IL-33: An Extracellular Arbiter of Macrophage Mediated Myogenesis

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Volumetric muscle loss (VML) is a debilitating injury associated with chronic disability, and existing treatment options remain unsatisfactory. The current therapeutic gold standard is autologous free-flap grafting; however, the efficacy of this treatment option is limited(Corona, Rivera, Owens, Wenke, & Rathbone, 2015). Biologic scaffolds composed of extracellular matrix (ECM) provide an inductive microenvironment that promotes functional, site appropriate tissue deposition(Dziki, Sicari, Wolf, Cramer, & Badylak, 2016; Dziki, Wang, et al., 2017) and modulation of local immune responses, in particular macrophages. ECM bioscaffolds promote a transition from a pro-inflammatory, M1-like to a pro-healing, M2-like macrophage phenotype that is critical for skeletal muscle tissue healing(Brown, Londono, et al., 2012). However, the component(s) of ECM that govern this phenotypic transition remain poorly understood. Recent studies suggest that matrix-bound nanovesicles (MBV), a component of ECM, may be responsible for ECM-mediated macrophage phenotype activation(L. Huleihel, J. G. Bartolacci, et al., 2017). MBV are an abundant source of extracellular IL-33. IL-33, signaling through the ST2 receptor, is an established mediator of macrophage phenotype(Joshi et al., 2010; H. Xu et al., 2019). Evidence supports non-canonical, ST2-independent IL-33 signaling as a mechanism by which MBV promote a pro-healing macrophage phenotype(G. S. Hussey et al., 2019). However, the nature of MBV-associated IL-33 signaling remains unexplored. The objectives of the present thesis were to determine the phenotypic response of macrophages and muscle stem cells to MBV-associated IL-33, to establish a mechanism for MBV uptake and the resulting localization of delivered MBV

cargo, and to interrogate the effects of IL-33 deletion on macrophage phenotype and functional recovery in a mouse model of muscle injury. Results show that ST2-independent IL-33 signaling results in an M2-like macrophage phenotype. MBV-associated IL-33 is internalized by clathrin-mediated endocytosis and is trafficked to the host cell nucleus. IL-33 deletion severely alters the macrophage response to injury and reduces functional recovery in a mouse model of acute skeletal muscle injury. Importantly, delivery of IL-33^{+} MBV reduced M1-like macrophages and increased force generation. Together, these data show that MBV-associated IL-33 is required for effective repair of skeletal muscle following injury and may represent an extracellular tissue homeostasis signaling molecule.

Table of Contents

List of Tables

List of Figures

Preface

The choice to pursue a doctoral degree in the Badylak laboratory was one I did not expect I would have the opportunity to make. The ability to accomplish this is in no way mine alone, but represents the collaborative efforts of many mentors, collaborators, and friends. I will be forever grateful for their contributions.

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1.0 The Skeletal Muscle Response to Injury

1.1 Clinical Significance of Skeletal Muscle Injury

Volumetric skeletal muscle loss (VML) as a result of traumatic injury is a significant clinical problem in both military and civilian populations with an estimated annual economic burden of $4 billion(Grasman, Zayas, Page, & Pins, 2015). Within military populations, VML underlies 65% of disability in discharged veterans(Corona et al., 2015), and are the most common non-fatal injuries in civilian populations(Corona et al., 2015; Grogan, Hsu, & Skeletal Trauma Research, 2011). Skeletal muscle has robust inherent regenerative capacity. However, defects greater than 20% of total muscle mass within a given muscle group overwhelms the ability of resident stem/progenitor cells to fully reconstitute the absent tissue and, instead, fibrotic tissue fills the void(Qazi, Mooney, Pumberger, Geissler, & Duda, 2015). Fibrosis negatively impacts muscle function and is a significant factor in the morbidity and reduced quality of life associated with VML(Cholok et al., 2017; Garg, Corona, & Walters, 2015). Current therapeutic standard of care includes rigorous physical therapy, use of orthotics, and/or autologous flap grafting, and, more recently, acellular bioscaffolds composed of extracellular matrix (ECM). Autologous flap grafting often has limited donor site availability, can incur donor site morbidity, and both grafting and orthotics have limited success in restoring appreciable function(Koul, Patil, & Nahar, 2013).

1.2 The Cellular Response to Acute Skeletal Muscle Injury

1.2.1 Muscle Stem Cell Response to Acute Muscle Injury

Skeletal muscle tissue frequently experiences injury as a result of normal function and physical exercise. Endogenous repair of muscle tissue is therefore required for the maintenance of proper function and for an organism to adapt to a changing environment. As a result, skeletal muscle tissue has a robust regenerative potential that is reliant upon resident muscle stem/progenitor cells, immune cells, and an intact extracellular matrix(Grasman et al., 2015). In particular, satellite cells, a resident population of muscle stem cells characterized by their expression of Pax7(Glass, 2003; Tidball, 2017), play an integral role in recovery from injury.

Satellite cells reside in a quiescent state between the basal lamina and sarcolemma of neighboring myofibers. In response to an acute injury, inflammatory signals promote satellite cell re-entry to the cell cycle and asymmetric proliferation, thereby expanding the pool of stem cells. A portion of activated satellite cells are subsequently directed to commit to the myogenic lineage and differentiate to myotubes and eventually myofibers that repair damaged muscle tissues in response to pro-regenerative signaling cues.

However, in injuries of great volume or severity, the traditional signaling mechanisms needed for the recruitment, activation, and differentiation of satellite cells are overwhelmed. Sustained pro-inflammatory signaling results in a failure of satellite maturation to myoblasts and integration with or replacement of myofibers. Instead, damaged muscle is replaced with fibrotic and adipogenic infiltrates that further compromise muscle structure and function, which can lead to significant reductions in quality of life(Greising, Dearth, & Corona, 2016; Grogan et al., 2011).

1.2.2 The Role of Host Immune Response to Skeletal Muscle Injury

Skeletal muscle is an immune cell dense tissue. Recent estimates show that muscle tissue is contains nearly 1 x 10^9 leukocytes/L of muscle, which, compared to ~1 x 10^{11} leukocytes/L of plasma, is high, is composed primarily of monocytes and macrophages(Tidball, 2017), and are critical for muscle homeostasis. Resident leukocytes in muscle connective tissue in a quiescent state, but can be activated in response to injury or physical exertion. The host immune response to skeletal muscle injury has been extensively characterized and is consistent regardless of injury origin, involving communication between immune cells and stem cells(Tidball & Villalta, 2010). The innate immune response, in particular the macrophage component, plays a central role in governing the behavior of stem/progenitor cells in the days and weeks following acute muscle injury. Macrophages, one of the earliest cell types to arrive in response to muscle injury, are a phenotypically plastic cell type, with the ability to transition between pro-inflammatory, M1-like and pro-healing, M2-like states in response to cues from the local microenvironment(Tidball, 2005, 2017; Tidball, Dorshkind, & Wehling-Henricks, 2014; Tidball & Villalta, 2010). Both macrophage phenotypes have been shown to be essential to the healing response, as a reduction of macrophage infiltration or appropriate phenotypic activation can severely delay and impair recovery from muscle injury(Cheng, Nguyen, Fantuzzi, & Koh, 2008; Londhe & Davie, 2013; Tidball & Wehling-Henricks, 2007).

Macrophage phenotype is spatially and temporally controlled and the earliest population to arrive after injury are M1-like macrophages within 1-3 days(Tidball, 2005; Tidball & Villalta, 2010; Warren et al., 2005), during which time these cells promote activation and expansion of the satellite cell pool. For successful repair, M1-like macrophages must transition to M2-like macrophages between 4- and 14-days post-injury(B. Deng, M. Wehling-Henricks, S. A. Villalta,

Y. Wang, & J. G. Tidball, 2012; J. L. Dziki et al., 2016). This transition is critical as M2-like macrophages help coordinate early stages of myogenic differentiation to myotubes(L. Arnold et al., 2007; St Pierre & Tidball, 1994a). Without an appropriate timed phenotypic transition, muscle healing can be severely delayed or impaired, as has been observed in large muscle injuries(B. Deng et al., 2012). Stated differently, the macrophage response is a central and indispensable component of the host response to muscle injury.

Although innate immune cells represent the majority of muscle leukocytes, multiple studies have shown that lymphoid cells, ulterior innate immune cell types, and mesenchymal cells play critical regulatory roles(W. Kuswanto et al., 2016; K. Sadtler, K. Estrellas, et al., 2016). A population of $CD8^+$ T cells was shown to be among the first responders to skeletal muscle injury, and ablation of this population delayed muscle healing, however more work is needed to determine what role these cells play. A large body of work is also emerging with respect to $FoxP3^+$ T_{REG} cells. T_{REG} cells follow similar kinetics to M2-like macrophages, arriving by ~4-10 days post-injury and secrete potent cytokines, IL-10 and amphiregulin, whose bioactivity on macrophages and activated satellite cells, respectively, promote the macrophage phenotype transition away from a predominantly M1-like macrophage population towards a predominantly M2-like macrophage population, and promote differentiation of activated satellite cells(Tidball, 2005; Tidball et al., 2014; Villalta et al., 2014). Eosinophils have also been reported to be important in the macrophage phenotype transition and in regulating the behavior of fibro-adipogenic precursors (FAPs) through the release of IL-4. Specifically, eosinophil-derived IL-4 sustains macrophage phenotype transition stimuli and increased FAP phagocytic capacity to increase debris clearance. FAPs have also been shown to interact with infiltrating macrophages(Heredia et al., 2013). Subsequently, M2-like macrophages release factors that promote differentiation of FAPs along the fibroblastic

lineage, resulting in the deposition of new muscle matrix and providing guidance to differentiating muscle stem cells(Heredia et al., 2013; Tidball, 2017). Taken together, muscle immunobiology is spatially and temporally controlled with several supporting cell types helping to orchestrate macrophage phenotype and modulate cytokine signaling in the muscle microenvironment.

1.2.3 Macrophage-Mesenchymal Cell Crosstalk

The central role of immune cells in tissue healing is derived from their ability to communicate with mesenchymal cell types, including fibroblasts and stem/progenitor cells, and modify their behavior. These macrophage-mesenchymal cell interactions have been reported in many tissue types, including skeletal muscle, and are thought to be critical for tissue homeostasis(Kumar, Alvarez-Croda, Stoica, Faden, & Loane, 2015; St Pierre & Tidball, 1994a, 1994b; J. G. Tidball & M. Wehling-Henricks, 2015; Tidball & Welc, 2015; Verheijden, Schepper, & Boeckxstaens, 2015; Villalta, Nguyen, Deng, Gotoh, & Tidball, 2009; Wei & Besner, 2015; Wu et al., 2015). For example, the role of macrophages in mediating satellite cell recruitment, proliferation, and differentiation in response to skeletal muscle injury(Tidball, 2005, 2017; Tidball et al., 2014; Tidball & Villalta, 2010; Tidball & Wehling-Henricks, 2007), is well characterized and is thought to be exemplary of repair processes in other organ systems, as well.

In skeletal muscle, macrophages direct the proliferation or differentiation of satellite cells, the native skeletal muscle stem cell type, as a function of macrophage phenotype. M1-like, pro-inflammatory macrophages are required for the expansion of the satellite cell pool in response to muscle injury, ensuring that sufficient cells are available for subsequent differentiation and replacement of lost myotubes. Following macrophage phenotype transition to a pro-healing, M2-

like phenotype, macrophages direct the differentiation of activated satellite cells to myotubes and, eventually, myofibers to replete muscle tissue lost to injury.

Macrophage-mesenchymal cell crosstalk is, however, bidirectional. Recent reports suggest that the secreted products of myoblasts exposed to serial strain promote the expression of M2-associated markers Arg1 and Fizz1 by bone marrow-derived macrophages (BMDM)(J. L. Dziki et al., 2016; Dziki, Wang, et al., 2017). Further, it has been reported that the secreted products of mesenchymal stem cell (MSC) can promote the expression of M2-associated marker Arg1 in treated BMDM(Zhou, Yamamoto, Xiao, & Ochiya, 2019). Separate studies have shown that co-culture of MSCs and BMDM resulted in a reduction in expression of M1-like markers TNF-a, IL-6, and iNOS, while promoting expression of M2-like markers CD206, IL-4, and IL-10(Cho et al., 2014). In vivo, it has been shown that MSCs exposed to LPS preferentially recruit M2-like macrophages, and reprogram M1-like macrophages towards an M2-biased phenotype through the production of prostaglandins. Targeting these interactions, either by focusing on macrophages or their communication counterparts, could be effective therapeutic strategies to improve tissue engineering outcomes.

1.3 Current Treatment Options for Large Muscle Injuries

1.3.1 Muscle Grafting

To date, the standard of care for large muscle injuries is autologous free-flap or rotational grafts, most commonly muscles of the thigh or back(Bertelli & Ghizoni, 2016; Klinkenberg et al., 2013). Muscle transposition has less than satisfactory success rates, in part due to a high rate of

graft infection and necrosis, and a limited availability of donor tissue without incurring donor site morbidity(Corona, Wenke, & Ward, 2016). As a result, such procedures often fail to provide efficient reconstruction or restoration of lost muscle function. Stated differently, the current standard of care does not address the underlying pathophysiology underlying the chronic functional deficits arising from large muscle injuries, and as a result do not address persistent strength and functional deficits which ultimately contribute to morbidity and and reduced patient quality of life(Grogan et al., 2011; C. H. Lin, Lin, Yeh, & Chen, 2007; Moneim & Omer, 1986; Tu et al., 2008).

1.3.2 Physical Rehabilitations and Orthotics

Physical rehabilitation and the use of orthotics can improve patients' ability to perform activities of daily living when muscle function has been severely compromised due to skeletal muscle injury, and can help augment endogenous healing. It has been shown that mechanically loading skeletal muscle can modify the post-injury remodeling process to improve strength maintenance, endurance, fatigue resistance, and regeneration(Shwartz, Blitz, & Zelzer, 2013). Studies have shown that early rehabilitation following skeletal muscle injury improved functional output(Hwang, Ra, Lee, Lee, & Ghil, 2006). Specifically, physical rehabilitation has been shown to modulate immune cell phenotypes(Dziki et al., 2018), activate satellite cells and improve alignment of nascent myotubes following acute injury(Ambrosio et al., 2009). The mechanisms underlying these cellular events are an active area of investigation and remain only partially understood. It has been shown that mechanotransduction can promote the expression of genes encoding extracellular matrix components by fibroblasts and promote satellite cell reentry to the cell cycle(Greising et al., 2018; Nuutila et al., 2017). These effects may, at least in part, be

accounted for by increasing the compliance of the skeletal muscle extracellular matrix, thereby increasing the ability of satellite cells and other stem cells to reconstitute missing tissue(Ambrosio et al., 2014; Stearns-Reider et al., 2017). However, it has been shown that physical rehabilitation alone following volumetric muscle loss injuries was not sufficient to improve muscle function across animal models, but that physical rehabilitation in combination with biomaterials and/or stem cell therapies were synergistic(Greising, Corona, McGann, Frankum, & Warren, 2019). Stated differently, physical rehabilitation is a powerful tool that can augment endogenous repair processes, but cannot recapitulate strength lost due to injury when used alone.

1.3.3 Cell-based Strategies

A Tissue engineering and regenerative medicine strategies to address VML have been primarily cell-centric. Exogenous delivery of stem cells to repopulate missing muscle tissue has shown moderate success in preclinical studies(Deasy, Jankowski, & Huard, 2001; Huard, Cao, & Qu-Petersen, 2003). However, technical, economic, and regulatory hurdles including low engraftment efficiency and low viability after injection have limited the success of clinical translation(Ezquer, Ezquer, Vicencio, & Calligaris, 2017; Schwartz, Brick, Nethercott, & Stover, 2011; Swijnenburg et al., 2008). In addition, cell isolation and maintenance requirements are costly, and allogeneic cell-sourcing necessitates concomitant immunosuppression(Vats, Tolley, Bishop, & Polak, 2005). Nonetheless, results of preclinical work show that some cell-seeded scaffolds can provide more significant gains of function in skeletal muscle regeneration than acellular materials alone(Borschel, Dennis, & Kuzon, 2004; Borschel, Dow, Dennis, & Brown, 2006; Garg, Ward, Rathbone, & Corona, 2014; Kin et al., 2007). The use of different cell types, different animal models, and different analyses have yielded conflicting results, however. The cell

types used in preclinical animal models do not have uniform characteristics and the ability of these cells to promote muscle repair varies widely. It is helpful to understand these differences to better compare results and guide future strategies for skeletal muscle tissue engineering.

Most cell types that have been investigated for skeletal muscle repair are autologous or allogeneic stem cells that have been isolated and expanded in vitro before seeding and reimplantation. Autologous cells are advantageous because there is no immune-mediated rejection, even in the absence of immunosuppression. Allogeneic cells require prolonged immunosuppressive therapy(Swijnenburg et al., 2008) that can adversely affect healing, foster fibrosis(Anderson & Hamm, 2012), and disrupt constructive macrophage phenotype transitions(Salehi & Reed, 2015). Autologous, induced pluripotent stem cells and allogeneic embryonic stem cells can theoretically be passaged indefinitely(Schwartz et al., 2011), affording much larger populations of cells for seeding compared to satellite cells, which are very limited in their passage potential(Danoviz & Yablonka-Reuveni, 2012). Cell survival is a concern as it has been shown that the vast majority of transplanted cells will fail to integrate with host tissue. Low engraftment efficiency is multifactorial(Ezquer et al., 2017), but a lack of a vascular supply and an inflammatory host environment have been implicated as key contributors to this phenomenon(S. Lee, Choi, Cha, & Hwang, 2015). The number of cell layers that can be seeded, for example, is limited by the ability of nutrients and gases to diffuse from a medium to the cells. Specifically, in the absence of a vascular network, cells in excess of 1 mm distance from a nutrient and gas rich medium will have difficulty surviving(Griffith et al., 2005).

Nutrient diffusion presents a particularly challenging obstacle for cell seeded scaffolds that lack a vascular network. Attempts have been made to design scaffolds with provisional vascular conduits(Lesman et al., 2011), but insufficient preclinical animal modeling has been conducted

and is an ongoing topic of research. Further, induced pluripotent stem cells (iPSCs) require complex growth factor cocktails to support appropriate differentiation, often have low viability, and there is concern surrounding the retroviruses used to induce pluripotency(Puri & Nagy, 2012). An appropriate level of safety must be considered, prior to large scale clinical application, which in itself can be particularly challenging since no technology exists that can sterilize a scaffold without killing embedded cells. Stated differently, all steps in the manufacture of a cell seeded scaffold must be conducted under sterile conditions, which adds time, cost, and potential risks to the development of these personalized devices. For these stated reasons, exogenously supplied autologous and allogeneic stem cells have achieved limited success.

1.3.4 Bioscaffolds

VML results in extensive damage to connective tissue and basement membrane architecture. Skeletal muscle tissue is a highly organized tissue and effective tissue repair requires that therapeutic strategies have a three-dimensional structure that resembles the native microenvironment. Ideally, scaffolds for the repair of skeletal muscle injuries should be immunomodulatory, allow for the transduction of forces, stimulate angiogenesis to support the requirements of tissue regeneration, and serve as a provisional matrix or substrate for host tissue integration, endogenous cell migration, proliferation, and differentiation during the remodeling process. Several biomaterial-based strategies have been investigated to reconstitute injured or missing soft tissue and restore lost function. Due to the highly dynamic cellular response to skeletal muscle injury, scaffolds are effective tools which can be designed to help regulate one or more components of the host response.

The formation of a vascular supply to sustain tissue healing is a particularly important need in tissue engineering. To address this need, growth factor-eluting materials have been developed to release VEGF over long periods of time to stimulate endogenous vascularization. Results of these studies have shown that the released growth factors can be temporally controlled to sustain angiogenesis and vessel maturation(Brudno, Ennett-Shepard, Chen, Aizenberg, & Mooney, 2013; Geer, Swartz, & Andreadis, 2005). Drug-eluting microspheres have also been used in conjunction with naturally derived polymers such as alginate, collagen, and fibrin, among others, to achieve functional restoration in animal models of volumetric muscle loss. One study found that the use of IGF-1 and VEGF-eluting PLG microspheres embedded in alginate hydrogels promoted early vasculogenesis and a significant increase in regenerating myotubes, resulting in near-complete functional recovery in a rodent model of ischemic muscle injury(Shvartsman et al., 2014). Other studies have evaluated vascular networks that were manufactured in vitro using three-dimensional fibrin-PGA/PLLA seeded with endothelial cells, fibroblasts, and myoblasts. Results showed that the engineered constructs were capable of supporting blood flow in vivo(Lesman et al., 2011), but functional testing was not performed. Determining the correct combinations, dosages, and dose regimens of growth factors and other bioactive molecules to obtain effective results is an active area of investigation(Lawrence & Diegelmann, 1994).Taken together, polymeric biomaterials are valuable research tools, but have not yet convincingly demonstrated translational potential in preclinical animal testing.

Bioscaffolds composed of acellular mammalian extracellular matrix (ECM) have shown notable success in promoting functional tissue remodeling in multiple anatomic sites(Agarwal et al., 2015; Atala, Bauer, Soker, Yoo, & Retik, 2006; Badylak et al., 2011; Gerdisch, Shea, & Barron, 2014; Kissane & Itani, 2012; Ladowski & Ladowski, 2011; Lecheminant & Field, 2012; B. M.

Sicari, J. P. Rubin, et al., 2014). ECM bioscaffolds retain local tissue ultrastructure and a variety of signaling factors that contribute to stem/progenitor cell recruitment(Agrawal, Tottey, et al., 2011; Reing et al., 2009; Vorotnikova et al., 2010), immunomodulation, angiogenesis(Hammond et al., 2011; Voytik-Harbin, Brightman, Kraine, Waisner, & Badylak, 1997), and more(Allen et al., 2010; Hammond et al., 2011; Reing et al., 2009; Vorotnikova et al., 2010). A recent meta-analysis of preclinical animal models, the use of ECM bioscaffolds with or without a cellular component resulted in the greatest improvement in functional remodeling of skeletal muscle injury(Greising et al., 2019). The beneficial effects on muscle healing are multifactorial, but rely on modulation of the host immune response towards a T_H2/M2-like pro-healing response and direct myogenic effects on muscle stem cells(J. Dziki et al., 2016; J. L. Dziki et al., 2016; L. Huleihel, J. L. Dziki, et al., 2017; K. Sadtler, K. Estrellas, et al., 2016). Importantly, the use of ECM bioscaffolds to treat VML in a human clinical cohort trial resulted in an average of ~250% gain of function and ~35% improvement in functional output(J. Dziki et al., 2016). Taken together, the complex variety of bioactive components make ECM bioscaffolds a viable option for the treatment of volumetric muscle loss.

1.4 Future Perspectives

The development of next-generation tissue engineering solutions for large muscle injuries will need to carefully consider the complex skeletal muscle injury microenvironment in order to be successful. To accomplish this, a multidisciplinary approach will be essential. In particular, combinatorial approaches that incorporate cells, scaffolds, and rehabilitation show functional muscle improvements beyond the use of cells or scaffolds alone(Corona, Ward, Baker, Walters, &

Christ, 2014). To meet these ends, there is a particular need for standardization in the animal models used, the metrics used to assess recovery, and the molecular characterization of transplanted cells. A rigorous, prospective study comparing the effect of different cell types and optimal scaffolds in functional recovery from large muscle injuries should be performed to provide an optimized platform for future work.

Clinical translation of tissue engineering approaches to skeletal muscle injury will require that these strategies be modular. Pre-existing medical conditions are common in the surgical patient population, and these risks increase in more advanced age patients(Hewitt et al., 2016). Although ECM bioscaffold use has shown successful restoration of function in humans, the cohort of patients was relatively small. Understanding the underlying changes to native extracellular matrix or the cellular responses to implanted biomaterials in patients with multiple comorbidities could expand the number of patients who could benefit from the products of tissue engineering and regenerative medicine research(Garg, Corona, & Walters, 2014). For example, the use of biomaterials in animal models of metabolic disease, one of the most common comorbidities, would be an important area for future investigation to achieve this end(Safdar, Saleem, & Tarnopolsky, 2016).

1.5 Conclusions

The host response to skeletal muscle healing is multifaceted and relies on a dynamic interaction between immune and stem cell players. In skeletal muscle, the critical balance between pro-inflammatory and pro-healing signals can quickly become disrupted in response to volumetric muscle loss, resulting in a sustained pro-inflammatory bias with accompanying fibrosis and loss

of function. Existing treatment options for large muscle injuries remain unsatisfactory. Although cell-centric regenerative medicine approaches have achieved some success, the use of an inductive scaffold, such as those composed of extracellular matrix, can help normalize aberrant endogenous repair processes, potentially circumventing the need for exogenously provided cells. Although tissue engineering efforts utilizing ECM bioscaffolds have shown promising early clinical translation, more work is needed to determine the mechanisms by which these materials induce functional, site-appropriate tissue remodeling.

2.0 Mechanisms of ECM Bioscaffold-mediated Endogenous Repair.

2.1 Overview

Bioscaffolds composed of acellular mammalian extracellular matrix (ECM) are complex materials with myriad bioactive components. ECM bioscaffolds have been used for decades to treat soft tissue pathology resulting from trauma or disease in a wide variety of anatomical sites. Host degradation of implanted ECM biomaterials is critically important for endogenous, functional and site appropriate tissue repair. Specifically, the use of ECM bioscaffolds has been associated with angiogenesis, stem/progenitor cell recruitment and differentiation, and immunomodulation. Several components have been attributed to these inductive effects, including the release of retained growth factors, cryptic peptides, and the mechanical properties of the material, however, a definitive mechanism for ECM bioscaffold-mediated immunomodulation has been established. The present manuscript will discuss the known bioactive components of the extracellular matrix as they pertain to tissue healing and remodeling, and discuss future avenues of investigation with respect to immunomodulatory mechanisms.

2.2 Introduction

The current standard of care for surgical scaffolds remains the use of synthetic, non-absorbable materials. However, non-absorbable polymers have been associated almost universally with the formation of a maladaptive host response termed the foreign body reaction (FBR),

comprising foreign body giant cells (FBGCs), chronic pro-inflammatory signaling, and fibrous encapsulation of implanted material(Roth, Brathwaite, Hacker, Fisher, & King, 2015; Schumpelick, Klinge, Rosch, & Junge, 2006). Over the long term, the FBR can result in damage to neighboring healthy tissue(Gandhi et al., 2011) and contracture of the fibrous capsule(Baylon et al., 2017; Nolfi et al., 2016), which can lead to reduced patient quality of life or even mandate surgical reintervention. These clinical observations obviate the need for biomaterials that can modulate immune cell phenotypes and promote tissue healing(Dort, Fabre, Molina, & Dumont, 2019; Mahdavian Delavary, van der Veer, van Egmond, Niessen, & Beelen, 2011; Tidball & Wehling-Henricks, 2007), stimulate angiogenesis to support the requirements of infiltrating cells(Aamodt & Grainger, 2016), and serve as a provisional matrix or substrate for host tissue integration, endogenous cell migration, proliferation, and differentiation(J. L. Dziki et al., 2016; Saldin et al., 2019) during the remodeling process.

ECM bioscaffolds have been investigated as a biomaterial for decades, at least in part due to the preservation of tissue architecture and cell signaling motifs, but numerous reports of tissue remodeling have resulted in a large body of research demonstrating the bioactivity of multiple ECM components. Studies have repeatedly demonstrated that the application of ECM bioscaffolds, when properly prepared, can promote angiogenesis(Billaud, Hill, Richards, Gleason, & Phillippi, 2018; Singelyn et al., 2009), recruitment of stem/progenitor cells(Agrawal, Tottey, et al., 2011; Brown, Chung, et al., 2012; J. L. Dziki et al., 2016), immunomodulation(Brown, Londono, et al., 2012; J. L. Dziki et al., 2016; Dziki, Wang, et al., 2017; L. Huleihel, J. L. Dziki, et al., 2017), and de novo, site appropriate, functional tissue deposition(J. Dziki et al., 2016). These outcomes have, to date, been linked to retained growth factors and cytokines, matricryptic peptides produced during scaffold degradation and remodeling, ligand-integrin interactions, and more recently,

matrix-bound nanovesicles (MBV). The objective of the present manuscript is to describe the components of ECM bioscaffolds as they contribute to endogenous repair of soft tissue defects, hypothesize new avenues of therapeutic intervention and identify underexplored components with a potential role in tissue repair.

2.3 The ECM Topographical Ligand Landscape

ECM bioscaffolds retain many of the structural and soluble components of the parent ECM from which they were derived, including ground matrix, structural proteins, embedded cytokines and growth factors, and basal lamina components(Saldin, Cramer, Velankar, White, & Badylak, 2017). These components, both alone and in combination, have been shown to promote cellular migration, proliferation, differentiation, largely through integrin and receptor-mediated signaling.

One of the earliest known roles for ECM in modifying cell behavior came that the discovery that growth factors are stably stored in the extracellular matrix, often bound to sugar moieties on proteoglycans. Growth factors are potent mitogens independently, however, their actions are often potentiated in the presence of proteoglycans such as heparan sulfate proteoglycan (HSPG) (Chintala, Miller, & McDevitt, 1995; Klagsbrun, 1992). For example, VEGF-mediated angiogenesis requires that the target cell have active integrin binding to ECM components(Jakobsson et al., 2006). Further, FGF-2 has been shown to promote proliferation of fibroblast, chondrocytes, myoblast, and endothelial cells, but only in the presence of heparan sulfate(Chintala et al., 1995; Klagsbrun, 1992; Rapraeger, Krufka, & Olwin, 1991; Yayon, Klagsbrun, Esko, Leder, & Ornitz, 1991). Free TGF-b promotes increased secretion of ECM components; however, it has been shown that when TGF-b is bound to decorin, a proteoglycan,

the production of ECM components elicited by TGF-b is reduced, suggesting a negative feedback loop may exist to limit overproduction of ECM(Yamaguchi, Mann, & Ruoslahti, 1990). Tissue epithelia secrete unique combinations of growth factors and their ECMs are comprised of different components, which may help explain the observation that ECM bioscaffolds derived from different tissues elicit different cellular responses(Dziki, Wang, et al., 2017).

In many cases, ECM components play a critical role in maintaining cell survival and differentiation, even in the absence of growth factors. In fact, cells are highly sensitive to their niche and respond to their microenvironment through integrins. Many contact-dependent epithelia require integrin binding for survival and maintenance following tissue injury(Ilic et al., 1998), and may become disrupted in disease states. In wound healing, the role of ECM-integrin signaling is arguably best characterized in epidermis. For example, keratinocyte b1-integrin binding to fibronectin maintains an adherent, undifferentiated state(Adams & Watt, 1989; Levy, Broad, Diekmann, Evans, & Watt, 2000) that is critical for the integrity of the epidermis. In response to wounding, keratinocytes upregulate integrins with an affinity for proteins deposited in wound provisional matrix (i.e. laminin, tenascin C, and fibrinogen), resulting in spatially controlled migration that facilitates reepithelialization(Mercurio, Rabinovitz, & Shaw, 2001; Nguyen, Ryan, Gil, & Carter, 2000; Tamaoki et al., 2005; Trebaul, Chan, & Midwood, 2007; Whitby, Longaker, Harrison, Adzick, & Ferguson, 1991). The role of integrin-ligand signaling is apparent in certain cancers such as melanoma, in which malignant cells increase production of tenascin C, affording higher motility and invasiveness(Grahovac, Becker, & Wells, 2013). In skeletal muscle tissue, basement membrane components have been implicated in promoting survival, migration and differentiation of myoblasts(Foster, Thompson, & Kaufman, 1987; Sanes, 2003). In fact, the role of the extracellular matrix in maintaining cellular survival and terminal differentiation states is

readily apparent in Duchenne's Muscular dystrophy and other muscular dystrophies. Loss of proteins (e.g. dystrophin) required for connection of the myofiber cytoskeleton to the extracellular matrix results in continuous muscle breakdown and replacement with fibrotic tissue(Nowak & Davies, 2004). In skin and vasculature, higher Advanced Glycation End (AGE) product burden on collagen fibers has been shown to promote increased oxidative stress(Vlassara, 2001) and increased inflammatory signaling through the acquired ability to activate the RAGE receptor(Pullerits, Brisslert, Jonsson, & Tarkowski, 2006). Taken together, ECM bioscaffolds represent a potent combination of trophic factors and structural cues that help guide and sustain tissue healing. The composition and integrity of applied ECM scaffolds must be discerningly chosen to resemble native architecture as much as possible. Tissue engineering strategies that fail to incorporate a biomimetic microenvironment will likely result in suboptimal, site-appropriate host cell integration.

2.4 Matricryptic Peptides

Matricryptic peptides, also known as matrikines, are a subcomponent of the ECM. Matricryptic peptides are typically inaccessible except in the event of a conformational change or proteolytic cleavage of the parent molecule, such as in a matrix turnover event(Davis, Bayless, Davis, & Meininger, 2000). Many ECM proteins are comprised of subdomains with similar motifs to known growth factors that, when released during ECM degradation, can bind cellular receptors and initiate signaling cascades. Laminin and tenascin C, for example, contain epidermal growth factor (EGF)-like repeats that are capable of binding and activating EGF receptors, albeit with lower affinity(Iyer et al., 2007; Swindle et al., 2001). During ECM bioscaffold degradation

following implantation, proteases released by infiltrating immune cells contribute to the production and release of these cryptic peptides and have been shown to mediate certain aspects of ECM-mediated endogenous repair(Giannelli, Falk-Marzillier, Schiraldi, Stetler-Stevenson, & Quaranta, 1997).

Matricryptic peptides are well characterized players in wound healing. For example, following tissue injury, the temporal release of matricryptic peptides by proteolytic enzymes is known to control the formation of new vasculature into the wound site(Bornstein, 2009; Valentin, Badylak, McCabe, & Badylak, 2006). Although xenogeneic, the principle components of ECM bioscaffolds (i.e. collagen I, collagen III, laminin, etc.) are highly phylogenetically conserved(Ozbek, Balasubramanian, Chiquet-Ehrismann, Tucker, & Adams, 2010), and, as a result, have not been shown to elicit rejection responses. In particular, the application of ECM bioscaffolds and their degradation products has been repeatedly shown to result in the migration of site-specific cells(Agrawal, Brown, Beattie, Gilbert, & Badylak, 2009; F. Li et al., 2004; Melman et al., 2011). A specific degradation product of collagen IIIa was shown to promote bone remodeling(Agrawal, Kelly, et al., 2011) and the recruitment of multipotent stem cells(Agrawal, Tottey, et al., 2011) in a mouse model of digit amputation. Further, although intact fibronectin is not chemotactic for monocytes, a 120 kDa produced through enzymatic cleavage has been shown to recruit monocytes and fibroblasts, but not neutrophils(Clark, Wikner, Doherty, & Norris, 1988; Fukai, Suzuki, Suzuki, Tsugita, & Katayama, 1991). Fibronectin is a major component of both the provisional wound matrix and the adsorbed protein layer following biomaterial implantation. In this way, the sequential recruitment of neutrophils, and subsequently monocytes, is molecularly encoded and promotes an appropriately timed cellular response to injury. Further, angiogenesis is another heavily regulated process that is critical for constructive tissue remodeling to support the

metabolic requirements of infiltrating cells. It is logical, then, that a significant portion of matricryptic peptides act on vascular endothelial cells(Davis et al., 2000). In fact, several peptides derived from the degradation of elastin and hyaluronic acid, both of which are found in ECM bioscaffolds, are known to promote vessel branching and chemotaxis of endothelial cells(D. Liu et al., 1996; Senior, Griffin, & Mecham, 1980).

Stated differently, ECM bioscaffold degradation is associated with the production of several biologically active peptides that can affect cellular function in a variety of ways. However, these effects are inherently transient and are not spatially orchestrated and may, therefore, have only a modest effect on functional remodeling response to ECM bioscaffolds.

2.5 Mechanical Microenvironment

Historically, tissue engineering strategies have focused on optimization of scaffold chemistry and the selection of biological additives including cells, growth factors, or guidance cues to achieve developmental control. However, the role of tissue or substrate mechanical properties in modulating cell behavior is an emerging field of investigation that has shown that local mechanical cues play a significant role in tissue development and homeostasis(Farge, 2011). Failure to account for mechanical forces may also help explain a failure of some in vitro assays to translate to preclinical animal models(Ingber, 2006).

An extraordinary number of cellular molecules are devoted to the transduction or generation of mechanical forces. Stretch sensitive ion channels(Sukharev & Corey, 2004), integrins(Evans, Leung, Heinrich, & Zhu, 2004), and cytoskeletal molecular motors(Veigel, Molloy, Schmitz, & Kendrick-Jones, 2003) have all been shown to regulate gene expression in

response to local variation in substrate stiffness and shear stress. Importantly, it has been repeatedly shown that 3-dimensional culture systems yield more translatable results than traditional 2-dimensional culture systems(Arslan-Yildiz et al., 2016; Baker & Chen, 2012; Magin, Alge, & Anseth, 2016). In vivo, high resolution imaging has revealed that abnormal flow rates and shear stress on vascular walls results in vascular wall ECM remodeling that is pathologic and permissive for the development of aortic aneurysm and atherosclerotic lesions(Billaud et al., 2018; Cunningham & Gotlieb, 2005; Majumdar et al., 2007). Further, increased cytoskeletal tension has been shown to promote physical alterations in the structure of the ECM in which the cell resides, increasing local tissue stiffness and increasing ECM production(Baneyx, Baugh, & Vogel, 2002). Stated differently, ECM-cellular mechanical signaling is bidirectional.

In skeletal muscle tissue, injuries that disrupt the high tissue organization can result in profound functional deficits due to the intimate relationship between muscle force generation and its conductance along the aponeurosis. In large muscle injuries, termed volumetric muscle loss (VML) injuries, functional deficits have been shown to be greater than what would be predicted as a function of lost muscle volume alone. In fact, untreated VML defects show greater fatty infiltrates and collagen abundance compared to uninjured muscle, but, perhaps more importantly, altered sarcomeric alignments in remaining tissue with compromised mechanics(Grogan et al., 2011). Further, studies have shown that age-associated declines in muscle healing may be due to microenvironmental changes rather than cellular dysfunction(Conboy et al., 2005). Importantly, changes in muscle stem cell behavior with aging were shown to be at least partially mediated by the mechanical ECM microenvironment, with stiffer substrates promoting stem cell fibrogenic conversion(Ambrosio et al., 2014; Brack et al., 2007; Stearns-Reider et al., 2017). Skeletal muscle ECM compliance is evident in studies involving physical exercise, too, where it has been shown

that repeated weight bearing exercise increases collagen turnover and lowers muscle ECM stiffness(Kjaer, 2004). Taken together, matrix stiffness and cellular behavior are tightly coupled and bidirectional.

Cellular responses to implanted materials largely mimic interactions with native connective tissue. It has been shown that mismatch between implanted biomaterials and local tissue mechanical properties can result in comorbidity and even the need for surgical reintervention. As an exemplary case, polypropylene surgical mesh materials have reported tensile strengths in the range of 150 N/cm, while the longitudinal tensile strength of native human abdominal wall was measured at 34 N/cm(Kalaba et al., 2016). Yet, despite theoretically providing excess mechanical support, nearly 1 in 3 patients will experience a hernia recurrence. The mismatch between native abdominal wall strength and material strength is non-trivial, and groups have found that larger molecular weight polymeric materials incur significantly greater patient discomfort and increased hernia recurrence(Anurov, Titkova, & Oettinger, 2012; Junge et al., 2001). The use of chemical crosslinking to increase mechanical parameters of ECM bioscaffolds has also resulted in lower tissue integration and an increased pro-inflammatory response(Aamodt & Grainger, 2016). Recently, stiff substrates were shown to promote an M1-like macrophage phenotype, in vitro, ostensibly providing an ulterior mechanism for the FBR to non-resorbable implants(Sridharan, Cavanagh, Cameron, Kelly, & O'Brien, 2019). However, hernia mesh materials with physiologically appropriate mechanical properties have shown reduced foreign body reaction (FBR) and superior host tissue integration(Klinge et al., 1998; Tran, Yang, & Ameer, 2015).

Together, these findings suggest that biomechanical forces are critical for the appropriate development and maintenance of tissues. Further, evidence from pathophysiological processes and surgical hernia repair processes suggest that biomaterials that proactively consider local tissue

microenvironment may have superior clinical outcomes. Stated differently, macroscopic material properties are an important consideration for the future design of engineered biomaterials for tissue engineering applications.

2.6 Matrix-bound Nanovesicles

The need for a fully functional immune system in normal development(Simon, Hollander, & McMichael, 2015), regeneration(Godwin, Pinto, & Rosenthal, 2016), tissue homeostasis(Okumura & Takeda, 2016; Rogier et al., 2014; Tidball et al., 2014; Zhan et al., 2014), and in the constructive remodeling properties of ECM bioscaffolds(S. F. Badylak, J. E. Valentin, A. K. Ravindra, G. P. McCabe, & A. M. Stewart-Akers, 2008; Brown, Ratner, Goodman, Amar, & Badylak, 2012; Valentin, Stewart-Akers, Gilbert, & Badylak, 2009) has been recognized. Although, no immunosuppressive agents are used with these bioscaffold materials, this does not imply immune-privilege. In fact, there is a distinct immune response as described below and in subsequent chapters.

ECM bioscaffolds have been associated with robust, pro-healing macrophage and T_H2 responses that are predictive of downstream constructive remodeling outcomes(Brown, Londono, et al., 2012). The promotion of a constructive immune response by ECM bioscaffolds was first reported nearly 20 years ago and have been extensively investigated since. More recently, it was shown that the promotion of an M2-like macrophage phenotype is required for constructive tissue remodeling(Sridharan et al., 2019). Interestingly, the ECM-elicited macrophage response is phenotypically unique with respect to IL-4-treated counterparts and with respect to the source tissue origin(K. Sadtler, B. W. Allen, et al., 2016). This immunomodulatory effect could be, at

least in part, attributable to matrix-bound nanovesicles (MBV), an extracellular vesicle component of the extracellular matrix(L. Huleihel, J. G. Bartolacci, et al., 2017; Huleihel et al., 2016; George S. Hussey et al., 2020).

MBV are nanometer sized extracellular vesicles found bound to the matrix with unique bioactive lipid, nucleic acid, and protein cargos(L. Huleihel, J. G. Bartolacci, et al., 2017; Huleihel et al., 2016; George S. Hussey et al., 2020). MBV miRNA cargos have been shown to contribute to cellular process important to tissue development and healing, and the profile of lipids contained within their membranes may promote activation of anti-inflammatory lipid receptors(George S. Hussey et al., 2020). Importantly, the macrophage phenotype elicited by MBV was nearly identical to that elicited by exposure to the parent ECM in vitro(L. Huleihel, J. G. Bartolacci, et al., 2017). In a rat model of optic nerve injury, van der Merwe et al. showed that exogenous provision of MBV and their miRNA cargos could successfully promote a pro-healing glial cell phenotype and M2-like microglia activation(van der Merwe et al., 2019; van der Merwe, Faust, & Steketee, 2017). Taken together, MBV are a viable mechanistic candidate for ECM-mediated macrophage phenotype activation, however, the specific mechanism underlying this activity are poorly understood.

2.7 Conclusions

Successful clinical translation of tissue engineering approaches requires that multiple tissue repair processes be modulated simultaneously, including modulation of the immune response. Native extracellular matrix and ECM bioscaffolds possess soluble and insoluble components that promote such events. It is obvious that scaffold degradation plays a central role in the generation

of bioactive components and, ostensibly, the successful induction of repair processes. The emerging role of MBV on macrophage phenotype suggests that the nature of these vesicles be characterized in greater detail. Further, although MBV miRNA cargos have been partially investigated, a systematic characterization of MBV cargos may offer new insights and potential therapeutic targets.

3.0 Strategies for Immunosuppression and Immunomodulation

3.1 Overview

Cells of the immune system have historically been considered one-dimensional, rigid cell types confined to inflammatory functions. However, it is now understood that immune cells, in particular innate immune cells such as macrophages, are highly phenotypically plastic, and play a role in homeostatic, developmental, and tissue repair processes(Wynn, Chawla, & Pollard, 2013). Macrophages, for example, have been shown to be critical components in the host response to injury, tissue regeneration, and organ development. Nonetheless, the majority of existing therapeutic options suppress immune cell function rather than harness it. The present manuscript will discuss the ways in which current pharmacological therapies affect immune cell function, the repercussions and advantages of these approaches, and present hypotheses for future therapeutic intervention.

3.2 Introduction

The development of immunosuppressants has led to a revolution in the number of patients who can benefit from whole organ transplantation. The focus of immunosuppression has been to reduce the host adaptive immune response to allogeneic antigens, thereby preventing destruction of the grafted organ(Duncan & Wilkes, 2005). However, an analogous approach to the innate immune response to implanted biomaterials is often inappropriate and can result in suboptimal

clinical outcomes(X. Liu et al., 2017). For example, it is now understood that the macrophage phenotype response to implanted materials is predictive of downstream clinical outcomes(Brown, Londono, et al., 2012). Despite the central role of innate immune cells in tissue healing and regeneration, few therapeutic options exist to modify their behavior rather than suppress their function.

The host immune response, in particular the macrophage component, to implanted materials is reproducible and well characterized. Within minutes of material implantation, proteins from blood adsorb to the material surface and provide a provisional matrix. Within hours, neutrophils arrive in response to chemotactic signals release due to tissue injury and platelet activation. By 1-3 days, pro-inflammatory M1-like macrophages are present and assist in the removal of necrotic cells and debris. If the foreign material can be degraded, M1-like macrophages gradually transition to a pro-healing M2-like macrophages that promote angiogenesis and matrix deposition(Corona et al., 2016; Dort et al., 2019; J. L. Dziki et al., 2016). If the foreign material cannot be degraded, the macrophage phenotype will fail to transition and a chronic foreign body reaction (FBR) will result with sustained matrix turnover and local tissue damage(Burger, Halm, Wijsmuller, ten Raa, & Jeekel, 2006; Elango, Perumalsamy, Ramachandran, & Vadodaria, 2017). This macrophage phenotype transition is critical, as disruption of either macrophage population can result in fibrosis and impaired tissue function. Stated differently, it is becoming increasingly clear that macrophages play a central role in both homeostatic and disease processes. The present manuscript will discuss the ways in which available therapeutic interventions affect adaptive and innate immune cell function, current understanding of the role of immune cells with respect to tissue healing and homeostasis, and the state of the art as it relates to immunomodulation.

3.3 Immunosuppressants

3.3.1 Non-steroidal Anti-inflammatory Drugs

Non-steroidal anti-inflammatory drugs are valuable analgesic tools following injury due to their relatively high safety profile and anti-inflammatory effects. NSAIDs reduce prostaglandin production through inhibition of the cyclooxygenase enzymes COX-1 and COX-2, although other targets have recently been reported. While COX-1 is constitutively expressed in tissues, COX-2 is primarily expressed by inflammatory cells, and a reduction in COX-2 mediated prostaglandin synthesis by immune cells is the primary mechanism of analgesia. NSAIDs vary in their selectivity for COX enzymes, and the ratio of COX-1:COX-2 selectivity is predictive of adverse event occurrence such as gastric ulceration.

Due to their high clinical usage rate, the role of NSAIDs in modifying the host response to tissue injury is an active area of investigation. In particular, impaired bone healing as a result of indomethacin (non-selective COX inhibitor) administration has been reported following orthopedic procedures(M. R. Chen & Dragoo, 2013). Retrospective clinical studies have identified reduced tendon-to-bone healing following rotator cuff repair when NSAIDs were administered for analgesia(D. B. Cohen, Kawamura, Ehteshami, & Rodeo, 2006), although the quality of these results has been disputed(Duchman et al., 2019).

The use of acetylsalicylic acid (also known as aspirin, a non-selective COX inhibitor) has been shown to reduce the immunomodulatory and functional remodeling effects characteristic of ECM bioscaffold implantation(Dearth et al., 2016). Multiple studies have demonstrated that inhibition of prostaglandin synthesis is associated with pro-inflammatory, M1-like phenotype activation, while administration of prostaglandins promotes an M2-like phenotype(Dearth et al.,

2016; Na, Jung, Yoon, Lee, & Seok, 2015; Na, Yoon, et al., 2015; Na, Yoon, Son, & Seok, 2013). Thus, although the consequences of NSAID use tissue repair in humans remains poorly understood, their effects on macrophage phenotype present challenges to existing tissue engineering strategies and should be carefully considered.

3.3.2 Steroidal Compounds

The discovery of glucocorticoid immunosuppressants (GCs) has allowed for organ transplantation to be commonplace. Immunosuppression strategies have gradually transitioned from cell-centric, non-specific GC-based regimens towards more targeted pharmaceuticals with improved side-effect profiles(Kahan, 2003). Yet, GCs are among the most commonly used treatments for inflammatory and autoimmune diseases due to their suppressive effects. GCs mediate a broad variety of effects on cells of the adaptive and innate immune system through direct transcriptional regulation at glucocorticoid response elements(Ashwell, Lu, & Vacchio, 2000), transrepression of NF-kB and AP1(John et al., 2008; Jonat et al., 1990), and even non-transcriptional effects(Stellato, 2004).

The impact of GCs on T cells, in particular, has been the subject of intensive investigation. The ability to rapidly and sustainably reduce T cell number and function in autoimmune disease has clear therapeutic value(John et al., 2008; Jonat et al., 1990), however the impact of GCs on innate immune cells is less well characterized. However, studies have shown that myeloid cells may, similar to T cells, undergo apoptosis in certain circumstances in response to GCs(J. Zhang et al., 2009). Interesting, animal models of contact allergy, a T cell-dependent process, deletion of the glucocorticoid receptor in macrophages, but not T cells, exacerbates the disease(Tuckermann et al., 2007). Further, it has been shown that GC exposure during differentiation yields a unique

macrophage phenotype that was highly phagocytic and resembled both tumor-associated macrophages and myeloid-derived suppressor cells. In these studies, GC-educated macrophages displayed increased apoptotic cell clearance and promoted resolution of inflammation(Ehrchen et al., 2007; Varga et al., 2008). It is of note, however, that tumor-associated macrophages and myeloid suppressor cells have been shown to be distinct from M2-like, pro-healing macrophages with respect to their protein expression and tissue remodeling functions(Wolf et al., 2019). Specifically, the macrophage phenotype transition characteristic of the healing response to muscle injury requires sequential and coordinated activation of p38, MPK-1, and Akt signaling cascade for functional repair(Perdiguero et al., 2011), all of which have been shown to be reduced in response to GCs(Coutinho & Chapman, 2011). As a result, it is important to consider that GC-educated macrophages may be functionally different than their IL-4-treated counterparts.

The presence of macrophages in response to tissue injury is a critical requirement for wound healing. In one model of wound healing, prednisolone administration resulted in reduced accumulation of macrophages at the site of injury(Geurtzen et al., 2017), and another demonstrated that GC administration reduced macrophage migratory capacity(Prokic & Vilic, 1983). It is of note that macrophage recruitment deficits may contribute to reduced functional tissue repair in aging(Hachim et al., 2019), suggesting that impaired macrophage recruitment may, in fact, not be desirable. The impact of GC on macrophage function may be compounded in tissue engineering applications that provide an allogeneic cell source, as it has been shown that GCs can promote apoptosis of stem cells(Singleton, Baker, & Thorburn, 2000). Yet, the impact of GCs on macrophages in tissue engineering contexts has not been evaluated, but may prove to be a rich avenue of investigation.

3.3.3 Non-steroidal Immunosuppressive Drugs and Anti-rheumatic Biologics

The metabolic and osteoporotic side effects of long-term glucocorticoid use prompted the development of more tailored immunosuppressive drugs that could similarly curb the rejection of allogeneic organs and reduce immune mediated tissue damage in autoimmunity(Briot & Roux, 2015). In both organ transplantation and autoimmunity, therapeutic targets have overwhelmingly been T cells and cytokines. However, a significant body of evidence suggests that cells of the innate immune system play a significant role in both transplant rejection and autoimmunity. Specifically, it is becoming clear that the role of antigen presenting cell (APC) phenotype and expression of co-stimulatory molecules are central in shaping downstream immune responses(Mannon, 2012; Rowshani & Vereyken, 2012). Efforts to understand the pathophysiologic mechanisms underlying rejection have provided some insight into the often-conflicting role that non-steroidal immunosuppressive drugs have on innate immune cells, in particular macrophages and dendritic cells.

Macrophages and other antigen presenting cells (APCs) produce cytokines, co-stimulatory molecules and antigens to cells of the adaptive immune system, thereby contributing to rejection processes(J. Li et al., 2019; Vereyken et al., 2013). In both cardiac and kidney allografts, the presence of macrophages is a poor prognostic indication(Rowshani & Vereyken, 2012; L. Xu, Collins, Drachenberg, Kukuruga, & Burke, 2014). To address the unmet clinical need of macrophage suppression in transplant, efforts have identified that several existing anti-rejection drugs have moderate immunosuppressive activity on macrophage recruitment and cytokine secretion, namely mycophenolate mofetil, rapamycin inhibitors, and calcineurin inhibitors(Allison & Eugui, 2000; Howell et al., 2013; Wenink et al., 2012). The activity of these drugs, however, was not associated with decreased chronic allograft rejection, the primary outcome measure. In

Chron's Disease and Rheumatoid Arthritis, antibodies specific to TNF-α have been remarkably successful in reducing disease severity(Atzeni et al., 2005). Unexpectedly, evidence suggests that biologics have secondary mechanisms of action on macrophages through binding of the Fc-g receptor to the Fc region of these antibodies that potentiate their effects. Further, activation of the Fc-g receptor on macrophages by humanized monoclonal antibodies was shown to promote increased T cell apoptosis and promotion of an M2-like macrophage phenotype in vivo(Louis et al., 2004; Vos et al., 2011). It has been reported that anti-TNF-α antibodies may have therapeutic value for chronic, non-healing wounds, however the role of these antibodies and others in tissue engineering and tissue healing has not been tested(Schreurs et al., 2020).

Interestingly, evidence has emerged that innate immune cells exposed to certain antigens and stimuli may be capable of 'antigen memory' that can promote tolerogenic immune responses following repeat antigen exposure(Kleinnijenhuis et al., 2012; Yoshida & Ishii, 2016). Importantly, this antigen memory is sustained following ex vivo exposure and subsequent transplantation. Antigen memory is, at least in part, predicated upon the phenotype of the APC, which may be induced through the careful use of selected anti-rejection agents(Hutchinson, Brem-Exner, et al., 2008). Ex vivo generation of tolerogenic dendritic cells and dendritic cell reprogramming in vivo have shown promise in in vitro and preclinical studies(Bluestone, Thomson, Shevach, & Weiner, 2007; McCurry, Colvin, Zahorchak, & Thomson, 2006; Rutella & Lemoli, 2004). In macrophages, two Phase I clinical trials have demonstrated that pre-operative treatment with a specific subset of donor-derived macrophages, termed tolerance-promoting cells (TAICs), is safe and, in select cases, patients were able to stop immunosuppressive therapies altogether for several months(Hutchinson, Riquelme, et al., 2008; Hutchinson, Roelen, et al., 2008). Lower doses of anti-rejection drugs, longer graft survival, and broader donor sources make

the use of immunomodulatory APC cell therapies an attractive therapeutic target. In tissue engineered constructs, co-delivery of M2-like macrophages with a scaffold improved tissue remodeling outcomes in preclinical animal models, although these macrophages were activated through exogenously provided cytokines(Riabov et al., 2017). Taken together, insufficient evidence exists to fully characterize the innate immune response to non-steroidal immunosuppressive drugs as either harmful or beneficial. However, tissue engineering strategies, specifically those that involve allogeneic cell components, may benefit from incorporation of immunosuppressive agents to promote a tolerogenic state to increase transplanted cell acceptance by the host.

3.4 Immunomodulatory Materials

The development of ECM-based biologic scaffold materials, the relatively recent elucidation of innate immune cell plasticity and their derivatives, and a more in-depth understanding of the role of the immune response during development, normal wound healing, tissue homeostasis, and tissue/organ regeneration have sparked renewed interest in biomaterials as facilitators of functional tissue repair. Historically, materials used for surgical repair of tissue injuries were appropriated due to their ease of manufacturing, relatively low systemic consequences, and amenable mechanichal properties. However, permanent mesh materials such as polypropylene (PP), expanded polytetrafluoroethylene (ePTFE), and polyethylenepterepthalate (PET) suffer from a predisposition to infection, a chronic inflammatory response at the site of implantation, tissue erosion, fistula formation, and premature device failure, among other complications(A. I. Gilbert, 1989; Klinge & Klosterhalfen, 2012; Roth et al., 2015). To address

these issues, significant resources have been devoted to the development of novel biomaterials that address one or more of these issues. Today, scaffolds composed of biodegradable, biosynthetic, and biologic materials can be found on the market as a testament to this effort. Beyond material origin, a growing body of evidence suggests that tissue engineering strategies that incorporate immunomodulatory, rather than immunosuppressive, therapies offer significant advantages to traditional approaches.

3.4.1 Exosomes

Exosomes, first discovered 50 years ago, were initially believed to be debris from nearby cells(Keshtkar, Azarpira, & Ghahremani, 2018). It is now understood that exosomes represent a fundamental intercellular signaling mechanism and have quickly become attractive therapeutic targets. Exosomes and other extracellular vesicles (EVs) are heterogeneous, nanometer-sized, lipid membrane-bound particles released by cells to extracellular fluids with multiple bioactive cargos including nucleic acid cargos (i.e. RNA and DNA), proteins, and lipids(Raposo & Stoorvogel, 2013). Therefore, exosomes have been to significantly impact processes in response to wound healing, including angiogenesis(C. Y. Chen et al., 2018), cell proliferation and differentiation(Lamichhane et al., 2015), and immunomodulation(Keshtkar et al., 2018; Lamichhane et al., 2015; Rani, Ryan, Griffin, & Ritter, 2015). The multifaceted effects of EVs have made their application to regenerative medicine and tissue engineering valuable therapeutic and research tools.

As stem cell therapy interest grew, a particular cell type stood out as a promising candidate for tissue engineering applications, namely mesenchymal stem cells (MSCs) and related cell types. MSCs have been applied to numerous pathologies due to their multipotency, ease of isolation, and

virtually endless passage potential(Kariminekoo et al., 2016). Despite low engraftment and survival, MSC therapies showed consistent reductions in pro-inflammatory signaling that was subsequently attributed, at least in part, to MSC-derived exosomes(Rani et al., 2015). Importantly, EV cargo are plastic largely reflect the state of the parent cell and have therefore been engineered as gene and drug carriers for regenerative medicine(Bunggulawa et al., 2018; Hannafon & Ding, 2013). Stated differently, EV may represent viable cell-free options for targeted tissue engineering applications, including immune cell phenotype modulation.

3.4.2 Biomaterials

The current standard of care for the repair of ventral hernia remains the use of scaffolds primary composed of polypropylene due to its ease of manufacture, low cost, and high mechanical properties(Faulk et al., 2014). However, as discussed above, non-resorbable mesh materials like polypropylene incur foreign body reactions associated with fibrous encapsulation, fistula formation, and tissue erosion. An improved understanding of the host response to implanted materials has led to a significant body of novel materials that address these sub-optimal clinical outcomes.

Acellular bioscaffolds composed of extracellular matrix (ECM) have been used for decades in a wide variety of anatomical locations and are one of the earliest materials recognized to have immunomodulatory, rather than immunosuppressive, effects. Although multiple mechanisms have been implicated in ECM-mediated tissue repair, modulation of immune cell phenotypes, in particular the macrophage component, have emerged as one of the primary mechanisms for site-appropriate tissue remodeling. Specifically, the use of ECM bioscaffolds in both preclinical animal models of muscle injury and a clinical cohort have demonstrated that these materials promote

improved function in treated patients through the recruitment of stem/progenitor cells and the promotion of an M2-like macrophage phenotype. Importantly, this effect was shown to be at least partially mediated by matrix-bound nanovesicles (MBV), a unique class of extracellular vesicles secreted to the matrix. Beyond macrophages, Sadtler et al. elegantly showed that ECM biomaterials also promote T_H2 cell responses and cytokine production, which reinforced the ECM-mediated promotion of an M2-like macrophage phenotype in a mouse model of volumetric muscle loss.

Macrophages have frequently been the target of surface modification biomaterial strategies, as well. ECM-coated synthetic polymers have been investigated as a method to promote a regulatory/anti-inflammatory host response as opposed to an otherwise pro-inflammatory response. Specifically, ECM hydrogel coatings have been shown to mitigate the chronic inflammatory response and associated downstream scar tissue formation after implantation of polyprolyene mesh, the most commonly used synthetic material used to manufacture surgical mesh devices(Faulk et al., 2014). Faulk et al. reported that the addition of an ECM hydrogel coating decreased the number of pro-inflammatory CD86+/CD68+ macrophages in the vicinity of the polypropylene fibers 2 weeks after implantation. Six months after implantation, the coated polypropylene was associated with less collagen deposition (i.e. fibrosis) than was associated with uncoated polypropylene. In similar work, the use of a porcine dermal ECM hydrogel to coat polypropylene mesh reduced macrophage accumulation and formation of foreign body giant cells(Wolf, Carruthers, et al., 2014)**.** At 35 days post-implantation, the ECM coating was fully degraded and replaced with a loose connective tissue.

Attempts have also been made to reduce the inflammatory reaction through the use of drug-eluting polymers. Polypropylene scaffolds that elute IL-4 have been investigated for their ability

to promote enhanced functional remodeling in a mouse model of muscle injury(Hachim, LoPresti, Yates, & Brown, 2017). Surface modification strategies have been developed that directly link cytokine(Hoque et al., 2009) or nucleic acid-containing nanoparticles to the surface(Boehler, Graham, & Shea, 2011; Tsianakas et al., 2012; Zelikin, 2010). Genes encoding IL-4(Butti et al., 2008), IL-10(Peranteau et al., 2008), IκBα(Wilson et al., 2005), or decoy TNF-α receptors(Ghivizzani et al., 1998) have been delivered using scaffolds to promote a local M2-like macrophage phenotype, however more work is needed to determine the viability of this approach. Recent pre-clinical data has shown that polycaprolactone microspheres can serve as a drug-elution mechanism with the potential to provide long term protection from infection(Guillaume et al., 2011), and steroid-eluting polymers have been developed that suppress the inflammatory reaction elicited by polymeric materials(Brandt, Kammer, Fiebeler, & Klinge, 2011). It is important to note that surface modification techniques are not uniformly effective, the immunomodulatory effects of surface-active agents can be short lived, and pharmacokinetics of long-term drug release can be challenging(Vishwakarma et al., 2016). Biomaterial based strategies for the modulation of the immune response in tissue engineering and regenerative medicine applications have been expansive. Although relatively few of these strategies currently have clinical evidence, it is clear that targeting innate and adaptive immune cell functions to improve functional remodeling of tissue injuries is a sensible avenue of investigation.

3.5 Conclusions and Future Directions

A rich understanding of T cell biology has led to the development of highly diverse small molecule and biologic therapies to prevent T cell activation, survival, and proliferation. However,

a growing appreciation of the role of macrophages in tissue healing has led to the advent of numerous biomaterial strategies to foster a favorable, immunomodulatory microenvironment. Further, it is now understood that immunosuppression has an as yet poorly understood impact on macrophages and other cells of the innate immune system. To help address these unanswered scientific questions, prospective studies evaluating the impact of NSAIDs on tissue healing should be rigorously performed. Further, as immunosuppressive strategies have become more tailored and widespread, it remains to be seen what impact these therapies have on cells of the innate immune system. However, work by Sadtler et al. suggests that perturbation of the adaptive immune system is likely to have unexpected, if not detrimental, effects on macrophage phenotype(K. Sadtler, K. Estrellas, et al., 2016). Future tissue engineering and regenerative strategies that incorporate cellular components must not underestimate the significance of immunosuppressant choice, or employ immunomodulatory strategies to reduce the need for immunosuppression as much as possible.

4.0 Objectives

Bioscaffolds composed of acellular mammalian extracellular matrix (ECM) have shown promise as a tissue engineering approach to the repair of skeletal muscle injuries. ECM bioscaffolds modulate multiple endogenous repair processes such as angiogenesis, stem/progenitor cell recruitment and differentiation, and immunomodulation to promote downstream functional, site appropriate tissue deposition. In particular, ECM bioscaffolds have been shown to promote a macrophage phenotype transition, away from a pro-inflammatory, M1-like macrophage phenotype towards a pro-healing, M2-like macrophage phenotype, a critical step in the functional repair of skeletal muscle injury. Although several components have been associated with aspects of ECM bioscaffold-mediated tissue remodeling, the mechanisms underlying ECM-mediated macrophage phenotype transition remain poorly understood. However, a recently discovered component of the ECM, matrix-bound nanovesicles (MBV), have been shown to partially recapitulate the effects of whole ECM on macrophage phenotype, but the mechanisms by which MBV promote an M2-like macrophage phenotype remain unexplored. The proposed study will investigate the role of MBV and recently identified protein cargo, IL-33, on macrophage phenotype both in vitro and in vivo, determine the mechanism of MBV-associated IL-33 uptake and intracellular localization, and will evaluate potential mechanisms of macrophage-stem cell cross-talk.

5.0 Central Hypothesis and Specific Aims

***Central Hypothesis*:** During the regenerative response to skeletal muscle injury, MBV-associated IL-33 promotes a macrophage phenotype transition that supports myogenesis.

Specific Aim 1: Establish the phenotypic and functional response in macrophages and myogenic precursor cells, respectively, to soluble vs. MBV-bound IL-33.

Subaim 1.1: Determine the potential of macrophage-derived exosomes to promote differentiation of muscle stem cells (MuSC).

Corollary Hypothesis: IL-33^{+} MBV will promote M2-like macrophage phenotype activation and differentiation of MuSC, but IL-33^{-} MBV and rIL-33 will not.

Corollary Hypothesis 1.1: M2-like macrophage-derived exosomes will promote differentiation of MuSC, but M1-like macrophage-derived exosomes will not.

Rationale: It has been shown that MBV are at least partially responsible for the bioactivity of whole ECM on macrophage phenotype(L. Huleihel, J. G. Bartolacci, et al., 2017), but the specific cargo(s) mediating this cellular response is not currently known. It has also been shown that M2-like macrophage secreted products promote differentiation of MuSC(Dziki et al., 2018); however, the specific product(s) underlying this effect is an active field of investigation. The secretion of growth factors, chemoattractants, and cytokines have been implicated in macrophage-mediated myogenesis (Novak, Weinheimer-Haus, & Koh, 2014; Panduro, Benoist, & Mathis, 2018), but studies that provided these cytokines via exogenous routes have failed to fully recapitulate what has been observed in vivo(Tidball et al., 2014; Zembron-Lacny, Krzywanski, Ostapiuk-Karolczuk, & Kasperska, 2012). The role of exosomes in immune cell signaling has been implicated as another mechanism by which cells, including macrophages, communicate with their

targets. Importantly, macrophage-derived exosome cargo is dependent on parent cell phenotype and has profound implications on target cell behavior(Ying et al., 2017; Zheng et al., 2018). It is plausible, therefore, that IL-33, an abundant cargo of MBV, may regulate macrophage phenotype, and MuSC fate by an exosome-mediated process.

Specific Aim 2: Determine the mechanism of MBV uptake and intracellular IL-33 trafficking in macrophages.

Corollary Hypothesis: MBV are taken up by an endocytic route and subsequently trafficked to the nucleus.

Rationale: Vesicular uptake has been shown to proceed by a variety of different mechanisms depending on the cell type being investigated and the origin of the vesicle(McKelvey, Powell, Ashton, Morris, & McCracken, 2015). Extracellular vesicle cargo must then be either be targeted away from the phagolysosomal system, or escape endocytic vesicles to the cytoplasm if it is to be bioactive on the target cell. Further, several studies have shown the that IL-33 was capable of modifying the gene expression profiles of certain cell types when it was in the nucleus, either through direct action on gene expression or by modification of the activity of signaling factors such as NFkB, but independent of ST2 activity(Ali et al., 2011; V. Carriere et al., 2007; Oshio et al., 2017). Stated differently, MBV-associated IL-33 may modify gene and protein expression through an ST2-independent mechanism that relies on the non-destructive uptake of MBV by target cells that has not been characterized.

Specific Aim 3: Determine the effect of IL-33 on macrophage phenotype in a mouse model of acute muscle injury.

Corollary Hypothesis: In the absence of IL-33, the macrophage response to acute skeletal muscle injury will be pro-inflammatory.

Rationale: The cellular response to muscle injury his highly orchestrated and is dependent upon macrophages to mobilize and subsequently differentiate the stem cell pool. Cardiotoxin injury models of muscle injury recapitulate the cellular responses to muscle injury, allowing for direct interrogation of immune cell phenotypes and progenitor cell dynamics in a controlled and reproducible setting. To date, a direct investigation of the role of IL-33 (either soluble or MBV-bound) on skeletal muscle regeneration has not yet been performed. Studies have shown that IL-33 plays a role in the recruitment and proliferation of T_{REG} cells in both heart(Matta et al., 2016) and muscle tissue(W. Kuswanto et al., 2016), these studies were limited in their scope. Given the established role of macrophages in directing muscle stem cell behavior, it is plausible that IL-33 may modulate macrophage phenotype in response to muscle injury. However, the impact of soluble and MBV-associated IL-33 on macrophage phenotype has not been described. It is possible that, during matrix degradation and turnover, macrophages release MBV and their IL-33 cargo during the remodeling process, which initiates a transition away from an M1-like phenotype and towards and M2-like, pro-remodeling phenotype.

6.0 Matrix-Bound Nanovesicle-Associated IL-33 Activates a Pro-remodeling Macrophage Phenotype Via a Non-Canonical, ST2-independent Pathway[1]

6.1 Overview

The regenerative healing response of injured skeletal muscle is dependent upon an appropriately timed switch from a local type-I to a type-II immune response. Biologic scaffolds derived from extracellular matrix (ECM) have been shown to facilitate a macrophage phenotype transition that leads to downstream site-appropriate functional tissue deposition and myogenesis. However, the mechanisms by which ECM directs the switching of immune cell phenotype are only partially understood. Herein, we provide the first evidence that matrix bound nanovesicles (MBV) embedded within ECM-scaffolds are a rich and stable source of interleukin-33 (IL-33), an alarmin/cytokine with emerging reparative properties. We show that IL-33 encapsulated within MBV bypass the classical IL33/ST2 receptor signaling pathway to direct macrophage differentiation into the reparative, pro-remodeling M2 phenotype, which in turn facilitates myogenesis of skeletal muscle progenitor cells. Our results suggest the potential of IL-33+ MBV as a clinical therapy to augment the restorative efficacy of existing ECM-based and non-ECM based approaches.

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[1]Portions of the following chapter have been adapted from the following publication:

Hussey, G. S., Dziki, J. L., Lee, Y. C., **Bartolacci, J. G.**, Behun, M., Turnquist, H. R., Badylak, S. F.. Matrix bound nanovesicle-associated IL-33 activates a pro-remodeling macrophage phenotype via a non-canonical, ST2-independent pathway. *J Immunol Regen Med* **3**, 26-35, doi:10.1016/j.regen.2019.01.001 (2019).

6.2 Introduction

Skeletal muscle has a remarkable capacity for repair in response to mild trauma(Turner & Badylak, 2012). However, skeletal muscle damage associated with volumetric muscle loss (VML) overwhelms the regenerative process, ultimately resulting in scar tissue deposition, loss of function, and aesthetic deformities. There are very limited therapeutic options for massive loss of skeletal muscle tissue subsequent to trauma, surgical excision of neoplasms, and related conditions. Autologous muscle grafts or muscle transposition represent optional salvage procedures for restoration of muscle tissue, but these approaches have shown limited success and are plagued by the associated morbidity at the donor site. Alternatively, cell-based therapies have been explored, but legitimate issues remain associated with ES/iPS cell therapies such as immunogenicity, the desirable requirement for source, and a favorable environment to maintain cell viability(Herberts, Kwa, & Hermsen, 2011). Moreover, development of a therapy that avoids the collection, isolation and/or ex-vivo expansion and purification of autologous stem cells with subsequent re-introduction to the patient would almost certainly reduce the regulatory hurdles for clinical translation, reduce the cost of treatment, and avoid the risks associated with cell-based approaches. There is an unmet need for therapeutic strategies that can enhance the innate regenerative ability of skeletal muscle following VML. Acellular biologic scaffolds composed of mammalian (typically porcine) extracellular matrix (ECM) have been investigated in in-vitro studies(Dziki, Wang, et al., 2017), preclinical in-vivo models of VML(Dziki, Giglio, et al., 2017; J. L. Dziki et al., 2016; B. M. Sicari, J. P. Rubin, et al., 2014) and in a recent cohort study involving the use of ECM bioscaffolds in 13 patients with VML(Jenna Dziki et al., 2016; B. M. Sicari, J. P. Rubin, et al., 2014). Outcomes have shown partial restoration of both structure and function, and support the translational aspects of an ECM-bioscaffold based approach. These ECM-based

materials are most commonly xenogeneic in origin, and are prepared by the decellularization of a source tissue such as dermis, urinary bladder or small intestinal submucosa (SIS), among others(Timothy J. Keane, Swinehart, & Badylak, 2015). A significant body of work has shown that xenogeneic ECM scaffolds do not elicit an adverse innate or adaptive immune response, and in fact, support an anti-inflammatory and reparative innate and adaptive immune response(Badylak, Dziki, Sicari, Ambrosio, & Boninger, 2016; Stephen F Badylak, Jolene E Valentin, Anjani K Ravindra, George P McCabe, & Ann M Stewart-Akers, 2008; Brown, Valentin, Stewart-Akers, McCabe, & Badylak, 2009; Dziki, Huleihel, Scarritt, & Badylak, 2017; Luai Huleihel et al., 2017; Kaitlyn Sadtler et al., 2016; Sadtler et al., 2017; Sicari, Dziki, & Badylak, 2015). Use of these naturally occurring biomaterials is typically associated with at least partial restoration of functional, site-appropriate tissue; a process referred to as "constructive remodeling"(Badylak, 2007). Arguably, the major determinant of downstream functional remodeling outcome is the early innate immune response to ECM bioscaffolds(Stephen F Badylak et al., 2008; Brown, Ratner, et al., 2012; Brown et al., 2009; Dziki, Huleihel, et al., 2017). ECM bioscaffolds, or more accurately, the degradation products of ECM bioscaffolds, have been shown to direct tissue repair by promoting a transition from a pro-inflammatory M1-like macrophage and Th1 T cell phenotype to a pro-remodeling M2-like macrophage and T helper Type 2 (T_H2) cell response(Agrawal et al., 2010; Beattie, Gilbert, Guyot, Yates, & Badylak, 2009; Brown, Ratner, et al., 2012; Kaitlyn Sadtler et al., 2016). Numerous studies have shown that an appropriately timed transition in macrophage activation state is required for promotion of tissue remodeling and wound healing processes rather than scar tissue formation in numerous anatomic sites including skeletal muscle(Ludovic Arnold et al., 2007; James G Tidball & Michelle Wehling-Henricks, 2015), and cardiovascular systems(Matthias Nahrendorf et al., 2007; Troidl et al., 2009). Importantly, this

transition is not immunosuppression, but rather a constructive form of immunomodulation that promotes a phenotypic change in local macrophage phenotype(T. J. Keane et al., 2017; Fernando O Martinez & Siamon Gordon, 2014).

Emerging evidence shows that degradation of the scaffold material and subsequent release of matrix-bound-nanovesicles (MBV), that harbor bioactive components which have been only partially defined, are critical for the activation of a reparative and anti-inflammatory M2 macrophage phenotype(L. Huleihel, J. Bartolacci, et al., 2017; Huleihel et al., 2016). MBV are nanometer-sized, membranous vesicles that are embedded within the collagen network of the ECM and protect biologically active signaling molecules (microRNAs and proteins) from degradation and denaturation(Huleihel et al., 2016). We have previously shown that ECM bioscaffolds and their resident MBV can activate macrophages toward a M2-like, pro-remodeling phenotype(L. Huleihel, J. Bartolacci, et al., 2017; Huleihel et al., 2016). However, the molecular components of MBV and the mechanisms by which these nanovesicles direct the switching of immune cell phenotype are poorly understood.

Herein, it is shown that MBV are a rich source of extra-nuclear interleukin-33 (IL-33). IL-33 is an IL-1 family member that is typically found in the nucleus of stromal cells and generally regarded as an alarmin, or a self-derived molecule that is released after tissue damage to activate immune cells via the IL-33 receptor, ST2(Liew, Girard, & Turnquist, 2016). Emerging evidence, however, indicates that IL-33 may function as a promoter of skeletal muscle repair by stimulating $ST2^{+}$ regulatory T cells (Treg)(Wilson Kuswanto et al., 2016). Intracellular IL-33 protein has been suggested to modulate gene expression through interactions with chromatin or signaling molecules via the IL-33 N-terminus(Liew et al., 2016; Serrels et al., 2017). As of yet, however, intracellular IL-33 activities have not been shown to control any immune cell functions. Herein, evidence is

provided that IL-33, protected from proteolytic cleavage by incorporation into the lumen of MBV, is a potent mediator of M2 macrophage activation through an uncharacterized, non-canonical ST2-independent pathway. Specifically, MBV isolated from *il33*$^{+/+}$ mouse tissue ECM, but not MBV from *il33*$^{-/-}$, direct *st2*$^{-/-}$ macrophage activation toward the reparative, pro-remodeling M2 activation state. Moreover, the secreted products from IL33+ MBV-treated macrophages promote myogenesis of skeletal muscle progenitor cells. In total, our results suggest that MBV can mediate receptor-free delivery of cytokine cargo to immune cells to orchestrate cell function.

6.3 Materials and Methods

6.3.1 Animals

C57BL/6 (B6) and Bm12 mice were purchased from Jackson Laboratories. The *il33*$^{-/-}$ mice were a gift from S. Nakae (University of Tokyo, Tokyo, Japan)(Oboki et al., 2010). *St2*$^{-/-}$ mice were originally generated on a BALB/c background as described(Townsend, Fallon, Matthews, Jolin, & McKenzie, 2000) and obtained from Dr. Anne Sperling (University of Chicago) after they were backcrossed 7 times onto the C57BL/6 background. These mice were then backcrossed 3 additional times onto the C57BL/6 background here at the University of Pittsburgh before use in our experiments. Animals were housed in a specific pathogen-free facility maintained by the University of Pittsburgh. The studies conformed to the principles set forth by the Animal Welfare Act and the National Institutes of Health guidelines for the care and use of animals in biomedical research.

6.3.2 Decellularization of Mouse Intestines

Mouse small intestine was decellularized as previously described(Oliveira et al., 2013) with minor modifications. Fresh small intestines were obtained from adult *wt* B6 mice or adult IL-$33^{-/-}$ B6 mice. Small intestines were washed in phosphate buffered saline (PBS) to completely remove all the intestinal contents, and cut into 1.5 cm-length fragments for immediate decellularization. Samples were immersed in 5M NaCl for 72 hr under continuous soft agitation. The decellularization solution was replaced every 24 hr. Decellularized intestines were then washed three times in distilled water. The native and decellularized tissue was prepared for histologic analysis to determine decellularization efficacy by fixing in 10% neutral buffered formalin, or lyophilized and powdered using a Wiley Mill with a #40 mesh screen.

6.3.3 Preparation of Small Intestine Submucosa (SIS)

A SIS was prepared as previously described(Badylak, Lantz, Coffey, & Geddes, 1989). Briefly, jejunum was harvested from 6-month-old market-weight (~110 to ~120 kg) pigs and split longitudinally. The superficial layers of the tunica mucosa were mechanically removed. Likewise, the tunica serosa and tunica muscularis externa were mechanically removed, leaving the tunica submucosa and basilar portions of the tunica mucosa. Decellularization and disinfection of the tissue were completed by agitation in 0.1% peracetic acid with 4% ethanol for 2 hours at 300 rpm. The tissue was then extensively rinsed with PBS and sterile water. The SIS was then lyophilized and milled into particulate using a Wiley Mill with a #60 mesh screen.

6.3.4 Isolation of Matrix-Bound Nanovesicles (MBV)

MBV were isolated as previously described(Huleihel et al., 2016) with minor modifications. Briefly, 100mg of powdered ECM was enzymatically digested with 100 ng/ml Liberase DL (Roche) in buffer (50mM Tris pH 7.5, 5mM $CaCl_2$, 150mM NaCl) for 12 hr at room temperature on an orbital rocker. The digested ECM was then subjected to centrifugation at 10,000 x g for 30 min to remove ECM debris, and the supernatant passed through a 0.22 µm filter (Millipore). The clarified supernatant containing the liberated MBV was then centrifuged at 100,000 x g (Beckman Coulter Optima L-90K Ultracentrifuge) at 4°C for 70 min to pellet the MBV. The MBV pellet was then washed and resuspended in 1X PBS. MBV were isolated as previously described(Huleihel et al., 2016) with minor modifications. Briefly, 100mg of powdered ECM was enzymatically digested with 100 ng/ml Liberase DL (Roche) in buffer (50mM Tris pH 7.5, 5mM $CaCl_2$, 150mM NaCl) for 12 hr at room temperature on an orbital rocker. The digested ECM was then subjected to centrifugation at 10,000 x g for 30 min to remove ECM debris, and the supernatant passed through a 0.22 µm filter (Millipore). The clarified supernatant containing the liberated MBV was then centrifuged at 100,000 x g (Beckman Coulter Optima L-90K Ultracentrifuge) at 4°C for 70 min to pellet the MBV. The MBV pellet was then washed and resuspended in 1X PBS. Purification of MBV by SEC was performed as previously described(Böing et al., 2014). Briefly, 15 ml of Sepharose CL-2B resin (Sigma Aldrich) was stacked in a 1cm x 20cm glass column and equilibrated with 1X PBS. 1 ml of MBV were loaded onto the column and fraction collection (0.35 ml per fraction, and a total of 30 fractions collected) started immediately under gravity flow using PBS as the elution buffer. Eluted fractions were continuously monitored by UV 280nm using the Biologic LP system (BioRad).

6.3.5 Isolation and Activation of Macrophages

Murine bone marrow-derived macrophages (BMDM) were isolated and characterized as previously described(Huleihel et al., 2016). Briefly, bone marrow was harvested from 6- to 8-week-old B6 mice, or B6 $ST2^{-/-}$ mice. Harvested cells from the bone marrow were washed and plated at 2×10^6 cells/mL and were allowed to differentiate into macrophages for 7 days in the presence of macrophage colony-stimulating factor (MCSF) with complete medium changes every 48 h. Macrophages were then activated for 24 h with one of the following: 1) 20 ng/mL Interferon-γ (IFNγ) and 100 ng/mL lipopolysaccharide (LPS) (Affymetrix eBioscience, Santa Clara, CA; Sigma Aldrich) to promote an $M_{IFN\gamma+LPS}$ phenotype (M1-like); 2) 20 ng/mL interleukin (IL)-4 (Invitrogen) to promote an $M_{IL\text{-}4}$ phenotype (M2-like); 3) 20 ng/ml IL-33 (Peprotech), or 4) 25 μg/mL of *wt* mouse MBV, IL-$33^{-/-}$ mouse MBV, or porcine SIS-MBV. After the incubation period at 37°C, cells were washed with sterile PBS and placed in blank DMEM for 5 hours to generate conditioned medias. After conditioned media was collected, lysates were prepared using RIPA buffer for immunoblot analysis or cells were fixed with 2% paraformaldehyde (PFA) for immunolabeling. Conditioned medias are defined by the treatment of the parent macrophage hereafter.

6.3.6 Macrophage Immunolabeling

To prevent nonspecific binding, the cells were incubated in a blocking solution composed of PBS, 0.1% Triton-X, 0.1% Tween-20, 4% goat serum, and 2% bovine serum albumin for 1 h at room temperature. The blocking buffer was then removed and cells were incubated in a solution of one of the following primary antibodies: 1) monoclonal anti-F4/80 (Abcam, Cambridge, MA)

at 1:200 dilution as a pan-macrophage marker; 2) polyclonal anti-inducible nitric oxide synthase (iNOS) (Abcam, Cambridge, MA) at 1:100 dilution as an M1-like marker, and 3) anti-Arginase1 (Abcam, Cambridge, MA) at 1:200 dilution, as an M2-like marker. The cells were incubated at 4°C for 16 h, the primary antibody was removed, and the cells washed with PBS. A solution of fluorophore-conjugated secondary antibody (Alexa donkey anti-rabbit 488 or donkey anti-rat 488; Invitrogen, Carlsbad, CA) was added to the appropriate well for 1 h at room temperature. The antibody was then removed, the cells washed with PBS, and the nuclei were counterstained using DAPI. Cytokine-activated macrophages were used to establish standardized exposure times (positive control), which were held constant throughout groups thereafter. CellProfiler (Broad Institute, Cambridge, MA) was used to quantify images. Data were analyzed for statistical significance using a one-way analysis of variance with Tukey's *post-hoc* test for multiple comparisons. Data are reported as mean ± standard deviation with a minimum of $N = 3$. p-values of <0.05 were considered to be statistically significant.

6.3.7 Macrophage-derived Exosome Isolation

Treatment-specific macrophage-derived exosomes were isolated as previously described(Hong, Funk, Muller, Boyiadzis, & Whiteside, 2016). Briefly, treatment-specific macrophage conditioned medias were concentrated using 100 kDa molecular weight cut-off spin columns (Millipore) to an approximate volume of ~1 mL. Concentrated samples were subsequently purified by size exclusion chromatography as described above. Collected fractions were subsequently collected and concentrated to ~250 ml using fresh 100 kDa molecular weight cut-off spin columns and their concentrations were determined using a NanoSight particle counter equipped with nanoparticle tracking analysis (NTA, NanoSight, Salisbury, UK).

6.3.8 Muscle Stem Cell (MuSC) Isolation and Culture

Muscle stem cells were isolated from the hindlimb skeletal muscle of C57BL/6 mice. Harvested muscle tissue was washed using Wash Medium consisting of HBSS with 10% horse serum and 1% penicillin/streptomycin. Hair and tendon tissue were removed and the muscle tissue was minced until it could pass through a 10 mL stripette. Minced muscle tissue was then subjected to serial enzymatic digestion, beginning with 750 U/mL Collagenase II in Wash Medium (Gibco, Grand Island, NY) for 60 minutes at 37ºC. Collagenase digested muscle was subsequently centrifuged at 900 rpm for 5 minutes, and the supernatant was removed. A solution of 2.4 U/mL Dispase neutral protease (Gibco) and 750 U/mL Collagenase II in Wash Medium was then added to the cell pellet. The cells were resuspended by repeated passage through a 10 mL stripette, and incubated for 45 minutes at 37°C. The mixture was then centrifuged at 900 rpm for 5 minutes, the supernatant removed, and a solution of 0.1% trypsin in Wash Medium added, and incubated at 37°C for 30 minutes. After completing the final enzymatic digestion, the cell mixture was centrifuged again at 900 rpm for 5 minutes, the supernatant removed and the cells resuspended in Wash Medium. The cell suspension was subdivided for flow sorting. The primary sort tube was incubated for 1h with all antibodies used for selection: CD31/CD45-FITC, Sca1-APC, and ITGA7-PeCy7 (all antibodies were purchased from Thermo Fisher Scientific, Waltham, MA). A second control tube was incubated with CD31/CD45-FITC alone, and a third tube was incubated with Propidium Iodide. Compensation beads (Ultracomp eBeads, ThermoFisher Scientific) were incubated for 30 minutes with individual antibodies and were used to establish gating parameters. MuSCs were isolated at $\geq$ 95% purity by selecting for the $CD31^{-}/45^{-}/Sca1^{-}/ITGA7^{+}$ population.

6.3.9 Myogenesis Assay

Upon reaching 80-90% confluence, medium was removed and primary murine MuSCs and murine C_2C_{12} myoblasts were cultured in differentiation medium (DMEM containing 10% FBS, 100 µg/ml streptomyocin and 100 U/ml penicillin) and one of the following treatments: macrophage conditioned media, 1E9 IL-33$^+$ MBV/mL, 1E9 IL-33$^-$ MBV, 20 ng/ml rIL-33, or 1E9 macrophage-derived exosomes/mL. Cells were maintained at 37 °C, 5% CO_2. Myogenic differentiation potential was determined by examining the skeletal muscle myoblast fusion index, defined as the number of nuclei within myotubes containing $\geq$ 3 nuclei per tube divided by the total of number of myotubes per field of view. For C_2C_{12}, a positive control group received 2% FBS 1% pen/strep in DMEM. For macrophage-mediated differentiation, MuSCs or C_2C_{12} were incubated with a 1:1 solution of macrophage supernatants and 20% FBS media (final concentration equivalent to differentiation medium). Following 2 and 5 days, cells were fixed for immunolabeling with 2% paraformaldehyde. Fixed cells were blocked according to the previous described protocol for 1 h at room temperature and incubated in anti-sarcomeric myosin primary antibody (Developmental Studies Hybridoma Bank) at a dilution of 1:500 for 16 h at 4 °C. Following primary incubation, cells were washed with PBS and incubated in Alexa Fluor donkey anti-mouse 488 secondary antibody at a dilution of 1:200 for 1 h at room temperature and counterstained with DAPI. Images of three 20× fields were taken for each well (n = 3 biological replicates) using a Zeiss Axiovert microscope, and the number of myotubes was quantified and averaged. The data were then analyzed by one-way ANOVA followed by Tukey's Multiple Comparison's Test, with a $p < 0.05$ considered significant.

6.4 Results

6.4.1 IL-33^{+} MBV Activate a Pro-Remodeling Macrophage Phenotype via a Non-Canonical, ST2-independent Pathway

It was hypothesized that encapsulation of IL-33 prevents binding to its cognate ST2 receptor, suggesting the presence of an ST2-independent transduction mechanism. To investigate this scenario, bone marrow-derived macrophages (BMDM) isolated from B6 *wt* or *st2*$^{-/-}$ mice were stimulated with interferon-γ (IFN-γ) and lipopolysaccharide (LPS) to induce an M1-like macrophage phenotype, interleukin-4 (IL-4) to induce an M2-like phenotype, recombinant IL-33, MBV isolated from decellularized *wt* (IL33^{+} MBV) or *il33*$^{-/-}$ (IL33^{-} MBV) mouse intestine, or MBV isolated from porcine small intestinal submucosa (SIS MBV). Results showed that macrophages expressed Arginase 1 (Arg-1) in response to SIS MBV and IL33^{+} MBV, similar to the expression pattern of the IL-4–stimulated (M2) cells. In contrast, IL33^{-} MBV induced the expression of iNOS but not Arg-1 (Figure 1A-C). A similar effect was observed with macrophages isolated from *st2*$^{-/-}$ mice. Specifically, IL33^{+} MBV, but not IL33^{-} MBV, directed *st2*$^{-/-}$ macrophage activation into the reparative, pro-remodeling M2-like phenotype (Figure 1A-C).

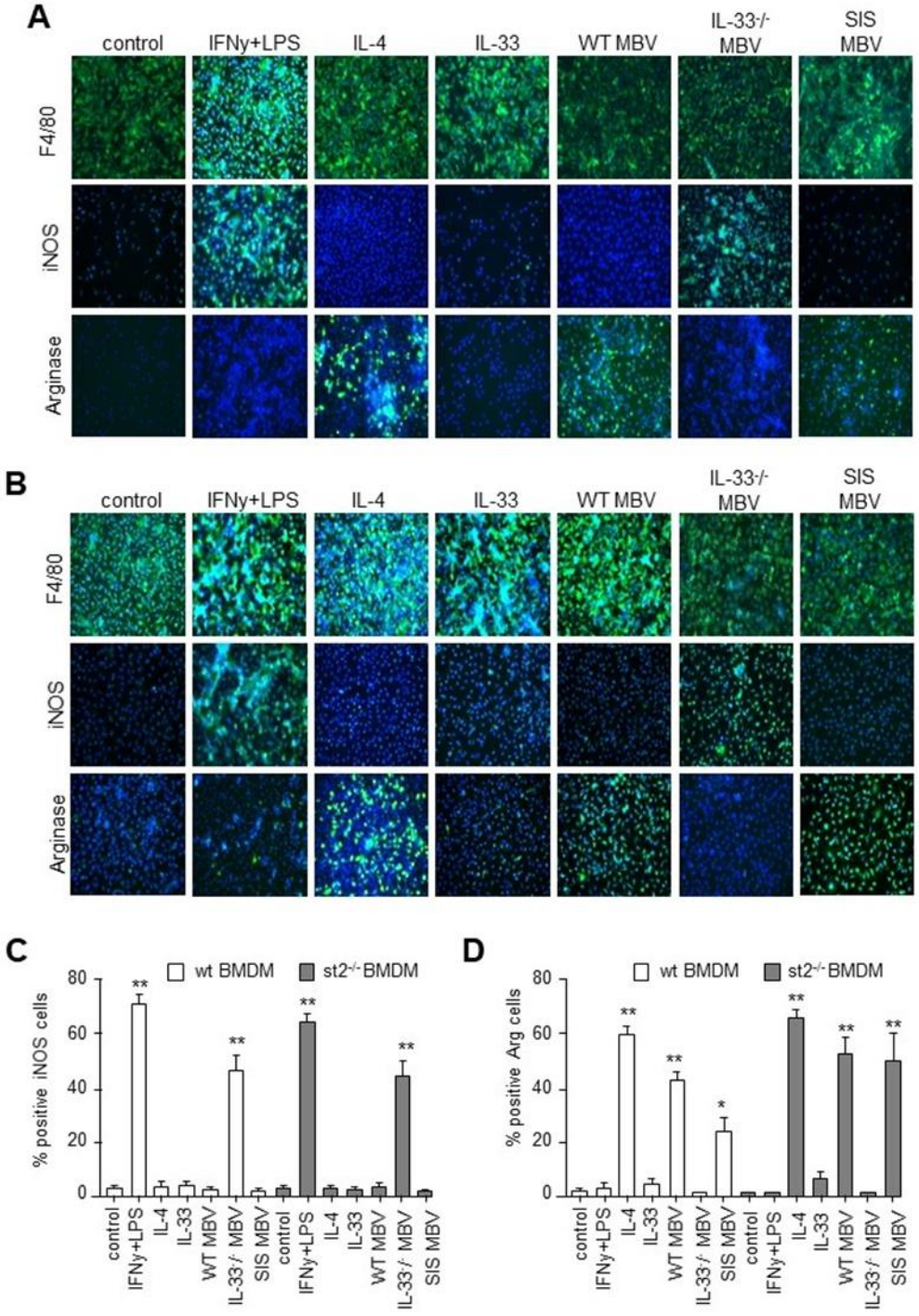

Figure 1. MBV containing luminal IL-33 activate a pro-remodeling macrophage phenotype (F4/80^{+}iNOS^{-} Arg^{+}) via a non-canonical ST2-independent pathway. (A) MBV containing luminal IL-33 activate a pro-remodeling macrophage phenotype (F4/80+iNOS-Arg+) via a non-canonical ST2-independent pathway. **(A, B)** Bone Marrow-Derived Macrophages (BMDM) harvested from wt (A) or st2−/− (B) mice were untreated (control) or

treated with the following test articles for 24 hours: IFNɣ+LPS, IL-4, IL-33, MBV isolated from decellularized wt mouse intestine (IL33+ MBV), MBV isolated from decellularized il33−/− mouse intestine (IL33- MBV), or MBV isolated from porcine small intestinal submucosa (SIS MBV). Cells were immunolabeled with F4/80 (macrophage marker), iNos (M1 marker), or Arg1 (M2 marker). **(C)** Quantification of iNOS immunolabeling (** indicates $p < 0.01$; compared to negative control, error bars represent SEM, n=3 biological replicates analyzed in triplicate). **(D)** Quantification of arginase immunolabeling (**indicates $p < 0.01$; * indicates $p < 0.05$ compared to negative control, error bars represent SEM, n=3 biological replicates analyzed in triplicate).

6.4.2 MBV-Associated IL-33 is Required for Macrophage-mediated Myogenesis

It has been shown that the secretome associated with alternatively activated M2 macrophages is myogenic for skeletal muscle myoblasts(Bo Deng, Michelle Wehling-Henricks, S Armando Villalta, Ying Wang, & James G Tidball, 2012; Daniela Ruffell et al., 2009). Previously, we have shown that media conditioned by ECM-treated macrophages promoted myotube formation and sarcomeric myosin expression of C_2C_{12} myoblasts(Brian M Sicari et al., 2014). The present study shows similar results in that media conditioned by macrophages stimulated with $IL33^+$ MBV, but not $IL33^-$ MBV, promoted myotube formation of C_2C_{12} myoblasts similar to the biologic activity of IL-4-induced M2-like macrophages (Figure 2A,B). Primary MuSCs exposed to macrophage conditioned media or direct exposure to rIL-33, $IL\text{-}33^+$ MBV, or IL-33 shows that direct MBV treatment results in no significant increase in myogenic differentiation compared to macrophage-mediated myogenesis (Figure 2C,D)

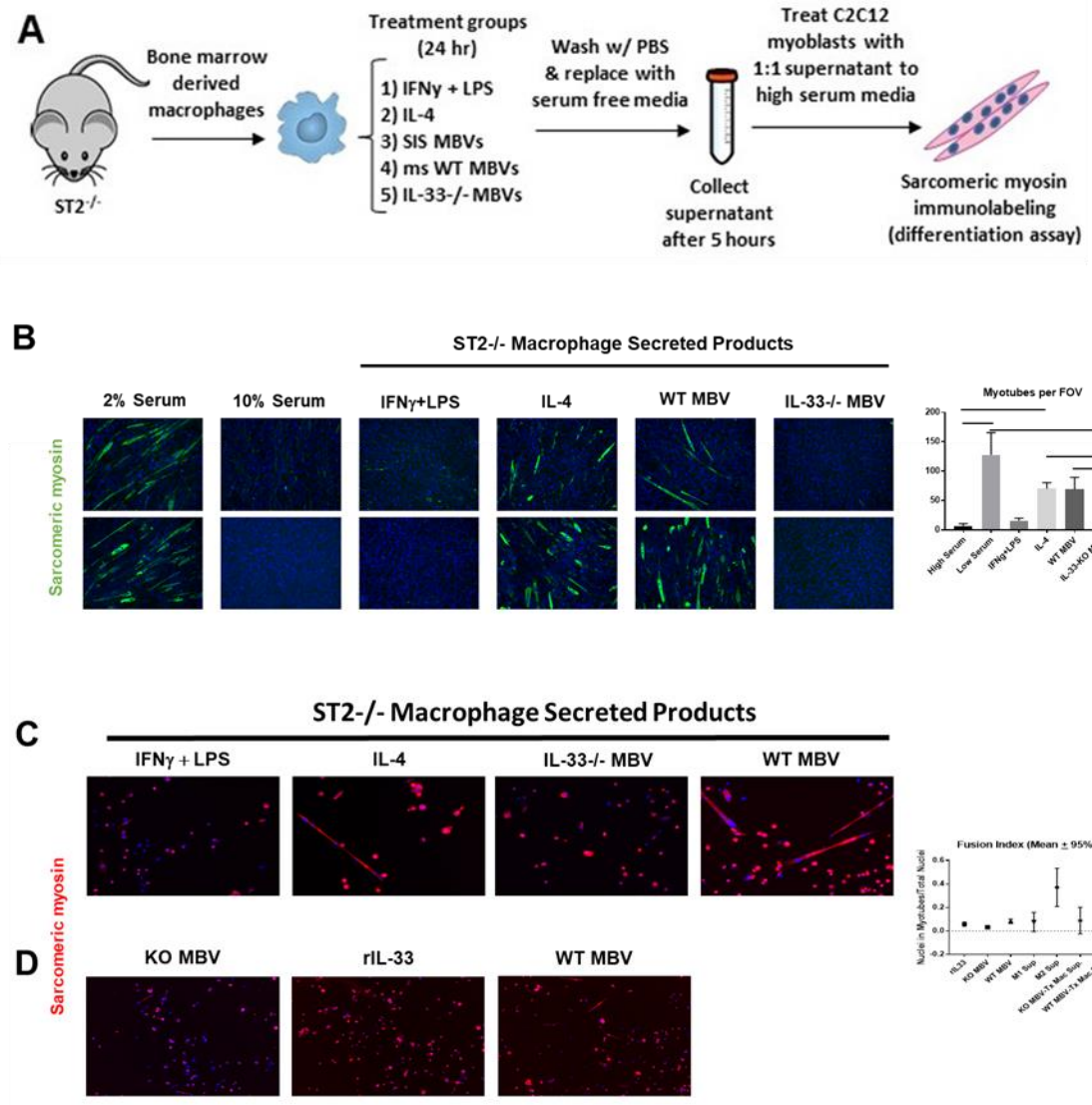

Figure 2. Secreted products of IL-33+ MBV-treated macrophages are pro-myogenic. (A) Experimental design. C2C12 myoblasts were cultured to confluence and treated with high serum proliferation media, low serum differentiation media, or proliferation media supplemented with media conditioned by st2-/-macrophages stimulated with the indicated test articles. **(B)** C2C12 cells were allowed to differentiate in the presence of macrophage secreted products for 2 or 5 days and were immunolabeled for sarcomeric myosin. **(C)** Primary MuSC were differentiated in the presence of macrophage secreted products and were immunolabeled for sarcomeric myosin. **(D)** Primary MuSC were differentiated in the presence of 20 ng/mL rIL-33, 1E9 IL-33- MBV, or 1E9 IL-33+ MBV and were immunolabeled for sarcomeric myosin. (*** indicates $p < 0.001$, ** indicates $p < 0.01$; * indicates $p < 0.05$, error

bars represent SEM (C2C12) or 95% CI of the mean (MuSC), n=3 biological replicates analyzed in triplicate (C2C12), and n = 1 biological replicate, technical triplicate (MuSC).

6.4.3 M2-like Macrophage-derived Exosomes are Pro-Myogenic

Although macrophages are known to interact with stem cells in a wide variety of clinical contexts, the secreted products that account for this cross-talk are incompletely understood. Exosomes have an accepted role in the maintenance and repair of skeletal muscle myofibers, and emerging evidence suggests that immune cell-derived exosomes may play a role in this process, but these studies were not definitive(Bittel & Jaiswal, 2019; Guescini et al., 2017). To assess the role of macrophage-derived exosomes in mediating myogenic differentiation, treatment-specific exosomes from *st2*$^{-/-}$ macrophages were added to MuSC in vitro. Results show that exosomes derived from M2-like macrophages (IL-4 and IL-33^{+} MBV-treated macrophages) promoted greater expression of sarcomeric myosin, a terminal differentiation marker, than those derived from M1-like macrophages ($p < 0.05$,)

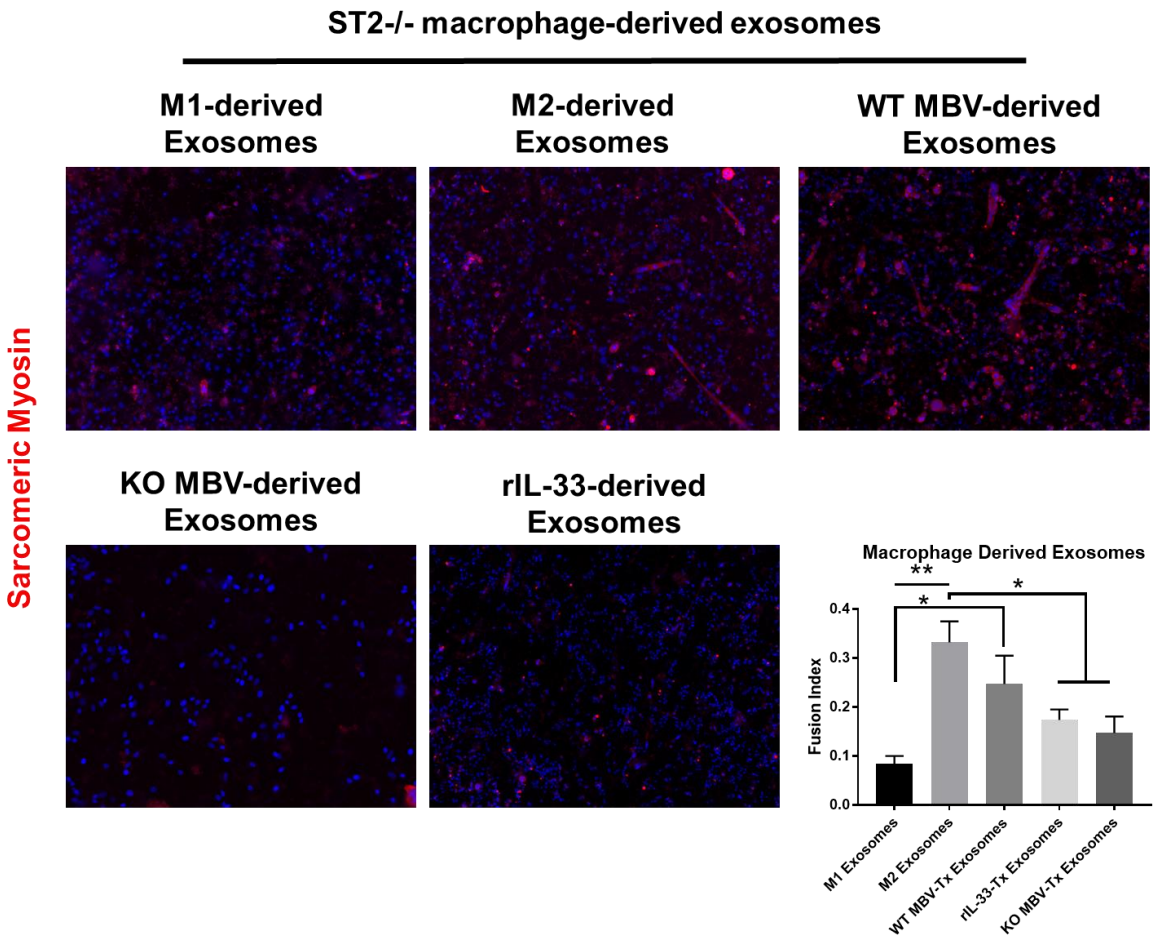

Figure 3. Macrophage-derived exosomes promote myogenesis in a phenotype-dependent manner M2-like macrophage-derived exosomes are pro-myogenice. Exosomes derived from M2-like macrophages (IL-4 and IL-33^{+} MBV-treated macrophages) increase expression of terminal myogenic differentiation marker, sarcomeric myosin (** indicates $p < 0.01$, * indicates $p < 0.05$, error bars represent SEM, n = 1 biological replicate, technical quadruplicate).

6.5 Discussion

In the present study, we provide the first evidence that IL-33, stably stored within the ECM and protected from proteolytic cleavage by incorporation into MBV, is a potent mediator of M2-

like macrophage activation through an uncharacterized, non-canonical ST2-independent pathway. Specifically, we show that $IL33^{+}$ MBV, but not $IL33^{-}$ MBV, direct $st2^{-/-}$ macrophage activation towards a reparative, pro-remodeling M2-like phenotype. $IL33^{-}$ MBV instead activated a pro-inflammatory M1-like phenotype. In total, our studies establish that IL-33 encapsulated within lipid-membrane nanovesicles mediates the differentiation of myeloid cells away from pro-inflammatory subsets independent of the classical ST2 receptor.

Biologic scaffold materials composed of ECM and configured as surgical meshes, powders, and hydrogels have been successfully used in a variety of tissue engineering/regenerative medicine and general surgery applications both in preclinical studies and in clinical applications(George S. Hussey, Dziki, & Badylak, 2018). If not chemically crosslinked, such scaffolds are readily degradable and associated with favorable tissue remodeling properties including angiogenesis, stem cell recruitment, and modulation of macrophage phenotype toward an anti-inflammatory effector cell type(George S. Hussey et al., 2018). However, the biologic mechanisms by which these events and functional tissue restoration are mediated are largely unknown. Separate from the mechanical and structural functions of ECM-scaffolds, the molecular components of ECM, including growth factors, cytokines, and matricryptic peptides, have been extensively investigated for their ability to confer bioactivity(George S Hussey, Keane, & Badylak, 2017). However, there is legitimate controversy concerning not only the relevant importance of these biochemical and structural components, but also the potential influence that the decellularization, disinfection, and sterilization methods used during the manufacturing process have on their biological activity. We have recently shown that MBV are a potent bioactive component of ECM scaffolds, and that these MBV shield their cargo from degradation and denaturation during the ECM-scaffold manufacturing process(Huleihel et al., 2016). Given their

location within the ECM of soft tissue, and their incorporation into the collagen network of the matrix itself, MBV can be separated from the matrix only after enzymatic digestion of the ECM-scaffold material, similar to the functional activity attributed to the degradation of implanted biologic scaffolds used in clinical applications. These findings suggest that MBV are only available for cellular uptake during matrix remodeling events, such as those that occur during normal physiologic processes like wound healing and mechanical stress, or during degradation initiated by the host response to implanted biologic scaffolds.

The early macrophage response to biomaterials has been shown to be a critical and predictive determinant of downstream outcomes(Brown, Londono, et al., 2012; Brown, Ratner, et al., 2012). When properly prepared (i.e. thoroughly decellularized and not chemically crosslinked), bioscaffolds composed of mammalian ECM have been shown to promote a transition in macrophage behavior from a pro-inflammatory to a regulatory/anti-inflammatory phenotype, which in turn has been associated with constructive and functional tissue repair(George S. Hussey et al., 2018). This macrophage phenotype transition is critically important in skeletal muscle as macrophages exert local paracrine effects on local stem/progenitor cell populations to promote their recruitment, proliferation, and subsequent differentiation as a function of their phenotype(L. Arnold et al., 2007; Bittel & Jaiswal, 2019; J. L. Dziki et al., 2016; Tidball, 2005). Although these phenomena are well known, the secreted product(s) by which macrophages exert these effects are incompletely understood.due to the phenotype-dependent We have previously shown that MBV are critical for the activation of macrophages towards a M2-like, pro-remodeling phenotype(L. Huleihel, J. Bartolacci, et al., 2017; Huleihel et al., 2016). Results from the present study support earlier findings and suggest that a single MBV cargo, IL-33, plays a critical role in this phenotypic transition, and lend credence to previous reports that macrophage-derived exosomes contribute to

differentiation of myogenic cells during the host response to injury. Further characterization of the role of these secreted extracellular vesicles in tissue remodeling may offer new avenues for therapeutic intervention.

Conceptually, the discovery that MBV embedded within ECM-scaffolds are a rich source of extra-nuclear IL-33, is novel in itself and has not been previously reported. Full length IL-33 is generated as an approximately 32 kDa protein with an IL-1 family C-terminal cytokine domain and an N-terminal domain consisting of a non-classical nuclear localization sequence and a DNA binding domain, as well as a chromatin-binding domain(Baekkevold et al., 2003; Lingel et al., 2009). Given this N-terminus and the lack of a secretion signaling sequence, current dogma is that IL-33 is found only in the nucleus and released upon necrotic cell death(Pichery et al., 2012). Thus, IL-33 is defined as an alarmin, or an endogenous molecule that alerts the immune system to cell injury and tissue damage. IL-33 released from necrotic cells has been shown to activate the canonical ST2 signaling pathway in a variety of $ST2^{+}$ immune cells to drive broad immune responses that include Type 1, Type 2, and regulatory immune responses(Kakkar & Lee, 2008; Liew et al., 2016). Full length IL-33 is fully capable of activating ST2-dependent signaling, but can be converted by inflammatory proteases to an 18 kDa cytokine that is a more potent activator of ST2 signaling(Lefrancais et al., 2014; Lefrancais et al., 2012). The ST2-dependent functions of IL-33 are negatively regulated by the apoptotic caspases-3 and -7, which cleave IL-33 in the cytokine domain, and a soluble and antagonistic form of ST2(Liew et al., 2016). More recently, it was revealed that soluble IL-33 released into the extracellular environment is rapidly oxidized to inactivate the cytokine domain and limit its range and duration of action(E. S. Cohen et al., 2015). Several studies have shown that cytokines that lack a signal peptide sequence can be packaged and secreted within extracellular vesicles, including the IL-1 family members IL-1β, IL-1α, and IL-

18(Berda-Haddad et al., 2011; Gulinelli et al., 2012; Hasegawa, Thomas, Schooley, & Born, 2011; MacKenzie et al., 2001). IL-33 has been reported to be present in exosomes released from Epstein-Barr Virus (EBV)-infected cells as a strategy to reduce innate immunity activation(Ansari et al., 2013). Furthermore, it was previously demonstrated that IL-33 can efflux from the nucleus where it is packaged within membrane vesicles in the cytosol(Rahul Kakkar, Hillary Hei, Stephan Dobner, & Richard T Lee, 2012). In the present study, we provide the first evidence that IL-33 can also be stably compartmentalized within the extracellular matrix through incorporation into the lumen of MBV thereby preventing IL-33 degradation and inactivation.

The findings of the present study show that MBV-mediated delivery of IL-33 to immune cells can upregulate Arginase-1 expression. Conversely, we show that IL-33 deficient MBV support M1-like macrophage activation, which suggests that the absence of IL-33 within MBV may either drive uncharacterized gene expression or that IL-33 may repress genes leading to M1 macrophage activation after MBV uptake. Previous reports have shown that epigenetic regulation of histone modification, including acetylation/deacetylation and chromatin remodeling are mechanistically important for activation of the M2 macrophage phenotype(Ishii et al., 2009; Kapellos & Iqbal, 2016). Although it has been reported that IL-33 binds chromatin and may act as a transcriptional repressor(Virginie Carriere et al., 2007), to date, no *bona fide* IL-33 regulated genes have been identified. Future studies aimed at a rigorous molecular characterization of IL-33 regulated genes will be required to fully explore the biology of not only MBV, but also IL-33, and will yield important information on their potential use in not only regenerative medicine applications, but also in the mitigation of adverse tissue responses; for example, to promote allograft survival following heart transplant. Given our finding that MBV can mediate receptor-free delivery of protein cargo to immune cells and orchestrate their function, it is easy to appreciate

how the present study may support the development of tailored vesicles (e.g., MBV, exosomes, or engineered liposomes) as next generation immunomodulatory therapeutics. Separately, the use of ECM-based therapies can now be examined by new directions and will help guide the design of next generation ECM-based materials.

6.6 Conclusions

Results of the present study suggest that IL-33 is a critical mediator of primary bone marrow derived macrophage phenotype activation. Specifically, MBV containing IL-33 promoted expression of the M2-associated marker, Arg1, while MBV deficient in IL-33 did not. Further, IL-33^{+} promoted a pro-myogenic macrophage secretome, which is in accordance with previously reported behavior of M2-like macrophages. These data suggest that MBV and their associated IL-33 cargo may underly the immunomodulatory bioactivity of whole ECM bioscaffolds observed in vitro and in vivo.

6.7 Acknowledgements

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7.0 Extracellular IL-33 Enters Target Cells via a Clathrin and PI3K-dependent Endocytic Mechanism and Translocates to the Nucleus

7.1 Overview

Numerous preclinical animal studies and clinical data have shown that bioscaffolds composed of extracellular matrix (ECM) effectively promote functional, site-appropriate tissue remodeling in a variety of clinical contexts(Badylak et al., 2011; J. Dziki et al., 2016; T. J. Keane et al., 2017; Saldin et al., 2019; Seif-Naraghi et al., 2013; Singelyn et al., 2009). Numerous ECM components have been associated with induction of endogenous repair processes; however, a mechanism by which ECM bioscaffolds promote modulation of immune cell phenotypes, in particular macrophages, has remained elusive. Recent studies suggest that matrix-bound nanovesicles (MBV), extracellular vesicle components of ECM, may be at least partially responsible for ECM-mediated macrophage phenotype activation(L. Huleihel, J. G. Bartolacci, et al., 2017). Although MBV contain a myriad of bioactive components, evidence supports non-canonical IL-33 signaling as a potential mechanism by which MBV promote a pro-healing macrophage phenotype(G. S. Hussey et al., 2019). Results of the present study show that MBV-associated IL-33 is functionally delivered to target cells by a clathrin and phosphatidylinositol 3-kinase (PI3K)-dependent mechanism. Within target cells, IL-33 rapidly translocates to the nucleus where it engages in multiple protein-protein interactions. A result of these interactions, IL-33 is required for the promotion of M2-associated marker, Arg1, and inhibition of M1-associated marker, iNOS. Stated differently, IL-33 is required for MBV-mediated macrophage phenotype activation.

7.2 Introduction

Biologic scaffolds derived from a variety of mammalian tissues and composed of extracellular matrix (ECM) have been used as surgical meshes to repair a variety of soft tissue defects (Agrawal et al., 2010; Badylak et al., 2011; J. Dziki et al., 2016; T. J. Keane et al., 2017). The in vivo degradation and remodeling of these ECM-based materials are associated with constructive and functional, site appropriate healing events such as angiogenesis(Bornstein, 2009; F. Li et al., 2004), immunomodulation (Brown, Londono, et al., 2012; J. L. Dziki et al., 2016; L. Huleihel, J. G. Bartolacci, et al., 2017; L. Huleihel, J. L. Dziki, et al., 2017; G. S. Hussey et al., 2019; B. M. Sicari, J. L. Dziki, et al., 2014), and stem cell recruitment and differentiation(Agrawal, Kelly, et al., 2011; J. L. Dziki et al., 2016; Huleihel et al., 2016) among others. The molecular signaling effectors responsible for these events have been attributed to embedded chemokines and cytokines(Mewhort et al., 2017; Michalopoulos, 2010; Peloso et al., 2015), cryptic peptides(Adams & Watt, 1989; Agrawal et al., 2010; Agrawal, Kelly, et al., 2011; Agrawal, Tottey, et al., 2011; Brennan et al., 2006), and various ligand-receptor interactions (Bissell & Aggeler, 1987; Dzobo, Vogelsang, & Parker, 2015; Grasman et al., 2015; Mammoto, Jiang, Jiang, & Mammoto, 2013), but convincing and definitive studies that show cause-effect relationships are lacking. Arguably, modulation of innate immune cell phenotypes, in particular the macrophage component, is the most critical of these processes but the least understood.

Recently, matrix bound nanovesicles (MBV) have been identified within the ECM(Huleihel et al., 2016). MBV are a distinct class of nanoscale, lipid-bound extracellular vesicles that contain a myriad of signaling molecules(L. Huleihel, J. G. Bartolacci, et al., 2017; Huleihel et al., 2016; G. S. Hussey et al., 2019). MBV are distinct from liquid-phase exosomes with respect to both their surface lipid membrane composition and intravesicular cargo(George S.

Hussey et al., 2020). MBV have been shown in vitro to modulate macrophage phenotype(L. Huleihel, J. G. Bartolacci, et al., 2017; Huleihel et al., 2016; G. S. Hussey et al., 2019), induce stem cell differentiation(J. L. Dziki et al., 2016; T. J. Keane et al., 2017; Schreurs et al., 2020), and to contain gene print transcripts that regulate an impressive array of constructive and tissue remodeling processes(L. Huleihel, J. G. Bartolacci, et al., 2017; Huleihel et al., 2016; G. S. Hussey et al., 2019; George S. Hussey et al., 2020; van der Merwe et al., 2019). Further, MBV have been shown to have favorable in vivo therapeutic effects in the preservation of retinal structure and function following injury through modulation of microglia and astrocyte phenotypes(van der Merwe et al., 2019) in a rat model of acute glaucoma. MBV miRNA cargo were shown to mediate, at least in part, the effects of MBV on neuronal cells(van der Merwe et al., 2017) and studies on primary murine bone marrow derived macrophages (BMDM) have shown that miRNA may contribute to MBV-mediated macrophage phenotype activation(L. Huleihel, J. G. Bartolacci, et al., 2017). However, the bioactivity of MBV protein cargo(s) is not fully understood. Specifically, recent in vitro studies have shown that MBV are an ample source of extracellular IL-33, an IL-1 superfamily member typically considered to be a pro-inflammatory alarmin(Ali et al., 2011; V. Carriere et al., 2007; Cayrol & Girard, 2014; G. S. Hussey et al., 2019; Milovanovic et al., 2012). Nonetheless, MBV-associated IL-33 has been shown to play a significant role in pro-healing, M2-like macrophage phenotype activation via a non-canonical, ST2-independent pathway(G. S. Hussey et al., 2019). Such in vitro and in vivo findings are logically dependent upon the attachment and intracellular trafficking of these information rich nanovesicles.

The objective of the present study was to determine the molecules involved in the uptake of MBV by macrophages, as well as the intracellular compartmentalization of delivered cargo. Results of fluorescently labeled MBV uptake studies show that the clathrin aggregation inhibitor,

Pitstop2, and a phosphatidylinositol-3-kinase (PI3K) inhibitor, LY294002, markedly reduced MBV uptake compared to uninhibited controls. To investigate the mechanism of IL-33 modulation of macrophage phenotype, the location of MBV-associated IL-33 was determined at several timepoints following exposure of *il33*$^{-/-}$ macrophages to IL-33^{+} MBV. The cytosolic and nuclear compartments were assayed by western blot directed against IL-33. Results show that IL-33 can be detected within the cytoplasm of exposed cells within 1 hour of exposure and within the nuclei within 3 hours. The appearance of IL-33 within target cells correlated with increased expression of Arg1 protein expression, an M2-like macrophage associated marker, that was not observed after exposure of macrophages to IL-33^{-} MBV. These results suggest that IL-33 is responsible for the expression of M2-associated genes. Co-immunoprecipitation against IL-33 at all timepoints showed that IL-33 has dynamic protein interactions in the nucleus by 3h that were maintained through the remainder of the investigation period. Together, these data suggest that MBV uptake is non-destructive to protein cargo(s) and may regulate an M2-like macrophage phenotype through direct protein-protein interactions. Stated differently, results of the present study suggest that MBV-associated IL-33 may represents a novel paradigm for gene expression regulation by a cytokine.

7.3 Materials and Methods

7.3.1 Preparation of SIS-ECM

The jejunum was harvested from market weight (~240 lbs) pigs (Animal Biotech Industries, Doylestown, PA) or from C57bl6-*arg1*^{gfp} or C57bl6-*arg1*^{gfp}/*il33*$^{-/-}$ mice and was

decellularized via mechanical and chemical methods as previously described(Badylak et al., 1989; Badylak et al., 1995; Prevel et al., 1995). Briefly, the tunica serosa, tunica muscularis externa, and the superficial layers of the tunica mucosa were mechanically removed, leaving only the tunica submucosa and basilar portions of the tunica mucosa. Decellularization and disinfection of the tissue were completed by agitation in 0.1% peracetic acid with 4% ethanol for 2 hours at 300 rpm. The tissue was then extensively rinsed with PBS and sterile water, frozen, and ultimately lyophilized. The resultant SIS-ECM was then milled into particulate using a Wiley Mill with a #60 mesh screen.

7.3.2 Matrix-Bound Nanovesicle (MBV) Isolation, Purification, and Quantification

SIS-MBV were isolated from SIS-ECM as previous described(L. Huleihel, J. G. Bartolacci, et al., 2017). Briefly, SIS-ECM was solubilized using Liberase DL (Sigma Aldrich, St Louis, MO), Immediately following solubilization, SIS-ECM samples were sequentially centrifuged at 500xg for 10 min (one time), 2,500xg for 20 min (one time), and 10,000xg for 30 min (3 times) to remove collagen fibrils and other insoluble remnants. The supernatants were recovered between each centrifugation step, while the pellets were discarded. Following centrifugation, the supernatants were filter-sterilized with a 0.22 μm filter (Millipore, Burlington, MA) and concentrated to a volume of 1 mL using 100 kDa molecular weight cut-off columns at 4,000xg. Concentrated samples were then subjected to SEC as previously described(Hong CS., 2017). Briefly, columns were prepared in 1.5 cm x 12 cm mini-columns (Bio-Rad, Hercules, CA, USA; Econo-Pack columns), to which Sepharose 2B (Sigma-Aldrich) was added to a final bed height of 10 ml, and finally by a porous frit to protect the column bed disruption during sample elution. The Sepharose column was maintained in 1X PBS at all times during column preparation,

and washed with 20 ml of 1X PBS prior to sample addition. Following column preparation, the concentrated digest (1 ml) was then loaded onto the column and 7 fractions of 1 mL were collected. Fractions 3, 4 and 5 were combined, concentrated using fresh 100 kDa cut-off columns until the final volume was ~500 mL. For MBV uptake assays, 6 ml of PKH26 (Sigma Aldrich) was added to the 1 mL and incubated in the dark for 5 minutes at room temperature. PKH-labeled MBV were then purified by SEC. MBV size and concentration were determined using a Nanoparticle Tracking Analysis (NTA) equipped Nanosight LM10 (Malvern Panalytical)(Filipe, Hawe, & Jiskoot, 2010) as previously described(Webber & Clayton, 2013).

7.3.3 Macrophage Isolation and Activation

Primary bone marrow-derived macrophages were isolated as previously described(Englen, Valdez, Lehnert, & Lehnert, 1995; B. M. Sicari, J. L. Dziki, et al., 2014). Briefly, female 6 to 8-week old C57bl/6 mice (Jackson Laboratories, Bar Harbor, ME) were euthanized via CO_2 inhalation and cervical dislocation. Femurs, tibia, and fibula were harvested and washed in 3x in macrophage Complete Medium consisting of 10% FBS (Invitrogen, Carlsbad, CA), 10% L929 supernatant, 10 mM non-essential amino acids (Gibco, Grand Island, NY), 10 mM HEPES (Gibco), 2 mM L-glutamine (Gibco), 100 U/mL penicillin (Gibco), 100 mg/mL streptomycin (Gibco) and 0.1% b-mercaptoethanol in DMEM high glucose (Gibco). Complete medium was flushed through the medullary space of harvested bones and cells plated at 2×10^6 cells/mL into 6 well plates (Corning) or 4×10^4 cells/mL into chambered slides (Nunc Lab-Tek II Chamber Slide System, Thermo Fisher Scientific, Waltham, MA). Medium was supplemented 24h after plating and complete media replacement every 48 hours thereafter for 7 days. For macrophage activation studies, mature macrophages were treated at 37^{o}C and 5% CO_2 with 1 ml of the following

treatments for in 10% FBS 1% pen/strep in DMEM: PBS (vehicle control), 1 x 10^9 IL-33^+ MBV (WT MBV), or 1 x 10^9 IL-33^- MBV (KO MBV). After 1.5h, an additional 1 ml of one of the following treatments was added for 16h: 20 ng/ml IFN-g + 100 ng/ml LPS (M1), or PBS. Cells were subsequently washed with PBS and lysed in RIPA buffer containing protease inhibitor cocktail. Lysates were stored at -20^{o}C until use for immunoblotting.

7.3.4 MBV Uptake Assay

Mature bone marrow derived macrophages cultured in chambered slides were exposed to inhibitors of cellular uptake processes as previously described(Carter, Bernstone, Baskaran, & James, 2011; Svensson et al., 2013; Yao et al., 2018). All treatments were delivered in DMEM containing 10% FBS and 1% pen/strep. Briefly, macrophages exposed to one of the following treatments at 37^{o}C and 5% CO_2: 0.5% DMSO (vehicle control), 25 mM Pitstop 2 (Clathrin aggregation inhibitor; Abcam), 25 mM LY294002 (PI3K inhibitor; Selleck Chemicals, Houston, TX), 25 mM Go 6894 (Protein Kinase C inhibitor; Selleck Chemicals), 5 mM methyl-b-cyclodextrin (lipid raft disruptor; Sigma), 100 mM amiloride (sodium ATPase inhibitor; Selleck Chemicals), 1 mg/ml Filipin iii (lipid raft disruptor; Selleck Chemicals), or 48 nM Latrunculin A (actin polymerization inhibitor; Abcam). After 1h, 1 x 10^9 PKH26-labeled MBV/well were added to each inhibitor group, or an equivalent volume of PBS was delivered (negative control). Following 1h of incubation with fluorescently labeled MBV, treatments were removed, the cells washed with PBS, and subsequently fixed in 4% paraformaldehyde (PFA) at room temperature for 20 minutes. PFA was then removed, the cells washed 3x in PBS, and the cytoskeleton and nuclei were counterstained using AF488-conjugated phalloidin (Invitrogen, Carslbad, CA) and 4′,6-diamidino-2-phenylindole (DAPI), respectively. Five images per treatment group were acquired

at 32X magnification using a Zeiss Axiovert microscope (Carl Zeiss, Oberkochen, GE). Fluorescence channel images were processed using CellProfiler (Broad Institute, Cambridge, MA) and cells with $\geq$ 1 fluorescently labeled MBV were considered positive. N = 3 biological replicates were performed and percent MBV-positive cells as a function of treatment was the primary outcome measure. Differences between groups was assessed using one-way ANOVA followed by post-hoc testing (GraphPad, San Diego, CA). Results are presented as mean $\pm$ standard error of the mean and the 95% confidence intervals for all comparisons are presented. A multiple comparisons-corrected $p \leq 0.05$ was considered significant.

7.3.5 Subcellular Fractionation

Mature *il33*$^{-/-}$ bone marrow-derived macrophages cultured in 6-well tissue culture plates (Corning) were exposed to 1 x 10^9 IL-33$^+$ MBV/ml in macrophage Complete Medium for 0 (negative control), 1, 3, 6, or 24h. At the designated endpoint, treatments were removed,1 ml of PBS was added and the cells were collected through mechanical scraping. Collected cells were subsequently centrifuged at 1500 xg for 5 minutes, the PBS removed and 100 ml of hypotonic lysis buffer (20 mM HEPES pH = 7.4, 10 mM KCl, 2 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, and protease inhibitor cocktail tablet) was added on ice with intermittent vortexing. Following 30 minutes, cell lysates were passed through a 27-gauge syringe ten times and then centrifuged at 4^{o}C for 20 minutes at 12000 xg. The cytosolic supernatant was collected and stored at -20^{o}C or used fresh. The nuclear pellet was washed 3x in ice cold PBS and then 0.1% SDS in TBS supplemented with 1 mM DTT and a protease inhibitor cocktail tablet was added to lyse nuclear membranes. After 30 minutes, samples underwent immersion sonication (Fisherbrand^{TM} Model 120 Sonic Dismembrator, Thermo Fisher) for 3 seconds at 30% amplitude to shear

chromatin. Sonicated nuclear lysates were then centrifuged at 12000 xg for 20 minutes at 4^{o}C. Nucleosol supernatants were collected and stored at -20^{o}C or used fresh.

7.3.6 Immunoblotting

Nucleosolic and cytosolic fractions were quantified by detergent compatible Bradford assay (Thermo Fisher) or BCA (Bio-Rad), respectively. 100 mg total protein in Laemmli buffer containing 5% b-mercaptoethanol were loaded to 4-20% polyacrylamide MiniPROTEAN TGX pre-cast gels (Bio-Rad) and run at 150 V for ~45 minutes in Tris-Glycine running buffer. Upon completion, wet transfer was performed using polyvinylidene difluoride membranes in Tris-Glycine transfer buffer with 20% methanol at 300 mAmp on ice. After 45 minutes, membranes were removed from the transfer chamber, washed for 10 minutes in TBST, and blocked for 1h in TBST with 5% bovine serum albumin (Sigma). After blocking, membranes were incubated overnight at 4^{o}C with: 0.1 mg/ml goat-anti-IL-33 (AF3626; R&D Biosystems, Minneapolis, MN), 0.1 mg/ml mouse-anti-b-actin (sc-47778, Santa Cruz Biotechnologies, Dallas, TX), 0.1 mg/ml rabbit-anti-histone H3 (ab18521, Abcam), 0.1 mg/ml rabbit-anti-arginase 1 (ab91279, Abcam), or 0.1 mg/ml rabbit-anti-iNOS (PA3-030A, Thermo Fisher). Following primary antibody incubation, membranes were washed 3x in TBST and subsequently incubated for 1h at room temperature with rabbit-anti-goat, goat-anti-mouse, or goat-anti-rabbit HRP-conjugated antibodies (Dako Affinity Purified; Agilent, Santa Clara, CA) diluted 1:1000 in TBST with 5% BSA. Membranes were then washed 3x with TBST and incubated in chemiluminescent substrate (Clarity ECL Substrate; Bio-Rad) for 5 minutes and subsequently imaged (ChemiDoc Touch; Bio-Rad). Acquired images were analyzed using ImageJ and all groups were normalized to the appropriate loading control. N = 2 biological replicates, densitometry results were averaged across replicates and means were

compared using two-way ANOVA (GraphPad) to determine row effects (time). Significance was set as $p \leq 0.05$.

7.3.7 Co-Immunoprecipitation

Co-immunoprecipitation of nucleosolic fractions was performed as previously described(C. Lee, 2007). Briefly, 10 mg/mg goat-anti-IL-33 (R&D Biosciences) or 10 mg/mg of isotype control antibody (Cell Signaling Technologies) were covalently conjugated to M-270 Epoxy Dynabeads (Thermo Fisher) according to manufacturer's instructions. 50 mg/reaction by total protein of nucleosol from each timepoint was incubated with dynabead-conjugated antibodies overnight at 4^{o}C with agitation. Samples were subsequently washed 3x in cold PBS and eluted in 50 ml of 2% SDS in PBS. 20 ml of each eluted sample was loaded in Laemmli buffer with 5% b-mercaptoethanol to 4-20% Mini-PROTEAN TGX pre-cast gels and run at 150 mV for ~45 minutes. Gels were then collected and washed in distilled H_2O. After washing, gels were stained with Pierce Silver Stain for Mass Spectroscopy kit (Thermo Fisher) according to manufacturer's instructions and imaged using ChemiDoc Touch (Bio-Rad). To confirm the identity of eluted products, 20 ml of eluate in Laemmli buffer were subjected to SDS-PAGE as described above and membranes were incubated with anti-IL-33 antibodies.

7.4 Results

7.4.1 MBV Uptake is Clathrin and PI3K Dependent

MBV-associated IL-33 was previously shown to signal through an ST2-independent, STAT6-independent mechanism(G. S. Hussey et al., 2019), however the nature of this non-canonical signaling mechanism was not completely elucidated. Determining the molecules by which MBV interact with target cells was, therefore, a critical starting point to begin to understand what cellular processes might be affected. Results of MBV uptake assays show that two inhibitors, Pitstop 2 and LY294002, significantly reduced MBV uptake with respect to uninhibited controls ($p < 0.001$ and $p < 0.01$, respectively, Figure 4). No significant changes in uptake were detected using other inhibitors.

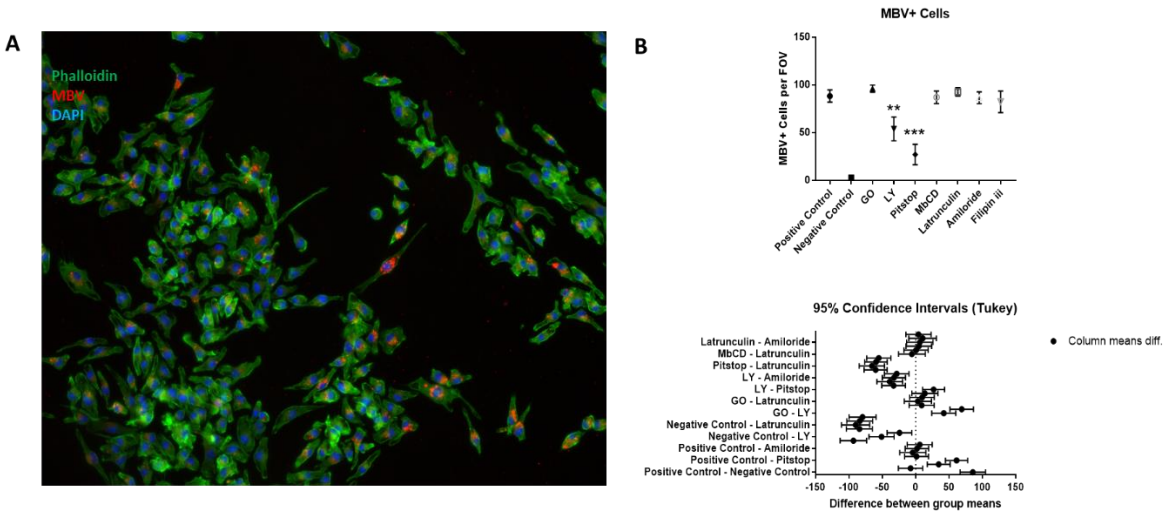

Figure 4. MBV uptake by macrophages is dependent upon clathrin and PI3K. (A) Representative 32x image showing fluorescently labeled MBV (red) within bone marrow-derived macrophages. **(B)** Quantification of three biological replicates of uptake experiments. (Top panel) Mean + SEM show that LY294002 (PI3K inhibitor) and PitStop2 (clathrin aggregation inhibitor) significantly reduce MBV uptake ($p < 0.01$ and $p < 0.001$, respectively). (Bottom panel) Post-hoc testing showing 95% CI about comparison means differences. (*** denotes $p < 0.001$, ** denotes $p < 0.01$ by one-way ANOVA followed by post-hoc testing, N = 3 biological replicates).

7.4.2 MBV-associated IL-33 Translocates to the Nucleus Promotes an M2-like Macrophage Phenotype, and Forms Protein-Protein Interactions

As a member of the IL-1 superfamily, IL-33 contains an N-terminus nuclear translocation sequence that promotes IL-33 localization to chromatin. Further, it has been suggested that IL-33 may have transcriptional repressor activity through interactions with NF-κB(V. Carriere et al., 2007). However, the behavior of MBV-associated IL-33 in target cells has not been characterized. Results of subcellular localization assays show that MBV are rapidly and non-destructively taken up by target cells, evidenced by detectable IL-33 in both cytoplasmic and nuclear compartments

by 1 hour after treatment. Cytoplasmic and nuclear concentration of delivered IL-33 peaked at 1 and 3 hours after treatment, respectively ($p < 0.05$, Figure 5B) and remained detectable by 24h.

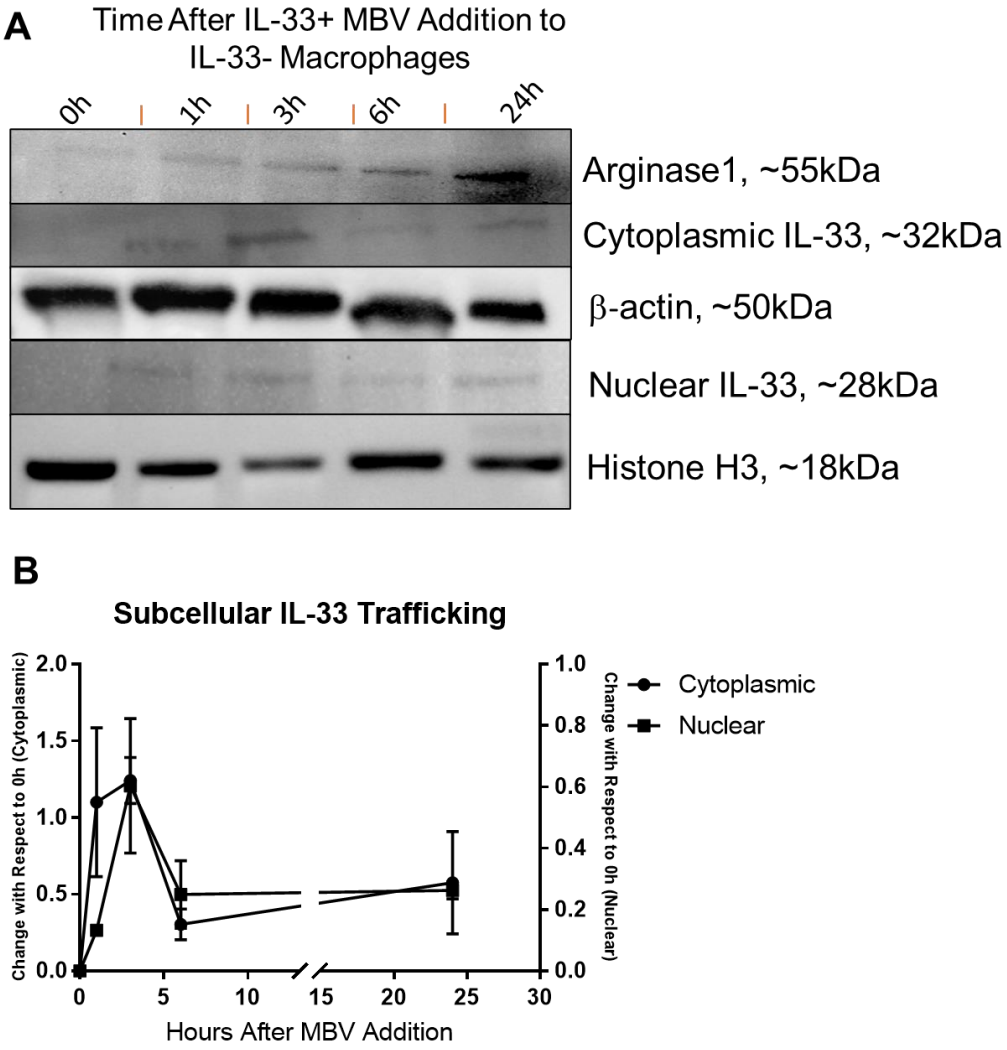

Figure 5. MBV-associated IL-33 is rapidly taken up by target cells and translocates to the nucleus. (A) Representative immunoblots of *il33-/-* bone marrow-derived macrophage cytosolic and nucleosolic fractions assessed for MBV-associated IL-33 by western immunoblotting and Arg1 expression. **(B)** Quantification of IL-33 in the cytoplasmic and nuclear compartments. Statistically significant increases in detected IL-33 by 3h with respect to 0h (data are presented as mean ± SEM, two-way ANOVA). (N = 3, * denotes $p < 0.05$ by two-way ANOVA).

As an important control, expression of Arg1, an M2-like macrophage phenotype marker, was measured as a function of time after treatment with MBV by immunoblotting. Results show that Arg1 expression increases with time after MBV (Figure 5A), reaching maximum expression by 24h. Further, results show that pretreatment of macrophages with IL-33^{+} MBV, but not IL-33^{-} MBV, reduced IFN-g and LPS-mediated iNOS expression and increased expression of Arg1, an M2-associated marker (Figure 6).

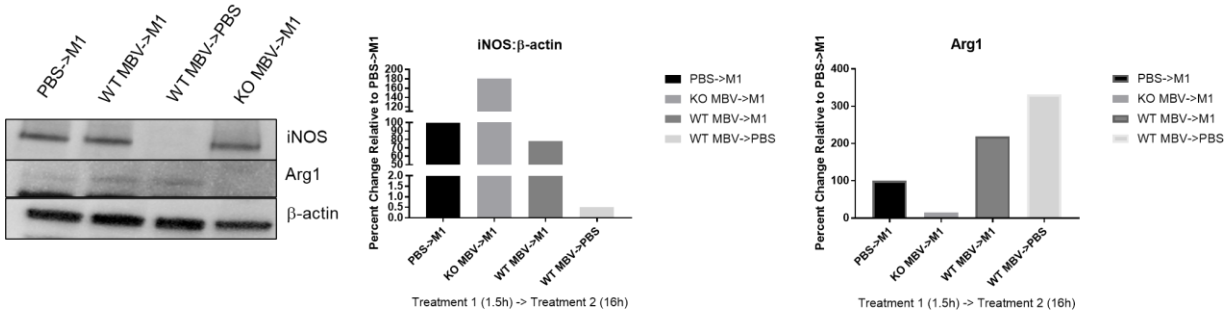

Figure 6. IL-33 is required for an M2-like macrophage phenotype in the presence of inflammatory cytokines.

Immunoblotting against iNOS, Arg1 and β-actin of il33+/+ macrophages exposures to PBS, IL-33+ MBV (WT MBV), or IL-33- MBV (KO MBV) for 1.5h followed by addition of IFNγ+LPS or PBS for 16h shows IL-33+ MBV promote reduced iNOS expression and increased Arg1 expression in the presence of inflammatory cytokines. (n=1, 3 pooled technical replicates).

In order to determine whether these IL-33 effects were mediated through interactions with intracellular transcription factors as has been suggested, co-immunoprecipitation of MBV-delivered IL-33 was performed. Results show that IL-33 undergoes direct protein interactions beginning at 3h in the nuclear compartment, and increasing protein associations were observed between hours 6-24 (Figure 7A).

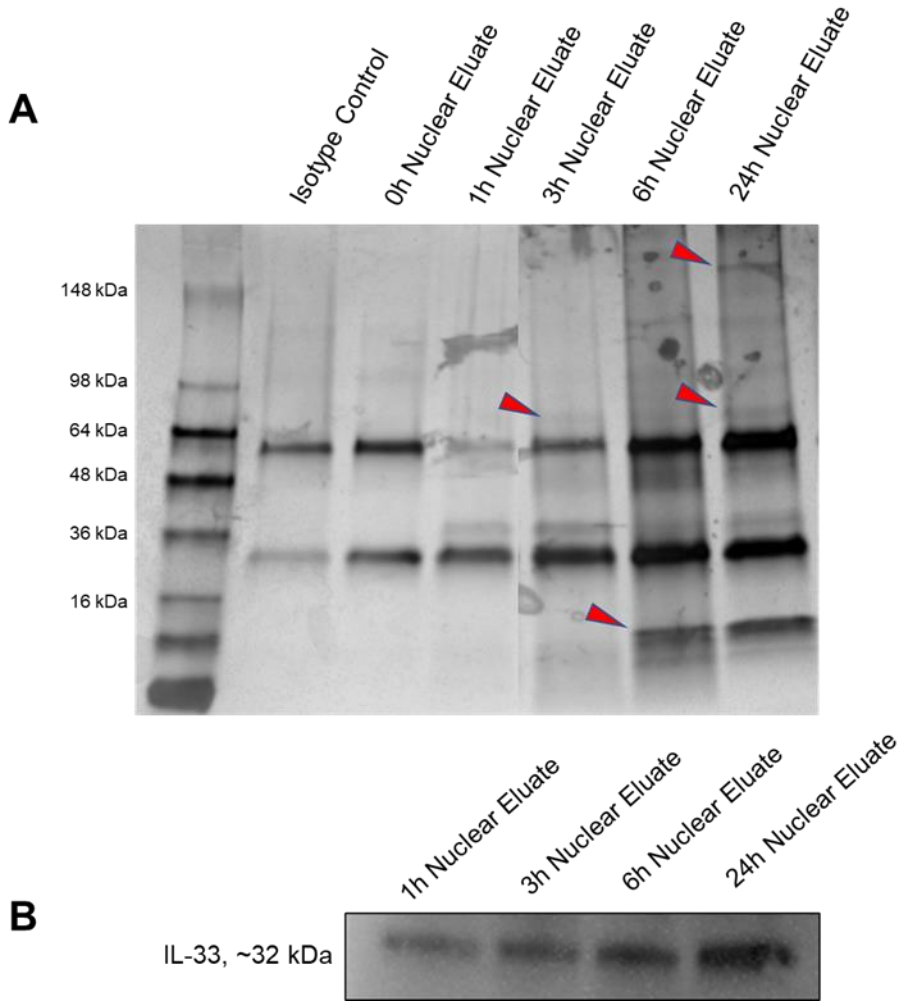

Figure 7. IL-33 interacts with target cell proteins. (A) Silver stain of proteins eluted from co-immunoprecipitation directed against IL-33 shows increasing complexity of interactions as a function of time (red arrowheads). **(B)** Confirmation of IL-33 presence in the eluted proteins.

7.5 Discussion

Modulation of immune cell phenotypes, in particular macrophages, is an established consequence of the use of ECM bioscaffolds, both in vitro and in vivo, and is predictive of downstream functional and constructive tissue remodeling(Brown, Londono, et al., 2012; J. L.

Dziki et al., 2016; L. Huleihel, J. L. Dziki, et al., 2017; K. Sadtler, K. Estrellas, et al., 2016). However, the mechanisms by which ECM bioscaffolds promote a pro-healing macrophage phenotype have remained poorly understood. Studies have shown that MBV play a significant role in ECM-mediated macrophage phenotype activation(L. Huleihel, J. G. Bartolacci, et al., 2017; G. S. Hussey et al., 2019; van der Merwe et al., 2019), and, more recently, that MBV-associated IL-33 may underly this bioactivity(G. S. Hussey et al., 2019). The present work shows that IL-33 rapidly enters target cells in a clathrin-dependent manner. Upon entering target macrophages, IL-33 forms direct interactions with other proteins, its intracellular distribution is dynamic, and may reduce pro-inflammatory cytokine-mediated iNOS expression. Stated differently, MBV-associated IL-33 promotes upregulation of M2-associated markers and may inhibit pro-inflammatory cytokine-mediated iNOS expression through protein-protein interactions in the nucleus.

The manner by which extracellular material enters cells in large part dictates its fate. Particulate taken up by phagocytosis, as is common for cellular debris after injury by macrophages, is rapidly degraded(Pauwels, Trost, Beyaert, & Hoffmann, 2017), while other pathways such as clathrin-mediated endocytosis, employed frequently following receptor activation, have more varied outcomes for internalized products(McKelvey et al., 2015). It was imperative to determine the mechanism by which MBV are taken up by target cells to subsequently postulate which cellular process(es) may be exploited for functional cargo delivery. It is logical, therefore, that endocytosis is the mechanism by which MBV enter target cells due to the potential for vesicle membrane fusion and preservation of protein function, as has been reported for exosomes(Svensson et al., 2013; Yao et al., 2018). Further, the role of PI3K in this process was unexpected, although not surprising, due to its known roles in modulating vesicular trafficking and internalization(Bhattacharya,

McElhanon, Gushchina, & Weisleder, 2016; Heath, Stahl, & Barbieri, 2003). The decision to choose 1 hour as the timepoint after which MBV were removed from cells was made on the observation that, in uninhibited cells, nearly 100% of cells had taken up at least one fluorescently labeled particle and would provide a reproducible rate against which to compare inhibited groups.

The current understanding of intracellular IL-33 behavior suggests that a dynamic balance of cytoplasmic and nuclear localization occurs in living cells. IL-33 has been reported to shuttle in and out of the nucleus through the nuclear pore complex and subsequently bind to euchromatin. However, within the cytoplasm, IL-33 has been shown to reside within secretory vesicles (R. Kakkar, H. Hei, S. Dobner, & R. T. Lee, 2012). Results of the present study show a rapid cellular uptake of MBV and associated cargo, with near simultaneous transport to the nucleus. Similar to uptake assays, MBV IL-33 cargo was detected within the nucleus and cytoplasm by 1h after treatment and remained detectable for the duration of the study period. It is of note that IL-33 levels in different cellular compartments was in a state of apparent flux, oscillating between peaks and nadirs from its maximum concentration in both compartments at 1h. MBV-associated IL-33 cargo is delivered in its functional, ~32 kDa form and promoted upregulation of Arg1, an M2-like macrophage phenotype marker. It is evident that the functional domains of IL-33 are preserved following incorporation into MBV, and future studies will be needed to determine the contribution of IL-33 subdomains, such as the N-terminus, on MBV-associated IL-33 intracellular trafficking. It is possible that Arg1 expression could be a result of internalization of extracellular material rather than the bioactivity of IL-33, however, this possibility is unlikely, as macrophages treated with IL-33^{+} MBV reduced IFN-g and LPS-induced iNOS expression, while IL-33^{-} augmented iNOS expression.

These findings are in accordance with previous studies in which it was shown that macrophages treated with MBV or parent ECM, following pre-treatment with LPS+IFNg, had lower expression of iNOS, an M1-like macrophage associated marker, and increased expression of M2-like macrophage associated markers, Arg1 and Fizz1(L. Huleihel, J. G. Bartolacci, et al., 2017; L. Huleihel, J. L. Dziki, et al., 2017). It has also been shown that when endothelial cells overexpressing IL-33 were treated with IL-1b, the production and secretion of TNF-a and IL-6, downstream of NF-kB signaling, were reduced. In these studies, IL-33 physically interacted with p65 on chromatin and reduced its ability to increase gene expression(V. Carriere et al., 2007). It is plausible, though untested, that p65 may represent one of the proteins co-eluted with IL-33. It is plausible, therefore, that IL-33, when delivered by MBV, may regulate the activity of p65, thereby reducing the expression of pro-inflammatory molecules.

Several studies suggest that IL-33 may promote changes in the epigenetic landscape of cells, either as a result of interaction with NFkB or indirectly through activation of ST2 signaling(Ali et al., 2011; Oshio et al., 2017; F. Zhang et al., 2014). Specifically, it was hypothesized that IL-33 may assist in the recruitment of, or alter the activity of, chromatin modifiers such as histone deacetylases or histone acetyltransferases (HDACs or HATs, respectively). Importantly, epigenetic landscape regulation is an emerging field of investigation with regards to macrophage polarization and has been shown, in particular, to play a role in macrophage phenotype transition from a pro-inflammatory M1-like macrophage towards a pro-healing M2-like macrophage phenotype(S. Chen, Yang, Wei, & Wei, 2020). Determination of the role of MBV-associated IL33 in regulating the epigenetic landscape of cells would be an important consideration for future investigations with significant implications for both tissue engineering and cytokine biology.

7.6 Conclusion

MBV and their cargo are immune active components of ECM bioscaffolds. MBV-associated IL-33 rapidly enters cells through an endocytic route that preserves its function and results in the promotion of an M2-like macrophage phenotype. Within target cells, IL-33 forms direct interactions with host cells molecules that may contribute to its immunomodulatory function. Further, IL-33 was required for MBV-mediated downregulation of iNOS expression induced by type 1 cytokines. These findings corroborate previously reported findings and offer mechanistic insight into ECM bioscaffold mediated immunomodulation in vitro.

8.0 Extracellular IL-33 Modulates Macrophage Phenotype in Response to Skeletal Muscle Injury

8.1 Overview

Injuries to skeletal muscle are among the most common injuries in civilian and military populations, accounting for nearly 60% of extremity injuries(Corona et al., 2015; J. Dziki et al., 2016; Grogan et al., 2011). During the wait period between injury and treatment, inflammation in the damaged tissues exacerbates the original injury, often leading to extensive fibrosis, increasing the possibility of necessary amputation. The standard of care for extremity injury has therefore been focused upon limb salvage procedures, utilizing grafts or orthotics in conjunction with rehabilitation in attempts to avoid amputation. Limb salvage approaches such as these do not address the underlying lack of muscle tissue and therefore, even with this standard of care treatment, patients are left with persistent strength and functional deficits that permanently impact their quality of life. However, results of clinical cohort studies have shown that restoration of contractile, skeletal muscle tissue and preservation of neuromuscular activity in volumetric muscle loss (VML) patients can be achieved by implantation of an extracellular matrix (ECM) biologic scaffold, which promotes endogenous stem/progenitor cell recruitment when combined with a targeted physical rehabilitation protocol (J. Dziki et al., 2016; Dziki et al., 2018). ECM-mediated skeletal muscle repair involves a beneficial spatiotemporal macrophage activation state switch corresponding to myogenic progenitor cell proliferation and differentiation, a process required for a switch from fibrosis to myogenesis (Tidball, 2005; Tidball & Villalta, 2010). Though the promise of regenerative rehabilitation protocols using ECM placement and physical therapy have reached

clinical cohort studies, to date only incremental improvements in overall patient population have been achieved. The present study addresses this shortcoming and aims to accelerate VML recovery by identifying a novel mechanistic driver behind the clinical use of ECM-based biomaterials: the immunomodulatory effects of ECM-based biomaterials and, more specifically, the effect of ECM subcomponents upon inflammation resolution. Results show nanosized vesicles embedded within ECM, matrix-bound nanovesicles (MBV) serve as a protected source of matrix-associated interleukin-33 (IL-33). MBV embedded in the ECM act as a stable and bioactive source of IL-33 which supports skeletal muscle regeneration by controlling local inflammation through ST2-independent macrophage activation towards a pro-remodeling phenotype. Genomic deletion of IL-33 profoundly alters the macrophage response to injury in an established mouse model of severe skeletal muscle injury. *il33*$^{-/-}$ mice showed a sustained pro-inflammatory macrophage response to injury that was absent in wild-type littermates, and culminated in impaired functional recovery (i.e. force generation) by 14 days post-injury. Importantly, exogenous provision of IL-33^{+} MBV successfully reduced the M1-like macrophage response to injury observed in untreated *il33*$^{-/-}$ mice, promoting an M2-like:M1-like macrophage phenotype ratio similar to wild-type animals. Taken together, these data suggest that MBV and their associated IL-33 cargo may represent a novel homeostatic signaling mechanism that contributes to skeletal muscle repair. Furthermore, IL-33-mediated events may represent an attractive therapeutic target for modulating macrophage phenotype following muscle injury.

8.2 Introduction

Skeletal muscle tissue inherently regenerates following injury(Appell, Forsberg, & Hollmann, 1988; Baghdadi & Tajbakhsh, 2018; Glass, 2003), a capacity that relies on the coordinated recruitment, activation, and differentiation of resident stem/progenitor cells(Tidball, 2005; Tidball & Villalta, 2010). Spatiotemporal control of this stem/progenitor cell activity is orchestrated in large part by several infiltrating immune cell types, which are themselves regulated by microenvironmental cues(Glass, 2003; Mammoto et al., 2013). The injury response begins with a robust infiltration of neutrophils within the first hours of injury, followed by the recruitment of pro-inflammatory, 'M1-like' macrophages. These pro-inflammatory macrophages promote expansion of the activated myoblast pool via the cytokine and chemokine effects of their secretome upon resident stem/progenitor cells(Novak et al., 2014; Tidball, 2005). These pro-inflammatory macrophages transition to a pro-regenerative, 'M2-like' macrophage phenotype between 4- and 14-days post-injury(Tidball, 2005). The secretome of the M2-like macrophages promotes myoblast differentiation into myotubes, in conjunction with the appearance of adaptive immune cells(B. Deng et al., 2012; J. L. Dziki et al., 2016; D. Ruffell et al., 2009; Tidball, 2005). It has been suggested that phagocytosis of apoptotic neutrophils may contribute to this macrophage phenotype transition, but a definitive mechanism for this process has not been convincingly established(Ariel & Serhan, 2012). The spatiotemporal switch from M1-like to M2-like macrophages is required for functional muscle repair(L. Arnold et al., 2007; Brown, Londono, et al., 2012), and a failure to transition has been associated with delayed healing(B. Deng et al., 2012).

The central role of macrophages in skeletal muscle healing is exemplified in the aberrant immune response observed following volumetric muscle loss (VML) injuries. The endogenous repair processes that underly skeletal muscle's characteristically regenerative response to injury

are overwhelmed in VML. Sustained pro-inflammatory signaling, a disrupted native extracellular matrix architecture, and reduced satellite cell availability all contribute to morbidity in VML patients. As a result, tissue engineering strategies for the repair of VML must, at a minimum, promote restoration of the skeletal muscle stem/progenitor cell niche and modulation of immune cell phenotypes. In particular, the M1-like to M2-like macrophage phenotype transition is notably absent, replaced instead by a persistent pro-inflammatory response that results in fibroadipogenic tissue deposition(Dziki et al., 2018; J. L. Dziki et al., 2016). It has been shown that the ratio of pro-inflammatory to pro-regenerative macrophages during the early post-injury phase is a predictor of clinical outcomes(Brown, Londono, et al., 2012). Preclinical studies show that acellular bioscaffolds composed of extracellular matrix (ECM) effectively promote an M1-like to M2-like macrophage phenotype transition in vivo with associated improvements in functional outcomes(J. L. Dziki et al., 2016; L. Huleihel, J. L. Dziki, et al., 2017; Huleihel et al., 2016; B. M. Sicari, J. L. Dziki, et al., 2014). Clinical cohort studies using ECM bioscaffolds to treat VML resulted in an average ~37% increase in force production and ~250% increase in functional activity(J. Dziki et al., 2016). Stated differently, ECM bioscaffolds promote endogenous functional repair of skeletal muscle and an associated robust immunomodulatory response through direct and indirect mechanisms.

Despite its known immunomodulatory properties, the bioactive component(s) within ECM that promote an M2-like pro-healing macrophage phenotype remain only partially understood. The topographical ligand landscape(Grasman et al., 2015), the release of growth factors and matricryptic peptides(Agrawal et al., 2010; Agrawal, Kelly, et al., 2011), and mechanical cues(Mammoto et al., 2013) have all been shown to play a role in ECM-mediated cell behavior. Recently, an extracellular vesicle subtype associated with the ECM, identified as matrix-bound

nanovesicles (MBV), was shown to recapitulate many of the immunomodulatory effects of the parent ECM on macrophage phenotype(L. Huleihel, J. G. Bartolacci, et al., 2017). MBV show tissue-specific profiles of miRNA, lipid, and protein cargo, many of which could plausibly contribute to the influence of MBV on target cell bioactivity(L. Huleihel, J. G. Bartolacci, et al., 2017; Huleihel et al., 2016; George S. Hussey et al., 2020; Saldin et al., 2019; van der Merwe et al., 2017). Specifically, MBV are a rich source of extracellular IL-33, an IL-1 superfamily member cytokine with known roles in skeletal muscle healing(G. S. Hussey et al., 2019; W. Kuswanto et al., 2016). Of note, MBV-associated IL-33 effects upon macrophage phenotype are independent of the ST2 receptor(G. S. Hussey et al., 2019), the canonical cognate receptor, suggesting an alternative intracellular signaling pathway with distinctive effects upon cell behavior.

The present study describes a role for MBV in ECM bioscaffold-mediated constructive tissue remodeling and homeostatic repair processes. Previous studies suggest that IL-33, signaling through the ST2 receptor, promote accumulation of regulatory T cells that contribute to endogenous repair processes(W. Kuswanto et al., 2016). However, results of the present work show that extracellular IL-33 plays a central role in modulation of the macrophage response during muscle healing. In vivo, genomic deletion of IL-33 profoundly altered the macrophage response and impaired functional recovery from acute muscle injury. Importantly, macrophage phenotype and functional recovery could be partially restored following exogenous delivery of IL-33^{+} MBV. Together, these data show that IL-33, an interleukin most commonly identified as a pro-inflammatory alarmin, can promote an anti-inflammatory macrophage phenotype that contributes to functional skeletal muscle repair when delivered in the form of MBV.

8.3 Materials and Methods

8.3.1 Animal Use

Congenic C57BL/6-*arg1*^{gfp} and C57BL/6-*il33*$^{-/-}$*arg1*^{gfp} mouse strains were raised in accordance with the University of Pittsburgh Institutional Animal Care and Use Committee guidelines. Colony maintenance, including bedding and cage changes, was provided by the University of Pittsburgh Division of Laboratory Animals in Research on a weekly basis. Litters were weaned at 21 days of age and intermittent genotyping of all breeding pairs was performed to maintain the integrity of the strains. Animals were used as experimental subjects or as a source of tissues and cells. For animals undergoing surgical manipulation, pain management was achieved using buprenorphine hydrochloride twice daily for 3 days and infection prevention was maintained for 3 days using Baytril (Henry Schein). Euthanasia was performed in accordance with guidelines using 20-30% v/v inhaled CO_2 for 8-10 minutes until signs of life were no longer detectable and death was ensured using cervical dislocation.

8.3.2 MBV Isolation and Quantification

Small intestine was isolated from experimentally naïve C57BL/6 and C57BL/6-*il33*$^{-/-}$ mice between 6-8 weeks of age. Contents were removed manually and the intestine cut into ~3 cm long segments, placed in phosphate buffered saline (PBS) (Sigma, St. Louis, MO) and placed on a rotating tube rack for 6h to remove remnant blood. The intestines were then removed and the

external muscular layers removed by manual scraping and placed in fresh PBS for 1h. PBS was then removed and the resulting tissue was subsequently digested overnight at 37^{o}C while rotating using 0.01 mg/mL solution of Liberase DL (Sigma) in a buffer consisting of 50 mM tris (pH 8), 5 mM $CaCl_2$, and 200 mM NaCl. Crude digest mixtures were then subjected to progressive centrifugation: 3x10 minutes at 500 xg, 3x20 minutes at 2500 xg, and 3x30 minutes at 10000 xg. Digests were then passed through a 0.22 mm syringe filter and ultracentrifugated at 100,000xg for 2h at 4 ^{o}C (Beckman Coulter Optima L-90K ultracentrifuge, Brea, CA). Following ultracentrifugation, supernatants were discarded and the pellet resuspended in 1 mL of particle-free PBS and further purified via size exclusion chromatography with a 10 cm column height and 1 mL fraction volume (Sepharose CL-2B beads, Sigma). Purified MBV, contained in fractions 3-5, were collected and concentrated using 100 kDa molecular weight cut-off spin columns (Millipore Sigma, Burlington, MA). Particle concentration of each sample was determined using a NanoSight particle counter equipped with nanoparticle tracking analysis (NTA, NanoSight, Salisbury, UK).

8.3.3 Cardiotoxin Injury Model

Cardiotoxin muscle injury model was used as previously described(Hardy et al., 2016). Briefly, a small incision was made in the skin overlying the tibia of anesthetized mice in the supine position. Blunt dissection of the adjacent subcutaneous region was used to expose the tibialis anterior (TA) muscles and 25 ml of 10 µM cardiotoxin (Naja Pallida, Sigma Aldrich) was administered to the left and right hindlimbs. The incision was subsequently closed using resorbable sutures and the animals allowed to recover.

8.3.4 Immune Cell Analysis by Flow Cytometry

Isolated infiltrating leukocytes were incubated with heat-inactivated goat-serum (5%) to block FcR, and then labelled with different combinations of fluorochrome-conjugated antibodies (BD Bioscience, Biolegend, eBioscience or MD Biosciences) to distinguish myeloid and T cell populations (CD45(30-F11), CD45.2(104), F4/80(BM8), CD8(53-6.7), CD38(90/CD38), CD4(RM4-5), Foxp3(FJK-16S), Ly6C(AL-21), B220(RA3-6B2), iNOS(CXNF7), CD206(MR5D3), CD3(17A2), CD11b(M1/70), MHCII(2G9), Ly6G(1A8), CD11c(N418), ST2(U29-93,DIH9), EGR2(erongr2), GFP(FM264G)). Data was acquired with an Aurora and LSRFortessa flow cytometer (BD, Bioscience) and analyzed using FlowJo, Version 10.1 (Tree Star).

8.3.5 Skeletal Muscle Immunolabeling

Harvesting of the tibialis anterior was performed at 3, 7, and 14-day post-injury. Each muscle was fixed in 10% neutral-buffered formalin and embedded in paraffin. 5 mm sections were cut and mounted onto glass slides. Slides were deparaffinized using xylene and ethanol gradients (100-70% EtOH). Slides were stained with Masson's trichrome staining or subjected to antigen retrieval and immunolabeling. Immunofluorescence was performed on serial sections for each subject and timepoint to assess the phenotypes of immune and satellite cell populations. After deparaffinization, the slides were placed in citrate antigen retrieval buffer (10 mM citric acid monohydrate, pH 6.0), microwaved at 100% power for 45 seconds, followed by 15 minutes at 20% power. The slides were then cooled in copper sulfate solution (10mM $CuSO_4$, 50mM ammonium acetate, pH 5.0) for 20 minutes. Sections were then rinsed three times in Tris buffered saline/Tween

20 solution (TBST) and then incubated for 1 hour at room temperature in blocking buffer containing 0.1% Triton-X 100, 0.1% Tween, 2% goat serum, and 1% bovine serum albumin. The blocking buffer was then removed and the sections were incubated overnight at 4^{o}C in a humidified chamber with 1:200 rabbit-anti-CD11b (Abcam), a pan-macrophage marker. Following overnight incubation, each slide was washed in TBST for 3x2 minutes. A 1:200 solution of goat-anti-rabbit horseradish peroxidase conjugated secondary antibody (DAKO) in blocking buffer was subsequently applied and microwaved at 40% power for 3 minutes in a humidified chamber and allowed to cool for 2 minutes before washing in TBST. After washing, sections were incubated with a 1:200 solution of red fluorescent HRP substrate (OPAL 570, Perkin Elmer) in 1x Amplification Diluent (Perkin Elmer) for 10 minutes and then washed in TBST. To remove anti-CD11b and anti-rabbit antibodies, sections were subjected to a second round of antigen retrieval in citrate antigen retrieval buffer, followed by cooling copper sulfate solution, and blocked as described above. For each slide, one section was incubated with a 1:200 solution of rabbit-anti-iNOS antibody (Invitrogen) in blocking buffer, and one section was incubated with a 1:200 solution of rabbit-anti-RELMa (PeproTech). Slides with the primary antibodies were then placed on a raised waterbath and microwaved at 40% power for 3 minutes, followed by 2 minutes of cooling. Slides were then washed in TBST solution and a 1:200 solution of goat-anti-rabbit HRP-conjugated secondary antibody was placed on the sections. Slides with secondary antibody solutions were then placed in the waterbath and microwaved at 40% power for 3 minutes, followed by 2 minutes of cooling. After cooling, slides were washed in TBST and a 1:200 solution of green fluorescent HRP substrate in 1x Amplification Diluent (520 Opal, Perkin Elmer) was placed over each section and incubated in a dark humidified chamber for 10 minutes at room temperature. The sections were then washed in TBST, incubated with 4′,6-diamidino-2-phenylindole (DAPI)

nuclear counterstain for 5 minutes. The sections were washed with TBST and subsequently mounted for imaging by fluorescence microscopy.

8.3.6 In Situ Contractile Testing

14 days post-cardiotoxin injury (POD-14), *in situ* contractile testing protocol was implemented to evaluate the muscle's force producing capacity, 14 days following injury(Sahu et al., 2018; C. Zhang et al., 2016). Contractile testing was performed using an *in situ* testing apparatus (Model 809B, Aurora Scientific Inc, Canada), stimulator (Model 701C, Aurora Scientific Inc, Canada), and force transducer (Aurora Scientific Inc, Canada). Animals were anesthetized with 2% isoflurane. Through a small incision lateral to the knee, the animal's peroneal nerve was isolated and exposed. The Achilles tendon was surgically cut using a scalpel prior to placing the animal supine on a 37ºC-heated platform. The foot being tested was taped to the footplate with a surgical cloth tape, with the ankle position at 20^{o} of plantarflexion (the position that we determined to result in the greatest force output). The needle electrodes were inserted beneath the skin, over the peroneal nerve. The single-twitch protocol was implemented to evaluate muscle cross-sectional area (CSA), muscle peak twitch, time to peak twitch, and half-relaxation time. Next, a force-frequency protocol was implemented by eliciting stimulations at 10, 30, 50, 80, 100, 120, 150 Hz, with a 2-minute rest between each contraction. Note that the output from the machine is torque (mN-m). Force was calculated by dividing the torque by the length of the foot plate (0.03 m). The mean CSA of the muscle was obtained using the formula: Mean CSA= (weight of TA in mg/ (length of TA in mm * density of the muscle)), where muscle density is assumed to be 1.06 g/cm^3. The specific force was then obtained by dividing the force by the mean CSA. The TA muscles were subsequently harvested and fixed in formalin for histological analysis.

8.3.7 Exogenous MBV Delivery

2 days post-cardiotoxin injury (POD-2), *il33*$^{-/-}$ mice were anesthetized using 2% inhaled isoflurane and the original incision used for CTX injection was reopened. 30 mL of IL-33^{+} MBV were gradually delivered to both hindlimb TAs intramuscularly while retracting the needle from distal to proximal tendon. Pressure was applied to prevent leakage and the skin was subsequently closed with resorbable sutures and the animals were allowed to recover under supervision on a heated surface.

8.3.8 Statistical Analysis

Dependent variables were assessed using one or two-way ANOVAs, or independent samples *t*-tests. Tukey's HSD post-hoc means comparisons were performed with an applied alpha of 0.05. Data are presented as means $\pm$ SEM unless otherwise specified. Statistical testing was performed using Graphpad Prism 8 (Graphpad, La Jolla, CA).

8.4 Results

8.4.1 Genomic Deletion of IL-33 Results in an Early Pro-Inflammatory Macrophage Response to Muscle Injury Associated with Reduced Functional Recovery from Injury

Cardiotoxin (CTX) injury of the anterior TA compartment musculature of mice between 6-8 weeks of age was used to induce a reproducible, regenerative muscle injury. The injured skeletal muscle of CTX-treated animals was harvested at sequential timepoints, namely post-

operative day (POD) 3, POD-7, and POD-14. The mononuclear immune cells present in injured muscle at these timepoints were separated by FACS. Results showed that the $CD45^{+}CD3^{-}B220^{-}CD11b^{+}Ly6G^{-}F480^{+}iNOS^{+}$ M1-like macrophage population was nearly 2x was greater in muscle tissue from *il33*$^{-/-}$ mice than *il33*$^{+/+}$ littermates at POD-3 (10% vs. 5%, respectively, $p \leq 0.01$). The macrophage infiltrate at POD-7 was also predominantly M1-like in *il33*$^{-/-}$ mice (~20%) compared to *il33*$^{+/+}$ mice (~15%), however these differences were not statistically significant (Figure 8). Notably, a population of $CD11b^{+}MHCII^{+}$, with variable expression of ST2, antigen presenting cells were absent macrophages (Figure 8), across two separate experiments, in *il33*$^{-/-}$ compared to *il33*$^{+/+}$ mice ($p \leq 0.01$). Finally, $CD45^{+}CD3^{+}B220^{-}CD4^{+}Ly6G^{-}FoxP3^{+}ST2^{-}$ T_{REG} cells were significantly less abundant in *il33*$^{-/-}$ mice than in *il33*$^{+/+}$ littermates at POD-3 (~2.5% vs. ~11%, $p < 0.05$).

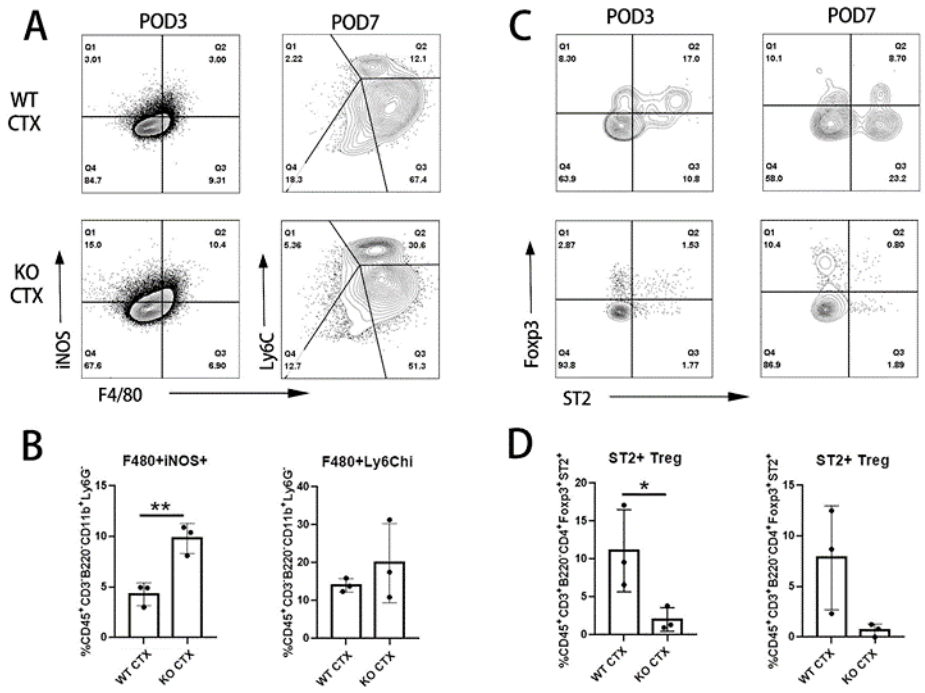

Figure 8. Genomic deletion of IL-33 promotes increased inflammatory macrophage presence and decreased ST2+ Treg accumulation. Cardiotoxin muscle injury surgeries were performed on IL-33 expressing Arg-1GFP (WT B6) or IL-33 deficient Arg-1GFP B6 (KO B6). On POD3 and POD7, injured TA muscles were harvested, and infiltrating leukocytes were assessed by flow cytometric analysis. **(A-B)** Representative dot plots and frequency for inflammatory macrophages in the CD45+CD3-B220-CD11b+Ly6G- gate. **(C-D)** Representative dot plots and frequency for ST2+ Treg in the CD45+CD3+B220-CD4+ gate. All p-values were calculated using one-way ANOVA. *P<0.05, **P<0.01, ***P<0.005, ****P<0.001.

Flow cytometry results were corroborated by immunolabeling in *situ*. Macrophages comprised ~5% of all cells within the tissue regardless of strain, however the phenotypic composition of the macrophage population was strikingly different. $CD11b^{+}Fizz1^{+}$ M2-like macrophages represented a greater percentage of macrophages at all timepoints in $il33^{+/+}$ mice compared to their $il33^{-/-}$ counterparts ($p < 0.001$, $p < 0.001$, and $p \leq 0.05$ for POD-3, POD-7, and POD-14, respectively). Further, the macrophage infiltrate in $il33^{-/-}$ mice consisted of

CD11b^{+}iNOS^{+} M1-like macrophages to a much greater extent than *il33*$^{+/+}$ mice at POD-3 and POD-7 ($p \leq 0.01$, and $p \leq 0.05$, respectively, Figure 9).

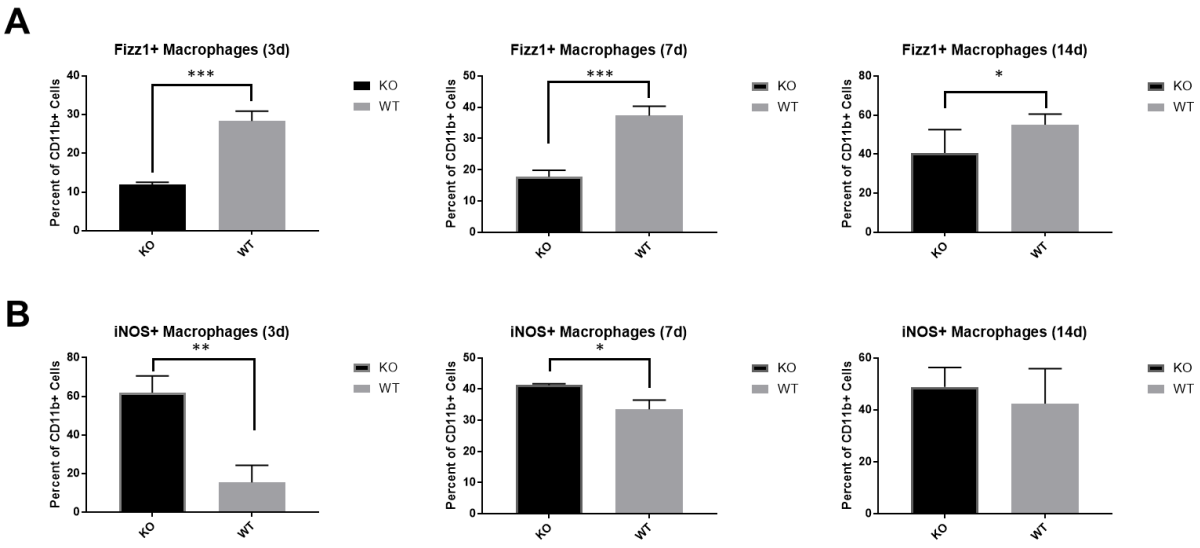

Figure 9. IL-33 deletion reduces M2-like:M1-like ratio at all timepoints. (A) Quantification of immunolabeling for CD11b+Fizz1+ M2-like macrophages at POD-3, 7, and 14 (N = 3 biological replicates for POD-3 and 7, N = 6 for POD-14, n > 5 images per replicate). **(B)** Quantification of CD11b+iNOS+ M1-like macrophages at POD-3, 7, and 14 (N = 3 biological replicates for POD-3 and 7, N = 6 for POD-14, $n \geq 3$ images per replicate). (*p<0.05, **p<0.01, ***p<0.001, p values were calculated using two-tail student's t-tests).

In situ contractile testing results show that loss of IL-33 severely impacts force generation after cardiotoxin injury, with *il33*$^{-/-}$ mice generating less specific and peak specific force ($p \leq 0.05$ and $p \leq 0.01$, respectively, Figure 10A-B). Further, the time to maximum contraction was shorter in *il33*$^{+/+}$ mice, with a similar trend in the half relaxation time, suggesting that the composition of regenerated fibers may be skewed toward Type 2A fast-twitch muscle fibers in *il33*$^{-/-}$ mice ($p \leq 0.01$, Figure 10C-D), rather than the Type 2B prevalent in the TA of wild-type animals. Unexpectedly, the physiologic CSA of *il33*$^{-/-}$ animal TAs was less than that measured in *il33*$^{+/+}$

littermates ($p \leq 0.01$). Taken together, these data show that depletion of IL-33 results in an inferior regenerative response compared to IL-33 containing animals.

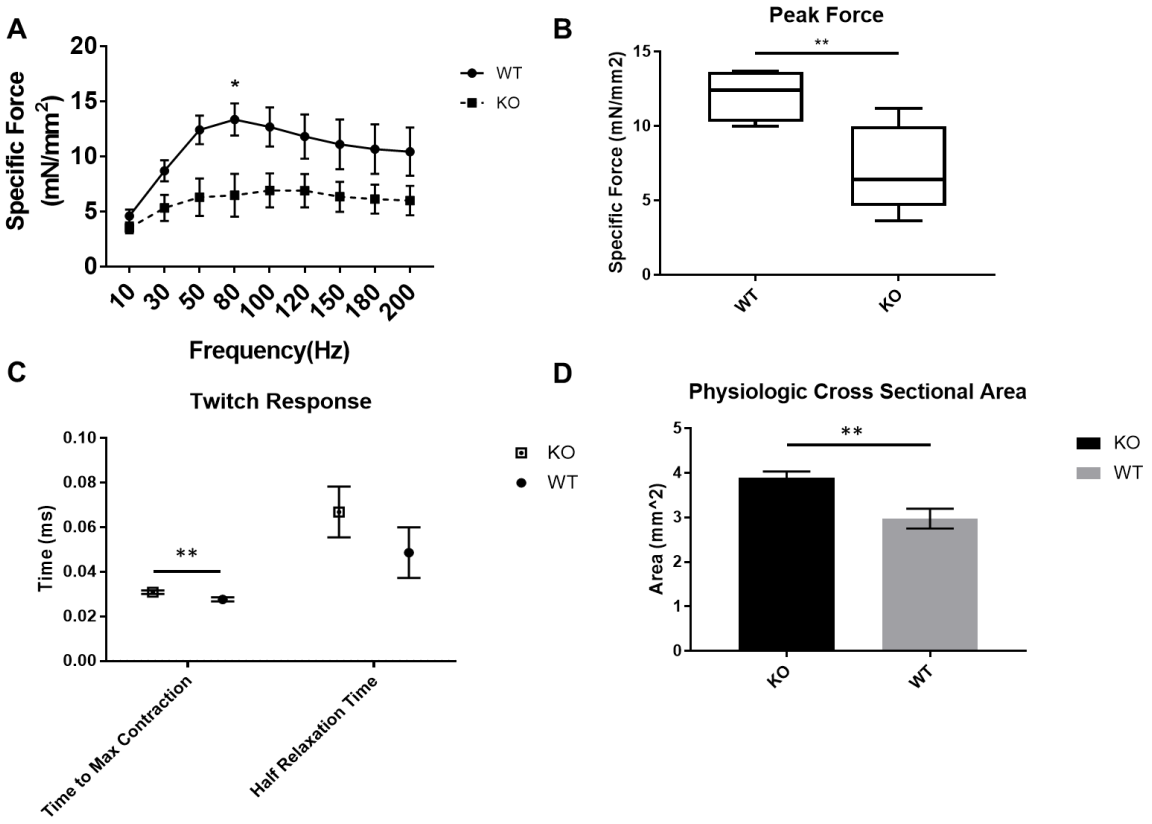

Figure 10. IL-33 is required for functional repair of injured skeletal muscle. (A-B) In situ contractile testing of TA tetanic force generation as a function of frequency at POD-14 post-cardiotoxin injury shows significant reductions in functional output in mice lacking IL-33 ($p<0.05$, $n = 6$). **(C-D)** Twitch response of TA muscles at POD-14 after muscle injury shows that healed muscle of *il33-/-* mice had significantly different properties compared to *il33+/+* littermates ($p < 0.01$, $n = 6$). **(D)** Comparison of the computed physiologic cross-sectional area of TA muscles of *il33-/-* vs *il33+/+* mice ($p < 0.01$, $n = 6$). * $p < 0.05$, ** $p < 0.01$, Force-Frequency analysis was analyzed by repeated measures two-way ANOVA with post-hoc testing, all other comparisons were performed using independent samples *t*-tests.

8.4.2 Exogenous Provision of MBV-Associated IL-33 Reduces M1-like Macrophage Response to Muscle Injury, Improves Function, and Normalizes Physiologic Cross-Sectional Area

To determine if MBV and their associated cargo play a role in modulation of macrophage phenotype in vivo, IL-33^{+} MBV were intramuscularly administered to the tibialis anterior muscles of cardiotoxin-injured *il33*$^{-/-}$ mice on POD-2. Results of macrophage immunolabeling show that injection of 5E10 MBV resulted in a peak specific force that was increased compared to their untreated *il33*$^{-/-}$ counterparts ($p \leq 0.05$) by POD-14 (Figure 11A). Twitch response analysis revealed that the time to half relaxation and physiologic cross-sectional area were significantly reduced in treated animals compared to untreated *il33*$^{-/-}$ littermates ($p < 0.0001$ and $p < 0.01$, respectively, Figure 11B).

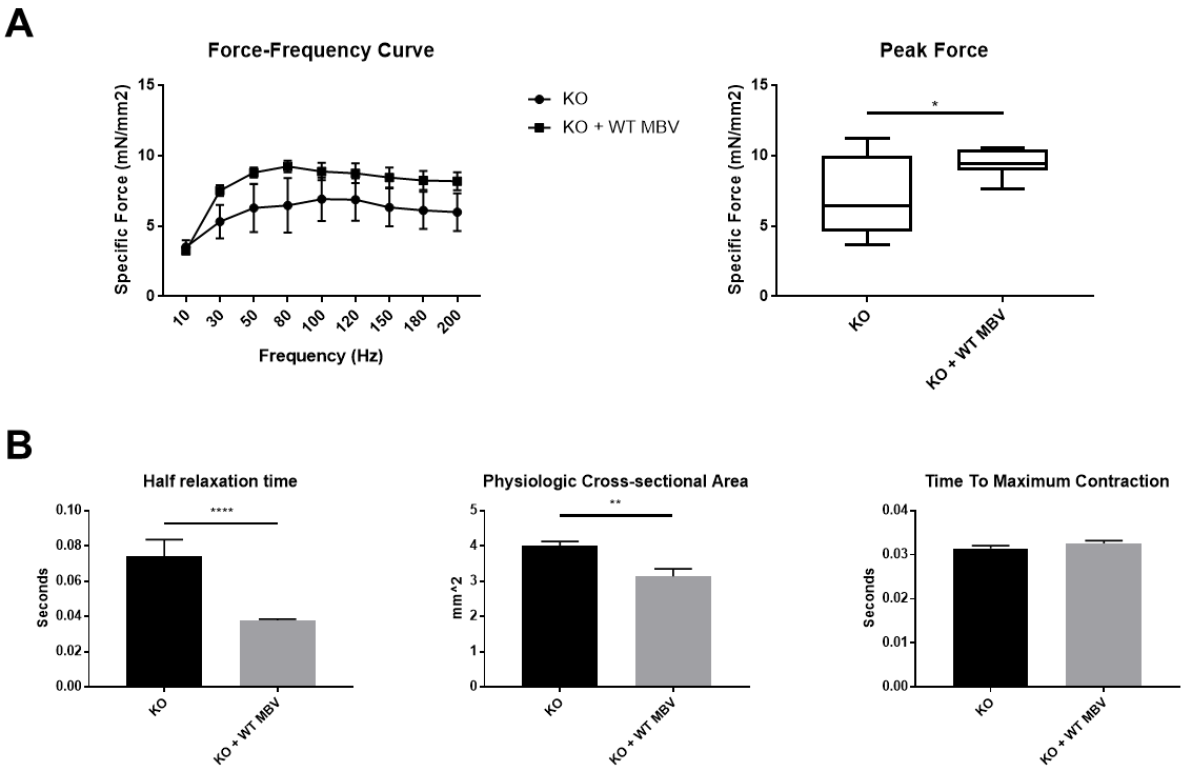

Figure 11. Exogenous provision of IL-33+ MBV improves functional recovery. (A) In situ contractile testing of TA tetanic force generation at POD-14 post-cardiotoxin injury shows improved peak force production following exogenous provision of IL-33+ MBV compared to untreated counterparts ($p \leq 0.05$, n = 5 (untreated *il33-/-*) and n = 6 (IL-33$^+$ MBV-treated animals, one-tailed *t*-test) **(B)** Twitch response of TA muscles at POD-14 after muscle injury shows that healed muscle of untreated *il33-/-* mice and *il33-/-* mice treated with IL-33+ MBV had significantly different half relaxation times and physiologic cross-sectional areas ($p < 0.0001$ and $p < 0.01$, respectively, one-tail *t*-test). **** $p < 0.0001$, ** $p < 0.01$, *$p < 0.05$.

Upon completion of the functional testing regimen, TA muscles used for in situ contractile testing were harvested and used for immunolabeling of macrophage infiltrate, activated satellite cells and FoxP3 T_{REG} cells. Results show that IL-33$^+$ MBV delivery results in significantly more macrophages overall and fewer CD11b$^+$iNOS$^+$ M1-like macrophages than in untreated *il33*$^{-/-}$ mice

($p \leq 0.01$ and $p \leq 0.05$, respectively). Finally, there were significantly fewer $CD11b^{+}Fizz1^{+}$ macrophages in IL-33^{+} MBV-treated animals compared to *il33*$^{+/+}$ mice ($p \leq 0.05$, Figure 12).

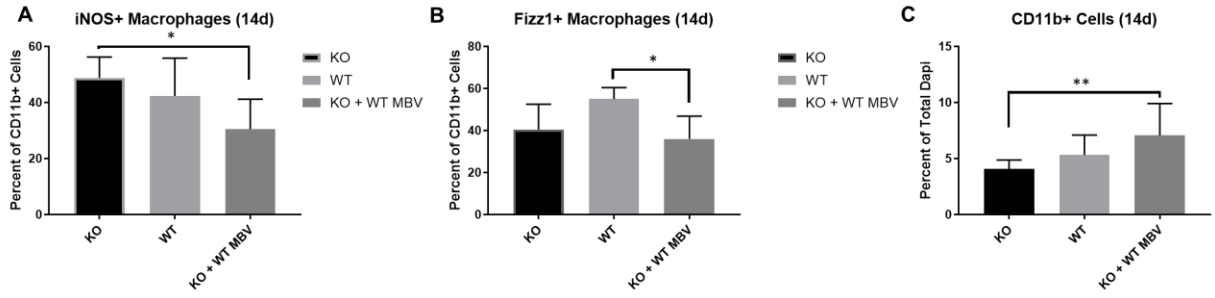

Figure 12. Exogenously delivered IL-33+ MBV increased total macrophages and partially normalize M2:M1 ratio. (A) Quantification of immunolabeling against CD11b+iNOS+ M1-like macrophage population shows significant reductions in total iNOS+ macrophages as a result of MBV administration (N = 6, one-way ANOVA). **(B)** Quantification of immunolabeling against CD11b+Fizz1+ M2-like macrophage population shows MBV do not increase total Fizz1+ cells (N = 6, one-way ANOVA). **(C)** Total CD11b+ macrophages were significantly elevated following MBV administration (N = 6, one-way ANOVA). **p < 0.01, *p < 0.05.

8.5 Discussion

IL-33 has long been considered to be a nuclear cytokine that functions as an alarmin upon necrotic cell death(Cayrol & Girard, 2014). Following loss of cell integrity, IL-33 is released to the extracellular space where it is cleaved by proteases to produce active soluble IL-33 that complexes with the ST2 receptor on infiltrating immune cells(Cayrol et al., 2018). In turn, MyD88 and p65 are activated with pro-inflammatory consequences(Milovanovic et al., 2012). The IL-33/ST2 axis is essential for the recruitment and activation of T_{REG} cells in skeletal muscle

healing(W. Kuswanto et al., 2016); however, recent reports show that MBV-associated IL-33 may mediate macrophage phenotype through an as yet undetermined mechanism(G. S. Hussey et al., 2019). Results of the present study suggest that MBV-associated IL-33 promotes a marked reduction in pro-inflammatory macrophage accumulation in vivo, which has clear implications for skeletal muscle repair.

In vivo results show that genomic deletion of IL-33 is associated with a profound pro-inflammatory state following skeletal muscle injury, especially in the acute post-injury phase. Notably, the *il33*$^{-/-}$ macrophage response to injury was comprised predominantly of pro-inflammatory M1-like macrophages, with lesser numbers of pro-reconstructive M2-like macrophages than their *il33*$^{+/+}$ littermates. As a consequence of this M2-like:M1-like distribution imbalance, *il33*$^{-/-}$ mice showed dysregulated satellite cell activation, culminating in reduced functional recovery from injury by POD-14. Moreover, twitch responses revealed faster times to maximum contraction in IL-33 deficient mice, suggesting enrichment in Type 2A fibers rather than the Type 2B fibers prevalent in wild-type mouse TA (Kammoun, Cassar-Malek, Meunier, & Picard, 2014). These results suggest that macrophages not only interact differentially with satellite cells depending on their phenotype, but that appropriate macrophage phenotype transition is necessary for site-appropriate skeletal muscle fiber subtyping following injury. Results of MBV-treated animals also suggest that suppression of the pro-inflammatory macrophage population alone is not sufficient to fully recapitulate the wild-type healing response, and promotion of an M2-like phenotype may be required for complete restoration of muscle function.

Although ECM bioscaffolds have been repeatedly shown to promote an M2-like macrophage response in mouse models of muscle injury(J. L. Dziki et al., 2016; Dziki, Wang, et al., 2017; K. Sadtler, K. Estrellas, et al., 2016; Wolf et al., 2019), the present study offers the first

evidence that a single component of the ECM can modulate macrophage phenotype in vivo independent of the parent biomaterial. These data strongly suggest that multiple mechanisms of action exist for IL-33 in the context of muscle healing, namely, an ST2-independent effect on macrophage phenotype and a previously reported ST2-dependent mechanism on T_{REG} cells (W. Kuswanto et al., 2016; Panduro et al., 2018). Evidence for an ST2-independent, direct effect on macrophage phenotype stems from the observation that the $CD11b^{+}iNOS^{+}$ macrophage population was reduced in the IL-33^{+} MBV-treated animals compared to untreated counterparts. Further, MBV treatment resulted in an increased recruitment of macrophages overall, suggesting that macrophages are a primary target of MBV, and their cargo have an impact on the phenotype of target cells.

Previous work has shown that macrophage phenotype is plastic and can transition from M1-like macrophages toward an M2-like macrophage phenotype in vitro upon stimulation with MBV(Dziki, Wang, et al., 2017; L. Huleihel, J. L. Dziki, et al., 2017; H. Liu et al., 2015), however the bioactive cargo responsible for this effect remained unidentified. The present study shows that a single MBV cargo component, IL-33, can modulate macrophage phenotype in vivo. The regulatory role of T_{REG} cells is likely synergistic with the functions of MBV-associated IL-33 observed in the present work, although given the effect that MBV and their cargo play at early timepoints following injury and the low frequency of T_{REG} cells until ~7-14 days, it is plausible that macrophages initiate remodeling. MBV were delivered two days after injury because previous studies showed a robust macrophage infiltration at this time and these M1-like, pro-inflammatory macrophages would have had an opportunity to promote expansion of the MuSC pool. The dose of 5E10 MBV/muscle was based on the observation that 1E9 MBV/mL was sufficient to activate

2E6 macrophages in vitro, but optimization of delivery dose and dose regimen will be important avenues of future work.

In summary, findings of the present study show the critical role for IL-33 in skeletal muscle healing. IL-33, contained within MBV, is required for the promotion of a constructive macrophage phenotype via an ST2-independent mechanism. Changes in macrophage phenotype at POD-2 were predictive of functional tissue remodeling outcomes at POD-14 and the healing deficiencies noted in *il33*$^{-/-}$ mice could be rescued by provision of IL-33-containing MBV. Taken together, these data suggest MBV and their IL-33 cargo are required for ECM-mediated immunomodulation and functional tissue remodeling.

8.6 Conclusion

The results of the present work substantiate the relevance and importance of IL-33 signaling in MBV-mediated, and by extension ECM bioscaffold-mediated, macrophage phenotype modulation in the context of skeletal muscle healing. Further, this work demonstrates that modulation of macrophage phenotypes in response to skeletal muscle injury is a viable approach that may narrow the gap between existing therapies and complete skeletal muscle regeneration with important implications for cytokine signaling and offers new avenues of investigation for tissue engineering and regenerative medicine approaches to volumetric muscle loss in clinical practice.

8.7 Acknowledgements

The author gratefully acknowledges the work of Lori Walton and Julia Hart for histology.

9.0 Summary of Milestones and Future Directions

The central hypothesis addressed in this thesis is that MBV-associated IL-33 promotes a macrophage phenotype transition that supports constructive remodeling of skeletal muscle tissue by a non-canonical mechanism. The following milestones were achieved:

Milestone 1: The macrophage phenotype and muscle stem cell response to IL-33 was determined.

Summary Milestone 1: ECM bioscaffolds treatment of skeletal muscle injury promotes a pro-inflammatory to pro-healing macrophage phenotype transition, which is notably absent in untreated cases of critically-sized injuries like volumetric muscle loss, and this switch is subsequently followed by an increase in functional tissue remodeling and functional output. The mechanism(s) underlying this macrophage phenotype transition, however, are poorly understood. Recent studies have shown that MBV recapitulate the effects of whole ECM on macrophage phenotype, but a causal mechanism for this effect was not described. Chapter 7 clearly shows that IL-33, bound within MBV, are central to MBV-mediated macrophage phenotype activation and for the secretion of pro-myogenic molecules, including macrophage-derived exosomes.

Future Directions Milestone 1: The present study was incapable of testing the effects of ECM deficient in MBV due to the inability to extract MBV from the parent ECM bioscaffold without fundamentally destroying the integrity of the material. As such, investigations of MBV biogenesis may offer new insights that could allow for the production of MBV-depleted ECM in order to test the contribution of MBV to whole ECM bioscaffold-mediated macrophage phenotype activation and constructive tissue remodeling. Further, the macrophages and muscle stem cells

used in these investigations were all derived from young mice. It is possible that MBV, whose cargo may also change as a function of aging, elicit a different response in cells derived from older animals. Interrogation of the role of MBV in aging may offer new mechanistic insights to reduced muscle healing and sarcopenia of aging.

Milestone 2: The mechanism of MBV uptake by macrophages and the subcellular localization of MBV-associated IL-33.

Summary Milestone 2: ECM-mediated constructive tissue remodeling has been associated with a modulation of macrophage phenotype at early time points and a growing body of work suggests that MBV contribute to this process. In general, MBV treatment promotes a pro-remodeling, M2-like macrophage phenotype and suppression of pro-inflammatory markers as evidenced through gene expression, surface marker and protein expression, and functional activity (Appendix 10.1,10.2). However, the molecules and mechanisms underlying this effect have not been described. Chapter 7 and 8 offer show that IL-33 is required for the promotion of an MBV-mediated M2-like macrophage phenotype, in an ST2-independent manner, through endocytic delivery of functional IL-33, which forms protein-protein interactions in the nucleus and modulates gene expression as a function of time.

Future Directions Milestone 2: An endocytic uptake mechanism is commonly utilized by ligand-activated receptors. Studies have also shown proteinase K treatment of MBV to remove surface antigens, reduces uptake by macrophages (manuscript under review). It is plausible, then, that molecules on the lipid membrane of MBV mediate target cell specificity and non-destructive uptake. It is also possible that molecules on the surface of MBV mediate endocytic vacuole release. Future investigations should focus on identification of the surface antigens on MBV that mediate

this process, identification of macrophage receptors that may mediate their uptake, and optimize isolation methods for the preservation of MBV surface antigens. Further, results shown in Chapter 7 suggest that MuSC are not a target of MBV, but this work did not quantify MBV uptake by these cells. Future experiments should focus on characterizing the cell types for which MBV have an affinity, for example through the use of co-culture systems. Results of Chapter 8 also suggest that, once inside target cells, IL-33 translocates to the nucleus where it forms protein-protein interactions. Characterization of these intracellular binding partners would help further understanding IL-33 biology and could provide new avenues for therapeutic intervention to modulate immune cell function.

Milestone 3: The effect of IL-33 deletion on macrophage phenotype and functional recovery in a mouse model of acute skeletal muscle injury, and the ability of MBV to modulate macrophage phenotype in vivo were determined.

Summary Milestone 3: The use of ECM bioscaffolds in preclinical animal studies and in clinical cohort studies shows that their application supports a macrophage phenotype transition that is critical to downstream functional remodeling outcomes, but the component of the ECM that mediates this effect is poorly understood. Chapter 9 shows that IL-33 deletion results in a profoundly altered macrophage response to skeletal muscle injury at early timepoints and impairs functional muscle recovery. These events were also associated with a reduced accumulation of FoxP3^{+} T_{REG} cells, in accordance with previous studies. Further, it is shown that exogenous provision of IL-33^{+} MBV reduce pro-inflammatory macrophage accumulation and improve functional recovery by 14d post-cardiotoxin injury compared to untreated *il33*$^{-/-}$ counterparts. These data show that MBV and their associated IL-33 cargo are a critical extracellular mediator

of macrophage phenotype and provides mechanistic insight to whole ECM bioscaffold-mediated macrophage phenotype transition.

Future Directions Milestone 3: Results of the present study show that MBV and their cargos reduce the expression of M1-associated marker, iNOS, and improve functional recovery in vivo. These findings suggest that MBV may be a viable therapeutic intervention for inflammatory-driven pathology such as psoriasis. Of note, it is interesting that a single dose of MBV resulted in a sustained reduction in M1-like macrophages up to 12 days. It is plausible, though untested, that this sustained phenotypic modulation could be due to MBV activity on a monocyte progenitor cell, or that the macrophage epigenetic landscape has been altered in response to MBV treatment. Further study is needed to determine an optimal dose and dose regimen, as well as the in vivo cellular targets and tissue distribution of exogenously delivered MBV. Further, it is now well accepted that a single macrophage phenotype marker may not fully capture the details of the macrophage activation state, and a growing body of literature suggests that macrophages, in response to skeletal muscle injury, do not fall into the traditional M1-like or M2-like macrophage classifications and are instead a unique subpopulation. Future studies should make use of in vivo single cell RNA-sequencing analysis to characterize MBV-treated macrophages in greater detail.

Appendix A : Matrix-bound nanovesicles recapitulate Extracellular Matrix effects on macrophage phenotype[2]

Appendix A.1 Overview

The early macrophage response to biomaterials has been shown to be a critical and predictive determinant of downstream outcomes. When properly prepared, bioscaffolds composed of mammalian extracellular matrix (ECM) have been shown to promote a transition in macrophage behavior from a proinflammatory to a regulatory/anti-inflammatory phenotype, which in turn has been associated with constructive and functional tissue repair. The mechanism by which ECM bioscaffolds promote this phenotypic transition, however, is poorly understood. The present study shows that matrix-bound nanovesicles (MBV), a component of ECM bioscaffolds, are capable of recapitulating the macrophage activation effects of the ECM bioscaffold from which they are derived. MBV isolated from two different source tissues, porcine urinary bladder and small intestinal submucosa, were found to be enriched in miRNA125b-5p, 143-3p, and 145-5p. Inhibition of these miRNAs within macrophages was associated with a gene and protein expression profile more consistent with a proinflammatory rather than an anti-inflammatory/regulatory

[2] Portions of this chapter were adapted from the following publication:

Huleihel L**, Bartolacci J**, Dziki J, Vorobyov T, Arnold B, Scarritt M, Pineda Molina C, LoPresti S, Brown B, Naranjo JD, Badylak SF. Matrix-bound nanovesicles recapitulate extracellular matrix effects on macrophage phenotype. *Tissue Engineering Part A.* November 2017.

phenotype. MBV and their associated miRNA cargo appear to play a significant role in mediating the effects of ECM bioscaffolds on macrophage phenotype.

Appendix A.2 Introduction

Biologic scaffolds composed of mammalian extracellular matrix (ECM) have been used in a variety of anatomic sites including the gastrointestinal tract(T. J. Keane et al., 2016), body wall(Alicuben & DeMeester, 2014), and cardiovascular system(Seif-Naraghi et al., 2013), among others, to promote the formation of site appropriate, functional tissue following injury. These bioscaffold materials influence the default tissue healing response by mitigating inflammation and scar tissue formation(J. L. Dziki et al., 2016; T. J. Keane et al., 2016), promoting an accumulation of endogenous stem/progenitor cells at the site of scaffold placement(Agrawal et al., 2012; Beattie et al., 2009), and perhaps most importantly, modulating the local innate and adaptive immune response(Brown, Londono, et al., 2012; K. Sadtler, K. Estrellas, et al., 2016; B. M. Sicari, J. L. Dziki, et al., 2014). Specifically, implantation of ECM bioscaffolds has been shown to enhance the ratio of anti-inflammatory/regulatory (M2-like) macrophages to pro-inflammatory (M1-like) macrophages at the site of implantation(K. Sadtler, K. Estrellas, et al., 2016). This effect upon macrophage phenotype has been positively correlated with constructive and functional tissue remodeling outcomes in animal models of soft tissue repair(Brown, Londono, et al., 2012; Valentin et al., 2009).

Macrophage phenotype plasticity is well established(Mantovani et al., 2004) and the importance of, in fact necessity of, a transition from a pro-inflammatory to an anti-inflammatory/regulatory phenotype for normal functional tissue repair has been shown in many

different body systems(L. Arnold et al., 2007; Daley, Brancato, Thomay, Reichner, & Albina, 2010; Mahdavian Delavary et al., 2011; M. Nahrendorf et al., 2007). However, the intercellular and intracellular signaling mechanisms responsible for macrophage phenotype transition in normal wound healing, tissue homeostasis and development, and biomaterial-mediated tissue repair are not fully understood. Reasonable explanations for cell-matrix interactions and the associated effects upon cell behavior include integrin-mediated responses to the topographical ligand landscape(Mammoto et al., 2013; Ruoslahti, 1996), mechanical cues(Bissell & Aggeler, 1987; T. W. Gilbert, Stolz, Biancaniello, Simmons-Byrd, & Badylak, 2005), and release of embedded growth factors/cytokines/cryptic peptides from the bioscaffold(Agrawal, Tottey, et al., 2011; Reing et al., 2010). While these factors are all suspected to contribute to the bioactivity attributed to the ECM, a specific mechanism by which ECM promotes a constructive macrophage phenotype has not been established. Recently, the presence of matrix-bound nanovesicles (MBV) within ECM bioscaffolds has been reported(Huleihel et al., 2016), and the miRNA cargo within these MBV has been associated with essential biologic processes such as normal tissue and organ development, inflammation, and immune cell regulation, among others(Adammek et al., 2013; Chaudhuri et al., 2011; Cordes et al., 2009; Y. S. Lee, Kim, Chung, Kim, & Dutta, 2005; Parisi et al., 2016; Rangrez, Massy, Metzinger-Le Meuth, & Metzinger, 2011; Xin et al., 2009). Three miRNA that are preferentially overexpressed in M2-like macrophages(Y. Zhang, Zhang, Zhong, Suo, & Lv, 2013) were chosen as targets in the present study and these miRNA have been shown to play a role in macrophage functions such as phagocytosis, NO production, and alteration of their secretome(Banerjee et al., 2013). The present study investigates the role of MBV in ECM bioscaffold-mediated macrophage activation.

Appendix A.3 Materials and Methods

Appendix A.3.1 Chemicals and Reagents

Pepsin (MP Biomedicals, Santa Ana, CA), collagenase from Clostridium histolyticum (Sigma-Aldrich, St. Louis, Missouri), proteinase-K, and RNase A (Thermo Fisher Scientific, Waltham, MA) were confirmed by transmission electron microscopy (TEM) to be free of contaminating extracellular vesicles.

Appendix A.3.2 ECM Bioscaffolds Production

Urinary bladder matrix (UBM) was prepared from market-weight pigs (Tissue Source; LLC, Lafayette, IN) as previously described(B. M. Sicari, J. L. Dziki, et al., 2014). Briefly, the tunica serosa, muscularis externa, submucosa, and muscularis mucosa were removed by mechanical delamination, and the urothelial cells of the tunica mucosa were dissociated from the basement membrane by washing with deionized water. The remaining basement membrane and the lamina propria (collectively referred to as UBM) were decellularized by agitation in 0.1% peracetic acid with 4% ethanol for 2 h at 300 rpm followed by phosphate-buffered saline (PBS) and type 1 water washes. UBM was then lyophilized and milled using a Wiley Mill with a #60 mesh screen. Preparation of small intestinal submucosa (SIS) bioscaffold has been previously described(Lantz, Badylak, Coffey, Geddes, & Blevins, 1990). Briefly, jejunum was harvested from market-weight pigs (Tissue Source; LLC). The superficial layers of the tunica mucosa, the tunica serosa, and tunica muscularis externa were mechanically removed after the jejunum was split longitudinally. The tunica submucosa, muscularis mucosa, and basilar portion of the tunica mucosa

(stratum compactum) remain intact (collectively referred to as SIS). The tissue was agitated in 0.1% peracetic acid with 4% ethanol for 2 h at 300 rpm and then extensively rinsed with PBS and sterile water. The SIS was then lyophilized and milled using a Wiley Mill with a #60 mesh screen.

Appendix A.3.3 Enzymatic Digestion of ECM Samples

Enzymatic digestion was performed by treating each sample (100 mg dry weight) with either proteinase K or collagenase (0.1 mg/mL) for 24 h at room temperature in 50 mM Tris (pH 8), 5 mM CaCl2, and 200 mM NaCl buffer. Pepsin (1 mg/mL) digestion was performed in 0.01 M HCl solution for 24 h in room temperature. Before addition of ECM, all enzymatic solutions were passed through a 0.22-μm filter (Millipore, Oak Brook, IL).

Appendix A.3.4 MBV Isolation

MBV were isolated as previously described175. Collagenase was used for isolating MBV that were used for treating cells. Proteinase-K was used for isolating MBV that were used for RNA isolation or for visualization by TEM. Enzymatically digested ECM was subjected to successive centrifugations at 500 g (10 min), 2500 g (20 min), and 10,000 g (30 min). Supernatant was then centrifuged at 100,000 g (Beckman Coulter Optima L-90K ultracentrifuge, Brea, CA) at 4°C for 70 min. Pellets were washed and suspended in 500 μL of PBS and passed through a 0.22-μm filter (Millipore).

Appendix A.3.5 MBV Imaging

TEM imaging was conducted on MBV loaded on carbon-coated grids and fixed in 1% uranyl acetate. Grids were imaged at 80 kV with a JEOL 1210 microscope.

Appendix A.3.6 RNA Isolation

RNA was isolated from 2×10^6 cells using the miRNeasy Mini Kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. Reverse transcription of 500 ng of RNA to cDNA was performed via a high-capacity RT kit (ABI, Foster City, CA) according to the manufacturer's instructions. RNA was isolated from, at a minimum, 50 μL of MBV using the SeraMir Kit (System Biosciences) according to the manufacturer's instructions. Before RNA isolation, MBV samples were treated with RNase A (10 μg/mL) (Applied Biosystems, Palo Alto, CA) at 37°C for 30 min to degrade any contaminating free RNA that may have remained as a result of the tissue decellularization process. RNA concentration was determined using a NanoDrop spectrophotometer (NanoDrop, Wilmington, DE). SYBR Green gene expression assays (premade sequences, ABI) were used to determine the relative expression levels of the following genes from DNA harvested from murine bone marrow-derived macrophages (BMDM): inos, tnf-α, stat1, stat2, stat5, irf3, irf4, irf5, il1rn, cd206, tgm2, stat3, stat6, klf4, klf6, fizz-1, arg1, bfkbf3, glut1, hif1α, hk3, pgk1, pdk4, rpia, ldha, pck2, g6pc3, and pparδ. Results were analyzed by the ΔΔCt method using glyceraldehyde 3-phosphate dehydrogenase (GAPDH) to normalize the results. Fold change was calculated using untreated macrophages (M0) as the baseline. Results are displayed in a heat map format created by Java Treeview (Oracle, Redwood City, California). TaqMan MicroRNA assays (ABI) were used to determine the relative levels of mmu-miR-145-5p, mmu-

miR-143-3p, and mmu-miR-125b-5p inhibitors. These three miRNAs were found to be enriched in MBV and have been suggested as mediators of macrophage activation364,370,372,374,375. mmu-SNO-55 was used to normalize the results.

Appendix A.3.7 RNA Sequencing and Data Analysis

RNA sequencing and data analysis were performed as previously described175. Briefly, small RNA libraries were prepared using Ion Total RNA-Seq Kit version 2, according to the manufacturer's instructions. Following bead-based size selection of RNA (10- to 20-nt range), cDNA was created. Amplified library was again size-selected using a bead-based method. Library size distribution was verified using Agilent Bioanalyzer (Agilent, Santa Clara, CA). The Ion One Touch 2 System was used to perform automated emulsion polymerase chain reaction of the prepared libraries and templated Ion Sphere Particle enrichment. Sequencing was performed on the Ion Proton platform using a single P1 sequencing chip. Obtained data were imported into CLC Genomics Workbench 8 (Qiagen). The adaptors were trimmed, and all reads that had two ambiguous nucleotides, had a Phred score <30, or were lower than 15 nt, or above 100 nt were removed. Conserved reads were then aligned to the human genome (hg38) to verify valid reads. Reads were extracted, counted, and then annotated on miRBase v.21 (human genome reference); a 2-nt mismatch was allowed per read. Only sequences that matched a mature miRNA were used for downstream analysis.

Appendix A.3.8 MBV Fluorescent Labeling

MBV nucleic acid cargo was labeled using Exo-Glow (System Biosciences), according to the manufacturer's instructions. Briefly, 500 μL of resuspended MBV was labeled with Exo-Glow and incubated at 37°C for 10 min. ExoQuick-TC (100 μL) was added to stop the reaction, and samples were placed on ice for 30 min. Samples were then centrifuged for 10 min at 14,000 g. The supernatant was removed and the pellet was resuspended with 500 μL of 1 × PBS, and 50 μL of this MBV suspension was added to BMDM cell culture. The cells were cultured for 4 h, and the transfer of the MBV cargo to the cells was determined by imaging using a 100 × objective and Axio Observer Z1 microscope.

Appendix A.3.9 Cell Culture

Murine BMDM were isolated and characterized as previously described319. Briefly, bone marrow was harvested from 6- to 8-week-old C57bl/6 mice. Harvested cells from the bone marrow were washed and plated at 1 × 106 cells/mL and were allowed to differentiate into macrophages for 7 days in the presence of macrophage colony-stimulating factor (MCSF) with complete medium changes every 48 h.

Appendix A.3.10 Macrophage Activation

Macrophages were activated for 24 h with one of the following: (1) 20 ng/mL interferon-γ (IFNγ) and 100 ng/mL lipopolysaccharide (LPS) (Affymetrix eBioscience, Santa Clara, CA; Sigma Aldrich) to promote an $M_{IFN\gamma+LPS}$ phenotype (M1-like), (2) 20 ng/mL interleukin (IL)-4

(Invitrogen) to promote an M_{IL-4} phenotype (M2-like), (3) 250 ug/mL of UBM-ECM, or SIS-ECM to promote an M_{ECM} phenotype, or (4) 25 μg/mL of UBM-MBV or SIS-MBV to promote an MMBV phenotype. The concentration of MBV was determined by bicinchoninic acid assay. Pepsin (1 mg/mL) and collagenase (0.1 mg/mL) were used as baseline controls for ECM and MBV, respectively. After the incubation period at 37°C, cells were washed with sterile PBS and fixed with 2% paraformaldehyde (PFA) for immunolabeling, or harvested with TRIzol lysis reagent (Thermo Fisher) for protein/RNA assessment, respectively. Macrophage function was evaluated by assays for phagocytosis and NO production.

Appendix A.3.11 Transfection

BMDM were transfected with 50 nM mmu-miR-154-5p, mmu-miR-143-3p, and mmu-miR-125b-5p inhibitor or a cocktail mix of the inhibitors (Thermo Fisher), as well as a scrambled negative control (Thermo Fisher), using Lipofectamine RNAiMAX (Thermo Fisher) according to the manufacturer's instructions. miRNA inhibitors were transfected for 4 h in Opti-MEM media. Cells were then washed and incubated overnight with Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin (P/S).

Appendix A.3.12 NO Quantification

Following 24 h of treatment with test articles, macrophage supernatants were transferred to a 96-well plate and frozen at −80°C. Fifty microliters of samples or internal assay standards consisting of sodium nitrite from 100 to 1.56 μM in a 1:2 serial dilution was added to the plate. The wells were treated with 50 μL of 1% sulfanilamide in 5% phosphoric acid for 10 min, followed

by addition of 50 μL of 0.1% N-1-napthylethylenediamine dihydrochloride in water for 10 min. The wells were then read at 540 nm and compared to the standard curve. Cells were counted using Cell Profiler software using 4'6-diamidino-2-phenylindole (DAPI) nuclear staining. NO values were normalized to the number of cells per well.

Appendix A.3.13 Phagocytosis Assay

Following 24h of treatment with the test articles, macrophages were incubated with the Vybrant Phagocytosis Kit (Thermo Fisher) FITC-labeled Escherichia coli beads for 2 h. Wells were then washed once with 1 × PBS and fixed with 2% PFA for 30 min. Wells were washed three times with PBS, then stained with DAPI for 10 min, and washed again three times with PBS. Wells were imaged using an automated Live Cell Scope and quantified for mean fluorescence intensity of the cells using Cell Profiler software.

Appendix A.3.14 Macrophage Immunolabeling

Cells were fixed with 2% PFA for 45 min at room temperature, then washed with PBS, followed by immunolabeling to determine surface marker expression. To prevent nonspecific binding, the cells were incubated in a blocking solution composed of PBS, 0.1% Triton-X, 0.1% Tween-20, 4% goat serum, and 2% bovine serum albumin for 1 h at room temperature. The blocking buffer was then removed and cells were incubated in a solution of one of the following primary antibodies: (1) monoclonal anti-F4/80 (Abcam, Cambridge, MA) at 1:200 dilution as a pan-macrophage marker, (2,3) polyclonal anti-inducible nitric oxide synthase (iNOS) and anti-tumor necrosis factor-α (TNF-α) (Abcam, Cambridge, MA) at 1:100 dilution, each as M1-like

markers, and (4,5) polyclonal anti-Fizz1 (Peprotech, Rocky Hill, NJ) and anti-Arginase1 (Abcam, Cambridge, MA) at 1:200 dilution, each as M2-like markers. The cells were incubated at 4°C for 16 h, the primary antibody was removed, and the cells washed with PBS. A solution of fluorophore-conjugated secondary antibody (Alexa donkey anti-rabbit 488 or donkey anti-rat 488; Invitrogen, Carlsbad, CA) was added to the appropriate well for 1 h at room temperature. The antibody was then removed, the cells washed with PBS, and the nuclei were counterstained using DAPI. Cytokine-activated macrophages were used to establish standardized exposure times (positive control), which were held constant throughout groups thereafter. CellProfiler (Broad Institute, Cambridge, MA) was used to quantify images.

Appendix A.3.15 Statistical Analysis

Data were analyzed for statistical significance using either an unpaired Student's t-test, through which treated macrophages were compared to the appropriate M0 media control, or a one-way analysis of variance with Tukey's post-hoc test for multiple comparisons. Data are reported as mean ± standard deviation with a minimum of $N = 3$. p-values of <0.05 were considered to be statistically significant. For gene expression data represented as a heat map, the p-values were generated by Student's t-tests comparing treated macrophages with M0 media control.

Appendix A.4 Results

Appendix A.4.1 MBV Imaging and Gene Expression Signature of MBV-treated Cells

Particulate UBM-ECM or SIS-ECM was enzymatically digested for 16 h at room temperature with proteinase-K or 0.1% collagenase solution. The solubilized ECM samples were then subjected to centrifugation at increasing g forces to isolate MBV. MBV were visualized at 100,000 × magnification using TEM (Figure 13A). Cellular uptake of MBV was determined by labeling MBV with acridine orange. Labeled MBV were visible within BMDM 2 h after their addition to the culture media (B). The effect of MBV on macrophage activation was evaluated by quantitative polymerase chain reaction (qPCR) analysis of more than 25 commonly used markers of macrophage activation, including surface markers, cytokines, transcription factors, and metabolic markers. The gene expression profile of macrophages treated with MBV was qualitatively very similar to the gene expression profile of macrophages treated with ECM (Figure 13C). Little to no effect on gene expression was observed after exposing the macrophages to pepsin or collagenase indicating that these enzymes, which are used to digest ECM before extraction of MBV, are not responsible for macrophage activation. Exposure of BMDM to IFNγ+LPS ($M_{IFN\gamma+LPS}$) or IL-4 ($M_{IL\text{-}4}$) led to distinct gene expression profiles consistent with previous studies(Mantovani et al., 2004; F. O. Martinez & S. Gordon, 2014; Martinez, Sica, Mantovani, & Locati, 2008; Ying, Cheruku, Bazer, Safe, & Zhou, 2013).

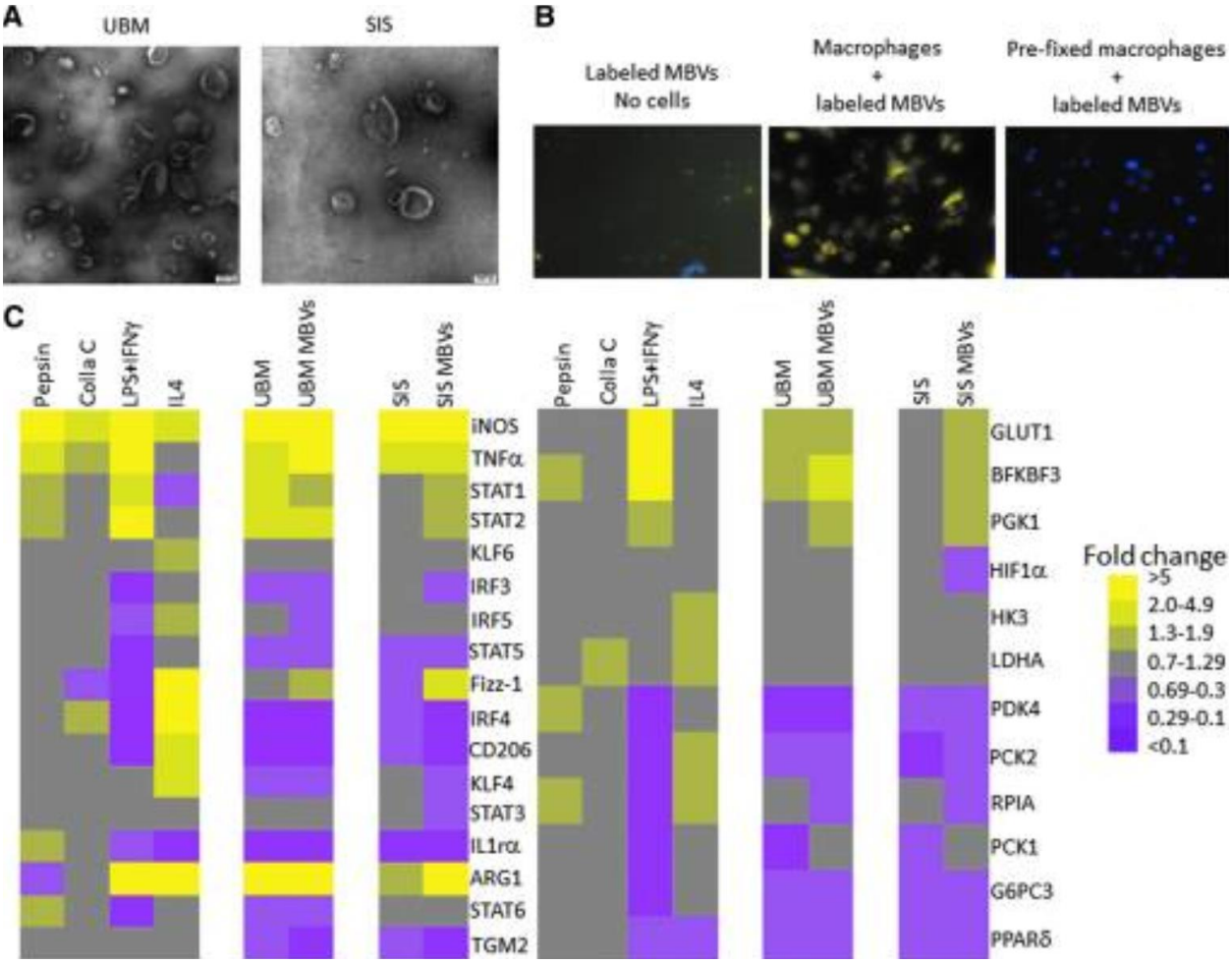

Figure 13. MBV imaging and gene expression signature of MBV-treated macrophages. (A) MBV isolated using proteinase-K digestion were visualized by transmission electron microscopy at 100,000 × magnification. **(B)** Following exposure of BMDMs (counterstained with DAPI) to MBVs whose nucleic acid content was labeled with acridine orange, the cells were visualized using fluorescence microscopy at 200 × magnification. MBV are seen within the cytosol of cells after a 2-h incubation. **(C)** Gene expression analysis of cells exposed to either ECM or their respective MBVs was evaluated using qPCR. Results are presented in a heatmap form that was generated using Treeview software; all fold changes are with respect to media control (N = 3). Scale bar scoring system is demonstrated as follows: less than 0.1-fold change (darkest purple), 0.1–0.29-fold change (intermediate purple), 0.3–0.69-fold change (light purple), 0.7–1.29-fold change (gray), 1.3–1.9-fold change (light yellow), 2.0–4.9-fold change (intermediate yellow), greater than 5.0-fold change (bright yellow).

Table 1. Statistically significant gene expression changes in MBV-treated BMDM. Gene expression changes in treated BMDM compared to untreated (M0) controls. Unpaired student's *t*-tests were used for all comparisons, * $p \leq 0.05$.

	Pepsin	Collagenase Control	LPS + IFNγ	IL4	UBM	UBM MBVs	SIS	SIS MBVs
iNOS	*	ns	*	*	*	*	*	*
TNFα	*	*	*	*	*	*	*	*
STAT1	ns	*	*	*	*	*	ns	ns
STAT2	ns	ns	*	ns	*	*	ns	ns
KLF6	ns	ns	ns	*	ns	ns	ns	ns
IRF3	ns	ns	*	*	*	*	ns	*
IRF5	ns	ns	*	*	ns	*	ns	*
STAT5	ns	ns	*	ns	*	*	*	*
Fizz1	ns	ns	*	*	ns	ns	ns	ns
IRF4	ns	*	*	*	*	*	*	*
CD206	ns	ns	*	*	*	*	*	*
KLF4	*	ns	ns	*	*	*	ns	*
STAT3	ns	ns	ns	ns	ns	ns	ns	*
IL1Ra	ns	ns	ns	ns	*	*	*	*
Arg 1	ns	ns	*	*	*	*	*	*
STAT6	*	ns	*	*	*	*	ns	ns
TGM2	*	ns	ns	*	*	*	*	*
GLUT1	ns	ns	*	*	*	ns	ns	*
BFKBF3	*	ns	*	ns	*	*	ns	ns
PGK1	ns	*	*	ns	ns	*	*	*
HIF1α	ns	ns	ns	*	ns	ns	ns	ns
HK3	ns	ns	ns	*	ns	ns	ns	ns
LDHA	ns	*	ns	*	ns	ns	ns	ns
PDK4	*	ns	*	ns	*	*	ns	*
PCK2	ns	ns	*	ns	*	*	*	*
RPIA	*	ns	*	ns	*	*	ns	*
PCK1	ns	ns	*	ns	*	ns	*	ns
G6PC3	ns	ns	*	ns	*	*	*	*
PPARδ	ns	*	*	ns	*	*	*	*

Appendix A.4.2 MBV Treatment Increases M2-like Protein Expression

Immunolabeling was performed to evaluate protein expression of BMDM (Figure 14). Similar to the gene expression results, MBV-treated groups had a qualitatively similar protein expression profile as the ECM-treated groups. Both ECM groups, as well as their corresponding MBV groups, had protein expression profiles similar to $M_{IL\text{-}4}$ cells. Macrophage treatment with SIS-ECM and UBM-ECM and their corresponding MBV resulted in expression of Fizz-1 and Arg-1 (markers that are associated with the $M_{IL\text{-}4}$ phenotype). Low levels of iNOS expression were detected in the SIS-MBV group. TNF-α was detectable only in the UBM-MBV group. Both TNF-α and iNOS are markers associated with the $M_{IFN\gamma+LPS}$ phenotype. No expression of these proteins was noted in the control groups. The majority of cells expressed F4/80, confirming their macrophage differentiation state. Quantification of immunolabeling images using CellProfiler software supported the qualitative interpretation of the results.

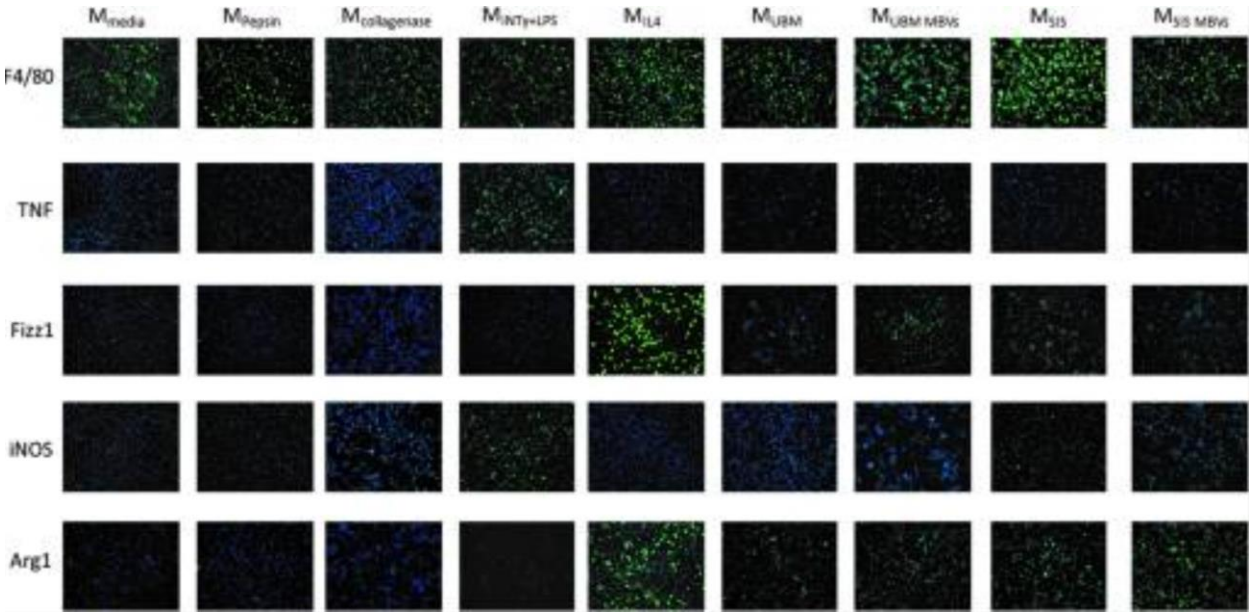

Figure 14. MBV treatment increases M2-like protein expression. BMDM were exposed for 24 h to media control, 1 mg/mL pepsin or 0.1 mg/mL collagenase controls, 250 μg/mL ECM, 25 μg/mL MBVs, or the cytokine controls IFNγ+LPS or interleukin (IL)-4. Cells were then fixed with 4% PFA .The cells were then incubated with anti-murine antibody for markers of the M1-like phenotype TNFα and iNOS, or markers of the M2-like phenotype Fizz1 and Arginase1. All images for the same antibody were taken at the same exposure time normalized to the positive control. Cell nuclei were stained with DAPI. Images were taken at 200 × magnification (N = 3).

Appendix A.4.3 MBV Treatment Affects BMDM Function More Than ECM Treatment

To determine the effect of MBV on macrophage function, NO production and phagocytosis were measured in macrophages exposed to MBV, ECM, or cytokines.

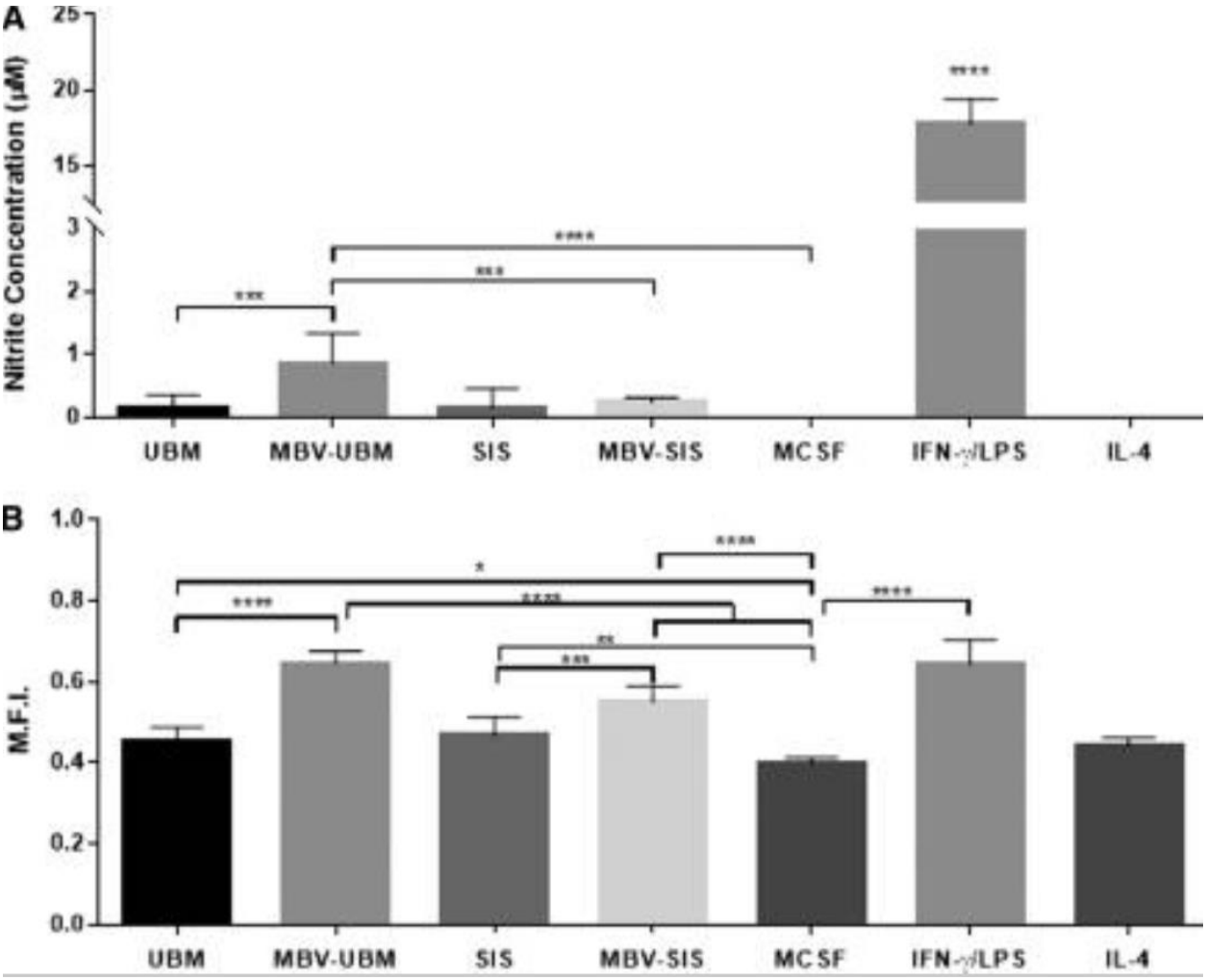

Figure 15. MBV treatment affects BMDM function more than ECM treatment. Macrophages were exposed for 24 h to MCSF control, 250 μg/mL ECM, 25 μg/mL MBVs, or the cytokine controls IFNγ+LPS or IL-4. **(A)** Macrophage supernatants were mixed with 1% sulfanilamide in 5% phosphoric acid for 10 min, followed by addition of 0.1% N-1-napthylethylenediamine dihydrochloride in water. The solutions were read in a spectrophotometer at 540 nm and compared to the standard curve of sodium nitrite to assess nitric oxide production levels. Values: mean ± standard deviation, $N = 6$, $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$ by one-way analysis of variance (ANOVA) with Tukey's post-hoc test. **(B)** Treated macrophages were incubated with Vybrant Phagocytosis Kit FITC-labeled Escherichia coli beads for 2 h. Cells were fixed and stained with DAPI. Using fluorescence microscopy, the cells were visualized and quantified for mean fluorescence intensity of the cells using Cell Profiler software. Values: mean ± standard deviation, $N = 6$, $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$ by one-way ANOVA with Tukey's post-hoc test.

Appendix A.4.4 Nitric Oxide Production

NO was assessed in MBV-exposed BMDM. NO production was not detectable in naive and $M_{IL\text{-}4}$ macrophages, consistent with the findings of previous studies(Hachim, LoPresti, et al., 2017; Hachim, Wang, et al., 2017). $M_{IFN\text{-}\gamma+LPS}$ macrophages produced a significant increase in NO. BMDM treated with MBV derived from UBM was the only treatment group to increase NO production (Figure 15B).

Appendix A.4.5 Phagocytosis

A basal level of phagocytosis as measured by uptake of FITC-*E. coli* particles was shown by all macrophages. Treatment with IFN-γ+LPS resulted in a significant increase in phagocytic activity compared to MCSF (M0). ECM and MBV treatment resulted in a significant increase in phagocytic uptake compared to MCSF (M0). When compared to their parent ECM bioscaffolds, exposure to MBV alone led to greater phagocytosis. Whereas there was no significant difference in phagocytosis between macrophages treated with UBM or SIS, UBM-MBV caused an increase in macrophage phagocytosis compared to macrophages treated with SIS-MBV (Figure 15B).

Appendix A.4.6 miRNA Inhibition Reverse Gene Expression Patterns Compared to MBV-exposed BMDM

Sequencing results revealed expression of 240 different miRNAs in UBM-MBV and 53 in SIS-MBV(Huleihel et al., 2016) (Figure 16A-C). miRNAs with the greatest expression in UBM-MBV and SIS-MBV are listed in Table 2. miRNAs highlighted in red were selected for

downstream analysis based on previous studies that showed their involvement in macrophage activation.

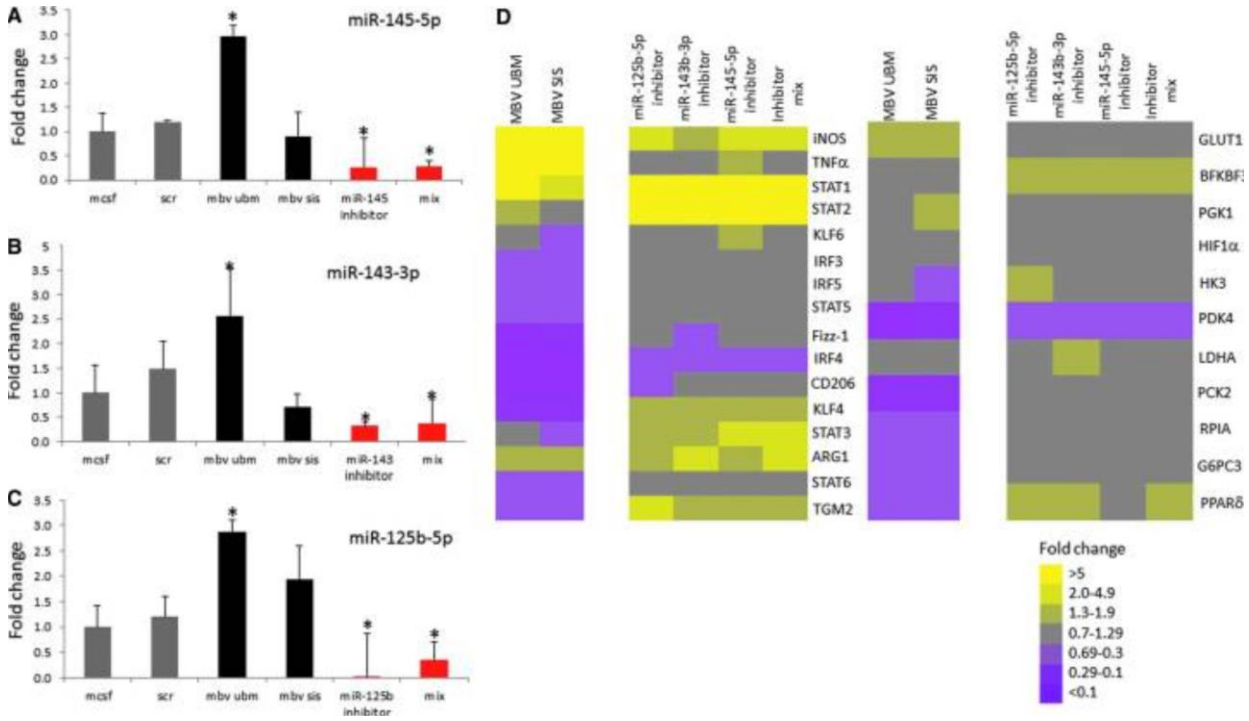

Figure 16. miRNA inhibition reverses gene expression patterns compared to MBV-exposed BMDM.

Macrophage miRNA inhibition. (A–C) Selective inhibition of specific miRNAs, miR-145-5p, miR-145-3p, and miR-125-b-5p using 50 nM of inhibitor for each. Relative abundance of miRNA levels following inhibition was determined by TaqMan miRNA qPCR assays. Values: mean ± standard deviation, N = 3, *p < 0.05 by Student's t-test. Data represents fold change in comparison to M0. **(D)** Gene expression analysis of cells exposed to MBVs, or transfected with scrambled control miRNA inhibitor, mmu-miR-154-5p inhibitor, mmu-miR-143-3p inhibitor, mmu-miR-125b-5p inhibitor, or a combination of all three inhibitors was evaluated using qPCR. Results are presented in a heatmap form that was generated using Treeview software; all fold changes are with respect to media control. Scale bar scoring system is demonstrated as follows: less than 0.1-fold change (darkest purple), 0.1–0.29-fold change (intermediate purple), 0.3–0.69-fold change (light purple), 0.7–1.29-fold change (gray), 1.3–1.9-fold change (light yellow), 2.0–4.9-fold change (intermediate yellow), and greater than 5.0-fold change (bright yellow).

Table 2. miRNA enriched in MBV

miRNA	UBM-MBV expression value	SIS-MBV expression value
mir-145	14848	93
mir-6087	113	61
mir-183	59	54
let-7b	338	31
mir-143	4108	30
mir-4792	49	18
mir-195	69	15
mir-192	12	15
mir-6803	5	13
mir-31	4	12
mir-320a	204	11
mir-125b	1204	10
mir-181a	23	10
mir-208a	22	9
mir-29a	206	7
mir-27b	341	4
mir-125a	320	4
mir-23b	405	2
mir-21	193	1
let-7g	189	1

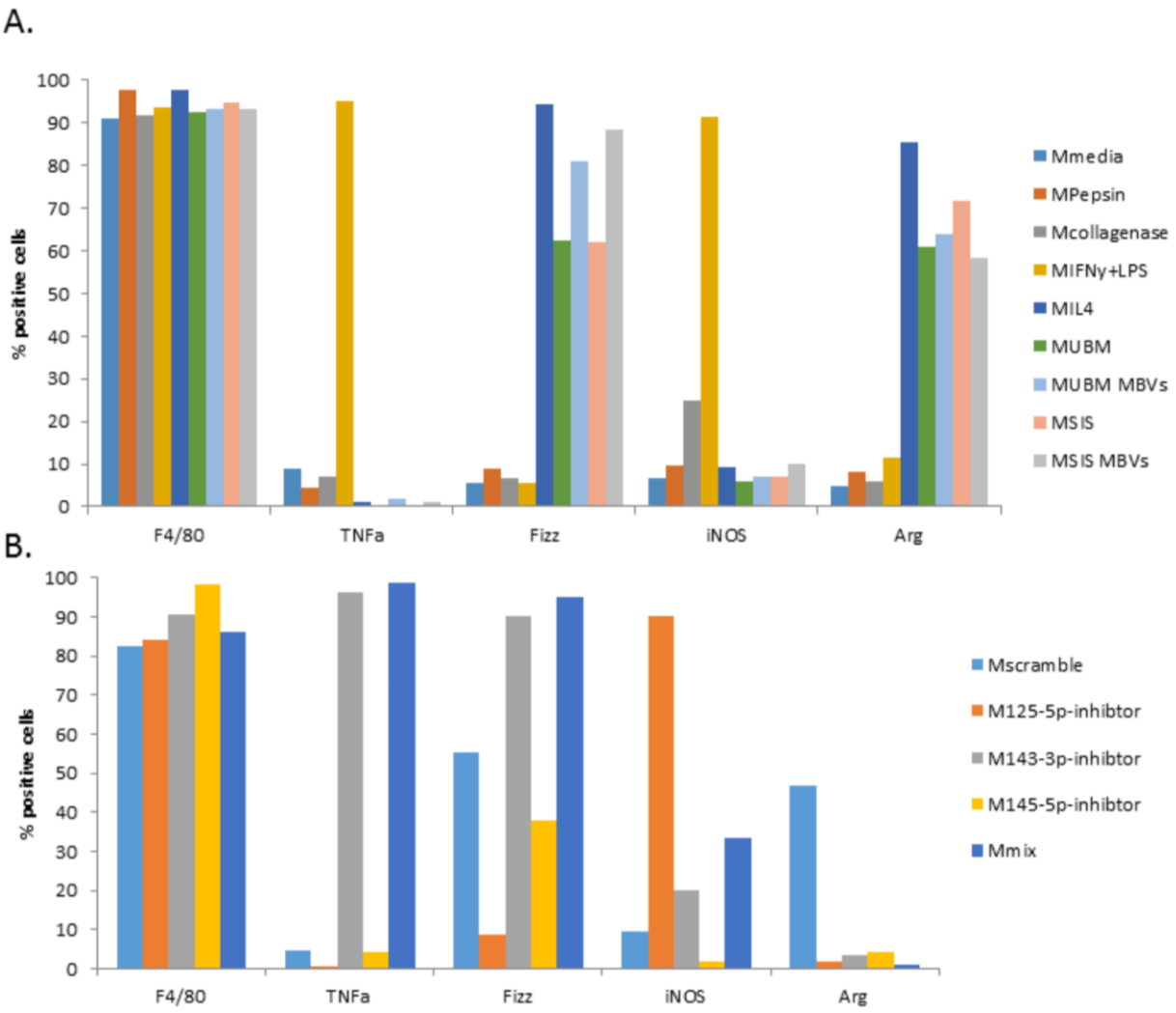

Figure 17. Quantification of macrophage immunolabeling. (A) Percent positive macrophages as a function of treatment were assessed using CellProfiler and compared to M0 controls. **(B)** Quantification of immunolabeled macrophages in which select miRNA were inhibited through siRNA.

Table 3. Significantly differentially expressed genes as a function of miRNA inhibition compared to M0 controls.

	MBV UBM	MBV SIS	125b	143	145	mix
iNOS	*	*	*	*	*	*
TNFα	*	*	ns	ns	ns	ns
STAT1	*	*	*	*	*	*
STAT2	ns	ns	*	*	*	*
KLF6	ns	*	*	ns	*	ns
IRF3	*	*	ns	ns	ns	ns
IRF5	*	*	ns	ns	ns	ns
STAT5	*	*	ns	ns	ns	ns
Fizz1	*	*	ns	ns	ns	ns
IRF4	*	*	*	*	*	*
CD206	*	*	*	*	*	*
KLF4	*	*	*	*	*	*
STAT3	ns	*	*	*	*	*
Arg 1	*	*	*	*	*	*
STAT6	*	*	ns	ns	ns	ns
TGM2	*	*	*	*	*	*
Glut1	*	*	ns	ns	ns	ns
BFKBK3	ns	ns	*	*	*	*
PGK1	*	*	ns	ns	*	ns
HIF1α	*	ns	ns	ns	ns	ns
HK3	*	*	*	ns	ns	ns
PDK4	*	*	*	*	*	*
LDHA	ns	ns	*	*	ns	ns
PCK2	*	*	ns	ns	ns	ns
RPIA	*	*	ns	ns	ns	ns
G6PC3	*	*	ns	ns	ns	ns
PPARδ	*	*	ns	ns	ns	*

miR-145-5p, miR-143-3p, and miR-125b-5p were inhibited in BMDM to determine their role in macrophage expression of activation markers. The degree of inhibition of each miRNA was determined by qPCR (Figure 16A-C). miR-145 expression was reduced by more than 70%, miR-143 expression was reduced by 65%, and miR-125b expression was reduced by more than 95%. qPCR analysis of MBV-treated and miRNA inhibitor-treated cells showed that 6 of 27 genes had a markedly different expression pattern (Figure 16D and Table 3). Interestingly, the six genes whose expression was increased by miRNA inhibition were the same as those that were decreased by MBV treatment. Importantly, KLF4, which is a transcription factor associated with macrophage activation and also a known target of miR-145-5p, shows an opposite expression pattern for all the inhibited miRNA treatment groups compared to both UBM-MBV- and SIS-MBV-treated cells. These results suggest that inhibition of KLF4 may be a target of the miRNA cargo within MBV.

Appendix A.4.7 miRNA Shows Opposite Protein Expression in BMDM

Immunolabeling was performed to evaluate protein expression of BMDM in which miRNA had been selectively inhibited. Similar to the gene expression results, treatment with miRNA inhibitors led to an opposite protein expression profile compared to MBV-treated groups (Figure 18). Inhibition of miRNA-125b-5p led to an increase in iNOS expression, whereas inhibition of miRNA-143-3p led to an increase in the expression of TNF-α and Fizz1 compared to uninhibited controls. The majority of cells expressed F4/80 consistent with a macrophage differentiation state. Quantification of immunolabeling images using CellProfiler software supported the qualitative interpretation of the results (Figure 18).

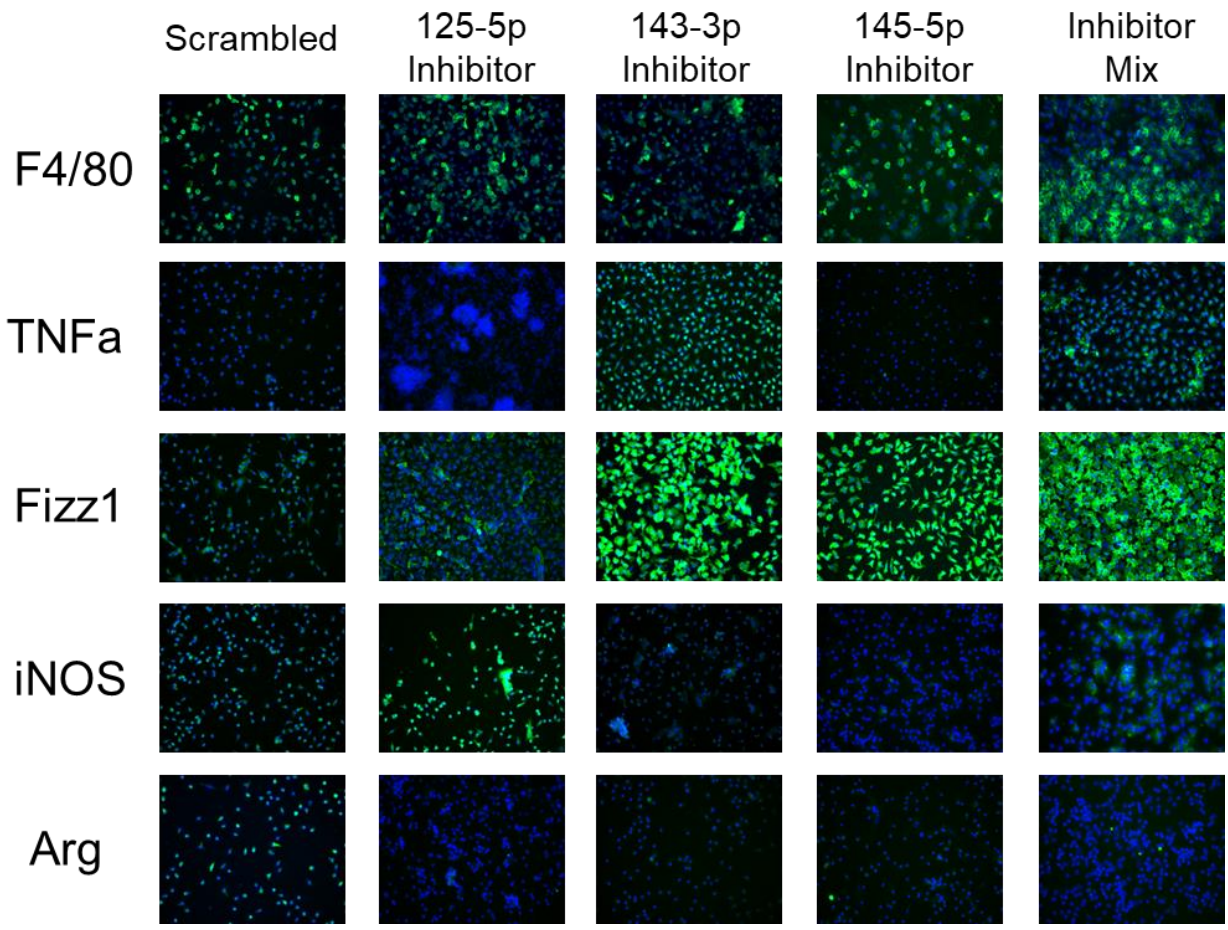

Figure 18. miRNA inhibition shows opposite protein expression compared to MBV. BMDM were exposed for 4 h to 50 nM of one of the following: scrambled control, mmu-miR-125b-5p inhibitor, mmu-miR-143-3p inhibitor, or miR-145-5p. Treatment media were then changed to normal growth media for an additional 18 h. Cells were then fixed with 4% PFA. Cells were then incubated with anti-murine antibody for markers of the M1-like phenotype TNFα and iNOS, or markers of the M2-like phenotype Fizz1 and Arginase1. Exposure times were established based on a negative isotype control and cytokine-treated controls and kept constant for each marker tested. Cell nuclei were stained with DAPI. Images were taken at 200 × magnification. The percentage of cells positive for each stain was quantified using Cell Profiler software and is presented in Supplementary Figure S1B. The results show that miRNA inhibition is capable of impacting the expression of several probed proteins, implicating the role of miR-125b-5p, miR-143-3p, and miR-145-5p in the formation of the MMBV phenotype.

Appendix A.5 Discussion

A The results of the present study show that MBV can largely recapitulate the effects of ECM upon macrophage phenotype. MBV were rapidly internalized by macrophages and direct inhibition of specific miRNAs cargo (found in high abundance in MBV) within these macrophages notably affected the macrophage phenotype. Macrophage gene and protein expression, cell surface markers, and functional capacity as determined by phagocytic activity, nitric oxide production, and antimicrobial activity were most representative of a regulatory/anti-inflammatory phenotype following treatment with MBV, which is consistent with previous reports describing the effects of ECM bioscaffolds upon macrophage phenotype(S. F. Badylak et al., 2008; Brown et al., 2009; Dziki, Wang, et al., 2017). These findings provide a plausible explanation of at least one mechanism by which MBV embedded within ECM bioscaffolds can regulate the macrophage component of the innate immune response during matrix remodeling events.

Macrophages have been shown to be important, in fact necessary, regulators of normal healing following injury, and/or in normal tissue development(Lavin, Mortha, Rahman, & Merad, 2015). Specifically, a transition in macrophage activation state from a pro-inflammatory to an anti-inflammatory and pro-remodeling phenotype is required for initiation and resolution of the healing process and a return to homeostasis(B. M. Sicari, J. L. Dziki, et al., 2014; Tidball, 2011; Tidball & Villalta, 2010; Wolf, Dearth, et al., 2014). Failure of this transition is associated with chronic inflammation, impaired wound healing, and dysregulation of the microenvironmental niche(B. Deng et al., 2012; Novak & Koh, 2013; Tidball et al., 2014), and has been suggested to play a causal role in conditions such as inflammatory bowel disease, muscular dystrophy, and kidney disease(Guiteras, Flaquer, & Cruzado, 2016; Novak & Koh, 2013; Rogler et al., 1997). While the importance of this phenotypic switch is recognized, the specific endogenous signals regulating spatiotemporal

patterns of macrophage phenotype are poorly understood. Phagocytosis of neutrophils as the cause of the phenotype switching in macrophages has been suggested(Fadok et al., 1998), but definitive studies have been lacking.

Extracellular matrix harvested from many different tissues and commercially available biomaterials composed of ECM consistently and reproducibly promote an M2-like regulatory macrophage phenotype(Brown, Londono, et al., 2012; J. L. Dziki et al., 2016; Dziki, Wang, et al., 2017; Piccoli et al., 2016), a process that drives downstream constructive and functional tissue remodeling. ECM bioscaffolds have thus far been shown to promote this phenotype activation by at least two mechanisms: 1) a direct transmembrane process involving intracellular pathways such as COX1/2(Dearth et al., 2016); and 2) an indirect effect through stem / progenitor cell paracrine signaling(J. L. Dziki et al., 2016). Macrophages have been shown to be necessary for the degradation of ECM bioscaffolds in vivo(Valentin et al., 2009). It is plausible that during degradation of the ECM, MBV are released, internalized by the local macrophage population, and facilitate transition to an anti-inflammatory/regulatory phenotype. It is also logical to hypothesize that these events occur not only in the presence of ECM bioscaffolds, but also during normal wound healing events; however additional work is required to test such a hypothesis. The profile of markers used in the present study to characterize macrophage activation state included transcription factors, surface markers, gene and protein expression, and functional assays. Stated differently, the activity of MBV was characterized on many levels, and it was shown that they mimic or even outperform ECM bioscaffolds with regard to macrophage activation in certain assays. These results suggest that MBV are a key bioactive component within ECM.

Sequencing of MBV nucleic acid cargo from UBM-ECM and SIS-ECM showed that specific miRNA are particularly enriched in these nanovesicles, depending upon the ECM tissue

source. Three of these miRNA, 125b-3p, 143-3p, 145-5p, were highly expressed within MBV and these specific miRNA have been implicated in macrophage activation and phenotypes(Banerjee et al., 2013; Chaudhuri et al., 2011; Y. Zhang et al., 2013). These miRNA were therefore targeted in the present study for closer scrutiny. There is not a consensus in the literature with respect to known functions of the particular miRNA investigated in the present study. It has been reported that, in murine Raw 264.7 macrophages(Tili et al., 2007), miRNA-125b-5p targets TNF-α and 5-lipoxygenase, negatively regulating the inflammatory response(H. M. Lee, Kim, & Jo, 2016). However, in isolated murine peritoneal macrophages, miRNA-125b-5p was shown to enhance the inflammatory response in certain contexts by targeting IRF4(Chaudhuri et al., 2011). In the present study, inhibition of miR-125b-5p did not lead to increases in TNF-α, however, a reduction in IRF4 gene expression levels was noted in both miR-125b-5p inhibition and MBV treated macrophages. This incongruity suggests that IRF4 regulation isn't dependent solely on miR-125-5p, and likely represents the net actions of various miRNA and proteins present in the MBV cargo. Discrepancies observed between the results of the current study and previous reports with respect to miRNA-125b-5p could arise from the use of macrophages derived from different tissue sources.

Additionally, it has been shown that miRNA-143/145 are elevated in M2-like macrophages and downregulated in M1-like macrophages, implying their role in macrophage activation(Y. Zhang et al., 2013). Moreover, it has been shown that miRNA-145-5p is capable of activating the epigenetic IL-10 gene silencer, HDAC11, in murine macrophage cell lines. The previous report showed that downregulation of miRNA-145-5p expression as a result of IFN-γ signaling directly contributes to pro-inflammatory macrophage activity and phenotype(L. Lin et al., 2013). However, miRNA-143-3p is less well studied in the context of innate immunity and its targets within macrophages are poorly described. Nonetheless, inhibition of both miRNA-143-3p and miRNA-

145-5p in naïve macrophages resulted in increased expression of the macrophage surface marker associated with an M1-like phenotype, TNF-α, which is consistent with the findings of the previous report(Y. Zhang et al., 2013). These results are further corroborated by the finding that MBV treatment led to increased expression of M2-like markers, Fizz1 and Arg1, which are associated with a reconstructive macrophage phenotype; however, there are a multitude of miRNA within MBV that could be similarly contributing to this downregulation of pro-inflammatory markers. Taken together, the results of the miRNA inhibition and MBV treatments corroborate previous reports that miRNA-145-5p contributes to a downregulation of the inflammatory phenotype and suggest that both miRNA-143/145 contribute to the macrophage phenotype in response to MBV and ECM bioscaffolds.

Further analysis of gene and protein expression assays of naïve BMDM in which these miRNA were inhibited showed that their inhibition resulted in opposite patterns of expression in nearly 25 percent of the genes that were investigated. Among these genes are members of the KLF, STAT, and IRF families of transcription factors. Interestingly, KLF4 was consistently downregulated by both ECM and MBV treatment. Inhibition of miR-145-5p resulted in an increase of KLF4. The regulation of KLF4 by miR-145-5p has been shown to be an effector of macrophage activation(Cordes et al., 2009; H. Liu et al., 2013; N. Xu, Papagiannakopoulos, Pan, Thomson, & Kosik, 2009), which together with findings of the present study, suggests its role in this process. Importantly, inhibition of the different miRNAs led to a similar gene expression profile. Using TargetScan software we identified 18 mutual genes that are predicted being regulated by miR-145-5p, miR-143-3p and miR125-5p. Among those genes is QKI, which was previously identified as a macrophage differentiation regulator(Fu et al., 2012; Wang et al., 2015). We postulate that mutual target genes are being regulated by these miRNAs, effecting specific pathways.

Overall, these results strongly suggest that MBV and their miRNA cargo are at least partially responsible for the macrophage response that is observed when ECM bioscaffolds facilitate functional tissue repair.

MBV treatment also impacted macrophage function, with effects that were greater in magnitude than the parent ECMs on all metrics. The rate of phagocytosis in UBM-MBV exposed macrophages rose to a level indistinguishable from that of $M_{IFN\gamma+LPS}$ macrophages, significantly greater than all other treatment groups. Labeling MBV with ExoRed showed that MBV were internalized by macrophages within 2 hours. This finding does not differentiate between phagocytosis and endocytosis as the major route of uptake however, and this represents an area of future study.

While miRNA-125/143/145 appear to modify macrophage activation state, there are greater than 200 miRNAs present within the MBV, which have the potential to affect macrophage activation or the behavior of other cell types(Huleihel et al., 2016). Additionally, the effect of MBV protein cargo upon macrophage activation has yet to be determined. The present work did not utilize an MBV-depleted ECM control due to the inability to remove MBV without destroying all remaining ECM constituents.

Appendix A.6 Conclusions

The results of the present study clearly show the ability of MBV to recapitulate many effects of ECM on macrophage activation, which is an important bioactive property of ECM bioscaffolds. Furthermore, specific miRNAs within MBV play a role in this process, which provides a plausible mechanism by which ECM promotes a transition in macrophage phenotype

and downstream constructive tissue remodeling not only with the use of ECM bioscaffolds but also during normal tissue repair processes. A more comprehensive understanding of the role of additional miRNAs present in the MBV cargo is an important area of future study.

Appendix B : Genes Uniquely Differentially Expressed in Response to MBV-associated IL-33 and Causal Network Analysis

Appendix B.1 Materials and Methods

Appendix B.1.1 Macrophage Isolation and Activation.

Bone marrow was isolated as previously described(L. Huleihel, J. L. Dziki, et al., 2017). Briefly, wild-type C57BL/6 or C57BL/6-*st2*$^{-/-}$ were sacrificed using inhaled carbon dioxide and cervical dislocation. Femurs, tibia, and fibula were then harvested and washed 3x in macrophage Complete Medium composed of 10% FBS (Invitrogen, Carlsbad, CA), 10% L929 supernatant, 10 mM non-essential amino acids (Gibco), 10 mM HEPES (Gibco), 2 mM L-glutamine (Gibco), 100 U/mL penicillin, 100 mg/mL streptomycin and 0.1% b-mercaptoethanol in DMEM high glucose (Gibco). Complete medium was flushed through the medullary space of harvested bones and plated at 2E6 cells/mL into 6 well plates (Corning) for 7 days until mature macrophages were obtained. Medium was supplemented 24h after plating and changed every 48 hours thereafter. Mature macrophages were subsequently treated at 37ºC and 5% CO_2 for 16h with Complete Medium containing one of the following treatments: 1E9 IL-33^{+} MBV/ml, 1E9 IL-33^{-} MBV/ml, 20 ng/ml rIL-33, or no treatment (control). Following treatment, cells were washed in PBS and used for RNA isolation.

Appendix B.1.2 RNA Isolation and RNA Sequencing

RNA isolated from wildtype or *st2*$^{-/-}$ macrophages treated with 20 ng/mL rIL-33, 1E9 MBV/mL IL-33^{-} MBV/mL, or IL-33^{+} MBV/mL using TRIzol reagent according to manufacturer's recommendations. Isolated RNA purity was then assessed using a Bioanalyzer and used for whole transcriptome sequencing using Illumina NovaSeq 6000 (Illumina, San Diego, CA). Following quality control and pre-processing, reads with $\geq$ 35 bp after trimming were aligned to the most recent murine genome, GRCm38. Downstream read count analysis was performed using R, version 3.6.1. For visual exploration of the data, the obtained read counts were normalized using the regularized log transformation function of R package DESeq2, version 1.24.0. For Ingenuity Pathway Analysis and Causal Network Analysis, genes uniquely differentially expressed by MBV-associated IL-33 was defined by removing genes common to IL-33^{+} and IL-33^{-} MBV treatment in *st2*$^{-/-}$ macrophages, and strain differences were accounted for by removing genes unique to IL-33^{+} MBV treatment of *st2*$^{-/-}$ macrophages compared to IL-33^{+} MBV treatment of wild-type macrophages. Three biological replicates were performed for each treatment group and Wald test was used for statistical testing with 0.05 set as the significance cutoff used for optimizing the independent filtering and with the null hypothesis being that the log2 fold changes between contrast groups are equal to zero. P-values were adjusted for multiple testing using the Benjamini-Hochberg procedure. The obtained results were then post-hoc filtered using thresholds of 0.05 for adjusted p-value and 1 for absolute log2 fold change.

Appendix B.2 Results

Appendix B.2.1 MBV-associated IL-33 Reduces M1-Associated Pathway Activation

Analysis of gene expression changes in treated macrophages showed 137 significantly differentially expressed genes (DEG) unique to IL-33^{+} MBV, of which 99 were downregulated and 48 were upregulated (adjusted p: $0.044 \leq p \leq 1E\text{-}18$), the most differentially upregulated and downregulated protein coding genes are presented in Table 4. IPA Core Analysis (IPA, Qiagen) of unique DEGs was used for causal network analysis. Results show that four pathways were significantly enriched, of which the top two related to immune cell mobilization and extravasation (Granulocyte Adhesion and Diapedesis ($p = 2.38E\text{-}5$), and Agranulocyte Adhesion and Diapedesis ($p = 3.73E\text{-}5$, Figure 19A). Using the Causal Networks algorithm, DEGs were used to predict master regulator and upstream regulator molecules whose combined activities could explain the observed expression changes. Results show that the top two causal networks were governed by IRF3 (activation z-score = -5.425, network bias-corrected $p < 0.0001$) and Trim21 (activation z-score = -5.048, network bias-corrected $p < 0.0001$, Figure 19B-C). Of note, the three upstream signaling molecules most significantly predicted to be inhibited by these master regulators were molecules associated with M1-like macrophage phenotype activation, namely: TLR4 (activation z-score = -3.202, $p = 1.26E\text{-}11$), IRF7 (activation z-score = -3.094, $p = 1.8E\text{-}9$), and NFkB (activation z-score = -3.440, $p = 4.21E\text{-}9$).

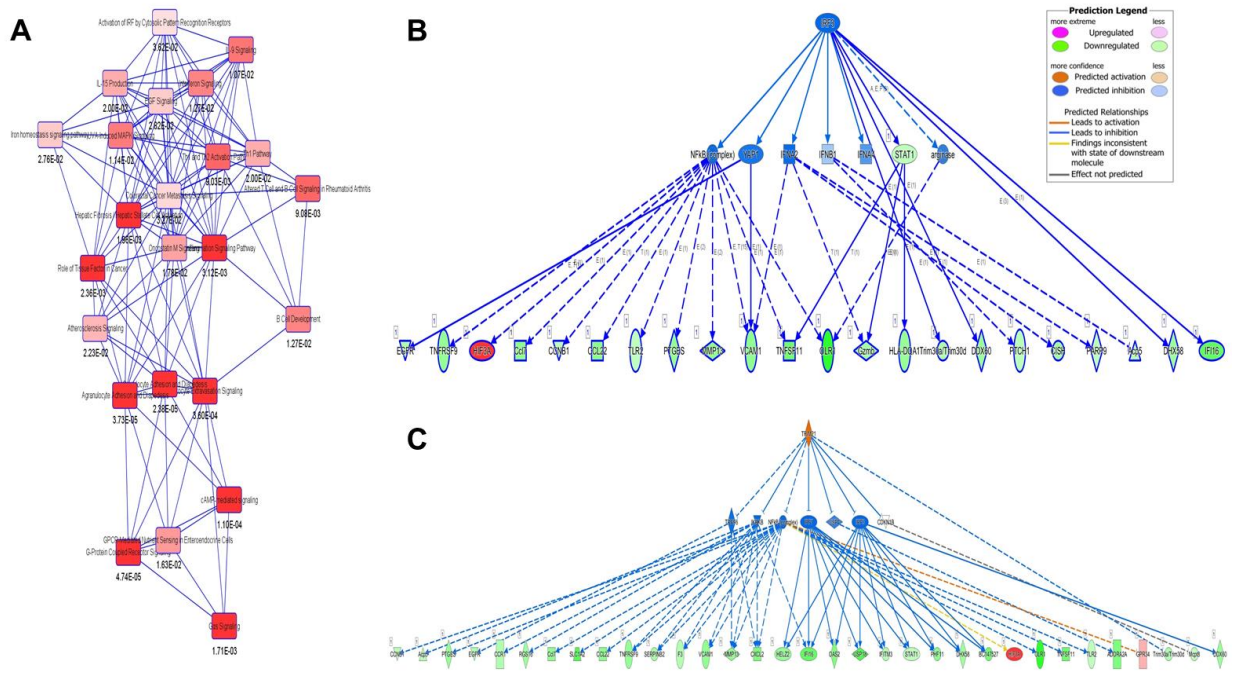

Figure 19. MBV-associated IL-33 inhibits M1-associated pathways and networks. (A) Pathways enriched by MBV-associated IL-33 uniquely differentially expressed genes (adj. p values shown beneath each pathway). **(B-C)** Top predicted causal networks for MBV-associated IL-33 differentially expressed genes.

Table 4. Top protein-encoding genes significantly regulated by MBV-associated IL-33.

Top Upregulated Genes (Z-score)	Top Downregulated Genes (Z-score)
HIF3a (4.352)	OLR1 (-3.308)
SPN (1.788)	USP18 (-2.886)
Shisa9 (1.769)	SLC1A2 (-2.780)
Zfyve28 (1.721)	Trim30c (-2.509)
HTRA4 (1.683)	IFI16 (-2.482)
GPR34 (1.631)	Art2b (-2.406)
S100B (1.523)	TNFSF11 (-2.291)
TMEM150B (1.467)	BC147527 (-2.231)

Appendix B.3 Discussion

A IL-33 has long been considered to be a nuclear cytokine that functions as an alarmin upon necrotic cell death(Cayrol & Girard, 2014). Following loss of cell integrity, IL-33 is released to the extracellular space where it is cleaved by proteases to produce active soluble IL-33 that complexes with the ST2 receptor on infiltrating immune cells(Cayrol et al., 2018). In turn, MyD88 and p65 are activated with pro-inflammatory consequences(Milovanovic et al., 2012). The IL-33/ST2 axis is essential for the recruitment and activation of T_{REG} cells in skeletal muscle healing(W. Kuswanto et al., 2016); however, recent reports show that MBV-associated IL-33 may mediate macrophage phenotype through an as yet undetermined mechanism(G. S. Hussey et al.,

2019). Results of the present study suggest that MBV-associated IL-33, which traffics into cells independent of the ST2 receptor, a markedly reduced pro-inflammatory macrophage phenotype than that propagated by the IL-33/ST2 pathway(G. S. Hussey et al., 2019), which has clear implications for macrophage phenotype modulation.

RNA sequencing analysis shows that MBV-associated IL-33 promotes a range of gene expression changes consistent with inhibition of M1-associated signaling pathways, including NFkB, TLR4, and IRF7, by way of activity on master regulators IRF3 and TRIM21. This gene expression profile could not be fully explained by the effect of MBV treatment or strain differences, demonstrating that IL-33 is capable of exerting modulatory effects in the absence of the ST2 receptor. The net macrophage phenotype elicited by IL-33^{+} MBV is broadly anti-inflammatory. It is of note that TLR4 and ST2 receptor signaling pathways share several secondary messengers, including MyD88, IRAKs, TRAFs, and p65, a component of NFkB(Kakkar & Lee, 2008; Kuzmich et al., 2017). It is plausible, though untested, that MBV-associated IL-33 may feedback on target cells to inhibit ST2-dependent signaling. In the context of muscle healing, these results strongly suggest ST2-independent IL-33 signaling may contribute to the timely transition from an M1-like macrophage phenotype towards an M2-like macrophage phenotype.

Appendix B.4 Conclusions

MBV-associated IL-33 elicits a unique pattern of gene expression that could not be accounted for by the effect of MBV treatment alone or due to source animal strain effects. The pathways and networks enriched in IL-33-unique genes are associated with an M1-like phenotype and are markedly inhibited as a result of MBV treatment.

Bibliography

Aamodt, J. M., & Grainger, D. W. (2016). Extracellular matrix-based biomaterial scaffolds and the host response. *Biomaterials, 86*, 68-82. doi:10.1016/j.biomaterials.2016.02.003

Adammek, M., Greve, B., Kassens, N., Schneider, C., Bruggemann, K., Schuring, A. N., . . . Gotte, M. (2013). MicroRNA miR-145 inhibits proliferation, invasiveness, and stem cell phenotype of an in vitro endometriosis model by targeting multiple cytoskeletal elements and pluripotency factors. *Fertil Steril, 99*(5), 1346-1355 e1345. doi:10.1016/j.fertnstert.2012.11.055

Adams, J. C., & Watt, F. M. (1989). Fibronectin inhibits the terminal differentiation of human keratinocytes. *Nature, 340*(6231), 307-309. doi:10.1038/340307a0

Agarwal, J. P., Mendenhall, S. D., Anderson, L. A., Ying, J., Boucher, K. M., Liu, T., & Neumayer, L. A. (2015). The breast reconstruction evaluation of acellular dermal matrix as a sling trial (BREASTrial): design and methods of a prospective randomized trial. *Plast Reconstr Surg, 135*(1), 20e-28e. doi:10.1097/PRS.0000000000000809

Agrawal, V., Brown, B. N., Beattie, A. J., Gilbert, T. W., & Badylak, S. F. (2009). Evidence of innervation following extracellular matrix scaffold-mediated remodelling of muscular tissues. *J Tissue Eng Regen Med, 3*(8), 590-600. doi:10.1002/term.200

Agrawal, V., Johnson, S. A., Reing, J., Zhang, L., Tottey, S., Wang, G., . . . Badylak, S. F. (2010). Epimorphic regeneration approach to tissue replacement in adult mammals. *Proc Natl Acad Sci U S A, 107*(8), 3351-3355. doi:10.1073/pnas.0905851106

Agrawal, V., Kelly, J., Tottey, S., Daly, K. A., Johnson, S. A., Siu, B. F., . . . Badylak, S. F. (2011). An isolated cryptic peptide influences osteogenesis and bone remodeling in an adult mammalian model of digit amputation. *Tissue Eng Part A, 17*(23-24), 3033-3044. doi:10.1089/ten.TEA.2011.0257

Agrawal, V., Siu, B. F., Chao, H., Hirschi, K. K., Raborn, E., Johnson, S. A., . . . Badylak, S. F. (2012). Partial characterization of the Sox2+ cell population in an adult murine model of digit amputation. *Tissue Eng Part A, 18*(13-14), 1454-1463. doi:10.1089/ten.TEA.2011.0550

Agrawal, V., Tottey, S., Johnson, S. A., Freund, J. M., Siu, B. F., & Badylak, S. F. (2011). Recruitment of progenitor cells by an extracellular matrix cryptic peptide in a mouse model of digit amputation. *Tissue Eng Part A, 17*(19-20), 2435-2443. doi:10.1089/ten.TEA.2011.0036

Ali, S., Mohs, A., Thomas, M., Klare, J., Ross, R., Schmitz, M. L., & Martin, M. U. (2011). The dual function cytokine IL-33 interacts with the transcription factor NF-kappaB to dampen

NF-kappaB-stimulated gene transcription. *J Immunol, 187*(4), 1609-1616. doi:10.4049/jimmunol.1003080

Alicuben, E. T., & DeMeester, S. R. (2014). Onlay ventral hernia repairs using porcine non-cross-linked dermal biologic mesh. *Hernia, 18*(5), 705-712. doi:10.1007/s10029-013-1054-2

Allen, R. A., Seltz, L. M., Jiang, H., Kasick, R. T., Sellaro, T. L., Badylak, S. F., & Ogilvie, J. B. (2010). Adrenal extracellular matrix scaffolds support adrenocortical cell proliferation and function in vitro. *Tissue Eng Part A, 16*(11), 3363-3374. doi:10.1089/ten.TEA.2010.0005

Allison, A. C., & Eugui, E. M. (2000). Mycophenolate mofetil and its mechanisms of action. *Immunopharmacology, 47*(2-3), 85-118. doi:10.1016/s0162-3109(00)00188-0

Ambrosio, F., Brown, E., Stolz, D., Ferrari, R., Goodpaster, B., Deasy, B., . . . Barchowsky, A. (2014). Arsenic induces sustained impairment of skeletal muscle and muscle progenitor cell ultrastructure and bioenergetics. *Free Radic Biol Med, 74*, 64-73. doi:10.1016/j.freeradbiomed.2014.06.012

Ambrosio, F., Kadi, F., Lexell, J., Fitzgerald, G. K., Boninger, M. L., & Huard, J. (2009). The effect of muscle loading on skeletal muscle regenerative potential: an update of current research findings relating to aging and neuromuscular pathology. *Am J Phys Med Rehabil, 88*(2), 145-155. doi:10.1097/PHM.0b013e3181951fc5

Anderson, K., & Hamm, R. L. (2012). Factors That Impair Wound Healing. *J Am Coll Clin Wound Spec, 4*(4), 84-91. doi:10.1016/j.jccw.2014.03.001

Ansari, M. A., Singh, V. V., Dutta, S., Veettil, M. V., Dutta, D., Chikoti, L., . . . Chandran, B. (2013). Constitutive interferon-inducible protein 16-inflammasome activation during Epstein-Barr virus latency I, II, and III in B and epithelial cells. *J Virol, 87*(15), 8606-8623. doi:10.1128/JVI.00805-13

Anurov, M. V., Titkova, S. M., & Oettinger, A. P. (2012). Biomechanical compatibility of surgical mesh and fascia being reinforced: dependence of experimental hernia defect repair results on anisotropic surgical mesh positioning. *Hernia, 16*(2), 199-210. doi:10.1007/s10029-011-0877-y

Appell, H. J., Forsberg, S., & Hollmann, W. (1988). Satellite cell activation in human skeletal muscle after training: evidence for muscle fiber neoformation. *Int J Sports Med, 9*(4), 297-299. doi:10.1055/s-2007-1025026

Ariel, A., & Serhan, C. N. (2012). New Lives Given by Cell Death: Macrophage Differentiation Following Their Encounter with Apoptotic Leukocytes during the Resolution of Inflammation. *Front Immunol, 3*, 4. doi:10.3389/fimmu.2012.00004

Arnold, L., Henry, A., Poron, F., Baba-Amer, Y., van Rooijen, N., Plonquet, A., . . . Chazaud, B. (2007). Inflammatory monocytes recruited after skeletal muscle injury switch into antiinflammatory macrophages to support myogenesis. *J Exp Med, 204*(5), 1057-1069. doi:10.1084/jem.20070075

Arnold, L., Henry, A., Poron, F., Baba-Amer, Y., Van Rooijen, N., Plonquet, A., . . . Chazaud, B. (2007). Inflammatory monocytes recruited after skeletal muscle injury switch into antiinflammatory macrophages to support myogenesis. *Journal of Experimental Medicine, 204*(5), 1057-1069.

Arslan-Yildiz, A., El Assal, R., Chen, P., Guven, S., Inci, F., & Demirci, U. (2016). Towards artificial tissue models: past, present, and future of 3D bioprinting. *Biofabrication, 8*(1), 014103. doi:10.1088/1758-5090/8/1/014103

Ashwell, J. D., Lu, F. W., & Vacchio, M. S. (2000). Glucocorticoids in T cell development and function*. *Annu Rev Immunol, 18*, 309-345. doi:10.1146/annurev.immunol.18.1.309

Atala, A., Bauer, S. B., Soker, S., Yoo, J. J., & Retik, A. B. (2006). Tissue-engineered autologous bladders for patients needing cystoplasty. *Lancet, 367*(9518), 1241-1246. doi:10.1016/S0140-6736(06)68438-9

Atzeni, F., Turiel, M., Capsoni, F., Doria, A., Meroni, P., & Sarzi-Puttini, P. (2005). Autoimmunity and anti-TNF-alpha agents. *Ann N Y Acad Sci, 1051*, 559-569. doi:10.1196/annals.1361.100

Badylak, S. F. (2007). The extracellular matrix as a biologic scaffold material. *Biomaterials, 28*(25), 3587-3593. doi:10.1016/j.biomaterials.2007.04.043

Badylak, S. F., Dziki, J. L., Sicari, B. M., Ambrosio, F., & Boninger, M. L. (2016). Mechanisms by which acellular biologic scaffolds promote functional skeletal muscle restoration. *Biomaterials, 103*, 128-136. doi:10.1016/j.biomaterials.2016.06.047

Badylak, S. F., Hoppo, T., Nieponice, A., Gilbert, T. W., Davison, J. M., & Jobe, B. A. (2011). Esophageal preservation in five male patients after endoscopic inner-layer circumferential resection in the setting of superficial cancer: a regenerative medicine approach with a biologic scaffold. *Tissue Eng Part A, 17*(11-12), 1643-1650. doi:10.1089/ten.TEA.2010.0739

Badylak, S. F., Lantz, G. C., Coffey, A., & Geddes, L. A. (1989). Small intestinal submucosa as a large diameter vascular graft in the dog. *J Surg Res, 47*(1), 74-80.

Badylak, S. F., Tullius, R., Kokini, K., Shelbourne, K. D., Klootwyk, T., Voytik, S. L., . . . Simmons, C. (1995). The use of xenogeneic small intestinal submucosa as a biomaterial for Achilles tendon repair in a dog model. *J Biomed Mater Res, 29*(8), 977-985. doi:10.1002/jbm.820290809

Badylak, S. F., Valentin, J. E., Ravindra, A. K., McCabe, G. P., & Stewart-Akers, A. M. (2008). Macrophage phenotype as a determinant of biologic scaffold remodeling. *Tissue Engineering Part A, 14*(11), 1835-1842.

Badylak, S. F., Valentin, J. E., Ravindra, A. K., McCabe, G. P., & Stewart-Akers, A. M. (2008). Macrophage phenotype as a determinant of biologic scaffold remodeling. *Tissue Eng Part A, 14*(11), 1835-1842. doi:10.1089/ten.tea.2007.0264

Baekkevold, E. S., Roussigné, M., Yamanaka, T., Johansen, F.-E., Jahnsen, F. L., Amalric, F., . . . Girard, J.-P. (2003). Molecular characterization of NF-HEV, a nuclear factor preferentially expressed in human high endothelial venules. *Am J Pathol, 163*(1), 69-79.

Baghdadi, M. B., & Tajbakhsh, S. (2018). Regulation and phylogeny of skeletal muscle regeneration. *Dev Biol, 433*(2), 200-209. doi:10.1016/j.ydbio.2017.07.026

Baker, B. M., & Chen, C. S. (2012). Deconstructing the third dimension: how 3D culture microenvironments alter cellular cues. *J Cell Sci, 125*(Pt 13), 3015-3024. doi:10.1242/jcs.079509

Banerjee, S., Cui, H., Xie, N., Tan, Z., Yang, S., Icyuz, M., . . . Liu, G. (2013). miR-125a-5p regulates differential activation of macrophages and inflammation. *J Biol Chem, 288*(49), 35428-35436. doi:10.1074/jbc.M112.426866

Baneyx, G., Baugh, L., & Vogel, V. (2002). Fibronectin extension and unfolding within cell matrix fibrils controlled by cytoskeletal tension. *Proc Natl Acad Sci U S A, 99*(8), 5139-5143. doi:10.1073/pnas.072650799

Baylon, K., Rodriguez-Camarillo, P., Elias-Zuniga, A., Diaz-Elizondo, J. A., Gilkerson, R., & Lozano, K. (2017). Past, Present and Future of Surgical Meshes: A Review. *Membranes (Basel), 7*(3). doi:10.3390/membranes7030047

Beattie, A. J., Gilbert, T. W., Guyot, J. P., Yates, A. J., & Badylak, S. F. (2009). Chemoattraction of progenitor cells by remodeling extracellular matrix scaffolds. *Tissue Eng Part A, 15*(5), 1119-1125. doi:10.1089/ten.tea.2008.0162

Berda-Haddad, Y., Robert, S., Salers, P., Zekraoui, L., Farnarier, C., Dinarello, C. A., . . . Kaplanski, G. (2011). Sterile inflammation of endothelial cell-derived apoptotic bodies is mediated by interleukin-1alpha. *Proc Natl Acad Sci U S A, 108*(51), 20684-20689. doi:10.1073/pnas.1116848108

Bertelli, J. A., & Ghizoni, M. F. (2016). Nerve and Free Gracilis Muscle Transfers for Thumb and Finger Extension Reconstruction in Long-standing Tetraplegia. *J Hand Surg Am, 41*(11), e411-e416. doi:10.1016/j.jhsa.2016.08.003

Bhattacharya, S., McElhanon, K. E., Gushchina, L. V., & Weisleder, N. (2016). Role of phosphatidylinositol-4,5-bisphosphate 3-kinase signaling in vesicular trafficking. *Life Sci, 167*, 39-45. doi:10.1016/j.lfs.2016.10.018

Billaud, M., Hill, J. C., Richards, T. D., Gleason, T. G., & Phillippi, J. A. (2018). Medial Hypoxia and Adventitial Vasa Vasorum Remodeling in Human Ascending Aortic Aneurysm. *Front Cardiovasc Med, 5*, 124. doi:10.3389/fcvm.2018.00124

Bissell, M. J., & Aggeler, J. (1987). Dynamic reciprocity: how do extracellular matrix and hormones direct gene expression? *Prog Clin Biol Res, 249*, 251-262.

Bittel, D. C., & Jaiswal, J. K. (2019). Contribution of Extracellular Vesicles in Rebuilding Injured Muscles. *Front Physiol, 10*, 828. doi:10.3389/fphys.2019.00828

Bluestone, J. A., Thomson, A. W., Shevach, E. M., & Weiner, H. L. (2007). What does the future hold for cell-based tolerogenic therapy? *Nat Rev Immunol, 7*(8), 650-654. doi:10.1038/nri2137

Boehler, R. M., Graham, J. G., & Shea, L. D. (2011). Tissue engineering tools for modulation of the immune response. *Biotechniques, 51*(4), 239-240, 242, 244 passim. doi:10.2144/000113754

Böing, A. N., Van Der Pol, E., Grootemaat, A. E., Coumans, F. A., Sturk, A., & Nieuwland, R. (2014). Single-step isolation of extracellular vesicles by size-exclusion chromatography. *Journal of extracellular vesicles, 3*(1), 23430.

Bornstein, P. (2009). Thrombospondins function as regulators of angiogenesis. *J Cell Commun Signal, 3*(3-4), 189-200. doi:10.1007/s12079-009-0060-8

Borschel, G. H., Dennis, R. G., & Kuzon, W. M., Jr. (2004). Contractile skeletal muscle tissue-engineered on an acellular scaffold. *Plast Reconstr Surg, 113*(2), 595-602; discussion 603-594. doi:10.1097/01.PRS.0000101064.62289.2F

Borschel, G. H., Dow, D. E., Dennis, R. G., & Brown, D. L. (2006). Tissue-engineered axially vascularized contractile skeletal muscle. *Plast Reconstr Surg, 117*(7), 2235-2242. doi:10.1097/01.prs.0000224295.54073.49

Brack, A. S., Conboy, M. J., Roy, S., Lee, M., Kuo, C. J., Keller, C., & Rando, T. A. (2007). Increased Wnt signaling during aging alters muscle stem cell fate and increases fibrosis. *Science, 317*(5839), 807-810. doi:10.1126/science.1144090

Brandt, C. J., Kammer, D., Fiebeler, A., & Klinge, U. (2011). Beneficial effects of hydrocortisone or spironolactone coating on foreign body response to mesh biomaterial in a mouse model. *J Biomed Mater Res A, 99*(3), 335-343. doi:10.1002/jbm.a.33136

Brennan, E. P., Reing, J., Chew, D., Myers-Irvin, J. M., Young, E. J., & Badylak, S. F. (2006). Antibacterial activity within degradation products of biological scaffolds composed of extracellular matrix. *Tissue Eng, 12*(10), 2949-2955. doi:10.1089/ten.2006.12.2949

Briot, K., & Roux, C. (2015). Glucocorticoid-induced osteoporosis. *RMD Open, 1*(1), e000014. doi:10.1136/rmdopen-2014-000014

Brown, B. N., Chung, W. L., Almarza, A. J., Pavlick, M. D., Reppas, S. N., Ochs, M. W., . . . Badylak, S. F. (2012). Inductive, scaffold-based, regenerative medicine approach to reconstruction of the temporomandibular joint disk. *J Oral Maxillofac Surg, 70*(11), 2656-2668. doi:10.1016/j.joms.2011.12.030

Brown, B. N., Londono, R., Tottey, S., Zhang, L., Kukla, K. A., Wolf, M. T., . . . Badylak, S. F. (2012). Macrophage phenotype as a predictor of constructive remodeling following the

implantation of biologically derived surgical mesh materials. *Acta Biomater, 8*(3), 978-987. doi:10.1016/j.actbio.2011.11.031

Brown, B. N., Ratner, B. D., Goodman, S. B., Amar, S., & Badylak, S. F. (2012). Macrophage polarization: an opportunity for improved outcomes in biomaterials and regenerative medicine. *Biomaterials, 33*(15), 3792-3802. doi:10.1016/j.biomaterials.2012.02.034

Brown, B. N., Valentin, J. E., Stewart-Akers, A. M., McCabe, G. P., & Badylak, S. F. (2009). Macrophage phenotype and remodeling outcomes in response to biologic scaffolds with and without a cellular component. *Biomaterials, 30*(8), 1482-1491. doi:10.1016/j.biomaterials.2008.11.040

Brudno, Y., Ennett-Shepard, A. B., Chen, R. R., Aizenberg, M., & Mooney, D. J. (2013). Enhancing microvascular formation and vessel maturation through temporal control over multiple pro-angiogenic and pro-maturation factors. *Biomaterials, 34*(36), 9201-9209. doi:10.1016/j.biomaterials.2013.08.007

Bunggulawa, E. J., Wang, W., Yin, T., Wang, N., Durkan, C., Wang, Y., & Wang, G. (2018). Recent advancements in the use of exosomes as drug delivery systems. *J Nanobiotechnology, 16*(1), 81. doi:10.1186/s12951-018-0403-9

Burger, J. W., Halm, J. A., Wijsmuller, A. R., ten Raa, S., & Jeekel, J. (2006). Evaluation of new prosthetic meshes for ventral hernia repair. *Surg Endosc, 20*(8), 1320-1325. doi:10.1007/s00464-005-0706-4

Butti, E., Bergami, A., Recchia, A., Brambilla, E., Del Carro, U., Amadio, S., . . . Furlan, R. (2008). IL4 gene delivery to the CNS recruits regulatory T cells and induces clinical recovery in mouse models of multiple sclerosis. *Gene Ther, 15*(7), 504-515. doi:10.1038/gt.2008.10

Carriere, V., Roussel, L., Ortega, N., Lacorre, D.-A., Americh, L., Aguilar, L., . . . Girard, J.-P. (2007). IL-33, the IL-1-like cytokine ligand for ST2 receptor, is a chromatin-associated nuclear factor in vivo. *Proceedings of the National Academy of Sciences, 104*(1), 282-287.

Carriere, V., Roussel, L., Ortega, N., Lacorre, D. A., Americh, L., Aguilar, L., . . . Girard, J. P. (2007). IL-33, the IL-1-like cytokine ligand for ST2 receptor, is a chromatin-associated nuclear factor in vivo. *Proc Natl Acad Sci U S A, 104*(1), 282-287. doi:10.1073/pnas.0606854104

Carter, G. C., Bernstone, L., Baskaran, D., & James, W. (2011). HIV-1 infects macrophages by exploiting an endocytic route dependent on dynamin, Rac1 and Pak1. *Virology, 409*(2), 234-250. doi:10.1016/j.virol.2010.10.018

Cayrol, C., Duval, A., Schmitt, P., Roga, S., Camus, M., Stella, A., . . . Girard, J. P. (2018). Environmental allergens induce allergic inflammation through proteolytic maturation of IL-33. *Nat Immunol, 19*(4), 375-385. doi:10.1038/s41590-018-0067-5

Cayrol, C., & Girard, J. P. (2014). IL-33: an alarmin cytokine with crucial roles in innate immunity, inflammation and allergy. *Curr Opin Immunol, 31*, 31-37. doi:10.1016/j.coi.2014.09.004

Chaudhuri, A. A., So, A. Y., Sinha, N., Gibson, W. S., Taganov, K. D., O'Connell, R. M., & Baltimore, D. (2011). MicroRNA-125b potentiates macrophage activation. *J Immunol, 187*(10), 5062-5068. doi:10.4049/jimmunol.1102001

Chen, C. Y., Rao, S. S., Ren, L., Hu, X. K., Tan, Y. J., Hu, Y., . . . Xie, H. (2018). Exosomal DMBT1 from human urine-derived stem cells facilitates diabetic wound repair by promoting angiogenesis. *Theranostics, 8*(6), 1607-1623. doi:10.7150/thno.22958

Chen, M. R., & Dragoo, J. L. (2013). The effect of nonsteroidal anti-inflammatory drugs on tissue healing. *Knee Surg Sports Traumatol Arthrosc, 21*(3), 540-549. doi:10.1007/s00167-012-2095-2

Chen, S., Yang, J., Wei, Y., & Wei, X. (2020). Epigenetic regulation of macrophages: from homeostasis maintenance to host defense. *Cell Mol Immunol, 17*(1), 36-49. doi:10.1038/s41423-019-0315-0

Cheng, M., Nguyen, M. H., Fantuzzi, G., & Koh, T. J. (2008). Endogenous interferon-gamma is required for efficient skeletal muscle regeneration. *Am J Physiol Cell Physiol, 294*(5), C1183-1191. doi:10.1152/ajpcell.00568.2007

Chintala, S. K., Miller, R. R., & McDevitt, C. A. (1995). Role of heparan sulfate in the terminal differentiation of growth plate chondrocytes. *Arch Biochem Biophys, 316*(1), 227-234. doi:10.1006/abbi.1995.1032

Cho, D. I., Kim, M. R., Jeong, H. Y., Jeong, H. C., Jeong, M. H., Yoon, S. H., . . . Ahn, Y. (2014). Mesenchymal stem cells reciprocally regulate the M1/M2 balance in mouse bone marrow-derived macrophages. *Exp Mol Med, 46*, e70. doi:10.1038/emm.2013.135

Cholok, D., Lee, E., Lisiecki, J., Agarwal, S., Loder, S., Ranganathan, K., . . . Levi, B. (2017). Traumatic muscle fibrosis: From pathway to prevention. *J Trauma Acute Care Surg, 82*(1), 174-184. doi:10.1097/TA.0000000000001290

Clark, R. A., Wikner, N. E., Doherty, D. E., & Norris, D. A. (1988). Cryptic chemotactic activity of fibronectin for human monocytes resides in the 120-kDa fibroblastic cell-binding fragment. *J Biol Chem, 263*(24), 12115-12123.

Cohen, D. B., Kawamura, S., Ehteshami, J. R., & Rodeo, S. A. (2006). Indomethacin and celecoxib impair rotator cuff tendon-to-bone healing. *Am J Sports Med, 34*(3), 362-369. doi:10.1177/0363546505280428

Cohen, E. S., Scott, I. C., Majithiya, J. B., Rapley, L., Kemp, B. P., England, E., . . . Bond, N. J. (2015). Oxidation of the alarmin IL-33 regulates ST2-dependent inflammation. *Nature communications, 6*.

Conboy, I. M., Conboy, M. J., Wagers, A. J., Girma, E. R., Weissman, I. L., & Rando, T. A. (2005). Rejuvenation of aged progenitor cells by exposure to a young systemic environment. *Nature, 433*(7027), 760-764. doi:10.1038/nature03260

Cordes, K. R., Sheehy, N. T., White, M. P., Berry, E. C., Morton, S. U., Muth, A. N., . . . Srivastava, D. (2009). miR-145 and miR-143 regulate smooth muscle cell fate and plasticity. *Nature, 460*(7256), 705-710. doi:10.1038/nature08195

Corona, B. T., Rivera, J. C., Owens, J. G., Wenke, J. C., & Rathbone, C. R. (2015). Volumetric muscle loss leads to permanent disability following extremity trauma. *J Rehabil Res Dev, 52*(7), 785-792. doi:10.1682/JRRD.2014.07.0165

Corona, B. T., Ward, C. L., Baker, H. B., Walters, T. J., & Christ, G. J. (2014). Implantation of in vitro tissue engineered muscle repair constructs and bladder acellular matrices partially restore in vivo skeletal muscle function in a rat model of volumetric muscle loss injury. *Tissue Eng Part A, 20*(3-4), 705-715. doi:10.1089/ten.TEA.2012.0761

Corona, B. T., Wenke, J. C., & Ward, C. L. (2016). Pathophysiology of Volumetric Muscle Loss Injury. *Cells Tissues Organs, 202*(3-4), 180-188. doi:10.1159/000443925

Coutinho, A. E., & Chapman, K. E. (2011). The anti-inflammatory and immunosuppressive effects of glucocorticoids, recent developments and mechanistic insights. *Mol Cell Endocrinol, 335*(1), 2-13. doi:10.1016/j.mce.2010.04.005

Cunningham, K. S., & Gotlieb, A. I. (2005). The role of shear stress in the pathogenesis of atherosclerosis. *Lab Invest, 85*(1), 9-23. doi:10.1038/labinvest.3700215

Daley, J. M., Brancato, S. K., Thomay, A. A., Reichner, J. S., & Albina, J. E. (2010). The phenotype of murine wound macrophages. *J Leukoc Biol, 87*(1), 59-67.

Danoviz, M. E., & Yablonka-Reuveni, Z. (2012). Skeletal muscle satellite cells: background and methods for isolation and analysis in a primary culture system. *Methods Mol Biol, 798*, 21-52. doi:10.1007/978-1-61779-343-1_2

Davis, G. E., Bayless, K. J., Davis, M. J., & Meininger, G. A. (2000). Regulation of tissue injury responses by the exposure of matricryptic sites within extracellular matrix molecules. *Am J Pathol, 156*(5), 1489-1498. doi:10.1016/S0002-9440(10)65020-1

Dearth, C. L., Slivka, P. F., Stewart, S. A., Keane, T. J., Tay, J. K., Londono, R., . . . Badylak, S. F. (2016). Inhibition of COX1/2 alters the host response and reduces ECM scaffold mediated constructive tissue remodeling in a rodent model of skeletal muscle injury. *Acta Biomater, 31*, 50-60. doi:10.1016/j.actbio.2015.11.043

Deasy, B. M., Jankowski, R. J., & Huard, J. (2001). Muscle-derived stem cells: characterization and potential for cell-mediated therapy. *Blood Cells Mol Dis, 27*(5), 924-933. doi:10.1006/bcmd.2001.0463

Deng, B., Wehling-Henricks, M., Villalta, S. A., Wang, Y., & Tidball, J. G. (2012). IL-10 triggers changes in macrophage phenotype that promote muscle growth and regeneration. *The Journal of Immunology*, 1103180.

Deng, B., Wehling-Henricks, M., Villalta, S. A., Wang, Y., & Tidball, J. G. (2012). IL-10 triggers changes in macrophage phenotype that promote muscle growth and regeneration. *J Immunol, 189*(7), 3669-3680. doi:10.4049/jimmunol.1103180

Dort, J., Fabre, P., Molina, T., & Dumont, N. A. (2019). Macrophages Are Key Regulators of Stem Cells during Skeletal Muscle Regeneration and Diseases. *Stem Cells Int, 2019*, 4761427. doi:10.1155/2019/4761427

Duchman, K. R., Lemmex, D. B., Patel, S. H., Ledbetter, L., Garrigues, G. E., & Riboh, J. C. (2019). The Effect of Non-Steroidal Anti-Inflammatory Drugs on Tendon-to-Bone Healing: A Systematic Review with Subgroup Meta-Analysis. *Iowa Orthop J, 39*(1), 107-119.

Duncan, M. D., & Wilkes, D. S. (2005). Transplant-related immunosuppression: a review of immunosuppression and pulmonary infections. *Proc Am Thorac Soc, 2*(5), 449-455. doi:10.1513/pats.200507-073JS

Dziki, J., Badylak, S., Yabroudi, M., Sicari, B., Ambrosio, F., Stearns, K., . . . Rubin, J. P. (2016). An acellular biologic scaffold treatment for volumetric muscle loss: results of a 13-patient cohort study. *NPJ Regen Med, 1*, 16008. doi:10.1038/npjregenmed.2016.8

Dziki, J., Badylak, S., Yabroudi, M., Sicari, B., Ambrosio, F., Stearns, K., . . . Rubin, J. P. (2016). An acellular biologic scaffold treatment for volumetric muscle loss: results of a 13-patient cohort study. *Npj Regenerative Medicine, 1*, 16008. doi:10.1038/npjregenmed.2016.8

http://www.nature.com/articles/npjregenmed20168#supplementary-information

Dziki, J. L., Giglio, R. M., Sicari, B. M., Wang, D. S., Gandhi, R. M., Londono, R., . . . Badylak, S. F. (2017). The effect of mechanical loading upon extracellular matrix bioscaffold-mediated skeletal muscle remodeling. *Tissue Eng Part A*. doi:10.1089/ten.TEA.2017.0011

Dziki, J. L., Giglio, R. M., Sicari, B. M., Wang, D. S., Gandhi, R. M., Londono, R., . . . Badylak, S. F. (2018). The Effect of Mechanical Loading Upon Extracellular Matrix Bioscaffold-Mediated Skeletal Muscle Remodeling. *Tissue Eng Part A, 24*(1-2), 34-46. doi:10.1089/ten.TEA.2017.0011

Dziki, J. L., Huleihel, L., Scarritt, M. E., & Badylak, S. F. (2017). Extracellular Matrix Bioscaffolds as Immunomodulatory Biomaterials. *Tissue Eng Part A*. doi:10.1089/ten.TEA.2016.0538

Dziki, J. L., Sicari, B. M., Wolf, M. T., Cramer, M. C., & Badylak, S. F. (2016). Immunomodulation and Mobilization of Progenitor Cells by Extracellular Matrix Bioscaffolds for Volumetric Muscle Loss Treatment. *Tissue Eng Part A, 22*(19-20), 1129-1139. doi:10.1089/ten.TEA.2016.0340

Dziki, J. L., Wang, D. S., Pineda, C., Sicari, B. M., Rausch, T., & Badylak, S. F. (2017). Solubilized extracellular matrix bioscaffolds derived from diverse source tissues

differentially influence macrophage phenotype. *J Biomed Mater Res A, 105*(1), 138-147. doi:10.1002/jbm.a.35894

Dzobo, K., Vogelsang, M., & Parker, M. I. (2015). Wnt/beta-Catenin and MEK-ERK Signaling are Required for Fibroblast-Derived Extracellular Matrix-Mediated Endoderm Differentiation of Embryonic Stem Cells. *Stem Cell Rev, 11*(5), 761-773. doi:10.1007/s12015-015-9598-4

Ehrchen, J., Steinmuller, L., Barczyk, K., Tenbrock, K., Nacken, W., Eisenacher, M., . . . Roth, J. (2007). Glucocorticoids induce differentiation of a specifically activated, anti-inflammatory subtype of human monocytes. *Blood, 109*(3), 1265-1274. doi:10.1182/blood-2006-02-001115

Elango, S., Perumalsamy, S., Ramachandran, K., & Vadodaria, K. (2017). Mesh materials and hernia repair. *Biomedicine (Taipei), 7*(3), 16. doi:10.1051/bmdcn/2017070316

Englen, M. D., Valdez, Y. E., Lehnert, N. M., & Lehnert, B. E. (1995). Granulocyte/macrophage colony-stimulating factor is expressed and secreted in cultures of murine L929 cells. *J Immunol Methods, 184*(2), 281-283.

Evans, E., Leung, A., Heinrich, V., & Zhu, C. (2004). Mechanical switching and coupling between two dissociation pathways in a P-selectin adhesion bond. *Proc Natl Acad Sci U S A, 101*(31), 11281-11286. doi:10.1073/pnas.0401870101

Ezquer, F. E., Ezquer, M. E., Vicencio, J. M., & Calligaris, S. D. (2017). Two complementary strategies to improve cell engraftment in mesenchymal stem cell-based therapy: Increasing transplanted cell resistance and increasing tissue receptivity. *Cell Adh Migr, 11*(1), 110-119. doi:10.1080/19336918.2016.1197480

Fadok, V. A., Bratton, D. L., Konowal, A., Freed, P. W., Westcott, J. Y., & Henson, P. M. (1998). Macrophages that have ingested apoptotic cells in vitro inhibit proinflammatory cytokine production through autocrine/paracrine mechanisms involving TGF-beta, PGE2, and PAF. *J Clin Invest, 101*(4), 890-898. doi:10.1172/JCI1112

Farge, E. (2011). Mechanotransduction in development. *Curr Top Dev Biol, 95*, 243-265. doi:10.1016/B978-0-12-385065-2.00008-6

Faulk, D. M., Londono, R., Wolf, M. T., Ranallo, C. A., Carruthers, C. A., Wildemann, J. D., . . . Badylak, S. F. (2014). ECM hydrogel coating mitigates the chronic inflammatory response to polypropylene mesh. *Biomaterials, 35*(30), 8585-8595. doi:10.1016/j.biomaterials.2014.06.057

Filipe, V., Hawe, A., & Jiskoot, W. (2010). Critical evaluation of Nanoparticle Tracking Analysis (NTA) by NanoSight for the measurement of nanoparticles and protein aggregates. *Pharmaceutical research, 27*(5), 796-810. doi:10.1007/s11095-010-0073-2

Foster, R. F., Thompson, J. M., & Kaufman, S. J. (1987). A laminin substrate promotes myogenesis in rat skeletal muscle cultures: analysis of replication and development using antidesmin

and anti-BrdUrd monoclonal antibodies. *Dev Biol, 122*(1), 11-20. doi:10.1016/0012-1606(87)90327-7

Fu, H., Yang, G., Wei, M., Liu, L., Jin, L., Lu, X., . . . Lu, Z. (2012). The RNA-binding protein QKI5 is a direct target of C/EBPalpha and delays macrophage differentiation. *Mol Biol Cell, 23*(9), 1628-1635. doi:10.1091/mbc.E11-05-0412

Fukai, F., Suzuki, H., Suzuki, K., Tsugita, A., & Katayama, T. (1991). Rat plasma fibronectin contains two distinct chemotactic domains for fibroblastic cells. *J Biol Chem, 266*(14), 8807-8813.

Gandhi, D., Marcin, S., Xin, Z., Asha, B., Kaswala, D., & Zamir, B. (2011). Chronic abdominal pain secondary to mesh erosion into cecum following incisional hernia repair: a case report and literature review. *Ann Gastroenterol, 24*(4), 321-324.

Garg, K., Corona, B. T., & Walters, T. J. (2014). Losartan administration reduces fibrosis but hinders functional recovery after volumetric muscle loss injury. *J Appl Physiol (1985), 117*(10), 1120-1131. doi:10.1152/japplphysiol.00689.2014

Garg, K., Corona, B. T., & Walters, T. J. (2015). Therapeutic strategies for preventing skeletal muscle fibrosis after injury. *Front Pharmacol, 6*, 87. doi:10.3389/fphar.2015.00087

Garg, K., Ward, C. L., Rathbone, C. R., & Corona, B. T. (2014). Transplantation of devitalized muscle scaffolds is insufficient for appreciable de novo muscle fiber regeneration after volumetric muscle loss injury. *Cell Tissue Res, 358*(3), 857-873. doi:10.1007/s00441-014-2006-6

Geer, D. J., Swartz, D. D., & Andreadis, S. T. (2005). Biomimetic delivery of keratinocyte growth factor upon cellular demand for accelerated wound healing in vitro and in vivo. *Am J Pathol, 167*(6), 1575-1586. doi:10.1016/S0002-9440(10)61242-4

Gerdisch, M. W., Shea, R. J., & Barron, M. D. (2014). Clinical experience with CorMatrix extracellular matrix in the surgical treatment of mitral valve disease. *J Thorac Cardiovasc Surg, 148*(4), 1370-1378. doi:10.1016/j.jtcvs.2013.10.055

Geurtzen, K., Vernet, A., Freidin, A., Rauner, M., Hofbauer, L. C., Schneider, J. E., . . . Knopf, F. (2017). Immune Suppressive and Bone Inhibitory Effects of Prednisolone in Growing and Regenerating Zebrafish Tissues. *J Bone Miner Res, 32*(12), 2476-2488. doi:10.1002/jbmr.3231

Ghivizzani, S. C., Lechman, E. R., Kang, R., Tio, C., Kolls, J., Evans, C. H., & Robbins, P. D. (1998). Direct adenovirus-mediated gene transfer of interleukin 1 and tumor necrosis factor alpha soluble receptors to rabbit knees with experimental arthritis has local and distal anti-arthritic effects. *Proc Natl Acad Sci U S A, 95*(8), 4613-4618.

Giannelli, G., Falk-Marzillier, J., Schiraldi, O., Stetler-Stevenson, W. G., & Quaranta, V. (1997). Induction of cell migration by matrix metalloprotease-2 cleavage of laminin-5. *Science, 277*(5323), 225-228. doi:10.1126/science.277.5323.225

Gilbert, A. I. (1989). An anatomic and functional classification for the diagnosis and treatment of inguinal hernia. *Am J Surg, 157*(3), 331-333. doi:10.1016/0002-9610(89)90564-3

Gilbert, T. W., Stolz, D. B., Biancaniello, F., Simmons-Byrd, A., & Badylak, S. F. (2005). Production and characterization of ECM powder: implications for tissue engineering applications. *Biomaterials, 26*(12), 1431-1435. doi:10.1016/j.biomaterials.2004.04.042

Glass, D. J. (2003). Signalling pathways that mediate skeletal muscle hypertrophy and atrophy. *Nat Cell Biol, 5*(2), 87-90. doi:10.1038/ncb0203-87

Godwin, J. W., Pinto, A. R., & Rosenthal, N. A. (2016). Chasing the recipe for a pro-regenerative immune system. *Semin Cell Dev Biol*. doi:10.1016/j.semcdb.2016.08.008

Grahovac, J., Becker, D., & Wells, A. (2013). Melanoma cell invasiveness is promoted at least in part by the epidermal growth factor-like repeats of tenascin-C. *J Invest Dermatol, 133*(1), 210-220. doi:10.1038/jid.2012.263

Grasman, J. M., Zayas, M. J., Page, R. L., & Pins, G. D. (2015). Biomimetic scaffolds for regeneration of volumetric muscle loss in skeletal muscle injuries. *Acta Biomater, 25*, 2-15. doi:10.1016/j.actbio.2015.07.038

Greising, S. M., Corona, B. T., McGann, C., Frankum, J. K., & Warren, G. L. (2019). Therapeutic Approaches for Volumetric Muscle Loss Injury: A Systematic Review and Meta-Analysis. *Tissue Eng Part B Rev, 25*(6), 510-525. doi:10.1089/ten.TEB.2019.0207

Greising, S. M., Dearth, C. L., & Corona, B. T. (2016). Regenerative and Rehabilitative Medicine: A Necessary Synergy for Functional Recovery from Volumetric Muscle Loss Injury. *Cells Tissues Organs, 202*(3-4), 237-249. doi:10.1159/000444673

Greising, S. M., Warren, G. L., Southern, W. M., Nichenko, A. S., Qualls, A. E., Corona, B. T., & Call, J. A. (2018). Early rehabilitation for volumetric muscle loss injury augments endogenous regenerative aspects of muscle strength and oxidative capacity. *BMC Musculoskelet Disord, 19*(1), 173. doi:10.1186/s12891-018-2095-6

Griffith, C. K., Miller, C., Sainson, R. C., Calvert, J. W., Jeon, N. L., Hughes, C. C., & George, S. C. (2005). Diffusion limits of an in vitro thick prevascularized tissue. *Tissue Eng, 11*(1-2), 257-266. doi:10.1089/ten.2005.11.257

Grogan, B. F., Hsu, J. R., & Skeletal Trauma Research, C. (2011). Volumetric muscle loss. *J Am Acad Orthop Surg, 19 Suppl 1*, S35-37.

Guescini, M., Maggio, S., Ceccaroli, P., Battistelli, M., Annibalini, G., Piccoli, G., . . . Stocchi, V. (2017). Extracellular Vesicles Released by Oxidatively Injured or Intact C2C12 Myotubes Promote Distinct Responses Converging toward Myogenesis. *Int J Mol Sci, 18*(11). doi:10.3390/ijms18112488

Guillaume, O., Lavigne, J. P., Lefranc, O., Nottelet, B., Coudane, J., & Garric, X. (2011). New antibiotic-eluting mesh used for soft tissue reinforcement. *Acta Biomater, 7*(9), 3390-3397. doi:10.1016/j.actbio.2011.05.009

Guiteras, R., Flaquer, M., & Cruzado, J. M. (2016). Macrophage in chronic kidney disease. *Clin Kidney J, 9*(6), 765-771. doi:10.1093/ckj/sfw096

Gulinelli, S., Salaro, E., Vuerich, M., Bozzato, D., Pizzirani, C., Bolognesi, G., . . . Ferrari, D. (2012). IL-18 associates to microvesicles shed from human macrophages by a LPS/TLR-4 independent mechanism in response to P2X receptor stimulation. *Eur J Immunol, 42*(12), 3334-3345. doi:10.1002/eji.201142268

Hachim, D., Iftikhar, A., LoPresti, S. T., Nolfi, A. L., Ravichandar, S., Skillen, C. D., & Brown, B. N. (2019). Distinct release strategies are required to modulate macrophage phenotype in young versus aged animals. *J Control Release, 305*, 65-74. doi:10.1016/j.jconrel.2019.05.020

Hachim, D., LoPresti, S. T., Yates, C. C., & Brown, B. N. (2017). Shifts in macrophage phenotype at the biomaterial interface via IL-4 eluting coatings are associated with improved implant integration. *Biomaterials, 112*, 95-107. doi:10.1016/j.biomaterials.2016.10.019

Hachim, D., Wang, N., Lopresti, S. T., Stahl, E. C., Umeda, Y. U., Rege, R. D., . . . Brown, B. N. (2017). Effects of aging upon the host response to implants. *J Biomed Mater Res A*. doi:10.1002/jbm.a.36013

Hammond, J. S., Gilbert, T. W., Howard, D., Zaitoun, A., Michalopoulos, G., Shakesheff, K. M., . . . Badylak, S. F. (2011). Scaffolds containing growth factors and extracellular matrix induce hepatocyte proliferation and cell migration in normal and regenerating rat liver. *J Hepatol, 54*(2), 279-287. doi:10.1016/j.jhep.2010.06.040

Hannafon, B. N., & Ding, W. Q. (2013). Intercellular communication by exosome-derived microRNAs in cancer. *Int J Mol Sci, 14*(7), 14240-14269. doi:10.3390/ijms140714240

Hardy, D., Besnard, A., Latil, M., Jouvion, G., Briand, D., Thepenier, C., . . . Chretien, F. (2016). Comparative Study of Injury Models for Studying Muscle Regeneration in Mice. *PLoS One, 11*(1), e0147198. doi:10.1371/journal.pone.0147198

Hasegawa, H., Thomas, H. J., Schooley, K., & Born, T. L. (2011). Native IL-32 is released from intestinal epithelial cells via a non-classical secretory pathway as a membrane-associated protein. *Cytokine, 53*(1), 74-83. doi:10.1016/j.cyto.2010.09.002

Heath, C. M., Stahl, P. D., & Barbieri, M. A. (2003). Lipid kinases play crucial and multiple roles in membrane trafficking and signaling. *Histol Histopathol, 18*(3), 989-998. doi:10.14670/HH-18.989

Herberts, C. A., Kwa, M. S., & Hermsen, H. P. (2011). Risk factors in the development of stem cell therapy. *Journal of translational medicine, 9*(1), 29.

Heredia, J. E., Mukundan, L., Chen, F. M., Mueller, A. A., Deo, R. C., Locksley, R. M., . . . Chawla, A. (2013). Type 2 innate signals stimulate fibro/adipogenic progenitors to facilitate muscle regeneration. *Cell, 153*(2), 376-388. doi:10.1016/j.cell.2013.02.053

Hewitt, J., McCormack, C., Tay, H. S., Greig, M., Law, J., Tay, A., . . . Stechman, M. J. (2016). Prevalence of multimorbidity and its association with outcomes in older emergency general surgical patients: an observational study. *BMJ Open, 6*(3), e010126. doi:10.1136/bmjopen-2015-010126

Hong, C. S., Funk, S., Muller, L., Boyiadzis, M., & Whiteside, T. L. (2016). Isolation of biologically active and morphologically intact exosomes from plasma of patients with cancer. *J Extracell Vesicles, 5*, 29289. doi:10.3402/jev.v5.29289

Hong CS., F. S., Whiteside T.L. (2017). Isolation of Biologically Active Exosomes from Plasma of Patients with Cancer. In L. E. Fortina P., Park J., Kricka L (Ed.), *Acute Myeloid Leukemia. Methods in Molecular Biology* (Vol. 1633, pp. 257-265). New York, NY: Humana Press.

Hoque, M. E., San, W. Y., Wei, F., Li, S., Huang, M. H., Vert, M., & Hutmacher, D. W. (2009). Processing of polycaprolactone and polycaprolactone-based copolymers into 3D scaffolds, and their cellular responses. *Tissue Eng Part A, 15*(10), 3013-3024. doi:10.1089/ten.TEA.2008.0355

Howell, J., Sawhney, R., Testro, A., Skinner, N., Gow, P., Angus, P., . . . Visvanathan, K. (2013). Cyclosporine and tacrolimus have inhibitory effects on toll-like receptor signaling after liver transplantation. *Liver Transpl, 19*(10), 1099-1107. doi:10.1002/lt.23712

Huard, J., Cao, B., & Qu-Petersen, Z. (2003). Muscle-derived stem cells: potential for muscle regeneration. *Birth Defects Res C Embryo Today, 69*(3), 230-237. doi:10.1002/bdrc.10020

Huleihel, L., Bartolacci, J., Dziki, J. L., Vorobyov, T., Arnold, B., Scarritt, M., . . . Badylak, S. F. (2017). Matrix bound nanovesicles recapitulate extracellular matrix effects on macrophage phenotype. *Tissue Eng Part A*. doi:10.1089/ten.TEA.2017.0102

Huleihel, L., Bartolacci, J. G., Dziki, J. L., Vorobyov, T., Arnold, B., Scarritt, M. E., . . . Badylak, S. F. (2017). Matrix-Bound Nanovesicles Recapitulate Extracellular Matrix Effects on Macrophage Phenotype. *Tissue Eng Part A, 23*(21-22), 1283-1294. doi:10.1089/ten.TEA.2017.0102

Huleihel, L., Dziki, J. L., Bartolacci, J. G., Rausch, T., Scarritt, M. E., Cramer, M. C., . . . White, L. J. (2017). *Macrophage phenotype in response to ECM bioscaffolds.* Paper presented at the Seminars in immunology.

Huleihel, L., Dziki, J. L., Bartolacci, J. G., Rausch, T., Scarritt, M. E., Cramer, M. C., . . . Badylak, S. F. (2017). Macrophage phenotype in response to ECM bioscaffolds. *Semin Immunol, 29*, 2-13. doi:10.1016/j.smim.2017.04.004

Huleihel, L., Hussey, G. S., Naranjo, J. D., Zhang, L., Dziki, J. L., Turner, N. J., . . . Badylak, S. F. (2016). Matrix-bound nanovesicles within ECM bioscaffolds. *Sci Adv, 2*(6), e1600502. doi:10.1126/sciadv.1600502

Hussey, G. S., Dziki, J. L., & Badylak, S. F. (2018). Extracellular matrix-based materials for regenerative medicine. *Nature Reviews Materials*. doi:10.1038/s41578-018-0023-x

Hussey, G. S., Dziki, J. L., Lee, Y. C., Bartolacci, J. G., Behun, M., Turnquist, H. R., & Badylak, S. F. (2019). Matrix bound nanovesicle-associated IL-33 activates a pro-remodeling macrophage phenotype via a non-canonical, ST2-independent pathway. *J Immunol Regen Med, 3*, 26-35. doi:10.1016/j.regen.2019.01.001

Hussey, G. S., Keane, T. J., & Badylak, S. F. (2017). The extracellular matrix of the gastrointestinal tract: a regenerative medicine platform. *Nature Reviews Gastroenterology and Hepatology, 14*(9), 540.

Hussey, G. S., Pineda Molina, C., Cramer, M. C., Tyurina, Y. Y., Tyurin, V. A., Lee, Y. C., . . . Badylak, S. F. (2020). Lipidomics and RNA sequencing reveal a novel subpopulation of nanovesicle within extracellular matrix biomaterials. *Science Advances, 6*(12), eaay4361. doi:10.1126/sciadv.aay4361

Hutchinson, J. A., Brem-Exner, B. G., Riquelme, P., Roelen, D., Schulze, M., Ivens, K., . . . Fandrich, F. (2008). A cell-based approach to the minimization of immunosuppression in renal transplantation. *Transpl Int, 21*(8), 742-754. doi:10.1111/j.1432-2277.2008.00692.x

Hutchinson, J. A., Riquelme, P., Brem-Exner, B. G., Schulze, M., Matthai, M., Renders, L., . . . Fandrich, F. (2008). Transplant acceptance-inducing cells as an immune-conditioning therapy in renal transplantation. *Transpl Int, 21*(8), 728-741. doi:10.1111/j.1432-2277.2008.00680.x

Hutchinson, J. A., Roelen, D., Riquelme, P., Brem-Exner, B. G., Witzke, O., Philipp, T., . . . Fandrich, F. (2008). Preoperative treatment of a presensitized kidney transplant recipient with donor-derived transplant acceptance-inducing cells. *Transpl Int, 21*(8), 808-813. doi:10.1111/j.1432-2277.2008.00712.x

Hwang, J. H., Ra, Y. J., Lee, K. M., Lee, J. Y., & Ghil, S. H. (2006). Therapeutic effect of passive mobilization exercise on improvement of muscle regeneration and prevention of fibrosis after laceration injury of rat. *Arch Phys Med Rehabil, 87*(1), 20-26. doi:10.1016/j.apmr.2005.08.002

Ilic, D., Almeida, E. A., Schlaepfer, D. D., Dazin, P., Aizawa, S., & Damsky, C. H. (1998). Extracellular matrix survival signals transduced by focal adhesion kinase suppress p53-mediated apoptosis. *J Cell Biol, 143*(2), 547-560. doi:10.1083/jcb.143.2.547

Ingber, D. E. (2006). Cellular mechanotransduction: putting all the pieces together again. *FASEB J, 20*(7), 811-827. doi:10.1096/fj.05-5424rev

Ishii, M., Wen, H., Corsa, C. A., Liu, T., Coelho, A. L., Allen, R. M., . . . Kunkel, S. L. (2009). Epigenetic regulation of the alternatively activated macrophage phenotype. *Blood, 114*(15), 3244-3254. doi:10.1182/blood-2009-04-217620

Iyer, A. K., Tran, K. T., Borysenko, C. W., Cascio, M., Camacho, C. J., Blair, H. C., . . . Wells, A. (2007). Tenascin cytotactin epidermal growth factor-like repeat binds epidermal growth factor receptor with low affinity. *J Cell Physiol, 211*(3), 748-758. doi:10.1002/jcp.20986

Jakobsson, L., Kreuger, J., Holmborn, K., Lundin, L., Eriksson, I., Kjellen, L., & Claesson-Welsh, L. (2006). Heparan sulfate in trans potentiates VEGFR-mediated angiogenesis. *Dev Cell, 10*(5), 625-634. doi:10.1016/j.devcel.2006.03.009

John, S., Sabo, P. J., Johnson, T. A., Sung, M. H., Biddie, S. C., Lightman, S. L., . . . Hager, G. L. (2008). Interaction of the glucocorticoid receptor with the chromatin landscape. *Mol Cell, 29*(5), 611-624. doi:10.1016/j.molcel.2008.02.010

Jonat, C., Rahmsdorf, H. J., Park, K. K., Cato, A. C., Gebel, S., Ponta, H., & Herrlich, P. (1990). Antitumor promotion and antiinflammation: down-modulation of AP-1 (Fos/Jun) activity by glucocorticoid hormone. *Cell, 62*(6), 1189-1204. doi:10.1016/0092-8674(90)90395-u

Joshi, A. D., Oak, S. R., Hartigan, A. J., Finn, W. G., Kunkel, S. L., Duffy, K. E., . . . Hogaboam, C. M. (2010). Interleukin-33 contributes to both M1 and M2 chemokine marker expression in human macrophages. *BMC Immunol, 11*, 52. doi:10.1186/1471-2172-11-52

Junge, K., Klinge, U., Prescher, A., Giboni, P., Niewiera, M., & Schumpelick, V. (2001). Elasticity of the anterior abdominal wall and impact for reparation of incisional hernias using mesh implants. *Hernia, 5*(3), 113-118. doi:10.1007/s100290100019

Kahan, B. D. (2003). Individuality: the barrier to optimal immunosuppression. *Nat Rev Immunol, 3*(10), 831-838. doi:10.1038/nri1204

Kakkar, R., Hei, H., Dobner, S., & Lee, R. T. (2012). Interleukin 33 as a mechanically responsive cytokine secreted by living cells. *J Biol Chem, 287*(9), 6941-6948. doi:10.1074/jbc.M111.298703

Kakkar, R., Hei, H., Dobner, S., & Lee, R. T. (2012). Interleukin 33 as a mechanically responsive cytokine secreted by living cells. *Journal of Biological Chemistry, 287*(9), 6941-6948.

Kakkar, R., & Lee, R. T. (2008). The IL-33/ST2 pathway: therapeutic target and novel biomarker. *Nat Rev Drug Discov, 7*(10), 827-840. doi:10.1038/nrd2660

Kalaba, S., Gerhard, E., Winder, J. S., Pauli, E. M., Haluck, R. S., & Yang, J. (2016). Design Strategies and Applications of Biomaterials and Devices for Hernia Repair. *Bioact Mater, 1*(1), 2-17. doi:10.1016/j.bioactmat.2016.05.002

Kammoun, M., Cassar-Malek, I., Meunier, B., & Picard, B. (2014). A simplified immunohistochemical classification of skeletal muscle fibres in mouse. *Eur J Histochem, 58*(2), 2254. doi:10.4081/ejh.2014.2254

Kapellos, T. S., & Iqbal, A. J. (2016). Epigenetic Control of Macrophage Polarisation and Soluble Mediator Gene Expression during Inflammation. *Mediators Inflamm, 2016*, 6591703. doi:10.1155/2016/6591703

Kariminekoo, S., Movassaghpour, A., Rahimzadeh, A., Talebi, M., Shamsasenjan, K., & Akbarzadeh, A. (2016). Implications of mesenchymal stem cells in regenerative medicine. *Artif Cells Nanomed Biotechnol, 44*(3), 749-757. doi:10.3109/21691401.2015.1129620

Keane, T. J., Dziki, J., Sobieski, E., Smoulder, A., Castleton, A., Turner, N., . . . Badylak, S. F. (2016). Restoring Mucosal Barrier Function and Modifying Macrophage Phenotype with an Extracellular Matrix Hydrogel: Potential Therapy for Ulcerative Colitis. *J Crohns Colitis*. doi:10.1093/ecco-jcc/jjw149

Keane, T. J., Dziki, J., Sobieski, E., Smoulder, A., Castleton, A., Turner, N., . . . Badylak, S. F. (2017). Restoring Mucosal Barrier Function and Modifying Macrophage Phenotype with an Extracellular Matrix Hydrogel: Potential Therapy for Ulcerative Colitis. *J Crohns Colitis, 11*(3), 360-368. doi:10.1093/ecco-jcc/jjw149

Keane, T. J., Swinehart, I. T., & Badylak, S. F. (2015). Methods of tissue decellularization used for preparation of biologic scaffolds and in vivo relevance. *Methods, 84*, 25-34. doi:https://doi.org/10.1016/j.ymeth.2015.03.005

Keshtkar, S., Azarpira, N., & Ghahremani, M. H. (2018). Mesenchymal stem cell-derived extracellular vesicles: novel frontiers in regenerative medicine. *Stem Cell Res Ther, 9*(1), 63. doi:10.1186/s13287-018-0791-7

Kin, S., Hagiwara, A., Nakase, Y., Kuriu, Y., Nakashima, S., Yoshikawa, T., . . . Yamagishi, H. (2007). Regeneration of skeletal muscle using in situ tissue engineering on an acellular collagen sponge scaffold in a rabbit model. *ASAIO J, 53*(4), 506-513. doi:10.1097/MAT.0b013e3180d09d81

Kissane, N. A., & Itani, K. M. (2012). A decade of ventral incisional hernia repairs with biologic acellular dermal matrix: what have we learned? *Plast Reconstr Surg, 130*(5 Suppl 2), 194S-202S. doi:10.1097/PRS.0b013e318265a5ec

Kjaer, M. (2004). Role of extracellular matrix in adaptation of tendon and skeletal muscle to mechanical loading. *Physiol Rev, 84*(2), 649-698. doi:10.1152/physrev.00031.2003

Klagsbrun, M. (1992). Mediators of angiogenesis: the biological significance of basic fibroblast growth factor (bFGF)-heparin and heparan sulfate interactions. *Semin Cancer Biol, 3*(2), 81-87.

Kleinnijenhuis, J., Quintin, J., Preijers, F., Joosten, L. A., Ifrim, D. C., Saeed, S., . . . Netea, M. G. (2012). Bacille Calmette-Guerin induces NOD2-dependent nonspecific protection from reinfection via epigenetic reprogramming of monocytes. *Proc Natl Acad Sci U S A, 109*(43), 17537-17542. doi:10.1073/pnas.1202870109

Klinge, U., & Klosterhalfen, B. (2012). Modified classification of surgical meshes for hernia repair based on the analyses of 1,000 explanted meshes. *Hernia, 16*(3), 251-258. doi:10.1007/s10029-012-0913-6

Klinge, U., Klosterhalfen, B., Conze, J., Limberg, W., Obolenski, B., Ottinger, A. P., & Schumpelick, V. (1998). Modified mesh for hernia repair that is adapted to the physiology of the abdominal wall. *Eur J Surg, 164*(12), 951-960. doi:10.1080/110241598750005138

Klinkenberg, M., Fischer, S., Kremer, T., Hernekamp, F., Lehnhardt, M., & Daigeler, A. (2013). Comparison of anterolateral thigh, lateral arm, and parascapular free flaps with regard to donor-site morbidity and aesthetic and functional outcomes. *Plast Reconstr Surg, 131*(2), 293-302. doi:10.1097/PRS.0b013e31827786bc

Koul, A. R., Patil, R. K., & Nahar, S. (2013). Unfavourable results in free tissue transfer. *Indian J Plast Surg, 46*(2), 247-255. doi:10.4103/0970-0358.118600

Kumar, A., Alvarez-Croda, D. M., Stoica, B. A., Faden, A. I., & Loane, D. J. (2015). Microglial/macrophage polarization dynamics following traumatic brain injury. *J Neurotrauma*. doi:10.1089/neu.2015.4268

Kuswanto, W., Burzyn, D., Panduro, M., Wang, K. K., Jang, Y. C., Wagers, A. J., . . . Mathis, D. (2016). Poor repair of skeletal muscle in aging mice reflects a defect in local, interleukin-33-dependent accumulation of regulatory T cells. *Immunity, 44*(2), 355-367.

Kuswanto, W., Burzyn, D., Panduro, M., Wang, K. K., Jang, Y. C., Wagers, A. J., . . . Mathis, D. (2016). Poor Repair of Skeletal Muscle in Aging Mice Reflects a Defect in Local, Interleukin-33-Dependent Accumulation of Regulatory T Cells. *Immunity, 44*(2), 355-367. doi:10.1016/j.immuni.2016.01.009

Kuzmich, N. N., Sivak, K. V., Chubarev, V. N., Porozov, Y. B., Savateeva-Lyubimova, T. N., & Peri, F. (2017). TLR4 Signaling Pathway Modulators as Potential Therapeutics in Inflammation and Sepsis. *Vaccines (Basel), 5*(4). doi:10.3390/vaccines5040034

Ladowski, J. M., & Ladowski, J. S. (2011). Retrospective analysis of bovine pericardium (Vascu-Guard) for patch closure in carotid endarterectomies. *Ann Vasc Surg, 25*(5), 646-650. doi:10.1016/j.avsg.2010.11.008

Lamichhane, T. N., Sokic, S., Schardt, J. S., Raiker, R. S., Lin, J. W., & Jay, S. M. (2015). Emerging roles for extracellular vesicles in tissue engineering and regenerative medicine. *Tissue Eng Part B Rev, 21*(1), 45-54. doi:10.1089/ten.TEB.2014.0300

Lantz, G. C., Badylak, S. F., Coffey, A. C., Geddes, L. A., & Blevins, W. E. (1990). Small intestinal submucosa as a small-diameter arterial graft in the dog. *J Invest Surg, 3*(3), 217-227.

Lavin, Y., Mortha, A., Rahman, A., & Merad, M. (2015). Regulation of macrophage development and function in peripheral tissues. *Nat Rev Immunol, 15*(12), 731-744. doi:10.1038/nri3920

Lawrence, W. T., & Diegelmann, R. F. (1994). Growth factors in wound healing. *Clin Dermatol, 12*(1), 157-169.

Lecheminant, J., & Field, C. (2012). Porcine urinary bladder matrix: a retrospective study and establishment of protocol. *J Wound Care, 21*(10), 476, 478-480, 482. doi:10.12968/jowc.2012.21.10.476

Lee, C. (2007). Coimmunoprecipitation assay. *Methods Mol Biol, 362*, 401-406. doi:10.1007/978-1-59745-257-1_31

Lee, H. M., Kim, T. S., & Jo, E. K. (2016). MiR-146 and miR-125 in the regulation of innate immunity and inflammation. *BMB Rep, 49*(6), 311-318.

Lee, S., Choi, E., Cha, M. J., & Hwang, K. C. (2015). Cell adhesion and long-term survival of transplanted mesenchymal stem cells: a prerequisite for cell therapy. *Oxid Med Cell Longev, 2015*, 632902. doi:10.1155/2015/632902

Lee, Y. S., Kim, H. K., Chung, S., Kim, K. S., & Dutta, A. (2005). Depletion of human micro-RNA miR-125b reveals that it is critical for the proliferation of differentiated cells but not for the down-regulation of putative targets during differentiation. *J Biol Chem, 280*(17), 16635-16641. doi:10.1074/jbc.M412247200

Lefrancais, E., Duval, A., Mirey, E., Roga, S., Espinosa, E., Cayrol, C., & Girard, J. P. (2014). Central domain of IL-33 is cleaved by mast cell proteases for potent activation of group-2 innate lymphoid cells. *Proc Natl Acad Sci U S A, 111*(43), 15502-15507. doi:10.1073/pnas.1410700111

Lefrancais, E., Roga, S., Gautier, V., Gonzalez-de-Peredo, A., Monsarrat, B., Girard, J. P., & Cayrol, C. (2012). IL-33 is processed into mature bioactive forms by neutrophil elastase and cathepsin G. *Proc Natl Acad Sci U S A, 109*(5), 1673-1678. doi:10.1073/pnas.1115884109

Lesman, A., Koffler, J., Atlas, R., Blinder, Y. J., Kam, Z., & Levenberg, S. (2011). Engineering vessel-like networks within multicellular fibrin-based constructs. *Biomaterials, 32*(31), 7856-7869. doi:10.1016/j.biomaterials.2011.07.003

Levy, L., Broad, S., Diekmann, D., Evans, R. D., & Watt, F. M. (2000). beta1 integrins regulate keratinocyte adhesion and differentiation by distinct mechanisms. *Mol Biol Cell, 11*(2), 453-466. doi:10.1091/mbc.11.2.453

Li, F., Li, W., Johnson, S., Ingram, D., Yoder, M., & Badylak, S. (2004). Low-molecular-weight peptides derived from extracellular matrix as chemoattractants for primary endothelial cells. *Endothelium, 11*(3-4), 199-206. doi:10.1080/10623320490512390

Li, J., Li, C., Zhuang, Q., Peng, B., Zhu, Y., Ye, Q., & Ming, Y. (2019). The Evolving Roles of Macrophages in Organ Transplantation. *J Immunol Res, 2019*, 5763430. doi:10.1155/2019/5763430

Liew, F. Y., Girard, J.-P., & Turnquist, H. R. (2016). Interleukin-33 in health and disease. *Nature Reviews Immunology, 16*(11), 676-689.

Lin, C. H., Lin, Y. T., Yeh, J. T., & Chen, C. T. (2007). Free functioning muscle transfer for lower extremity posttraumatic composite structure and functional defect. *Plast Reconstr Surg, 119*(7), 2118-2126. doi:10.1097/01.prs.0000260595.85557.41

Lin, L., Hou, J., Ma, F., Wang, P., Liu, X., Li, N., . . . Cao, X. (2013). Type I IFN inhibits innate IL-10 production in macrophages through histone deacetylase 11 by downregulating microRNA-145. *J Immunol, 191*(7), 3896-3904. doi:10.4049/jimmunol.1203450

Lingel, A., Weiss, T. M., Niebuhr, M., Pan, B., Appleton, B. A., Wiesmann, C., . . . Fairbrother, W. J. (2009). Structure of IL-33 and its interaction with the ST2 and IL-1RAcP receptors—insight into heterotrimeric IL-1 signaling complexes. *Structure, 17*(10), 1398-1410.

Liu, D., Pearlman, E., Diaconu, E., Guo, K., Mori, H., Haqqi, T., . . . Sy, M. S. (1996). Expression of hyaluronidase by tumor cells induces angiogenesis in vivo. *Proc Natl Acad Sci U S A, 93*(15), 7832-7837. doi:10.1073/pnas.93.15.7832

Liu, H., Lin, H., Zhang, L., Sun, Q., Yuan, G., Zhang, L., . . . Chen, Z. (2013). miR-145 and miR-143 regulate odontoblast differentiation through targeting Klf4 and Osx genes in a feedback loop. *J Biol Chem, 288*(13), 9261-9271. doi:10.1074/jbc.M112.433730

Liu, H., Wu, X., Gang, N., Wang, S., Deng, W., Zan, L., & Yu, S. (2015). Macrophage functional phenotype can be consecutively and reversibly shifted to adapt to microenvironmental changes. *Int J Clin Exp Med, 8*(2), 3044-3053.

Liu, X., Liu, Y., Zhao, L., Zeng, Z., Xiao, W., & Chen, P. (2017). Macrophage depletion impairs skeletal muscle regeneration: The roles of regulatory factors for muscle regeneration. *Cell Biol Int, 41*(3), 228-238. doi:10.1002/cbin.10705

Londhe, P., & Davie, J. K. (2013). Interferon-gamma resets muscle cell fate by stimulating the sequential recruitment of JARID2 and PRC2 to promoters to repress myogenesis. *Sci Signal, 6*(305), ra107. doi:10.1126/scisignal.2004633

Louis, E., El Ghoul, Z., Vermeire, S., Dall'Ozzo, S., Rutgeerts, P., Paintaud, G., . . . Watier, H. (2004). Association between polymorphism in IgG Fc receptor IIIa coding gene and biological response to infliximab in Crohn's disease. *Aliment Pharmacol Ther, 19*(5), 511-519. doi:10.1111/j.1365-2036.2004.01871.x

MacKenzie, A., Wilson, H. L., Kiss-Toth, E., Dower, S. K., North, R. A., & Surprenant, A. (2001). Rapid secretion of interleukin-1beta by microvesicle shedding. *Immunity, 15*(5), 825-835.

Magin, C. M., Alge, D. L., & Anseth, K. S. (2016). Bio-inspired 3D microenvironments: a new dimension in tissue engineering. *Biomed Mater, 11*(2), 022001. doi:10.1088/1748-6041/11/2/022001

Mahdavian Delavary, B., van der Veer, W. M., van Egmond, M., Niessen, F. B., & Beelen, R. H. (2011). Macrophages in skin injury and repair. *Immunobiology, 216*(7), 753-762. doi:10.1016/j.imbio.2011.01.001

Majumdar, R., Miller, D. V., Ballman, K. V., Unnikrishnan, G., McKellar, S. H., Sarkar, G., . . . Sundt, T. M., 3rd. (2007). Elevated expressions of osteopontin and tenascin C in ascending aortic aneurysms are associated with trileaflet aortic valves as compared with bicuspid aortic valves. *Cardiovasc Pathol, 16*(3), 144-150. doi:10.1016/j.carpath.2006.12.001

Mammoto, T., Jiang, E., Jiang, A., & Mammoto, A. (2013). Extracellular matrix structure and tissue stiffness control postnatal lung development through the lipoprotein receptor-related protein 5/Tie2 signaling system. *Am J Respir Cell Mol Biol, 49*(6), 1009-1018. doi:10.1165/rcmb.2013-0147OC

Mannon, R. B. (2012). Macrophages: contributors to allograft dysfunction, repair, or innocent bystanders? *Curr Opin Organ Transplant, 17*(1), 20-25. doi:10.1097/MOT.0b013e32834ee5b6

Mantovani, A., Sica, A., Sozzani, S., Allavena, P., Vecchi, A., & Locati, M. (2004). The chemokine system in diverse forms of macrophage activation and polarization. *Trends Immunol, 25*(12), 677-686. doi:10.1016/j.it.2004.09.015

Martinez, F. O., & Gordon, S. (2014). The M1 and M2 paradigm of macrophage activation: time for reassessment. *F1000Prime Rep, 6*, 13. doi:10.12703/P6-13

Martinez, F. O., & Gordon, S. (2014). The M1 and M2 paradigm of macrophage activation: time for reassessment. *F1000prime reports, 6*.

Martinez, F. O., Sica, A., Mantovani, A., & Locati, M. (2008). Macrophage activation and polarization. *Front Biosci, 13*, 453-461.

Matta, B. M., Reichenbach, D. K., Zhang, X., Mathews, L., Koehn, B. H., Dwyer, G. K., . . . Turnquist, H. R. (2016). Peri-alloHCT IL-33 administration expands recipient T-regulatory cells that protect mice against acute GVHD. *Blood, 128*(3), 427-439. doi:10.1182/blood-2015-12-684142

McCurry, K. R., Colvin, B. L., Zahorchak, A. F., & Thomson, A. W. (2006). Regulatory dendritic cell therapy in organ transplantation. *Transpl Int, 19*(7), 525-538. doi:10.1111/j.1432-2277.2006.00306.x

McKelvey, K. J., Powell, K. L., Ashton, A. W., Morris, J. M., & McCracken, S. A. (2015). Exosomes: Mechanisms of Uptake. *J Circ Biomark, 4*, 7. doi:10.5772/61186

Melman, L., Jenkins, E. D., Hamilton, N. A., Bender, L. C., Brodt, M. D., Deeken, C. R., . . . Matthews, B. D. (2011). Early biocompatibility of crosslinked and non-crosslinked biologic meshes in a porcine model of ventral hernia repair. *Hernia, 15*(2), 157-164. doi:10.1007/s10029-010-0770-0

Mercurio, A. M., Rabinovitz, I., & Shaw, L. M. (2001). The alpha 6 beta 4 integrin and epithelial cell migration. *Curr Opin Cell Biol, 13*(5), 541-545. doi:10.1016/s0955-0674(00)00249-0

Mewhort, H. E. M., Svystonyuk, D. A., Turnbull, J. D., Teng, G., Belke, D. D., Guzzardi, D. G., . . . Fedak, P. W. M. (2017). Bioactive Extracellular Matrix Scaffold Promotes Adaptive Cardiac Remodeling and Repair. *JACC Basic Transl Sci, 2*(4), 450-464. doi:10.1016/j.jacbts.2017.05.005

Michalopoulos, G. K. (2010). Liver regeneration after partial hepatectomy: critical analysis of mechanistic dilemmas. *Am J Pathol, 176*(1), 2-13. doi:10.2353/ajpath.2010.090675

Milovanovic, M., Volarevic, V., Radosavljevic, G., Jovanovic, I., Pejnovic, N., Arsenijevic, N., & Lukic, M. L. (2012). IL-33/ST2 axis in inflammation and immunopathology. *Immunol Res, 52*(1-2), 89-99. doi:10.1007/s12026-012-8283-9

Moneim, M. S., & Omer, G. E. (1986). Latissimus dorsi muscle transfer for restoration of elbow flexion after brachial plexus disruption. *J Hand Surg Am, 11*(1), 135-139.

Na, Y. R., Jung, D., Yoon, B. R., Lee, W. W., & Seok, S. H. (2015). Endogenous prostaglandin E2 potentiates anti-inflammatory phenotype of macrophage through the CREB-C/EBP-beta cascade. *Eur J Immunol, 45*(9), 2661-2671. doi:10.1002/eji.201545471

Na, Y. R., Yoon, Y. N., Son, D., Jung, D., Gu, G. J., & Seok, S. H. (2015). Consistent inhibition of cyclooxygenase drives macrophages towards the inflammatory phenotype. *PLoS One, 10*(2), e0118203. doi:10.1371/journal.pone.0118203

Na, Y. R., Yoon, Y. N., Son, D. I., & Seok, S. H. (2013). Cyclooxygenase-2 inhibition blocks M2 macrophage differentiation and suppresses metastasis in murine breast cancer model. *PLoS One, 8*(5), e63451. doi:10.1371/journal.pone.0063451

Nahrendorf, M., Swirski, F. K., Aikawa, E., Stangenberg, L., Wurdinger, T., Figueiredo, J.-L., . . . Pittet, M. J. (2007). The healing myocardium sequentially mobilizes two monocyte subsets with divergent and complementary functions. *Journal of Experimental Medicine, 204*(12), 3037-3047.

Nahrendorf, M., Swirski, F. K., Aikawa, E., Stangenberg, L., Wurdinger, T., Figueiredo, J. L., . . . Pittet, M. J. (2007). The healing myocardium sequentially mobilizes two monocyte subsets with divergent and complementary functions. *J Exp Med, 204*(12), 3037-3047. doi:10.1084/jem.20070885

Nguyen, B. P., Ryan, M. C., Gil, S. G., & Carter, W. G. (2000). Deposition of laminin 5 in epidermal wounds regulates integrin signaling and adhesion. *Curr Opin Cell Biol, 12*(5), 554-562. doi:10.1016/s0955-0674(00)00131-9

Nolfi, A. L., Brown, B. N., Liang, R., Palcsey, S. L., Bonidie, M. J., Abramowitch, S. D., & Moalli, P. A. (2016). Host response to synthetic mesh in women with mesh complications. *Am J Obstet Gynecol, 215*(2), 206 e201-208. doi:10.1016/j.ajog.2016.04.008

Novak, M. L., & Koh, T. J. (2013). Phenotypic transitions of macrophages orchestrate tissue repair. *Am J Pathol, 183*(5), 1352-1363. doi:10.1016/j.ajpath.2013.06.034

Novak, M. L., Weinheimer-Haus, E. M., & Koh, T. J. (2014). Macrophage activation and skeletal muscle healing following traumatic injury. *J Pathol, 232*(3), 344-355. doi:10.1002/path.4301

Nowak, K. J., & Davies, K. E. (2004). Duchenne muscular dystrophy and dystrophin: pathogenesis and opportunities for treatment. *EMBO Rep, 5*(9), 872-876. doi:10.1038/sj.embor.7400221

Nuutila, K., Sakthivel, D., Kruse, C., Tran, P., Giatsidis, G., & Sinha, I. (2017). Gene expression profiling of skeletal muscle after volumetric muscle loss. *Wound Repair Regen, 25*(3), 408-413. doi:10.1111/wrr.12547

Oboki, K., Ohno, T., Kajiwara, N., Arae, K., Morita, H., Ishii, A., . . . Matsumoto, K. (2010). IL-33 is a crucial amplifier of innate rather than acquired immunity. *Proceedings of the National Academy of Sciences, 107*(43), 18581-18586.

Okumura, R., & Takeda, K. (2016). Maintenance of gut homeostasis by the mucosal immune system. *Proc Jpn Acad Ser B Phys Biol Sci, 92*(9), 423-435. doi:10.2183/pjab.92.423

Oliveira, A. C., Garzón, I., Ionescu, A. M., Carriel, V., de la Cruz Cardona, J., González-Andrades, M., . . . Campos, A. (2013). Evaluation of small intestine grafts decellularization methods for corneal tissue engineering. *PLoS One, 8*(6), e66538.

Oshio, T., Komine, M., Tsuda, H., Tominaga, S. I., Saito, H., Nakae, S., & Ohtsuki, M. (2017). Nuclear expression of IL-33 in epidermal keratinocytes promotes wound healing in mice. *J Dermatol Sci, 85*(2), 106-114. doi:10.1016/j.jdermsci.2016.10.008

Ozbek, S., Balasubramanian, P. G., Chiquet-Ehrismann, R., Tucker, R. P., & Adams, J. C. (2010). The evolution of extracellular matrix. *Mol Biol Cell, 21*(24), 4300-4305. doi:10.1091/mbc.E10-03-0251

Panduro, M., Benoist, C., & Mathis, D. (2018). Treg cells limit IFN-gamma production to control macrophage accrual and phenotype during skeletal muscle regeneration. *Proc Natl Acad Sci U S A, 115*(11), E2585-E2593. doi:10.1073/pnas.1800618115

Parisi, C., Napoli, G., Amadio, S., Spalloni, A., Apolloni, S., Longone, P., & Volonte, C. (2016). MicroRNA-125b regulates microglia activation and motor neuron death in ALS. *Cell Death Differ, 23*(3), 531-541. doi:10.1038/cdd.2015.153

Pauwels, A. M., Trost, M., Beyaert, R., & Hoffmann, E. (2017). Patterns, Receptors, and Signals: Regulation of Phagosome Maturation. *Trends Immunol, 38*(6), 407-422. doi:10.1016/j.it.2017.03.006

Peloso, A., Petrosyan, A., Da Sacco, S., Booth, C., Zambon, J. P., O'Brien, T., . . . Orlando, G. (2015). Renal Extracellular Matrix Scaffolds From Discarded Kidneys Maintain

Glomerular Morphometry and Vascular Resilience and Retains Critical Growth Factors. *Transplantation, 99*(9), 1807-1816. doi:10.1097/TP.0000000000000811

Peranteau, W. H., Zhang, L., Muvarak, N., Badillo, A. T., Radu, A., Zoltick, P. W., & Liechty, K. W. (2008). IL-10 overexpression decreases inflammatory mediators and promotes regenerative healing in an adult model of scar formation. *J Invest Dermatol, 128*(7), 1852-1860. doi:10.1038/sj.jid.5701232

Perdiguero, E., Sousa-Victor, P., Ruiz-Bonilla, V., Jardi, M., Caelles, C., Serrano, A. L., & Munoz-Canoves, P. (2011). p38/MKP-1-regulated AKT coordinates macrophage transitions and resolution of inflammation during tissue repair. *J Cell Biol, 195*(2), 307-322. doi:10.1083/jcb.201104053

Piccoli, M., Urbani, L., Alvarez-Fallas, M. E., Franzin, C., Dedja, A., Bertin, E., . . . Pozzobon, M. (2016). Improvement of diaphragmatic performance through orthotopic application of decellularized extracellular matrix patch. *Biomaterials, 74*, 245-255. doi:10.1016/j.biomaterials.2015.10.005

Pichery, M., Mirey, E., Mercier, P., Lefrancais, E., Dujardin, A., Ortega, N., & Girard, J.-P. (2012). Endogenous IL-33 is highly expressed in mouse epithelial barrier tissues, lymphoid organs, brain, embryos, and inflamed tissues: in situ analysis using a novel Il-33–LacZ gene trap reporter strain. *The Journal of Immunology, 188*(7), 3488-3495.

Prevel, C. D., Eppley, B. L., Summerlin, D. J., Jackson, J. R., McCarty, M., & Badylak, S. F. (1995). Small intestinal submucosa: utilization for repair of rodent abdominal wall defects. *Ann Plast Surg, 35*(4), 374-380.

Prokic, L. M., & Vilic, I. M. (1983). Effect of prednisolone in vitro on the migration ability of peritoneal macrophages in rats. *Experientia, 39*(10), 1133-1134. doi:10.1007/bf01943145

Pullerits, R., Brisslert, M., Jonsson, I. M., & Tarkowski, A. (2006). Soluble receptor for advanced glycation end products triggers a proinflammatory cytokine cascade via beta2 integrin Mac-1. *Arthritis Rheum, 54*(12), 3898-3907. doi:10.1002/art.22217

Puri, M. C., & Nagy, A. (2012). Concise review: Embryonic stem cells versus induced pluripotent stem cells: the game is on. *Stem Cells, 30*(1), 10-14. doi:10.1002/stem.788

Qazi, T. H., Mooney, D. J., Pumberger, M., Geissler, S., & Duda, G. N. (2015). Biomaterials based strategies for skeletal muscle tissue engineering: existing technologies and future trends. *Biomaterials, 53*, 502-521. doi:10.1016/j.biomaterials.2015.02.110

Rangrez, A. Y., Massy, Z. A., Metzinger-Le Meuth, V., & Metzinger, L. (2011). miR-143 and miR-145: molecular keys to switch the phenotype of vascular smooth muscle cells. *Circ Cardiovasc Genet, 4*(2), 197-205. doi:10.1161/CIRCGENETICS.110.958702

Rani, S., Ryan, A. E., Griffin, M. D., & Ritter, T. (2015). Mesenchymal Stem Cell-derived Extracellular Vesicles: Toward Cell-free Therapeutic Applications. *Mol Ther, 23*(5), 812-823. doi:10.1038/mt.2015.44

Raposo, G., & Stoorvogel, W. (2013). Extracellular vesicles: exosomes, microvesicles, and friends. *J Cell Biol, 200*(4), 373-383. doi:10.1083/jcb.201211138

Rapraeger, A. C., Krufka, A., & Olwin, B. B. (1991). Requirement of heparan sulfate for bFGF-mediated fibroblast growth and myoblast differentiation. *Science, 252*(5013), 1705-1708. doi:10.1126/science.1646484

Reing, J. E., Brown, B. N., Daly, K. A., Freund, J. M., Gilbert, T. W., Hsiong, S. X., . . . Badylak, S. F. (2010). The effects of processing methods upon mechanical and biologic properties of porcine dermal extracellular matrix scaffolds. *Biomaterials, 31*(33), 8626-8633. doi:10.1016/j.biomaterials.2010.07.083

Reing, J. E., Zhang, L., Myers-Irvin, J., Cordero, K. E., Freytes, D. O., Heber-Katz, E., . . . Badylak, S. F. (2009). Degradation products of extracellular matrix affect cell migration and proliferation. *Tissue Eng Part A, 15*(3), 605-614. doi:10.1089/ten.tea.2007.0425

Riabov, V., Salazar, F., Htwe, S. S., Gudima, A., Schmuttermaier, C., Barthes, J., . . . Kzhyshkowska, J. (2017). Generation of anti-inflammatory macrophages for implants and regenerative medicine using self-standing release systems with a phenotype-fixing cytokine cocktail formulation. *Acta Biomater, 53*, 389-398. doi:10.1016/j.actbio.2017.01.071

Rogier, E. W., Frantz, A. L., Bruno, M. E., Wedlund, L., Cohen, D. A., Stromberg, A. J., & Kaetzel, C. S. (2014). Secretory antibodies in breast milk promote long-term intestinal homeostasis by regulating the gut microbiota and host gene expression. *Proc Natl Acad Sci U S A, 111*(8), 3074-3079. doi:10.1073/pnas.1315792111

Rogler, G., Andus, T., Aschenbrenner, E., Vogl, D., Falk, W., Scholmerich, J., & Gross, V. (1997). Alterations of the phenotype of colonic macrophages in inflammatory bowel disease. *Eur J Gastroenterol Hepatol, 9*(9), 893-899.

Roth, J. S., Brathwaite, C., Hacker, K., Fisher, K., & King, J. (2015). Complex ventral hernia repair with a human acellular dermal matrix. *Hernia, 19*(2), 247-252. doi:10.1007/s10029-014-1245-5

Rowshani, A. T., & Vereyken, E. J. (2012). The role of macrophage lineage cells in kidney graft rejection and survival. *Transplantation, 94*(4), 309-318. doi:10.1097/TP.0b013e318250c10f

Ruffell, D., Mourkioti, F., Gambardella, A., Kirstetter, P., Lopez, R. G., Rosenthal, N., & Nerlov, C. (2009). A CREB-C/EBPbeta cascade induces M2 macrophage-specific gene expression and promotes muscle injury repair. *Proc Natl Acad Sci U S A, 106*(41), 17475-17480. doi:10.1073/pnas.0908641106

Ruffell, D., Mourkioti, F., Gambardella, A., Kirstetter, P., Lopez, R. G., Rosenthal, N., & Nerlov, C. (2009). A CREB-C/EBPβ cascade induces M2 macrophage-specific gene expression and promotes muscle injury repair. *Proceedings of the National Academy of Sciences, 106*(41), 17475-17480.

Ruoslahti, E. (1996). RGD and other recognition sequences for integrins. *Annu Rev Cell Dev Biol, 12*, 697-715. doi:10.1146/annurev.cellbio.12.1.697

Rutella, S., & Lemoli, R. M. (2004). Regulatory T cells and tolerogenic dendritic cells: from basic biology to clinical applications. *Immunol Lett, 94*(1-2), 11-26. doi:10.1016/j.imlet.2004.04.015

Sadtler, K., Allen, B. W., Estrellas, K., Housseau, F., Pardoll, D. M., & Elisseeff, J. H. (2016). The Scaffold Immune Microenvironment: Biomaterial-Mediated Immune Polarization in Traumatic and Nontraumatic Applications. *Tissue Eng Part A*. doi:10.1089/ten.TEA.2016.0304

Sadtler, K., Estrellas, K., Allen, B. W., Wolf, M. T., Fan, H., Tam, A. J., . . . Wagner, K. R. (2016). Developing a pro-regenerative biomaterial scaffold microenvironment requires T helper 2 cells. *Science, 352*(6283), 366-370.

Sadtler, K., Estrellas, K., Allen, B. W., Wolf, M. T., Fan, H., Tam, A. J., . . . Elisseeff, J. H. (2016). Developing a pro-regenerative biomaterial scaffold microenvironment requires T helper 2 cells. *Science, 352*(6283), 366-370. doi:10.1126/science.aad9272

Sadtler, K., Sommerfeld, S. D., Wolf, M. T., Wang, X., Majumdar, S., Chung, L., . . . Elisseeff, J. H. (2017). Proteomic composition and immunomodulatory properties of urinary bladder matrix scaffolds in homeostasis and injury. *Semin Immunol*. doi:10.1016/j.smim.2017.05.002

Safdar, A., Saleem, A., & Tarnopolsky, M. A. (2016). The potential of endurance exercise-derived exosomes to treat metabolic diseases. *Nat Rev Endocrinol, 12*(9), 504-517. doi:10.1038/nrendo.2016.76

Sahu, A., Mamiya, H., Shinde, S. N., Cheikhi, A., Winter, L. L., Vo, N. V., . . . Ambrosio, F. (2018). Age-related declines in alpha-Klotho drive progenitor cell mitochondrial dysfunction and impaired muscle regeneration. *Nat Commun, 9*(1), 4859. doi:10.1038/s41467-018-07253-3

Saldin, L. T., Cramer, M. C., Velankar, S. S., White, L. J., & Badylak, S. F. (2017). Extracellular matrix hydrogels from decellularized tissues: Structure and function. *Acta Biomater, 49*, 1-15. doi:10.1016/j.actbio.2016.11.068

Saldin, L. T., Patel, S., Zhang, L., Huleihel, L., Hussey, G. S., Nascari, D. G., . . . Badylak, S. F. (2019). Extracellular Matrix Degradation Products Downregulate Neoplastic Esophageal Cell Phenotype. *Tissue Eng Part A, 25*(5-6), 487-498. doi:10.1089/ten.TEA.2018.0105

Salehi, S., & Reed, E. F. (2015). The divergent roles of macrophages in solid organ transplantation. *Curr Opin Organ Transplant, 20*(4), 446-453. doi:10.1097/MOT.0000000000000209

Sanes, J. R. (2003). The basement membrane/basal lamina of skeletal muscle. *J Biol Chem, 278*(15), 12601-12604. doi:10.1074/jbc.R200027200

Schreurs, M., Suttorp, C. M., Mutsaers, H. A. M., Kuijpers-Jagtman, A. M., Von den Hoff, J. W., Ongkosuwito, E. M., . . . Wagener, F. (2020). Tissue engineering strategies combining molecular targets against inflammation and fibrosis, and umbilical cord blood stem cells to improve hampered muscle and skin regeneration following cleft repair. *Med Res Rev, 40*(1), 9-26. doi:10.1002/med.21594

Schumpelick, V., Klinge, U., Rosch, R., & Junge, K. (2006). Light weight meshes in incisional hernia repair. *J Minim Access Surg, 2*(3), 117-123. doi:10.4103/0972-9941.27722

Schwartz, P. H., Brick, D. J., Nethercott, H. E., & Stover, A. E. (2011). Traditional human embryonic stem cell culture. *Methods Mol Biol, 767*, 107-123. doi:10.1007/978-1-61779-201-4_8

Seif-Naraghi, S. B., Singelyn, J. M., Salvatore, M. A., Osborn, K. G., Wang, J. J., Sampat, U., . . . Christman, K. L. (2013). Safety and efficacy of an injectable extracellular matrix hydrogel for treating myocardial infarction. *Sci Transl Med, 5*(173), 173ra125. doi:10.1126/scitranslmed.3005503

Senior, R. M., Griffin, G. L., & Mecham, R. P. (1980). Chemotactic activity of elastin-derived peptides. *J Clin Invest, 66*(4), 859-862. doi:10.1172/JCI109926

Serrels, B., McGivern, N., Canel, M., Byron, A., Johnson, S. C., McSorley, H. J., . . . Anderton, S. M. (2017). IL-33 and ST2 mediate FAK-dependent antitumor immune evasion through transcriptional networks. *Sci. Signal., 10*(508), eaan8355.

Shvartsman, D., Storrie-White, H., Lee, K., Kearney, C., Brudno, Y., Ho, N., . . . Mooney, D. J. (2014). Sustained delivery of VEGF maintains innervation and promotes reperfusion in ischemic skeletal muscles via NGF/GDNF signaling. *Mol Ther, 22*(7), 1243-1253. doi:10.1038/mt.2014.76

Shwartz, Y., Blitz, E., & Zelzer, E. (2013). One load to rule them all: mechanical control of the musculoskeletal system in development and aging. *Differentiation, 86*(3), 104-111. doi:10.1016/j.diff.2013.07.003

Sicari, B. M., Dziki, J. L., & Badylak, S. F. (2015). Strategies for functional bioscaffold-based skeletal muscle reconstruction. *Ann Transl Med, 3*(17), 256. doi:10.3978/j.issn.2305-5839.2015.09.45

Sicari, B. M., Dziki, J. L., Siu, B. F., Medberry, C. J., Dearth, C. L., & Badylak, S. F. (2014). The promotion of a constructive macrophage phenotype by solubilized extracellular matrix. *Biomaterials, 35*(30), 8605-8612. doi:10.1016/j.biomaterials.2014.06.060

Sicari, B. M., Dziki, J. L., Siu, B. F., Medberry, C. J., Dearth, C. L., & Badylak, S. F. (2014). The promotion of a constructive macrophage phenotype by solubilized extracellular matrix. *Biomaterials, 35*(30), 8605-8612.

Sicari, B. M., Rubin, J. P., Dearth, C. L., Wolf, M. T., Ambrosio, F., Boninger, M., . . . Badylak, S. F. (2014). An acellular biologic scaffold promotes skeletal muscle formation in mice

and humans with volumetric muscle loss. *Sci Transl Med, 6*(234), 234ra258. doi:10.1126/scitranslmed.3008085

Simon, A. K., Hollander, G. A., & McMichael, A. (2015). Evolution of the immune system in humans from infancy to old age. *Proc Biol Sci, 282*(1821), 20143085. doi:10.1098/rspb.2014.3085

Singelyn, J. M., DeQuach, J. A., Seif-Naraghi, S. B., Littlefield, R. B., Schup-Magoffin, P. J., & Christman, K. L. (2009). Naturally derived myocardial matrix as an injectable scaffold for cardiac tissue engineering. *Biomaterials, 30*(29), 5409-5416. doi:10.1016/j.biomaterials.2009.06.045

Singleton, J. R., Baker, B. L., & Thorburn, A. (2000). Dexamethasone inhibits insulin-like growth factor signaling and potentiates myoblast apoptosis. *Endocrinology, 141*(8), 2945-2950. doi:10.1210/endo.141.8.7621

Sridharan, R., Cavanagh, B., Cameron, A. R., Kelly, D. J., & O'Brien, F. J. (2019). Material stiffness influences the polarization state, function and migration mode of macrophages. *Acta Biomater, 89*, 47-59. doi:10.1016/j.actbio.2019.02.048

St Pierre, B. A., & Tidball, J. G. (1994a). Differential response of macrophage subpopulations to soleus muscle reloading after rat hindlimb suspension. *J Appl Physiol (1985), 77*(1), 290-297. doi:10.1152/jappl.1994.77.1.290

St Pierre, B. A., & Tidball, J. G. (1994b). Macrophage activation and muscle remodeling at myotendinous junctions after modifications in muscle loading. *Am J Pathol, 145*(6), 1463-1471.

Stearns-Reider, K. M., D'Amore, A., Beezhold, K., Rothrauff, B., Cavalli, L., Wagner, W. R., . . . Ambrosio, F. (2017). Aging of the skeletal muscle extracellular matrix drives a stem cell fibrogenic conversion. *Aging Cell, 16*(3), 518-528. doi:10.1111/acel.12578

Stellato, C. (2004). Post-transcriptional and nongenomic effects of glucocorticoids. *Proc Am Thorac Soc, 1*(3), 255-263. doi:10.1513/pats.200402-015MS

Sukharev, S., & Corey, D. P. (2004). Mechanosensitive channels: multiplicity of families and gating paradigms. *Sci STKE, 2004*(219), re4. doi:10.1126/stke.2192004re4

Svensson, K. J., Christianson, H. C., Wittrup, A., Bourseau-Guilmain, E., Lindqvist, E., Svensson, L. M., . . . Belting, M. (2013). Exosome uptake depends on ERK1/2-heat shock protein 27 signaling and lipid Raft-mediated endocytosis negatively regulated by caveolin-1. *J Biol Chem, 288*(24), 17713-17724. doi:10.1074/jbc.M112.445403

Swijnenburg, R. J., Schrepfer, S., Govaert, J. A., Cao, F., Ransohoff, K., Sheikh, A. Y., . . . Wu, J. C. (2008). Immunosuppressive therapy mitigates immunological rejection of human embryonic stem cell xenografts. *Proc Natl Acad Sci U S A, 105*(35), 12991-12996. doi:10.1073/pnas.0805802105

Swindle, C. S., Tran, K. T., Johnson, T. D., Banerjee, P., Mayes, A. M., Griffith, L., & Wells, A. (2001). Epidermal growth factor (EGF)-like repeats of human tenascin-C as ligands for EGF receptor. *J Cell Biol, 154*(2), 459-468. doi:10.1083/jcb.200103103

Tamaoki, M., Imanaka-Yoshida, K., Yokoyama, K., Nishioka, T., Inada, H., Hiroe, M., . . . Yoshida, T. (2005). Tenascin-C regulates recruitment of myofibroblasts during tissue repair after myocardial injury. *Am J Pathol, 167*(1), 71-80. doi:10.1016/S0002-9440(10)62954-9

Tidball, J. G. (2005). Inflammatory processes in muscle injury and repair. *Am J Physiol Regul Integr Comp Physiol, 288*(2), R345-353. doi:10.1152/ajpregu.00454.2004

Tidball, J. G. (2011). Mechanisms of muscle injury, repair, and regeneration. *Compr Physiol, 1*(4), 2029-2062. doi:10.1002/cphy.c100092

Tidball, J. G. (2017). Regulation of muscle growth and regeneration by the immune system. *Nat Rev Immunol, 17*(3), 165-178. doi:10.1038/nri.2016.150

Tidball, J. G., Dorshkind, K., & Wehling-Henricks, M. (2014). Shared signaling systems in myeloid cell-mediated muscle regeneration. *Development, 141*(6), 1184-1196. doi:10.1242/dev.098285

Tidball, J. G., & Villalta, S. A. (2010). Regulatory interactions between muscle and the immune system during muscle regeneration. *Am J Physiol Regul Integr Comp Physiol, 298*(5), R1173-1187. doi:10.1152/ajpregu.00735.2009

Tidball, J. G., & Wehling-Henricks, M. (2007). Macrophages promote muscle membrane repair and muscle fibre growth and regeneration during modified muscle loading in mice in vivo. *J Physiol, 578*(Pt 1), 327-336. doi:10.1113/jphysiol.2006.118265

Tidball, J. G., & Wehling-Henricks, M. (2015). Shifts in macrophage cytokine production drive muscle fibrosis. *Nat Med, 21*(7), 665-666. doi:10.1038/nm.3896

Tidball, J. G., & Wehling-Henricks, M. (2015). Shifts in macrophage cytokine production drive muscle fibrosis. *Nature medicine, 21*(7), 665.

Tidball, J. G., & Welc, S. S. (2015). Macrophage-Derived IGF-1 Is a Potent Coordinator of Myogenesis and Inflammation in Regenerating Muscle. *Mol Ther, 23*(7), 1134-1135. doi:10.1038/mt.2015.97

Tili, E., Michaille, J. J., Cimino, A., Costinean, S., Dumitru, C. D., Adair, B., . . . Croce, C. M. (2007). Modulation of miR-155 and miR-125b levels following lipopolysaccharide/TNF-alpha stimulation and their possible roles in regulating the response to endotoxin shock. *J Immunol, 179*(8), 5082-5089.

Townsend, M. J., Fallon, P. G., Matthews, D. J., Jolin, H. E., & McKenzie, A. N. (2000). T1/ST2-deficient mice demonstrate the importance of T1/ST2 in developing primary T helper cell type 2 responses. *Journal of Experimental Medicine, 191*(6), 1069-1076.

Tran, R. T., Yang, J., & Ameer, G. A. (2015). Citrate-Based Biomaterials and Their Applications in Regenerative Engineering. *Annu Rev Mater Res, 45*, 277-310. doi:10.1146/annurev-matsci-070214-020815

Trebaul, A., Chan, E. K., & Midwood, K. S. (2007). Regulation of fibroblast migration by tenascin-C. *Biochem Soc Trans, 35*(Pt 4), 695-697. doi:10.1042/BST0350695

Troidl, C., Möllmann, H., Nef, H., Masseli, F., Voss, S., Szardien, S., . . . Troidl, K. (2009). Classically and alternatively activated macrophages contribute to tissue remodelling after myocardial infarction. *Journal of cellular and molecular medicine, 13*(9b), 3485-3496.

Tsianakas, A., Varga, G., Barczyk, K., Bode, G., Nippe, N., Kran, N., . . . Sunderkoetter, C. (2012). Induction of an anti-inflammatory human monocyte subtype is a unique property of glucocorticoids, but can be modified by IL-6 and IL-10. *Immunobiology, 217*(3), 329-335. doi:10.1016/j.imbio.2011.10.002

Tu, Y. K., Yen, C. Y., Ma, C. H., Yu, S. W., Chou, Y. C., Lee, M. S., & Ueng, S. W. (2008). Soft-tissue injury management and flap reconstruction for mangled lower extremities. *Injury, 39 Suppl 4*, 75-95. doi:10.1016/j.injury.2008.08.034

Tuckermann, J. P., Kleiman, A., Moriggl, R., Spanbroek, R., Neumann, A., Illing, A., . . . Schutz, G. (2007). Macrophages and neutrophils are the targets for immune suppression by glucocorticoids in contact allergy. *J Clin Invest, 117*(5), 1381-1390. doi:10.1172/JCI28034

Turner, N. J., & Badylak, S. F. (2012). Regeneration of skeletal muscle. *Cell Tissue Res, 347*(3), 759-774. doi:10.1007/s00441-011-1185-7

Valentin, J. E., Badylak, J. S., McCabe, G. P., & Badylak, S. F. (2006). Extracellular matrix bioscaffolds for orthopaedic applications. A comparative histologic study. *J Bone Joint Surg Am, 88*(12), 2673-2686. doi:10.2106/JBJS.E.01008

Valentin, J. E., Stewart-Akers, A. M., Gilbert, T. W., & Badylak, S. F. (2009). Macrophage participation in the degradation and remodeling of extracellular matrix scaffolds. *Tissue Eng Part A, 15*(7), 1687-1694. doi:10.1089/ten.tea.2008.0419

van der Merwe, Y., Faust, A. E., Sakalli, E. T., Westrick, C. C., Hussey, G., Chan, K. C., . . . Steketee, M. B. (2019). Matrix-bound nanovesicles prevent ischemia-induced retinal ganglion cell axon degeneration and death and preserve visual function. *Sci Rep, 9*(1), 3482. doi:10.1038/s41598-019-39861-4

van der Merwe, Y., Faust, A. E., & Steketee, M. B. (2017). Matrix bound vesicles and miRNA cargoes are bioactive factors within extracellular matrix bioscaffolds. *Neural Regen Res, 12*(10), 1597-1599. doi:10.4103/1673-5374.217324

Varga, G., Ehrchen, J., Tsianakas, A., Tenbrock, K., Rattenholl, A., Seeliger, S., . . . Sunderkoetter, C. (2008). Glucocorticoids induce an activated, anti-inflammatory monocyte subset in mice that resembles myeloid-derived suppressor cells. *J Leukoc Biol, 84*(3), 644-650. doi:10.1189/jlb.1107768

Vats, A., Tolley, N. S., Bishop, A. E., & Polak, J. M. (2005). Embryonic stem cells and tissue engineering: delivering stem cells to the clinic. *J R Soc Med, 98*(8), 346-350. doi:10.1258/jrsm.98.8.346

Veigel, C., Molloy, J. E., Schmitz, S., & Kendrick-Jones, J. (2003). Load-dependent kinetics of force production by smooth muscle myosin measured with optical tweezers. *Nat Cell Biol, 5*(11), 980-986. doi:10.1038/ncb1060

Vereyken, E. J., Kraaij, M. D., Baan, C. C., Rezaee, F., Weimar, W., Wood, K. J., . . . Rowshani, A. T. (2013). A shift towards pro-inflammatory CD16+ monocyte subsets with preserved cytokine production potential after kidney transplantation. *PLoS One, 8*(7), e70152. doi:10.1371/journal.pone.0070152

Verheijden, S., Schepper, S. D., & Boeckxstaens, G. E. (2015). Neuron-macrophage crosstalk in the intestine: a "microglia" perspective. *Front Cell Neurosci, 9*, 403. doi:10.3389/fncel.2015.00403

Villalta, S. A., Nguyen, H. X., Deng, B., Gotoh, T., & Tidball, J. G. (2009). Shifts in macrophage phenotypes and macrophage competition for arginine metabolism affect the severity of muscle pathology in muscular dystrophy. *Hum Mol Genet, 18*(3), 482-496. doi:10.1093/hmg/ddn376

Villalta, S. A., Rosenthal, W., Martinez, L., Kaur, A., Sparwasser, T., Tidball, J. G., . . . Bluestone, J. A. (2014). Regulatory T cells suppress muscle inflammation and injury in muscular dystrophy. *Sci Transl Med, 6*(258), 258ra142. doi:10.1126/scitranslmed.3009925

Vishwakarma, A., Bhise, N. S., Evangelista, M. B., Rouwkema, J., Dokmeci, M. R., Ghaemmaghami, A. M., . . . Khademhosseini, A. (2016). Engineering Immunomodulatory Biomaterials To Tune the Inflammatory Response. *Trends Biotechnol, 34*(6), 470-482. doi:10.1016/j.tibtech.2016.03.009

Vlassara, H. (2001). The AGE-receptor in the pathogenesis of diabetic complications. *Diabetes Metab Res Rev, 17*(6), 436-443. doi:10.1002/dmrr.233

Vorotnikova, E., McIntosh, D., Dewilde, A., Zhang, J., Reing, J. E., Zhang, L., . . . Braunhut, S. J. (2010). Extracellular matrix-derived products modulate endothelial and progenitor cell migration and proliferation in vitro and stimulate regenerative healing in vivo. *Matrix Biol, 29*(8), 690-700. doi:10.1016/j.matbio.2010.08.007

Vos, A. C., Wildenberg, M. E., Duijvestein, M., Verhaar, A. P., van den Brink, G. R., & Hommes, D. W. (2011). Anti-tumor necrosis factor-alpha antibodies induce regulatory macrophages in an Fc region-dependent manner. *Gastroenterology, 140*(1), 221-230. doi:10.1053/j.gastro.2010.10.008

Voytik-Harbin, S. L., Brightman, A. O., Kraine, M. R., Waisner, B., & Badylak, S. F. (1997). Identification of extractable growth factors from small intestinal submucosa. *J Cell Biochem, 67*(4), 478-491.

Wang, S., Zan, J., Wu, M., Zhao, W., Li, Z., Pan, Y., . . . Zhu, J. (2015). miR-29a promotes scavenger receptor A expression by targeting QKI (quaking) during monocyte-macrophage differentiation. *Biochem Biophys Res Commun, 464*(1), 1-6. doi:10.1016/j.bbrc.2015.05.019

Warren, G. L., Hulderman, T., Mishra, D., Gao, X., Millecchia, L., O'Farrell, L., . . . Simeonova, P. P. (2005). Chemokine receptor CCR2 involvement in skeletal muscle regeneration. *FASEB J, 19*(3), 413-415. doi:10.1096/fj.04-2421fje

Webber, J., & Clayton, A. (2013). How pure are your vesicles? *Journal of extracellular vesicles, 2*. doi:10.3402/jev.v2i0.19861

Wei, J., & Besner, G. E. (2015). M1 to M2 macrophage polarization in heparin-binding epidermal growth factor-like growth factor therapy for necrotizing enterocolitis. *J Surg Res, 197*(1), 126-138. doi:10.1016/j.jss.2015.03.023

Wenink, M. H., Santegoets, K. C., Platt, A. M., van den Berg, W. B., van Riel, P. L., Garside, P., . . . McInnes, I. B. (2012). Abatacept modulates proinflammatory macrophage responses upon cytokine-activated T cell and Toll-like receptor ligand stimulation. *Ann Rheum Dis, 71*(1), 80-83. doi:10.1136/annrheumdis-2011-200348

Whitby, D. J., Longaker, M. T., Harrison, M. R., Adzick, N. S., & Ferguson, M. W. (1991). Rapid epithelialisation of fetal wounds is associated with the early deposition of tenascin. *J Cell Sci, 99 (Pt 3)*, 583-586.

Wilson, H. M., Chettibi, S., Jobin, C., Walbaum, D., Rees, A. J., & Kluth, D. C. (2005). Inhibition of macrophage nuclear factor-kappaB leads to a dominant anti-inflammatory phenotype that attenuates glomerular inflammation in vivo. *Am J Pathol, 167*(1), 27-37.

Wolf, M. T., Carruthers, C. A., Dearth, C. L., Crapo, P. M., Huber, A., Burnsed, O. A., . . . Badylak, S. F. (2014). Polypropylene surgical mesh coated with extracellular matrix mitigates the host foreign body response. *J Biomed Mater Res A, 102*(1), 234-246. doi:10.1002/jbm.a.34671

Wolf, M. T., Dearth, C. L., Ranallo, C. A., LoPresti, S. T., Carey, L. E., Daly, K. A., . . . Badylak, S. F. (2014). Macrophage polarization in response to ECM coated polypropylene mesh. *Biomaterials, 35*(25), 6838-6849. doi:10.1016/j.biomaterials.2014.04.115

Wolf, M. T., Ganguly, S., Wang, T. L., Anderson, C. W., Sadtler, K., Narain, R., . . . Elisseeff, J. H. (2019). A biologic scaffold-associated type 2 immune microenvironment inhibits tumor formation and synergizes with checkpoint immunotherapy. *Sci Transl Med, 11*(477). doi:10.1126/scitranslmed.aat7973

Wu, X., Zhang, J., Ge, H., Gupte, J., Baribault, H., Lee, K. J., . . . Li, Y. (2015). Soluble CLEC2 Extracellular Domain Improves Glucose and Lipid Homeostasis by Regulating Liver Kupffer Cell Polarization. *EBioMedicine, 2*(3), 214-224. doi:10.1016/j.ebiom.2015.02.013

Wynn, T. A., Chawla, A., & Pollard, J. W. (2013). Macrophage biology in development, homeostasis and disease. *Nature, 496*(7446), 445-455. doi:10.1038/nature12034

Xin, M., Small, E. M., Sutherland, L. B., Qi, X., McAnally, J., Plato, C. F., . . . Olson, E. N. (2009). MicroRNAs miR-143 and miR-145 modulate cytoskeletal dynamics and responsiveness of smooth muscle cells to injury. *Genes Dev, 23*(18), 2166-2178. doi:10.1101/gad.1842409

Xu, H., Sun, L., He, Y., Yuan, X., Niu, J., Su, J., & Li, D. (2019). Deficiency in IL-33/ST2 Axis Reshapes Mitochondrial Metabolism in Lipopolysaccharide-Stimulated Macrophages. *Front Immunol, 10*, 127. doi:10.3389/fimmu.2019.00127

Xu, L., Collins, J., Drachenberg, C., Kukuruga, D., & Burke, A. (2014). Increased macrophage density of cardiac allograft biopsies is associated with antibody-mediated rejection and alloantibodies to HLA antigens. *Clin Transplant, 28*(5), 554-560. doi:10.1111/ctr.12348

Xu, N., Papagiannakopoulos, T., Pan, G., Thomson, J. A., & Kosik, K. S. (2009). MicroRNA-145 regulates OCT4, SOX2, and KLF4 and represses pluripotency in human embryonic stem cells. *Cell, 137*(4), 647-658. doi:10.1016/j.cell.2009.02.038

Yamaguchi, Y., Mann, D. M., & Ruoslahti, E. (1990). Negative regulation of transforming growth factor-beta by the proteoglycan decorin. *Nature, 346*(6281), 281-284. doi:10.1038/346281a0

Yao, Z., Qiao, Y., Li, X., Chen, J., Ding, J., Bai, L., . . . Yuan, Z. (2018). Exosomes Exploit the Virus Entry Machinery and Pathway To Transmit Alpha Interferon-Induced Antiviral Activity. *J Virol, 92*(24). doi:10.1128/JVI.01578-18

Yayon, A., Klagsbrun, M., Esko, J. D., Leder, P., & Ornitz, D. M. (1991). Cell surface, heparin-like molecules are required for binding of basic fibroblast growth factor to its high affinity receptor. *Cell, 64*(4), 841-848. doi:10.1016/0092-8674(91)90512-w

Ying, W., Cheruku, P. S., Bazer, F. W., Safe, S. H., & Zhou, B. (2013). Investigation of macrophage polarization using bone marrow derived macrophages. *J Vis Exp*(76). doi:10.3791/50323

Ying, W., Riopel, M., Bandyopadhyay, G., Dong, Y., Birmingham, A., Seo, J. B., . . . Olefsky, J. M. (2017). Adipose Tissue Macrophage-Derived Exosomal miRNAs Can Modulate In Vivo and In Vitro Insulin Sensitivity. *Cell, 171*(2), 372-384 e312. doi:10.1016/j.cell.2017.08.035

Yoshida, K., & Ishii, S. (2016). Innate immune memory via ATF7-dependent epigenetic changes. *Cell Cycle, 15*(1), 3-4. doi:10.1080/15384101.2015.1112687

Zelikin, A. N. (2010). Drug releasing polymer thin films: new era of surface-mediated drug delivery. *ACS Nano, 4*(5), 2494-2509. doi:10.1021/nn100634r

Zembron-Lacny, A., Krzywanski, J., Ostapiuk-Karolczuk, J., & Kasperska, A. (2012). Cell and molecular mechanisms of regeneration and reorganization of skeletal muscles. *Ortop Traumatol Rehabil, 14*(1), 1-11.

Zhan, Y., Paolicelli, R. C., Sforazzini, F., Weinhard, L., Bolasco, G., Pagani, F., . . . Gross, C. T. (2014). Deficient neuron-microglia signaling results in impaired functional brain connectivity and social behavior. *Nat Neurosci, 17*(3), 400-406. doi:10.1038/nn.3641

Zhang, C., Ferrari, R., Beezhold, K., Stearns-Reider, K., D'Amore, A., Haschak, M., . . . Ambrosio, F. (2016). Arsenic Promotes NF-Kappab-Mediated Fibroblast Dysfunction and Matrix Remodeling to Impair Muscle Stem Cell Function. *Stem Cells, 34*(3), 732-742. doi:10.1002/stem.2232

Zhang, F., Tossberg, J. T., Spurlock, C. F., Yao, S. Y., Aune, T. M., & Sriram, S. (2014). Expression of IL-33 and its epigenetic regulation in Multiple Sclerosis. *Ann Clin Transl Neurol, 1*(5), 307-318. doi:10.1002/acn3.47

Zhang, J., Ge, R., Matte-Martone, C., Goodwin, J., Shlomchik, W. D., Mamula, M. J., . . . Geller, D. (2009). Characterization of a novel gain of function glucocorticoid receptor knock-in mouse. *J Biol Chem, 284*(10), 6249-6259. doi:10.1074/jbc.M807997200

Zhang, Y., Zhang, M., Zhong, M., Suo, Q., & Lv, K. (2013). Expression profiles of miRNAs in polarized macrophages. *Int J Mol Med, 31*(4), 797-802. doi:10.3892/ijmm.2013.1260

Zheng, P., Luo, Q., Wang, W., Li, J., Wang, T., Wang, P., . . . Shen, L. (2018). Tumor-associated macrophages-derived exosomes promote the migration of gastric cancer cells by transfer of functional Apolipoprotein E. *Cell Death Dis, 9*(4), 434. doi:10.1038/s41419-018-0465-5

Zhou, Y., Yamamoto, Y., Xiao, Z., & Ochiya, T. (2019). The Immunomodulatory Functions of Mesenchymal Stromal/Stem Cells Mediated via Paracrine Activity. *J Clin Med, 8*(7). doi:10.3390/jcm8071025