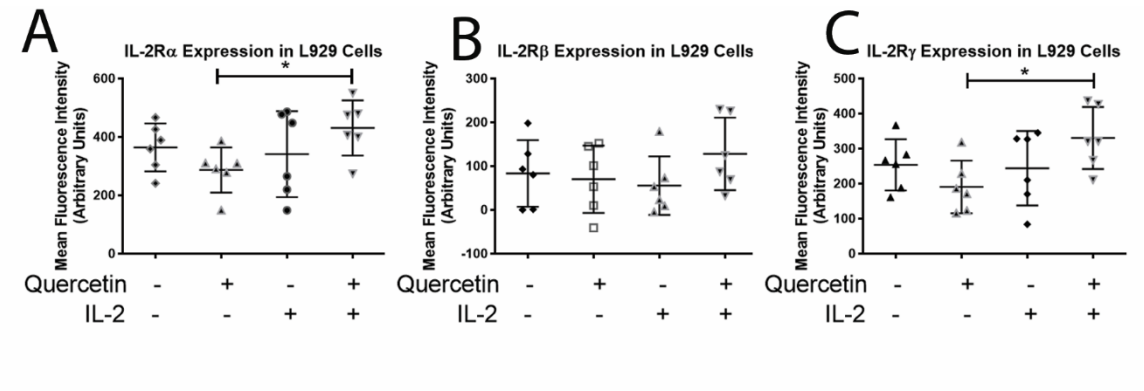

Figure 10: IL-2R Expression on L929 Fibroblast Cell Surface. A. IL-2Rα does not change in response to IL-2 treatment compared to control. However, quercetin and IL-2 together significantly increase IL-2Rα compared to quercetin alone. B. IL-2Rβ levels do not change significantly in any of the groups. C. IL-2Rγ levels do not change in response to IL-2 treatment alone. However, a combination of IL-2 and quercetin increase IL-2Rγ levels compared to quercetin alone. *, $p<0.05$.

producing cells, which could include cells of the skin and immune system, may communicate at wound sites.

Given the alterations in the expression of IL-2R subunits following IL-2 and quercetin treatment, we then sought to examine the expression of IL-2Rα at the wound site. In the 10 day old wounds, IL-2Rα was present at the wound site in both WT and *lpr* mice (Figure 11). However, there was a substantially higher expression of IL-2Rα in the *lpr* mouse wounds compared to the wound in the WT mice. Quercetin treatment diminished IL-2Rα expression at the wound site in *lpr* mice compared to control-treated *lpr* wounds. Together, these findings indicate that a high level of IL-2Rα in the skin of *lpr* mice, and possibly SLE patients, may play a role in wound repair. However, decreasing the levels of IL-2Rα may be part of the mechanism by which quercetin

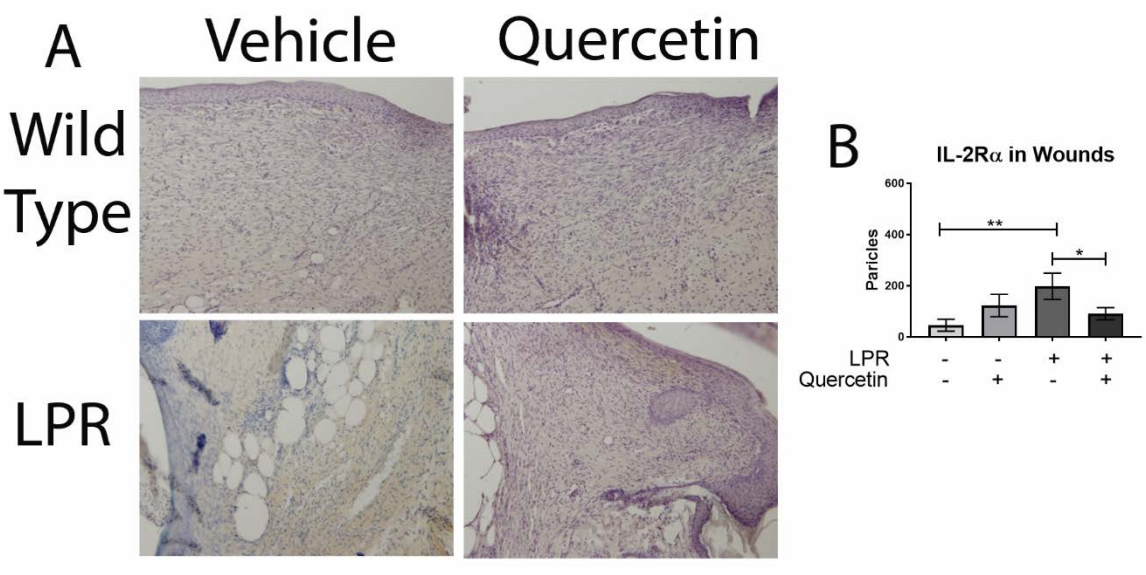

Figure 11: IL-2Rα Expression at the Wound Sites of Mice. A. Characteristic image of IL-2Rα staining WT and *lpr* mice treated with either quercetin or vehicle. B. Quantification of IL-2Rα in the wound sites of mice was performed using ImageJ and measuring density of brown particles. IL-2Rα levels are low in WT mice regardless of quercetin treatment. However, IL-2Rα levels are substantially higher in the control *lpr* mice compared to control WT. However, in the quercetin-treated *lpr* mice IL-2Rα levels are significantly lower. *, $p<0.05$, **, $p<0.01$.

impairs wound healing. It is possible that IL-2 signaling increases ECM formation in *lpr* mice and that when this signaling is reduced, wound healing is delayed. The finding that IL-2Rα is reduced at the wound site in association with a healing delay thus indicates that IL-2-IL-2Rα signaling is important for wound healing. The proposed mechanism is that reduced IL-2R in the skin prevents IL-2 from acting as a growth factor to increase skin cell proliferation and ECM fiber formation.

4.8 Impact on Immune Cell Percentages in the Systemic Lupus Erythematosus-Model Mice in Response to Quercetin Treatment

The finding that IL-2Rα levels in the *lpr* mice treated with quercetin are reduced was unexpected given that fibroblast surface expression of IL-2Rα was increased by the combination of IL-2 treatment and quercetin. This led us to consider whether immune cells, which likely mediate the majority of IL-2 signaling in the *lpr* mice and are likely responsible for depositing IL-2 and sIL-2Rα at the wound site, might be impacted by quercetin in ways that diminish sIL-2Rα production. These changes in immune cell behavior could substantially impact other immune cells, skin cells, and ECM production in ways that would alter the content of the wound site during the healing process.

We sought an immune cell source of the altered IL-2 and IL-2Rα in the wounds of *lpr* mice compared to WT mice. We examined the frequency of various immune cells known to contribute to IL-2 signaling at the draining LNs and spleens of the wounded animals. We performed a similar wounding experiment and then, at 10 days post-wounding, we examined the contents of the proper and accessory axillary LNs, which drain the back region of the mice. We also stained splenic cells for the same markers. Because T-cell dysregulation plays a substantial role in the pathogenesis of SLE, we stained for T-cell markers to identify CD4+ THCs, CD8+ CTLs, and T-regs, which were identified as CD4+CD25^{hi}FoxP3+ (5, 23, 89, 151). We also analyzed a cell population, the CD3+CD4-CD8- cells, also called double-negative (DN) cells. These cells are known to be present in high quantities in *lpr* mice and are capable of signaling. In the WT mice, few of this cell type escape the thymus and therefore they contribute

negligibly to the immune response. However, in *lpr* mice and some SLE patients, these so-called DN T-cells are present in excess and contribute substantially to immune function (153). DN cells appear to be pro-inflammatory and produce IL-17, which promotes pro-inflammatory T-helper-17 (TH_{17}) cell development. Finally, we used CD11c+ to identify dendritic cells, which are known to be involved in skin homeostasis and are also intimately involved in IL-2 signaling (45, 154, 155).

We next examined surface expression of IL-2Rα on the immune cells found in the LN and spleen to compare the expression of the IL-2-specific surface receptor in the *lpr* mice to WT in the quercetin-treated and control groups. We sought to illuminate the role that the various immune cells studied might play in the wound healing deficit in the quercetin-treated LPR mice by understanding IL-2Rα changes in the *lpr* and WT in response to quercetin.

Lymph Node Proportions of Cells Involved in Interleukin-2 Signaling

In the *lpr* mouse, the percentage of THCs, which are identified by being CD3+CD4+, is reduced compared to WT mice (Figure 12a). Quercetin treatment in the *lpr* mouse raises the percentage of THCs, although the percentages in the quercetin-treated *lpr* are still reduced compared to the quercetin-treated WT LN. WT and *lpr* mice have equivalent percentages of T-regs in the draining LN (Figure 12b). The percentages of T-regs in both mouse strains were not impacted by quercetin treatment. CTL and B-cell populations also showed no statistically significant differences (data not shown). *Lpr* mice have a substantial increase in the number of DN cells in their axillary LN compared

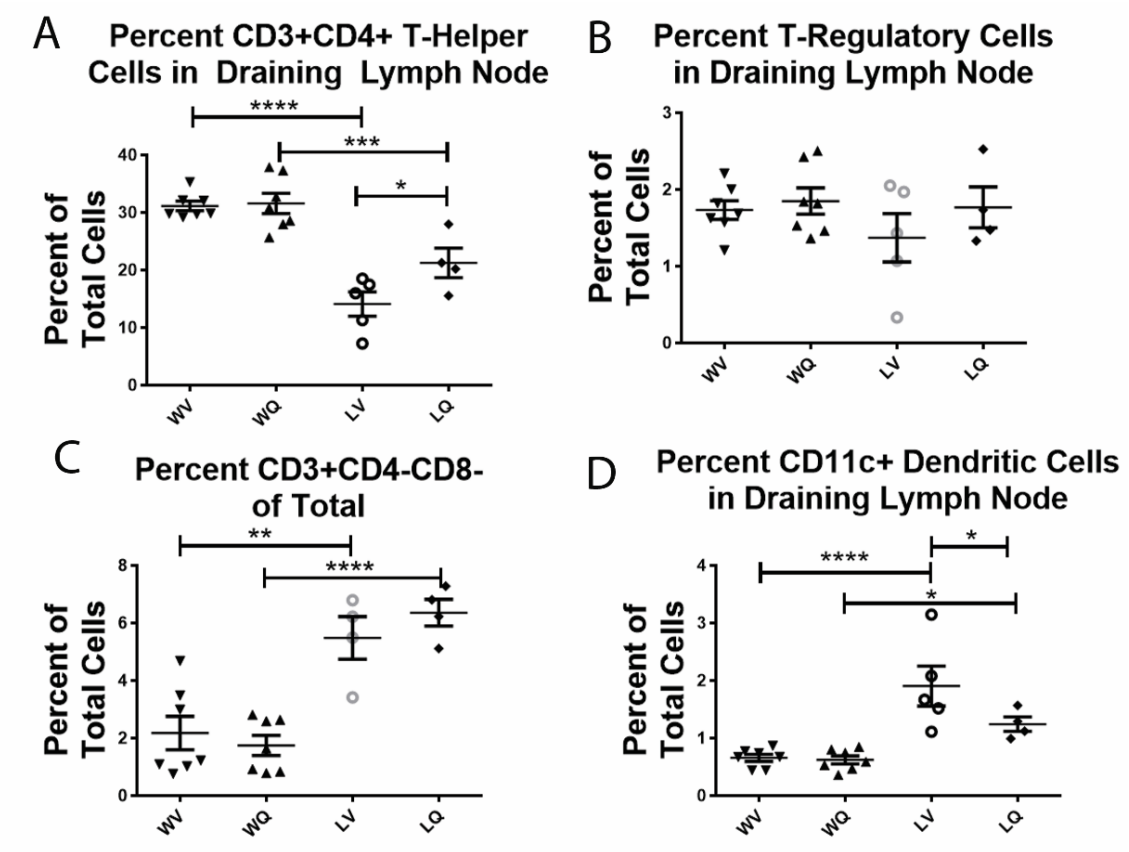

Figure 12: Immune Cells in the Wound-Draining LN. A. There are substantial differences between CD3+CD4+ THCs. Quercetin-treated and control WT had no differences in their THC levels. However, in both quercetin-treated and control animals, CD3+CD4+ cells represent a smaller percentage of the total cell population in *lpr* mice than in the corresponding WT mice. Quercetin treatment increases the fraction of THCs in the *lpr* mice compared to control *lpr* but not back to the level in the WT mouse groups. B. T-reg levels are unchanged in any of the groups. C. *Lpr* mice have more CD3+CD4-CD8- T-cells than WT in both the quercetin-treated and control groups. D. *Lpr* mice also have more CD11c+ dendritic cells compared to WT in both the quercetin-treated and control groups. In the *lpr* mice, quercetin treatment reduces the percentage of CD11c+ cells in the draining LN. WV, WT Control; WQ, WT with quercetin; LV, *lpr* control; LQ, *lpr* with quercetin. *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$; ****, $p<0.0001$.

to WT mice in both the quercetin-treated and control groups (Figure 12c). Finally, dendritic cells identified by CD11c, exhibit a very interesting pattern (Figure 12d). These cells were more frequent in the LN of the *lpr* mice compared to WT mice. Additionally, quercetin treatment in the *lpr* mice diminished the percentage of dendritic cells present in the LN compared to control *lpr* mice. This diminution in dendritic cells at the wound-draining LN of the *lpr* mice during quercetin treatment, coupled with the poor healing and the decreased expression of IL-2Rα in the wounds, may indicate a role for dendritic cells in promoting IL-2 signaling to aid in wound healing.

The analysis of the cells found in the LN indicate that the quercetin-treated *lpr* mice, the mice that have delayed wound healing, had enrichment of THCs and a diminution of dendritic cells compared to the control *lpr* mice that healed at the normal rate. Furthermore, they had proportionally more dendritic cells and DN T-cells and proportionally fewer THCs compared to quercetin-treated WT. These cells, especially the THCs and dendritic cells, are likely responsible for IL-2 production locally at the wound, given their known propensity to produce IL-2 and their proximity to the wound in the wound-draining LNs (45, 47, 156). Given that IL-2 levels are equivalent between quercetin-treated and control-treated *lpr* mice, these cells are likely capable of producing IL-2. However, IL-2Rα levels at the wound sites of quercetin-treated *lpr* mice are lower, indicating that cells at the wound sites have a down-regulation of their potential to respond to IL-2. This may mean that immune cells in the quercetin-treated *lpr* compared to control may be taking up and sequestering the IL-2. A lack of ability to signal via IL-2 may explain the reduced percentage of dendritic cells in the quercetin-treated *lpr*

compared to control. Furthermore, in other models, dendritic cells have been shown to be responsible for sIL-2Rα production (157). Thus, decreased dendritic cell fraction in the quercetin-treated *lpr* may lead to reduced sIL-2Rα production, which then impacts the amount of this factor and its ability to aid in IL-2 signaling at the wound site. The lack of IL-2 signaling may further be modified by the THCs, which are present at a higher percentage in the wound-draining LN, or by behavior of the DN T-cells, which will be explored later in this work.

Splenic Proportions of Cells Involved in Interleukin-2 Signaling

Splenic immune cell populations were also analyzed in the mice to understand the systemic impact of quercetin treatment on *lpr* mice compared to WT mice and how this impact might alter wound healing. The percentage of THCs was decreased significantly in the spleen of the control-group *lpr* compared to the control-group WT, a finding similar to the changes in percentages seen in the LN, indicating that these mice have a global decrease in the proportion of THCs compared to other immune cell types (Figure 13a). Quercetin treatment abolished this difference between the WT and the *lpr*. T-regs were increased in the quercetin-treated *lpr* mice compared to the quercetin-treated WT mice (Figure 13b). The increased percentage of T-regs was not present in the control groups, indicating that quercetin led to an enrichment of T-regs in the spleens in the *lpr* mice. CTL and B-cell populations showed no statistically significant differences (data not shown). Additionally, the DN cell subset was increased in the spleens of *lpr* mice treated with quercetin compared with the quercetin- treated WT mice (Figure 13c).

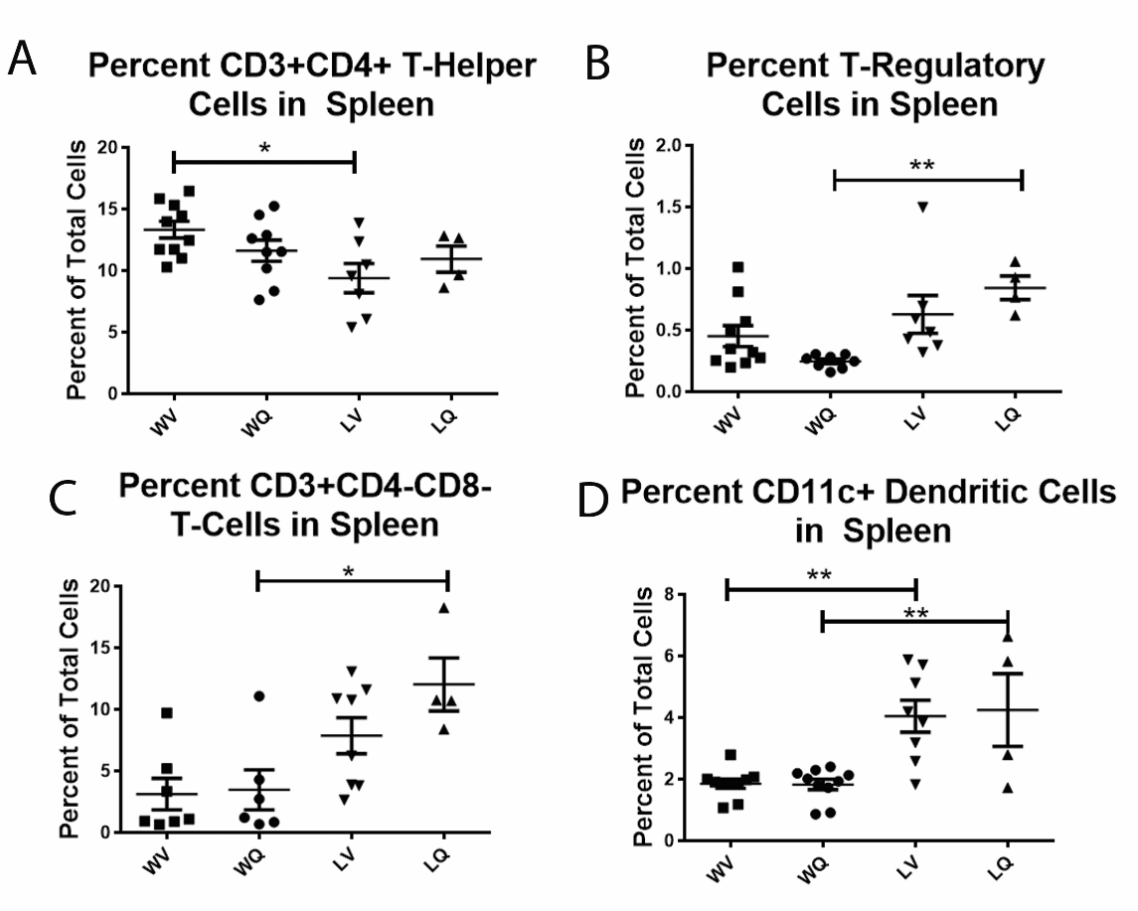

Figure 13: Immune Cells in the Spleen. A. Quercetin-treated and control WT had no differences in their CD3+CD4+ THC levels. However, in the control group only, THCs represented a reduced fraction of the total cell population in the *lpr* compared to WT. B. T-regs increased in percentage in the spleen only in the quercetin-treated *lpr* compared to quercetin-treated WT. There were no differences in the control *lpr* and WT groups. C. CD3+CD4-CD8- cells mirrored the T-regulatory cell frequencies with the only difference being an increased percentage in the quercetin-treated *lpr* group compared to quercetin-treated. D. *lpr* mice also have more CD11c+ dendritic cells compared to WT in both the quercetin-treated and control groups. WV, WT Control; WQ, WT with quercetin; LV, *lpr* control; LQ, *lpr* with quercetin. *, $p<0.05$; **, $p<0.01$.

Control-treated *lpr* mice trended toward an increase in this cell population over WT mice, although the difference did not reach statistical significance (p=0.0857). These findings, coupled with those from the LN, indicate that *lpr* mice have an enrichment of DN T-cells, which may, in the context of changes in percentages of other cell types that impact IL-2 signaling, further contribute to aberrant IL-2 signaling in the *lpr* mice. The findings regarding the 3 T-cell subtypes studied may indicate that quercetin treatment increased the percentages of T-cells compared to other cell types following an injury in the *lpr* mice. Additionally, the *lpr* spleens in both the quercetin-treated and control groups had higher proportions of CD11c+ dendritic cells compared to WT mice, a finding that was also seen in the LN (Figure 13d).

Thus, *lpr* mice seem to possess increased percentages of their dendritic cell population, which may underlie some of the pathology demonstrated in these mice and highlight an underappreciated role for dendritic cells in SLE (23). The results of these studies demonstrate that immune aberrations seen in the *lpr* mouse are impacted by quercetin treatment. They also demonstrate that quercetin treatment of this mouse strain after wounding leads to substantial changes in the immune cells found at the wound-draining LN. Given that many of the immune cells examined in this study impact IL-2 signaling, it is likely that these changes play a role in the IL-2 signaling at the wound site. Furthermore, changes in percentages of these cells following quercetin treatment in the *lpr* mice may play a role in the wound healing deficit experienced by the *lpr* mice following quercetin treatment.

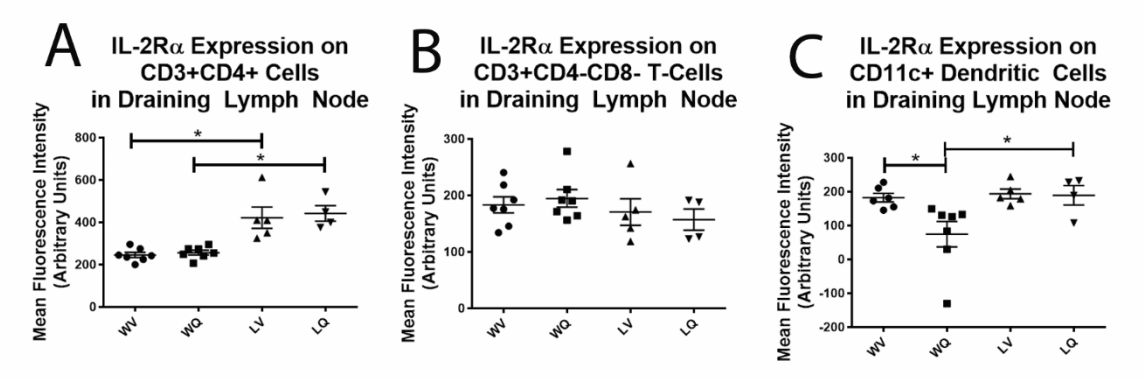

Figure 14: Surface IL-2Rα Expression on LN Immune Cell Populations. A. CD3+CD4+ THC populations have increased levels of surface IL-2Rα in both the quercetin-treated and control *lpr* groups compared to the corresponding WT groups. B. There is no change in surface IL-2Rα in any of the groups' CD3+CD4-CD8- population, although the expression of this receptor on these cells is high. C. CD11c+ cells exhibit diminished surface IL-2Rα levels in response to quercetin treatment. This change in expression is lost in the *lpr* mice.

4.9 Interleukin-2-Receptor-α Expression on Immune Cells in the Axillary Lymph Node and Spleen

To further understand the behavior of the cells that might contribute to IL-2 signaling, we examined their surface expression of IL-2Rα. CD3+CD4+ THCs in the LN of both the quercetin-treated and control *lpr* mice had enriched surface IL-2Rα levels compared to quercetin-treated or control WT mice (Figure 14a). Thus, while this cell population is present at a lower frequency in the *lpr* mice, they likely contribute substantially to IL-2 signaling given their high expression of IL-2Rα. Furthermore, quercetin treatment increases the percentage of THCs in the *lpr* mice, and, given their

high expression of IL-2Rα, they may be sequestering IL-2, reducing its signaling potential, and leading to lower production of sIL-2Rα by other cells or lower IL-2Rα expression by cells in the skin. LN DN T-cells had equivalent IL-2Rα levels across all the groups (Figure 14b). However, these cells expressed relatively high levels of IL-2Rα compared with other cell types and, given the high percentage of these cells in the LPR mice, they may be contributing more substantially to IL-2 signaling in these mice than in the WT mice. In the WT mice, CD11c+ cells had a decrease in the IL-2Rα levels in response to quercetin (Figure 14c). However, this change was not seen in the *lpr* mice and thus the quercetin-treated *lpr* mice had greater surface IL-2Rα than the quercetin-treated WT mice. Given that dendritic cell percentages were reduced in *lpr* mice treated with quercetin compared to the control group, this may indicate that there were fewer of these cells capable of producing sIL-2Rα, leading to lower IL-2Rα levels at the wound site. Together, these findings hint that there is an overall increase in IL-2Rα on several LN immune cell subtypes that may play a role in the wound healing deficit demonstrated in quercetin-treated *lpr* mice.

Splenic cells also demonstrated changes in surface IL-2Rα levels. CD3+CD4+ THCs from the spleen expressed increased levels of IL-2Rα in the *lpr* compared to WT, an increase that was preserved with quercetin treatment of both groups and was similar to that seen in the LN (Figure 15a). DN T-cells from the spleen exhibited lower levels of IL-2Rα than they had at the draining LN, as demonstrated by MFI, that was not different

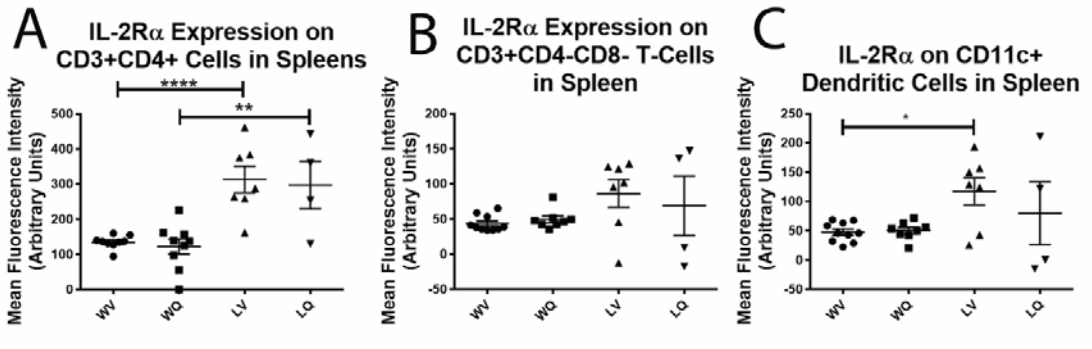

Figure 15: Surface IL-2Rα Levels on Spleen Immune Cell Populations. A. CD3+CD4+ THC populations have increased levels of surface IL-2Rα in both the quercetin-treated and control *lpr* groups compared to the corresponding WT groups. B. There is no change in surface IL-2Rα in any of the groups' CD3+CD4-CD8- population. C. CD11c+ cells exhibit higher surface IL-2Rα levels in the *lpr* control group compared to the WT control group. In the quercetin-treated WT and *lpr* groups this difference is not present.

between any of the groups, just as in the LN (Figure 15b). CD11c+ cells demonstrated higher IL-2Rα on their surfaces in the *lpr* mice compared to the WT in the control groups (Figure 15c). However, quercetin treatment did not mirror this increase compared to the quercetin-treated WT mice ($p=0.7137$). Additionally, there was no difference between the quercetin-treated and control *lpr* groups in the spleen. These low splenic IL-2Rα levels on DN and CD11c+ cells in the *lpr* mice may reflect the relatively low systemic IL-2 levels seen in these mice. Furthermore, the fact that THCs in the spleen had high levels of IL-2Rα may indicate that these cells are experiencing high levels of TCR engagement or other stimulation that increases their potential for IL-2R signaling. The fact that the pattern of IL-2Rα expression on splenic cells is different from that of the cells in the wound-draining LN indicates that the cells in the LN are likely reacting to the altered cytokine milieu at the wound site, including the balance of IL-2 and IL-

2Rα found in the wound. These data hint that there is a difference between IL-2 signaling in the blood versus tissues, especially the skin, in the *lpr* mice and possibly in human SLE. Additionally, these data indicate that IL-2 signaling may be responsible for wound healing deficits demonstrated with quercetin treatment.

4.10 Humans with Cutaneous Lupus Exhibit Altered Skin Interleukin-2 Signaling

To better understand how IL-2 might contribute to the excess scarring seen in SLE, we used data from a database available online to analyze skin samples from CLE patients, who had discoid lesions, and compare them to control patients. Samples in this dataset include mRNA isolated from biopsy punches of discoid lesions from CLE patients and biopsy punches from separate patients without CLE. We selected genes of interest that participate in IL-2 signaling from the database. Hierarchical clustering based on these genes of the IL-2 pathway demonstrates that the CLE patients cluster together and control patients cluster separately, based upon changes in the expression of transcripts for genes in this pathway (Figure 16). These data indicate that IL-2 signaling is likely associated with discoid lesions in CLE patients. This cluster analysis, in addition to the evidence collected *in vitro* and in mice, serves as evidence that IL-2 signaling in the skin may play a substantial role in the wound healing deficits that occur as part of SLE.

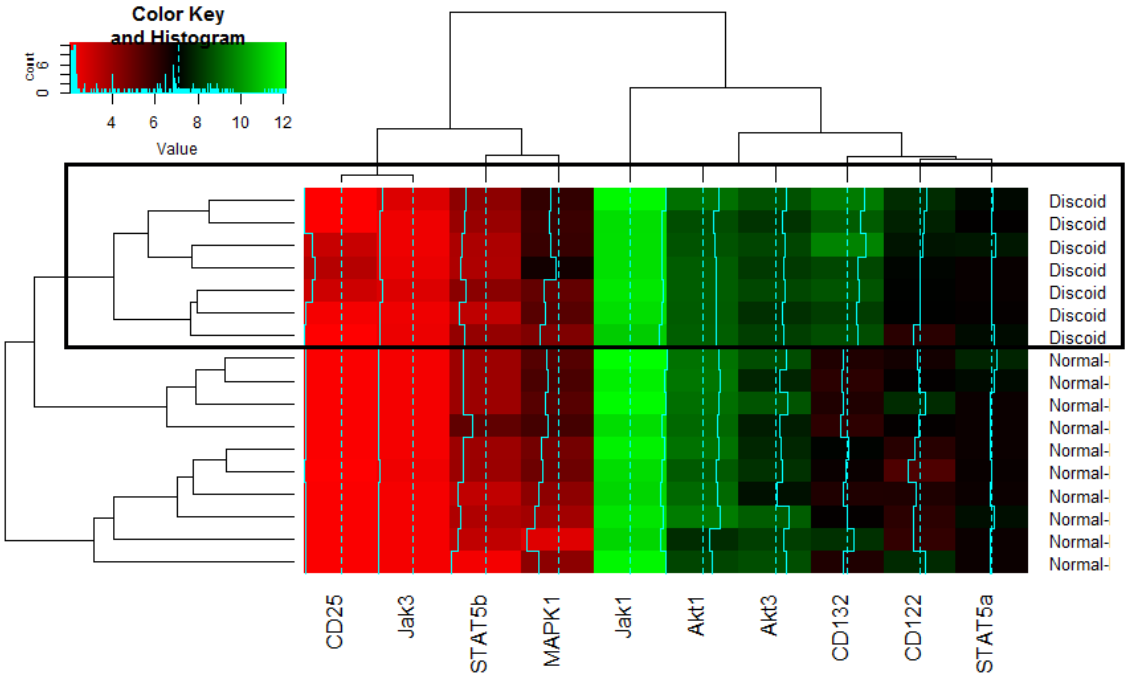

Figure 16: Human Microarray Data. A hierarchical clustering analysis was performed on skin samples from either discoid lesions of SLE patients or skin biopsies from healthy controls. Genes of interest in the IL-2 signaling pathway were selected and a hierarchical clustering analysis was performed. This analysis demonstrates a clustering of the SLE patients separate from control lesions.

5. DISCUSSION*

5.1 Summary of Results

We first tested the hypothesis that an anti-fibrotic treatment could improve wound healing. The purpose of this study was to explore the use of a flavonoid, quercetin, which is currently available over the counter, to improve wound healing. The hypothesis was tested by using an *in vitro* model of wound healing to demonstrate that quercetin decreased fibroblast survival, which may impact the development of fibrosis, and that quercetin decreases β1 and increases αV integrin expression only in the absence of artificial ECM. We also tested the use of quercetin to prevent fibrosis in an *in vivo* wound healing model and demonstrated a normal healing rate with a diminished occurrence of fibrosis at the wound site based on Masson's Trichrome stain. This work demonstrates that quercetin can be used to decrease the formation of fibrosis at a wound site without delaying the closure *in vivo*, ideal characteristics for a therapeutic intervention. Additionally, quercetin treatment does not alter the *in vitro* growth of fibroblasts in the absence of ECM.

We next tested the hypothesis that IL-2 signaling mediates the deficits in wound healing seen in SLE. This hypothesis was tested using an SLE-model mouse strain, the *lpr* mouse. These mice were wounded and treated with quercetin and wounds were analyzed for the expression of IL-2 and IL-2Rα. In the *lpr* mice, quercetin treatment led to a decrease in fibrosis at the wound site and a wound healing delay. These findings were associated with a decrease in IL-2Rα in the *lpr* wounds, while the level of IL-2 mal. To further study wound healing, we examined L929 cultured fibroblasts and

*Parts of this chapter reprinted with permission from:
"The impact of quercetin on wound healing relates to changes in αV and β1 integrin expression" by Doersch KM, Newell-Rogers MK, 2017. *Exp Biol Med*, 242(14), 1424-1431, Copyright 2017 by the Society for Experimental Biology and Medicine.

demonstrated that a combination of IL-2 and quercetin increases IL-2Rα and γ expression on these cells and promotes IL-2 signaling. Thus, we considered the possibility that immune cells were contributing to the mechanism by which IL-2Rα is reduced at the wound site. We demonstrated that the draining LNs for the wound sites contain a reduction in dendritic cells and an increase in THCs and DN T-cells, all of which are known to impact IL-2 signaling. These cells may play a role in the mechanism by which IL-2 levels are increased in *lpr* mice. Furthermore, human skin samples demonstrate evidence of increased signaling in the pathway downstream of IL-2 in the context of SLE, similar to the control *lpr* mice. Together, these findings serve as evidence that, in SLE, IL-2 may contribute to skin repair, but that signaling via this pathway is pro-fibrotic. Thus, decreasing fibrosis in the *lpr* mice may have led to a wound healing deficit that could help guide future therapeutic strategies to improve healing in SLE patients. Furthermore, this work implicates immune cell signaling in the IL-2-dependent alterations in wound healing seen in SLE. Targeting these immune cells may represent a therapeutic strategy for improving wound healing in SLE and possibly other disease states.

5.2 Proposed Mechanism for the Action of Quercetin on Integrin Signaling

The fact that quercetin treatment increases αV and decreases β1 integrin expression may reflect an anti-fibrotic and pro-adhesion phenotype in which cells require less ECM for migration and proliferation. The fact that the effect on αV, but not β1 integrin, is ameliorated in the presence of ECM may indicate that with the ECM already present, high αV integrin is no longer needed to augment migration. Growth on

artificial ECM is associated with decreased fibroblast proliferation, decreased survival, and delayed *in vitro* wound closure. This may mean that αV integrin provides important survival signaling in the context of quercetin treatment, and that without this increase, cell growth is impaired when quercetin is present. On the other hand, β1 integrin is decreased by quercetin treatment regardless of whether ECM is present or absent. This may indicate that β1 subunit-containing integrins are a result of quercetin's impact on internal cellular signaling, leading to a decrease in β1 levels regardless of the amount of ECM present.

Additional factors that mediate the effects of quercetin demonstrated in this work remain to be elucidated. However, integrin signaling appears to be involved in the decrease in fibrosis in response to quercetin. Integrins must be activated to participate in ECM adhesion and their behavior is mediated by both "inside-out" and "outside-in" signaling (32). Inside-out signaling occurs when integrin activation occurs in response to intracellular changes while outside-in signaling occurs when integrin activation is modulated by the content of the ECM. Unfortunately, this study does not determine whether the integrins on the surface of the fibroblasts were activated or not. More work is required to demonstrate whether quercetin impacts the activation of integrins on the surface of skin cells and, if so, how this impacts the proliferation and migration of these cells. However, it is clear that altering integrin expression and activation impacts fibrosis. Integrins, specifically αV-containing integrins, but also some β1-containing integrins are known to cleave TGF-β in the ECM, a behavior that is often, but not always, pro-fibrotic (28). By altering the integrin content on the cell surface, quercetin

may diminish signaling via pro-fibrotic pathways while maintaining cell adhesion and the ability of the cells to migrate. These results hint that more exploration of quercetin's impact on fibrosis may yield promising treatment strategies.

5.3 Proposed Mechanism for the Involvement of Interleukin-2 in Wound Healing and Systemic Lupus Erythematosus

Improving wound healing in SLE would substantially improve the quality of life for SLE patients and thus warrants exploration. Wound healing appears to involve IL-2 signaling, suggesting that the IL-2 pathway may be an important therapeutic target, although this relationship needs more study before it can be exploited clinically. As shown in Figure 3, it appears that early in the wound healing process, IL-2 signaling may play a role in attracting immune mediators for initiating the healing process and preventing colonization of the wound site by microbes. It may also contribute to skin and blood vessel cell proliferation. Later, decreasing IL-2 may help promote resolution of inflammation by attracting and expanding regulatory immune cells. Immunologic derangements, especially those involving the IL-2 signaling pathways, may impair wound healing. Evidence for this role for IL-2 in wound healing comes from studies that explore the cytokine mediators of wound repair and from evidence in diseases involving both aberrant wound healing and derangements of IL-2. Taken together, the evidence indicates that the role IL-2 plays in wound healing needs further exploration but that IL-2 signaling is a promising therapeutic target in improving wound healing rate and quality.

SLE is a complicated autoimmune disease in which immune dysregulation contributes to numerous pathologies, including poor cutaneous wound healing and excess scarring. The association of the immune system and the wound healing process implies that immunologic mediators may contribute to the impaired wound closure seen in SLE. Thus, it seems logical to explore the contributions of immunologic mediators to the wound healing process in this disease state. The known deficits in serum IL-2 in SLE and the contribution of IL-2-responsive immune cell types to wound healing led us to consider the contribution of IL-2 to the wound healing defect. These relationships could form the basis for future clinical recommendations and thus should be explored.

5.4 Mechanisms by Which Interleukin-2 May Impact Healing in Systemic Lupus Erythematosus

This study sought to understand the reason that quercetin treatment delayed healing in LPR mice and found that IL-2 may play a role in this process. IL-2 a ppeared enriched at wound sites in *lpr* mice regardless of quercetin treatment. However, IL-2Rα was enriched only at the wound site in the control *lpr* mouse but not in the quercetin-treated *lpr* mouse. These results also demonstrated that treatment of fibroblasts with both IL-2 and quercetin sensitizes these cells to IL-2, as demonstrated by increases in surface IL-2Rα and IL-2Rγ. Furthermore, quercetin-treated *lpr* mice have altered immune cell complements in their draining LNs, with increases in THCs and decreases in dendritic cells compared to control-treated *lpr* mice. These cells, which express high levels of IL-2Rα, may play a role in the mechanism by which IL-2 signaling is altered in the *lpr* mice and may contribute to the impaired healing demonstrated when these mice are treated with quercetin. Finally, human data analyzed for mediators of IL-2 signaling

demonstrated an altered pattern in the expression of IL-2 signaling pathway participants in the lesioned skin of SLE patients compared to healthy controls. These findings suggest that IL-2 and IL-2Rα at the wound sites of SLE patients may contribute to wound healing in this disease state and that blocking these factors may impair wound healing.

5.5 Strengths, Weaknesses, and Alternative Interpretations

Strengths of this study include the use of *in vitro* and *in vivo* models to explore wound healing and the wound healing deficits experienced by SLE patients. Furthermore, this study tested a therapeutic strategy that could potentially be translated to a clinical setting. This study also tested, albeit unsuccessfully, the use of an anti-fibrotic agent in a disease in which cutaneous fibrosis following wounding presents a problem, SLE. Additionally, the use of human samples to corroborate the finding that IL-2 signaling is altered in the context of CLE strengthens the quality of the results. Finally, the extensive examination in the LN and spleen of the immune cells that likely contribute to the findings helps identify potential key players in the SLE-mediated skin symptoms and wound healing deficits.

Weaknesses of this study include the fact that much of the data is correlative. In the study of quercetin's impact on wound healing, the mechanism is not fully explored. It is unclear whether the integrins are activated in this model. Additionally, adhesion studies were not performed to demonstrate the impact of the integrin expression altered by quercetin treatment on the ability of cells to adhere. Only migration of cells into simulated wounds was examined. Furthermore, it is unclear whether downstream

signaling via the integrin pathways is impacting the integrin-mediated behavior. Thus, the mechanism by which quercetin, and likely other anti-fibrotic treatments, impacts integrins and integrin signaling warrants further exploration.

The data regarding IL-2 signaling in the SLE-model mice does not definitively identify the cells responsible for the IL-2 alterations in the *lpr* mice. Given the ubiquity of signaling via IL-2 among immune cells and skin cells, it is likely that a combination of players contributed to causing the excess of IL-2 signaling seen in the skin of SLE-model mice and likely SLE patients. Additionally, the IL-2Rα identified at the wound sites is not definitively identified. It is unclear whether this represents IL-2Rα on the surface of cells or whether it is sIL-2Rα in the ECM. Furthermore, quercetin's impact on the immune cells and IL-2Rα production may have contributed to the wound healing deficit uncovered in these mice. However, it is unclear which cells most strongly contribute to this process. Again, it is likely that multiple cell types played a role in contributing to the findings. Thus, further exploration, especially in the context of *lpr* mice that are also missing certain immune cell subsets or components of the IL-2 signaling pathway, is warranted. The wound healing deficit could also be further explored by adding IL-2Rα, either locally or systemically, or by adoptive transfer of immune cells from WT mice. These experiments might help tease out the key players in this process.

Finally, IL-2Rα can both contribute to IL-2 signaling and also block it. More understanding of downstream mediators is needed to understand whether the IL-2 in the *lpr* mice is signaling or not. If it is not, then it may be serving as a chemotactic factor or

have some other function. However, given that the quercetin-treated *lpr* mice had a decrease in IL-2Rα and an associated impairment in wound healing, it seems most logical that IL-2-IL-2R signaling is acting as an alternative growth factor pathway and promoting wound healing in the *lpr* mice. In the quercetin-treated *lpr* mice, the decrease in IL-2Rα leads to impaired IL-2 signaling, which means that IL-2 cannot compensate for the growth factors already deficient in these mice. Thus, wound healing fails to occur. Even though our findings favor this mechanism, IL-2's role at the wound site still warrants further exploration in the *lpr* mouse model and in human SLE.

An additional alternative interpretation concerns the mechanism by which quercetin improves fibrosis. We attributed the altered ECM deposition following quercetin treatment to integrin expression. We believe, based upon our findings, that improved efficiency in migration, mediated by αV integrin, causes cells to require less ECM to migrate. However, it is possible that a quercetin-mediated decrease in ECM production impacted both integrin expression and the quality of the healed wounds independently. This is unlikely, however, given that the ECM and integrins interact substantially and given that when ECM was already present at the wound site, wound healing was impaired and quercetin's effect on αV integrin was lost. Even so, more research is needed to determine the mediators by which quercetin permits wound healing in the context of less ECM.

5.6 Future Directions for the Study of Fibrosis in Wound Healing

Integrin Signaling in Wound Healing

The impact of quercetin and other anti-fibrotic treatments on the complement of integrins on the surface of fibroblast cells warrants further investigation. Fibroblasts express a variety of integrins on their surface. Furthermore, the α and β subunits of the integrins form many different combinations, each with different ECM ligands. These various integrins and their ligands warrant further study in this model to better understand how anti-fibrotic treatment alters integrin expression. Additionally, the use of techniques to demonstrate whether the integrins expressed on the cell surface are activated is also warranted; while expressing more on the surface prepares the cells for an increase in outside-in signaling in response to ECM components, inside-out signaling may also play a role in the mechanism by which cells are able to close a wound in the context of diminished fibrosis. Additionally, to that end, determining the activity of both upstream mediators of integrin activation and downstream mediators of integrin signaling in the context of anti-fibrotic treatment is warranted. Understanding the regulation of these factors may improve our understanding of wound healing in general and allow for the development of more efficient targeted therapies.

Additionally, *in vivo* studies should be used to better understand the mechanisms by which quercetin impacts fibrosis. Firstly, the decrease in fibrosis should be correlated with specific ECM components, such as collagen and fibronectin, which serve as ligands for many αV- and β1-containing integrins. Additionally, integrin expression in the cells at the wound site and their upstream and downstream mediators should be explored in

the mouse wounds to understand how quercetin interacts with these factors. This approach would help demonstrate whether quercetin's action *in vivo* occurs via outside-in or inside-out signaling in the integrins. Furthermore, mice that either overexpress or have reduced expression of various integrin subunits could help further tease out which integrins are most important in quercetin's ability to reduce fibrosis. Thus, more work is needed to understand how quercetin impacts integrins and identify the mediators of this impact. Better understanding this process might aid in the design of targeted therapies for reducing scar formation and might help us better understand the complex process of wound healing.

Finally, the use of quercetin and other anti-fibrotic agents should be applied to a disease model. While it is obvious that quercetin was not beneficial in the *lpr* model of SLE, there are numerous other diseases in which fibrosis during wound healing is a problem that warrants further exploration. For example, scleroderma and systemic sclerosis, two related diseases that involve substantial scarring, may benefit from modulation of integrin signaling. In fact, modulation of integrins with a β1 integrin-activating antibody has been shown to be beneficial in a systemic sclerosis model (158). While these findings differ from our findings, in which quercetin-mediated diminution of β1 integrin was associated with decreased fibrosis, the complexity of integrin signaling means that both may be beneficial in certain situations (28, 158). Additionally, scarring of organs other than the skin might also be an attractive therapeutic target (36, 159). For example, integrins have been shown to play a role in cardiac fibrosis and, in cardiac muscle, β1 integrin has been shown to be the dominant β subunit (159). This

integrin subunit is also highly present on cardiac fibroblasts. Thus, modulating the integrins on these cells may have an impact on the cardiac fibrosis that occurs following MI, exposure to chemotherapeutic agents or in various other disease states (159, 160). Therefore, more exploration of quercetin might allow for its use in certain disease states to diminish fibrosis and improve quality of life.

Interleukin-2 Signaling in Systemic Lupus Erythematosus

One important experiment that was not done in this study was to administer recombinant IL-2Rα to the *lpr* mice treated with quercetin to determine whether the reduced IL-2Rα found in the wounds of these mice contributes to the wound pathology. If administration of IL-2Rα, either locally or systemically, improves the wound healing rate, then this could represent a treatment strategy for improving wound healing. Additionally, whether the IL-2Rα in the wounds is bound to cell surfaces or part of the ECM needs to be determined. Furthermore, assuming IL-2Rα does improve wound healing, it is unclear how it works. It could assist in IL-2 signaling, promoting IL-2 production and a feedforward signaling mechanism that mimics growth factors. If this were the case, the cells involved, whether immune or skin cells, would need to be identified. Alternatively, it could be that IL-2 and/or IL-2Rα serve as components of the ECM and are capable of acting as motility factor(s) through an undiscovered mechanism. These possible roles in the skin and wound site for IL-2Rα warrant further exploration in the context of SLE to improve our understanding of the skin pathologies seen in SLE and provide insight into potential therapeutic strategies.

SLE is a multifactorial disease and, in many patients, the genetic basis for their disease is unclear. The mutation seen in *lpr* mice is one that affects about 1% of humans with SLE (161). However, there are many other known mutations and likely many unknown mutations that lead to SLE. Thus, while we found that IL-2 signaling is involved in SLE in the *lpr* mice and in some patients with discoid lesions, this may not be true of all patients with SLE. More work needs to be done, especially in patients with other mutations or in whom no known mutation has been found, to determine whether the findings herein apply to all SLE patients or just subsets. Other animal models of SLE should also be used to corroborate these findings. Other animal models include the New Zealand mouse models, which mirror several SLE phenotypes relevant to IL-2 signaling, including a paucity of T-cells (162). Additionally, the *lpr* mutation on the Murphy Roths Large (MRL) mouse background, leads to the development of a syndrome similar to SLE. The MRL mice also have an additional deficit in IL-2 signaling compared to the C57Bl/6J background, and may be a useful model for further understanding of the role IL-2 may play in SLE and specifically in SLE-mediated skin pathologies (86, 163). Another model is the induction of SLE by administering pristane to SLE-susceptible mice, which leads to autoantibody production and an overabundance of Type I IFNs (163). These and other models could serve to further test the findings regarding IL-2 in wound healing in SLE to better understand IL-2's contribution to pathology. Thus, there are many possible experiments that could be performed to further explore how IL-2 impacts wound healing in SLE and, given the importance of wound healing and the

potential for wound healing complications in this disease, it is important to continue research in this area.

5.7 Clinical Relevance of the Results

Anti-fibrotic Therapies to Improve Wound Healing

Undesirable scarring following wounding is a common problem, making it attractive to try to manipulate the formation of, and skin cell reaction to, ECM. Treatment strategies that decrease the formation of ECM without substantially impairing cell growth are ideal for improving the appearance of healed skin without delaying closure. This study demonstrated that the use of a flavonoid, quercetin, which is currently available over the counter, could improve wound healing. This work demonstrates that quercetin can be used to decrease the formation of fibrosis at a wound site without delaying the closure *in vivo*, ideal characteristics for a therapeutic intervention. Additionally, quercetin treatment does not alter the *in vitro* growth of fibroblasts in the absence of an artificial ECM. Thus, quercetin could represent a strategy by which wound healing in healthy patients or those with certain scar-forming diseases might be improved.

Other studies have employed quercetin treatment and have demonstrated improvements in wound healing (37). Additionally, there is substantial interest in incorporating quercetin into ECM-like scaffolds as part of a bioengineering strategy to improve wound healing (164). While this is an attractive strategy, our work suggests that quercetin may be more effective without the use of artificial ECM. Furthermore, our results add to the evidence that quercetin is anti-fibrotic and may be an effective therapy

for patients in whom excess scarring is likely to be a problem, such as patients with hypertrophic scarring or keloids, or patients in whom skin injuries cross a joint and may impair function. Thus, more work should be done to improve our understanding of the mechanism by which quercetin improves fibrosis in order to employ this medication, which is currently available in the United States, as a therapeutic.

The Interleukin-2 Pathway is an Attractive Therapeutic Target in Improving Wound Healing in Systemic Lupus Erythematosus

While it is known that serum IL-2 is diminished in SLE patients, the mechanisms behind this finding are not well-understood. Furthermore, as demonstrated in this study, the IL-2 level may not be diminished in other tissues; in fact, tissue levels of IL-2 may be higher than they would be in a patient without SLE. These findings elude to an imbalance, rather than a true deficiency of IL-2 in SLE patients. Furthermore, the findings that IL-2 signaling may be enriched in the skin and that diminishing this IL-2 signaling capacity impairs wound healing implies a role for IL-2 in the wound healing deficits found in SLE patients. It is possible that IL-2 is involved in the pathogenesis of the excess scarring, discoid lesions, and rashes associated with SLE. More work is needed to further examine IL-2's role in the cutaneous symptoms of this disease state; however, if its involvement could be better understood, alterations in IL-2 signaling could be a novel therapeutic strategy for improving the skin manifestations of SLE.

6. CONCLUSIONS

Delayed wound healing and excess scarring represent substantial problems for clinicians and patients. Exploring potential treatment strategies is important in improving outcomes for patients, decreasing the morbidity and mortality associated with these disease states. This work demonstrates that quercetin decreases fibrosis during wound healing and modulates fibroblast integrin expression. However, quercetin treatment in a model of SLE delayed wound healing, and this delay was associated with an impairment in IL-2 signaling, specifically a decrease in expression of IL-2Rα. Thus, IL-2 signaling may play a substantial role in modulating wound healing in SLE.

REFERENCES

1. Diegelmann RF, Evans MC. Wound healing: An overview of acute, fibrotic and delayed healing. Frontiers in Bioscience. 2004;9:283-9.

2. Broughton G, 2nd, Janis JE, Attinger CE. The basic science of wound healing. Plast Reconstr Surg. 2006;117(7 Suppl):12S-34S.

3. Lopez N, Cervero S, Jimenez MJ, Sanchez JF. Cellular characterization of wound exudate as a predictor of wound healing phases. Wounds. 2014;26(4):101-7.

4. Leoni G, Neumann PA, Sumagin R, Denning TL, Nusrat A. Wound repair: role of immune-epithelial interactions. Mucosal Immunol. 2015;8(5):959-68.

5. Doersch KM, DelloStritto DJ, Newell-Rogers MK. The contribution of interleukin-2 to effective wound healing. Exp Biol Med (Maywood). 2016;242(4):384-396.

6. Fu RH, Weinstein AL, Chang MM, Argenziano M, Ascherman JA, Rohde CH. Risk factors of infected sternal wounds versus sterile wound dehiscence. J Surg Res. 2016;200(1):400-7.

7. Khalil H, Cullen M, Chambers H, Carroll M, Walker J. Elements affecting wound healing time: An evidence based analysis. Wound Repair Regen. 2015;23(4):550-6.

8. Hanssen AD, Cabanela ME, Michet CJ. Hip arthroplasty in patients with systemic lupus erythematosus. Journal of Bone and Joint Surgery. 1987;69-A(6):807-14.

9. Owens PL, Barrett ML, Raetzman S, Maggard-Gibbons M, Steiner CA. Surgical site infections following ambulatory surgery procedures. JAMA. 2014;311(7):709-16.

10. Leaper D, Assadian O, Edmiston CE. Approach to chronic wound infections. Br J Dermatol. 2015;173(2):351-8.

11. Sen CK, Gordillo GM, Roy S, Kirsner R, Lambert L, Hunt TK, et al. Human skin wounds: a major and snowballing threat to public health and the economy. Wound Repair Regen. 2009;17(6):763-71.

12. Reinholz M, Poetschke J, Schwaiger H, Epple A, Ruzicka T, Gauglitz GG. The dermatology life quality index as a means to assess life quality in patients with different scar types. J Eur Acad Dermatol Venereol. 2015;29(11):2112-9.

13. Wolfram D, Tzankov A, Pulzl P, Piza-Katzer H. Hypertrophic scars and keloids--a review of their pathophysiology, risk factors, and therapeutic management. Dermatol Surg. 2009;35(2):171-81.

14. Gauglitz GG, Korting HC, Pavicic T, Ruzicka T, Jeschke MG. Hypertrophic scarring and keloids: pathomechanisms and current and emerging treatment strategies. Mol Med. 2011;17(1-2):113-25.

15. Eming SA, Martin P, Tomic-Canic M. Wound repair and regeneration: Mechanisms, signaling, and translation. Science Translational Medicine. 2015;6(256):1-16.

16. Tracy LE, Minasian RA, Caterson EJ. Extracellular Matrix and Dermal Fibroblast Function in the Healing Wound. Adv Wound Care (New Rochelle). 2016;5(3):119-36.

17. Toulon A, Breton L, Taylor KR, Tenenhaus M, Bhavsar D, Lanigan C, et al. A role for human skin-resident T cells in wound healing. J Exp Med. 2009;206(4):743-50.

18. Nishio N, Ito S, Suzuki H, Isobe K. Antibodies to wounded tissue enhance cutaneous wound healing. Immunology. 2009;128(3):369-80.

19. Komori HK, Witherden DA, Kelly R, Sendaydiego K, Jameson JM, Teyton L, et al. Cutting edge: dendritic epidermal gammadelta T cell ligands are rapidly and locally expressed by keratinocytes following cutaneous wounding. J Immunol. 2012;188(7):2972-6.

20. Lehmann P, Homey B. Clinic and pathophysiology of photosensitivity in lupus erythematosus. Autoimmunity Reviews. 2009;8:456-61.

21. Kuhn A, Sontheimer RD. Cutaneous lupus erythematosus: molecular and cellular basis of clinical findings. Curr Dir Autoimmun. 2008;10:119-40.

22. Solomou EE, Juang YT, Gourley MF, Kammer GM, Tsokos GC. Molecular Basis of Deficient IL-2 Production in T Cells from Patients with Systemic Lupus Erythematosus. The Journal of Immunology. 2001;166(6):4216-22.

23. Robinson ES, Werth VP. The role of cytokines in the pathogenesis of cutaneous lupus erythematosus. Cytokine. 2015;73:326-34.

24. Mok CC, Lau CS. Pathogenesis of systemic lupus erythematosus. J Clin Pathol. 2003;56:481-90.

25. Bolduc C, Sperling LC, Shapiro J. Primary cicatricial alopecia: Lymphocytic primary cicatricial alopecias, including chronic cutaneous lupus erythematosus, lichen planopilaris, frontal fibrosing alopecia, and Graham-Little syndrome. J Am Acad Dermatol. 2016;75(6):1081-99.

26. Ogunsanya ME, Kalb SJ, Kabaria A, Chen S. A systematic review of patient-reported outcomes in patients with cutaneous lupus erythematosus. Br J Dermatol. 2017;176(1):52-61.

27. Garza-Mayers AC, McClurkin M, Smith GP. Review of treatment for discoid lupus erythematosus. Dermatologic Therapy. 2016;29:274-83.

28. Koivisto L, Heino J, Hakkinen L, Larjava H. Integrins in Wound Healing. Adv Wound Care (New Rochelle). 2014;3(12):762-83.

29. Alberts B, Johnson A, Lewis J, Raff M, Roberts K, Walter P. Molecular Biology of the Cell. Anderson M, Granum S, editors. New York, NY: Garland Science; 2008.

30. Giancotti FG. A strucutural view of integrin activation and signaling. Dev Cell. 2003;4(2):149-51.

31. Giancotti FG. Complexity and specificity of integrin signaling. Nat Cell Biol. 2000;2:E13-E4.

32. Giancotti FG, Ruoslahti E. Integrin signaling. Science. 1999;285:1028-32.

33. Doersch KM, Newell-Rogers MK. The impact of quercetin on wound healing relates to changes in αV and β1 integrin expression. Exp Biol Med. 2017; 242(14): 1424-1431.

34. Almeida ME, Monteiro KS, Kato EE, Sampaio SC, Braga TT, Camara NO, et al. Hyperglycemia reduces integrin subunits alpha v and alpha 5 on the surface of dermal fibroblasts contributing to deficient migration. Mol Cell Biochem. 2016;421(1-2):19-28.

35. Duperret EK, Natale CA, Monteleon C, Dahal A, Ridky TW. The integrin alphav-TGF-beta signaling axis is necessary for epidermal proliferation during cutaneous wound healing. Cell Cycle. 2016;15(15):2077-86.

36. Zhao XK, Cheng Y, Liang Cheng M, Yu L, Mu M, Li H, et al. Focal Adhesion Kinase Regulates Fibroblast Migration via Integrin beta-1 and Plays a Central Role in Fibrosis. Sci Rep. 2016;6:19276.

37. Gopalakrishnana A, Ram M, Kumawat S, Tandan SK, Kumar D. Quercetin accelerated cutaneous wound healing in rats by increasing levels of VEGF and TGF-b1. Indian Journal of Experimental Biology. 2016;54:187-95.

38. Dinda M, Mazumdar S, Das S, Ganguly D, Dasgupta UB, Dutta A, et al. The Water Fraction of Calendula officinalis Hydroethanol Extract Stimulates In Vitro and In Vivo Proliferation of Dermal Fibroblasts in Wound Healing. Phytother Res. 2016;30(10):1696-707.

39. Horton JA, Li F, Chung EJ, Hudak K, White A, Krausz K, et al. Queretin inhibits radiation-induced skin fibrosis. Radiation Resistance. 2013;180(2):205-15.

40. El Goweini MF, Nour El Din NM. Effect of quercetin on excessive dermal scarring. EDOJ. 2005;1(1:5):1-10.

41. Liao W, Lin JX, Wang L, Li P, Leonard WJ. Modulation of cytokine receptors by IL-2 broadly regulates differentiation into helper T cell lineages. Nat Immunol. 2011;12(6):551-9.

42. Lin JX, Leonard WJ. The role of Stat5a and Stat5b in signaling by IL-2 family cytokines. Oncogene. 2000;19:2566-76.

43. Lin JX, Leonard WJ. Signaling from the IL-2 receptor to the nucleus. Cytokine and Growth Factor Reviews. 1997;8(4):313-32.

44. Lan RY, Selmi C, Gershwin ME. The regulatory, inflammatory, and T cell programming roles of interleukin-2 (IL-2). J Autoimmun. 2008;31(1):7-12.

45. Granucci F, Feau S, Angeli V, Trottein F, Ricciardi-Castagnoli P. Early IL-2 Production by Mouse Dendritic Cells Is the Result of Microbial-Induced Priming. The Journal of Immunology. 2003;170(10):5075-81.

46. Zhu J, Paul WE. CD4 T cells: Fates, functions, and faults. Blood. 2008;112(5):1557-69.

47. Liao W, Lin JX, Leonard WJ. Interleukin-2 at the crossroads of effector responses, tolerance, and immunotherapy. Immunity. 2013;38(1):13-25.

48. Nelson BH, Willerford DM. Biology of the interleukin-2 receptor. Advances in Immunology. 1998;70:1-82.

49. Khan SH, Martin MD, Starbeck-Miller GR, Xue HH, Harty JT, Badovinac VP. The Timing of Stimulation and IL-2 Signaling Regulate Secondary CD8 T Cell Responses. PLoS Pathog. 2015;11(10):e1005199.

50. Wang LH, Kirken RA, Erwin RA, Yu C, Farrar WL. JAK3, STAT, and MAPK signaling pathways as novel molecular targets for the tyrphostin AG-490 regulation of IL-2-mediated T cell response. J Immunol. 1999;162:3897-904.

51. Orton RJ, Sturm OE, Vyshemirsky V, Calder M, Gilbert DR, Kolch W. Computational modelling of the receptor-tyrosine-kinase-activated MAPK pathway. Biochem J. 2005;392(Pt 2):249-61.

52. Downward J. Targeting RAS and PI3K in lung cancer. Nature Medicine. 2008;14(12):1315-6.

53. Long SA, Cerosaletti K, Wan JY, Ho JC, Tatum M, Wei S, et al. An autoimmune-associated variant in PTPN2 reveals an impairment of IL-2R signaling in CD4(+) T cells. Genes Immun. 2011;12(2):116-25.

54. Rubin LA, Kurman CC, Fritz ME, Biddison WE, Boutin B, Yarchoan R, et al. Souble interleukin-2 receptors are released from activated human lymphoid cells in vitro. J Immunol. 1985;135(3):3172-7.

55. Kondo N, Kondo S, Shimizu A, Honjo T, Hamuro J. A soluble 'Anchorminus' interleukin 2 receptor suppresses in vitro interleukin 2-mediated immune responses. Immunology Letters. 1988;19:299-308.

56. Jacques Y, Le Mauff B, Boeffard F, Godard A, Soulillou JP. A soluble interleukin 2 receptor produced by a normal alloreactive human T cell clone binds interleukin 2 with low affinity. J Immunol. 1987;139:2308-16.

57. Symons JA, Wood NC, Di Giovine FS, Duff GW. Soluble IL-2 receptor in rheumatoid arthritis. Correlation with disease activity, IL-1 and IL-2 inhibition. J Immunol. 1988;141:2612-8.

58. Furtado GC, de Lafaille MAC, Kutchukhidze N, Lafaille JJ. Interleukin 2 Signaling Is Required for CD4+Regulatory T Cell Function. The Journal of Experimental Medicine. 2002;196(6):851-7.

59. Ray JP, Staron MM, Shyer JA, Ho PC, Marshall HD, Gray SM, et al. The Interleukin-2-mTORc1 Kinase Axis Defines the Signaling, Differentiation, and Metabolism of T Helper 1 and Follicular B Helper T Cells. Immunity. 2015;43(4):690-702.

60. Dooms H, Wolslegel K, Lin P, Abbas AK. Interleukin-2 enhances CD4+ T cell memory by promoting the generation of IL-7R alpha-expressing cells. J Exp Med. 2007;204(3):547-57.

61. Leclercq G, De Smedt M, Tison B, Plum J. Preferential proliferation of T cell receptor V gamma 3-positive cells in IL-2 stimulated fetal thymocytes. J Immunol. 1990;145:3992-7.

62. Kjeldsen-Kragh J, Quayle AJ, Skalhegg BS, Sioud M, Forre O. Selective activation of resting human γδ T λψμπηοχψτεσ βψ ιντερλευκιν–2. Eur J Immunol. 1993;23:2092-9.

63. Orsini DLM, Kooy YMC, Van Der Tol MA, Struyk L, Van Den Elsen P. T-cell receptor usage of interleukin-2-responsive peripheral gd T cells. Immunology. 1995;86(385-391).

64. Litwin SD. Product: Proleukin (aldesleukin), Proleukin, Interleukin-2 (IL-2). Food and Drug Administration; 1998.

65. McDermott DF, Regan MM, Atkins MB. Interleukin-2 therapy of metastatic renal cell carcinoma: update of phase III trials. Clin Genitourin Cancer. 2006;5(2):114-9.

66. Atkins MB, Lotze MT, Dutcher JP, Fisher RI, Weiss G, Margolin K, et al. High-dose recombinant interleukin 2 therapy for patients with metastatic melanoma: Analysis of 270 patients treated between 1985 and 1993. Journal of Clinical Oncology. 1999;17(7):2105-16.

67. Siegel RL, Puri RK. Interleukin-2 toxicity. Journal of Clinical Oncology. 1991;9(4):694-704.

68. Thompson JA, Lee DJ, Cox WW, Lindgren CG, Collins C, Neraas KA, et al. Recombinant interleukin 2 toxicity, pharmacokinetics, and immunomodulatory effects in a phase I trial. Cancer Research. 1987;47:4202-7.

69. Edwards MJ, Heniford BT, Klar EA, Doak KW, Miller FN. Pentoxifylline inhibits interleukin-2-induced toxicity in C57BL/6 mice but preserves antitumor efficacy. J Clin Invest. 1992;90:637-41.

70. Gillies SD, Lan Y, Hettmann T, Brunkhorst B, Sun Y, Mueller SO, et al. A low-toxicity IL-2-based immunocytokine retains antitumor activity despite its high degree of IL-2 receptor selectivity. Clin Cancer Res. 2011;17(11):3674-85.

71. Shi VY, Tran K, Patel F, Leventhal J, Konia T, Fung MA, et al. 100% Complete response rate in patients with cutaneous metastatic melanoma treated with intralesional

interleukin (IL)-2, imiquimod, and topical retinoid combination therapy: results of a case series. J Am Acad Dermatol. 2015;73(4):645-54.

72. Barbul A, Knud-Hansen J, Wasserkrug HL, Efron G. Interleukin 2 enhances wound healing in rats. Journal of Surgical Reserach. 1986;40:315-9.

73. Sakakibara S, Inouye K, Shudo K, Kishida Y, Kobayashi Y, Prockop DJ. Synthesis of $(Pro\text{-}Hyp\text{-}Gly)_n$ of defined molecular weights. Evidence for the stabilization of collagen triple helix by hydroxyproline. . Biochemica et Biophysica Acta. 1973;303:198-202.

74. Byrd VM, Ballard DW, Miller GG, Thomas JW. Fibroblast growth factor-1 (FGF-1) enhances IL-2 production and nuclear translocation of NF-kB in FGF receptor-bearing Jurkat T Cells. J Immunol. 1999;162:5853-9.

75. Cozzolino F, Torcia M, Lucibello M, Morbidelli L, Ziche M, Platt J, et al. Interferon-alpha and interleukin 2 synergistically enhance basic fibroblast growth factor synthesis and induce release, promoting endothelial cell growth. J Clin Invest. 1993;91(6):2504-12.

76. Breslin RJ, Barbul A, Kupper TS, Knud-Hansen J, Wasserkrug HL, Efron G. Generation of an anti-interleukin-2 factor in healing wounds. Archives of Surgery. 1988;123:305-8.

77. Boyman O, Sprent J. The role of interleukin-2 during homeostasis and activation of the immune system. Nat Rev Immunol. 2012;12:180-90.

78. Boyman O, Kolios AGA, Raeber ME. Modulation of T cell responses by IL-2 and IL-2 complexes. Clin Exp Rheumatol. 2015;33(Suppl. 92):S54-S7.

79. Kowal-Vern A, Walenga JM, Hoppensteadt D, Sharp-Pucci M, Gamelli RL. Interleukin-2 and interleukin-6 in relation to burn wound size in the acute phase of thermal injury. Journal of the American College of Surgeons. 1994;178(4):357-62.

80. Mikhal'chik EV, Piterskaya JA, Budkevich LY, Pen'kov LY, Facchiano A, De Luca C. Comparative study of cytokine content in the plasma and wound exudate from

children with severe burns. Bulletin of Experimental Biology and Medicine. 2009;148(5):771-5.

81. Ozawa A, Tada H, Tamai R, Uehara A, Watanabe K, Yamaguchi T, et al. Expression of IL-2 receptor B and Γ chains by human gingival fibroblasts and up-regulation of adhesion to neutrophils in response to IL-2. Journal of Leukocyte Biology. 2003;74:352-9.

82. Corrigall VM, Arastu M, Khan S, Shah C, Fife M, Smeets T, et al. Functional IL-2 receptor B (CD122) and Γ (CD132) chains are expressed by fibroblast-like synoviocytes: Activation by IL-2 stimulates monocyte chemoattractant protein-1 production. Journal of Immunology. 2001;166:4141-7.

83. Kang R, Tang D, Lotze MT, Zeh Iii HJ. Autophagy is required for IL-2-mediated fibroblast growth. Exp Cell Res. 2013;319(4):556-65.

84. Serban AI, Stanca L, Geicu OI, Munteanu MC, Dinischiotu A. RAGE and TGF-beta1 Cross-Talk Regulate Extracellular Matrix Turnover and Cytokine Synthesis in AGEs Exposed Fibroblast Cells. PLoS One. 2016;11(3):e0152376.

85. Heydemann A. The super super-healing MRL mouse strain. Front Biol (Beijing). 2012;7(6):522-38.

86. Petitto JM, Huang Z, Lo J, Beck RD, Rinker C, Hartemink DA. Relationship between the development of autoimmunity and sensorimotor gating in MRL-lpr mice with reduced IL-2 production. Neuroscience Letters. 2002;328:304-8.

87. Kench JA, Russell DM, Fadok VA, Young SK, Worthen GS, Jones-Carson J, et al. Aberrant wound healing and TGF-β production in the autoimmune-prone MRL/+ mouse. Clinical Immunology. 1999;92(3):300-10.

88. Carneiro JRM, Fuzii HT, Kayser C, Alberto FL, Soares FA, Sato EI, et al. IL-2, IL-5, TNF-α and IFN-γ mRNA expression in epidermal keratinocytes of systemic lupus erythematosus skin lesions. Clinics. 2011;66(1):77-82.

89. Solomou EE, Juang Y, Gourley MF, Kammer GM, Tsokos GC. Molecular basis of deficient IL-2 production in T cells from patients with systemic lupus erythematosus. J Immunol. 2011;166:4216-22.

90. Linker-Israeli M, Bakke AC, Kitridou RC, Gendler S, Gillis S, Horwitz DA. Defective production of interleukin 1 and interleukin 2 in patients with systemic lupus erythematosus (SLE). J Immunol. 1983;130:2651-5.

91. Alcocer-Varela J, Alarcon-Segovia D. Longitudinal study of the production of and cellular response to interleukin-2 in patients with systemic lupus erythematosus. Rheumatol Int. 1995;15:57-63.

92. Miyagi J, Minato N, Sumiya M, Kasahara T, Kano S. Two typesof antibodies inhibiting interleukin-2 production by normal lymphocytes in patients with systemic lupus erythematosus. Arthritis & Rheumatism. 1989;32(11):1356-64.

93. Wofsy D, Murphy ED, Roths RB, Dauphinee MJ, Kipper SB, Talal N. Deficient Interleukin 2 activity in MRL/Mp and C57BL6/J mice bearing the lpr gene. Journal of Experimental Medicine. 1981;154:1671-80.

94. Sawada S, Hashimoto H, Iijma S, Tokano Y, Matsukawa Y, Takei M, et al. Increased soluble Il-2 receptor in serum of patients with systemic lupus erythematosus. Clinical Rheumatology. 1993;12(2):204-9.

95. Iannuzzi MC, Rybicki BA, Teirstein AS. Sarcoidosis. New England Journal of Medicine. 2007;357:2153-65.

96. Buechner SA, Winkelmann RK, Banks PM. T-cell subsets in cutaneous sarcoidosis. Arch Dermatol. 1983;119:728-32.

97. Badgwell C, Rosen T. Cutaneous sarcoidosis therapy updated. J Am Acad Dermatol. 2007;56(1):69-83.

98. Saltini C, Spurzem JR, Lee JJ, Pinkston P, Crystal RG. Spontaneous release of interleukin 2 by lung T lymphocytes in active pulmonary sarcoidosis is primarily from the Leu3+DR+ T cell subset. J Clin Invest. 1986;77(6):1962-70.

99. Pinkston P, Bitterman PB, Crystal RG. Spontaneous release of interleukin-2 by lung T lymphocytes in active pulmonary sarcoidosis. New England Journal of Medicine. 1983;308(14):793-800.

100. Pinkston P, Saltini C, Muller-Quernheim J, Crystal RG. Corticosteroid therapy suppresses spontaneous interleukin 2 release and spontaneous proliferation of lung T lymphocytes of patients with active pulmonary sarcoidosis. J Immunol. 1987;139:755-60.

101. Su R, Nguyen MT, Agarwal MR, Kirby C, Nguyen CP, Ramstein J, et al. Interferon-inducible chemokines reflect severity and progression in sarcoidosis. Respiratory Research. 2013;14(121).

102. Gundlach E, Hoffmann MM, Prasse A, Heinzelmann S, Ness T. Interleukin-2 Receptor and Angiotensin-Converting Enzyme as Markers for Ocular Sarcoidosis. PLoS One. 2016;11(1):e0147258.

103. Vorselaars AD, van Moorsel CH, Zanen P, Ruven HJ, Claessen AM, van Velzen-Blad H, et al. ACE and sIL-2R correlate with lung function improvement in sarcoidosis during methotrexate therapy. Respir Med. 2015;109(2):279-85.

104. Doersch KM, Moses KA, Zimmer WE. Synergistic immunologic targets for the treatment of prostate cancer. Exp Biol Med (Maywood). 2016;241(17):1900-10.

105. Mercalli A, Calavita I, Dugnani E, Citro A, Cantarelli E, Nano R, et al. Rapamycin unbalances the polarization of human macrophages to M1. Immunology. 2013;140(2):179-90.

106. Wu H, Chang C, Wan L, Wu C, Tsai F, Chen W. IL-2 gene C/T polymorphism is associated with prostate cancer. J Clin Lab Anal. 2006;20:245-9.

107. Dreicer R, Stadler WM, Ahmann FR, Whiteside T, Bizouarne N, Acres B, et al. MVA-MUC1-IL2 vaccine immunotherapy (TG4010) improves PSA doubling time in patients with prostate cancer with biochemical failure. Invest New Drugs. 2008;27:379-86.

108. Quiox E, Lena H, Losonczy G, Forget F, Chouaid C, Papai Z, et al. TG4010 immunotherapy and first-line chemotherapy for advanced non-small-cell lung cancer (TIME): results from the phase 2b part of a randomised, double-blind, placebo-controlled, phase 2b/3 trial. Lancet Oncol. 2016;17:212-23.

109. Dieli F, Vermijlen D, Fulfaro F, Caccamo N, Meraviglia S, Cicero G, et al. Targeting human {gamma}delta} T cells with zoledronate and interleukin-2 for immunotherapy of hormone-refractory prostate cancer. Cancer Res. 2007;67(15):7450-7.

110. Brill TH, Kubler HR, Van Randenborgh H, Fend F, Pohla H, Breul J, et al. Allogenic retrovirally transduced, IL-2- and IFN-g-secreting cancer cell vaccine in patients with hormone refractory prostate cancer - a phase I clinical trial. The Journal of Gene Medicine. 2007;9:547-60.

111. Brill TH, Kubler HR, Pohla H, Buchner A, Fend F, Schuster T, et al. Therapeutic vaccination with an interleukin-2-interferon-g-secreting allogenic tumor vaccine in patients with progressive castration-resistant prostate cancer: A phase I/II trial. Human Gene Therapy. 2009;20:1641-51.

112. Belldegrun A, Tso C, Zisman A, Naitoh J, Said J, Pantuck A, et al. Interleukin 2 gene therapy for prostate cancer: Phase I clinical trial and basic biology. Human Gene Therapy. 2001;12:883-92.

113. Sugimoto Y, Hirota M, Yoshikawa K, Sumitomo M, Nakamura K, Ueda R, et al. The therapeutic potential of a novel PSMA antibody and its IL-2 conjugate in prostate cancer. Anticancer Research. 2014;34:89-98.

114. Wu Z, Xu Y. IL-15Ra-IgG1-Fc enhances IL-2 and IL-15 anti-tumor action through NK and CD8+T cells proliferation and activation. Journal of Molecular Cell Biology. 2010;2:217-22.

115. ClinicalTrials.gov: A service of the U.S. National Institutes of Health https://clinicaltrials.gov/ct2/results?term=interleukin-2+prostate&Search=Search: U.S. National Library of Medicine; 2012 [updated 26 November 2012.

116. Nanus DM, Milowsky MI, Kostakoglu L, Smith-Jones PM, Vallabahajosula S, Goldsmith SJ, et al. Clinical use of monoclonal antibody HuJ591 therapy: targeting prostate specific membrane antigen. J Urol. 2003;170(6 Pt 2):S84-8; discussion S8-9.

117. Madan RA, Gulley JL, Schlom J, Steinberg SM, Liewehr DJ, Dahut WL, et al. Analysis of overall survival in patients with nonmetastatic castration-resistant prostate cancer treated with vaccine, nilutamide, and combination therapy. Clin Cancer Res. 2008;14(14):4526-31.

118. Arlen PM, Gulley JL, Todd N, Lieberman R, Steinberg SM, Morin S, et al. Antiandrogen, vaccine and combination therapy in patients with nonmetastatic hormone refractory prostate cancer. J Urol. 2005;174(2):539-46.

119. Agah R, Malloy B, Kerner M, Mazumder A. Generation and characterization of IL-2-activated bone marrow cells as a potent graft vs tumor effector in transplantation. J Immunol. 1989;143:3093-9.

120. Redmond WL, Gough MJ, Charbonneau B, Ratliff TL, Weinberg AD. Defects in the acquisition of CD8 T cell effector function after priming wtih tumor or soluble antigen can be overcome by the addition of an OX40 agonist. J Immunol. 2007;179:7244-53.

121. Linch SN, Kasiewicz MJ, McNamara MJ, Hilgart-Martiszus IF, Farhad M, Redmond WL. Combination OX40 agonism/CTLA-4 blockade with HER2 vaccination reverses T-cell anergy and promotes survival in tumor-bearing mice. Proc Natl Acad Sci U S A. 2016;113(3):E319-27.

122. Abdul M, Hoosein N. Differences in the expression and effects of interleukin-1 and -2 on androgen-sensitive and -insensitive human prostate cancer cell lines. Cancer Letters. 2000;149:37-42.

123. Forbes JM, Cooper ME. Mechanisms of diabetic complications. Physiol Rev. 2013;93(1):137-88.

124. Ackermann PW, Hart DA. Influence of Comorbidities: Neuropathy, Vasculopathy, and Diabetes on Healing Response Quality. Adv Wound Care (New Rochelle). 2013;2(8):410-21.

125. Lowe CE, Cooper JD, Brusko T, Walker NM, Smyth DJ, Bailey R, et al. Large-scale genetic fine mapping and genotype-phenotype associations implicate polymorphism in the IL2RA region in type 1 diabetes. Nat Genet. 2007;39(9):1074-82.

126. van Belle TL, Coppieters KT, von Herrath MG. Type 1 diabetes: etiology, immunology, and therapeutic strategies. Physiol Rev. 2011;91(1):79-118.

127. Garg G, Tyler JR, Yang JH, Cutler AJ, Downes K, Pekalski M, et al. Type 1 diabetes-associated IL2RA variation lowers IL-2 signaling and contributes to diminished CD4+CD25+ regulatory T cell function. J Immunol. 2012;188(9):4644-53.

128. Long SA, Cerosaletti K, Bollyky PL, Tatum M, Shilling H, Zhang S, et al. Defects in IL-2R signaling contribute to diminished maintenance of FOXP3 expression in CD4+CD25+ regulatory T-cells of type 1 diabetic subjects. Diabetes. 2010;59:407-15.

129. Lagman M, Ly J, Saing T, Kaur Singh M, Vera Tudela E, Morris D, et al. Investigating the causes for decreased levels of glutathione in individuals with type II diabetes. PLoS One. 2015;10(3):e0118436.

130. Cai B, Zhang J, Zhang M, Li L, Feng W, An Z, et al. Micro-inflammation characterized by disturbed Treg/Teff balance with increasing sIL-2R in patients with type 2 diabetes. Exp Clin Endocrinol Diabetes. 2013;121(4):214-9.

131. Pereira FO, Frode TS, Medeiros YS. Evaluation of tumour necrosis factor alpha, interleukin-2 soluble receptor, nitric oxide metabolites, and lipids as inflammatory markers in type 2 diabetes mellitus. Mediators Inflamm. 2006;2006(1):39062.

132. Grinberg-Bleyer Y, Baeyens A, You S, Elhage R, Fourcade G, Gregoire S, et al. IL-2 reverses established type 1 diabetes in NOD mice by a local effect on pancreatic regulatory T cells. J Exp Med. 2010;207(9):1871-8.

133. Flores RR, Zhou L, Robbins PD. Expression of IL-2 in beta cells by AAV8 gene transfer in pre-diabetic NOD mice prevents diabetes through activation of FoxP3-positive regulatory T cells. Gene Ther. 2014;21(8):715-22.

134. Johnson MC, Garland AL, Nicolson SC, Li C, Samulski RJ, Wang B, et al. b-cell-specific IL-2 therapy increases islet Foxp3+Treg and suppresses type 1 diabetes in NOD mice. Diabetes. 2013;62:3775-84.

135. Diaz-de-Durana Y, Lau J, Knee D, Filippi C, Londei M, McNamara P, et al. IL-2 immunotherapy reveals potential for innate beta cell regeneration in the non-obese diabetic mouse model of autoimmune diabetes. PLoS One. 2013;8(10):e78483.

136. Baeyens A, Perol L, Fourcade G, Cagnard N, Carpentier W, Woytschak J, et al. Limitations of IL-2 and rapamycin in immunotherapy of type 1 diabetes. Diabetes. 2013;62(3120-3131).

137. Long SA, Reick M, Sanda S, Bollyky JB, Samuels PL, Goland R, et al. Rapamycin/IL-2 combination therapy in patients with type 1 diabetes augments Tregs yet transiently impairs b-cell function. Diabetes. 2012;61:2340-8.

138. Hartemann A, Bensimon G, Payan CA, Jacqueminet S, Bourron O, Nicolas N, et al. Low-dose interleukin 2 in patients with type 1 diabetes: a phase 1/2 randomised, double-blind, placebo-controlled trial. The Lancet Diabetes & Endocrinology. 2013;1(4):295-305.

139. Frangogiannis NG. Regulation of the inflammatory response in cardiac repair. Circ Res. 2012;110(1):159-73.

140. Cao CM, Xia Q, Tu J, Chen M, Wu S, Wong TM. Cardioprotection of interleukin-2 is mediated via kappa-opioid receptors. J Pharmacol Exp Ther. 2004;309(2):560-7.

141. Bouchentouf M, Williams P, Forner KA, Cuerquis J, Michaud V, Paradis P, et al. Interleukin-2 enhances angiogenesis and preserves cardiac function following myocardial infarction. Cytokine. 2011;56(3):732-8.

142. Koch M, Savvatis K, Scheeler M, Dhayat S, Bonaventura K, Pohl T, et al. Immunosuppression with an interleukin-2 fusion protein leads to improved LV function in experimental ischemic cardiomyopathy. Int Immunopharmacol. 2010;10(2):207-12.

143. Zeng Z, Yu K, Chen L, Li W, Xiao H, Huang Z. Interleukin-2/Anti-Interleukin-2 Immune Complex Attenuates Cardiac Remodeling after Myocardial Infarction through Expansion of Regulatory T Cells. J Immunol Res. 2016;2016:8493767.

144. Vial T, Descotes J. Clinical toxicity of interleukin-2. Drug Saf. 1992;7(6):417-33.

145. Kragel AH, Travis WD, Steis RG, Rosenberg SA, Roberts WC. Myocardial or acute myocardial infarction associated with interleukin-2 therapy for cancer. Cancer. 1990;66:1513-6.

146. Blum A, Sclarovsky S, Rehavia E, Shohat B. Levels of T-lymphocytes subpopulations, interleukin-1b, and soluble interleukin-2 receptor in acute myocardial infarction. Am Heart J. 1994;127:1226-30.

147. Zhang D, Wang S, Guan Y, Wang L, Xie W, Li N, et al. Effect of oral atorvastatin on CD4+CD25+ regulatory T cells, FoxP3 expression, and prognosis in patients with ST-segment elevated myocardial infarction before primary percutaneous coronary intervention. J Cardiovasc Pharmacol. 2011;57:536-41.

148. Wikramanayake TC, Villasante AC, Mauro LM, Perez CI, Schachner LA, Jimenez JJ. Prevention and treatment of alopecia areata with quercetin in the C3H/HeJ mouse model. Cell Stress Chaperones. 2012;17(2):267-74.

149. de la Rosa M, Rutz S, Dorninger H, Scheffold A. Interleukin-2 is essential for CD4+CD25+ regulatory T cell function. Eur J Immunol. 2004;34:2480-8.

150. Schindelin J, Arganda-Carreras I, Frise E, Kaynig V, Longair M, Pietzsch T, et al. Fiji: an open-source platform for biological-image analysis. Nature Methods. 2012;9(7):676-82.

151. Jabbari A, Suarez-Farinas M, Fuentes-Duculan J, Gonzalez J, Cueto I, Franks AG, Jr., et al. Dominant Th1 and minimal Th17 skewing in discoid lupus revealed by transcriptomic comparison with psoriasis. J Invest Dermatol. 2014;134(1):87-95.

152. Margadant C, Sonnenberg A. Integrin-TGF-beta crosstalk in fibrosis, cancer and wound healing. EMBO Rep. 2010;11(2):97-105.

153. Tsokos GC, Lo MS, Costa Reis P, Sullivan KE. New insights into the immunopathogenesis of systemic lupus erythematosus. Nat Rev Rheumatol. 2016;12(12):716-30.

154. Ramalingam R, Larmonier CB, Thurston RD, Midura-Kiela MT, Zheng SG, Ghishan FK, et al. Dendritic cell-specific disruption of TGF-beta receptor II leads to

altered regulatory T cell phenotype and spontaneous multiorgan autoimmunity. J Immunol. 2012;189(8):3878-93.

155. Tran DQ, Glass DD, Uzel G, Darnell DA, Spalding C, Holland SM, et al. Analysis of adhesion molecules, target cells, and role of IL-2 in human FOXP3+ regulatory T cell suppressor function. J Immunol. 2009;182(5):2929-38.

156. Busse D, de la Rosa M, Hobiger K, Thurley K, Flossdorf M, Scheffold A, et al. Competing feedback loops shape IL-2 signaling between helper and regulatory T lymphocytes in cellular microenvironments. Proc Natl Acad Sci U S A. 2010;107(7):3058-63.

157. Li J, Lu E, Yi T, Cyster JG. EBI2 augments Tfh cell fate by promoting interaction with IL-2-quenching dendritic cells. Nature. 2016;533(7601):110-4.

158. Gerber EE, Gallo EM, Fontana SC, Davis EC, Wigley FM, Huso DL, et al. Integrin-modulating therapy prevents fibrosis and autoimmunity in mouse models of scleroderma. Nature. 2013;503(7474):126-30.

159. Chen C, Li R, Ross RS, Manso AM. Integrins and integrin-related proteins in cardiac fibrosis. J Mol Cell Cardiol. 2015.

160. Mancilla TA, Andrews T, Aune G. Doxorubicin-induced chronic upregulation of transforming growth factor-beta. FASEB. 2016;30(1):6.

161. Rieux-Laucat F, Le Diest F, Hivroz C, Roberts IAG, Debatin KM, Fischer A, et al. Mutations in Fas associated with human lymphoproliferative syndrome and autoimmunity. Science. 1995;268:1347-9.

162. Huston DP, Steinberg AD. Animal models of human systemic lupus erythematosus. The Yale Journal of Biology and Medicine. 1979;52:289-305.

163. Perry D, Sang A, Yin Y, Zheng YY, Morel L. Murine models of systemic lupus erythematosus. J Biomed Biotechnol. 2011;2011:271694.

164. Gomathi K, Gopinath D, Ahmed MR, Jayakumar R. Quercetin incorporated collagen matricies for dermal wound healing processes in rat. Biomaterials. 2003;24:2767-72.