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Macrophage mechanosensing of the tissue environment and signal integration through the cytoskeleton

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Abstract

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The extracellular matrix (ECM) is an organized assembly of proteins and polysaccharides that is produced by cells and forms the physical environment in which cells reside. Together, diverse cell types and their surrounding extracellular matrix form units of organization known as tissues, which make up organs. The ECM gives rise to the particular architecture and mechanical properties of each organ. During tissue repair, cells known as myofibroblasts deposit large quantities of ECM, in order to reconstruct the injured tissue. In normal tissue repair, that reparative phase is followed by a resolution phase, in which cells such as macrophages degrade and remodel excess extracellular matrix, returning the tissue to a homeostatic state. In fibrotic diseases, however, tissue repair persists, leading to the progressive accumulation of dense, stiff extracellular matrix that prevents normal organ function and leads to organ failure.

Macrophages are immune cells that reside within all tissues and have important non-immunologic functions, including sensing and regulating features of the tissue environment. They often act as sensors within a homeostatic circuit, monitoring a variable of interest and communicating with other cells, known as effectors, that can correct the variable when it deviates from the desired range. During tissue repair, monocytes from the blood enter the tissue and differentiate into macrophages, where they play a critical role both in driving fibroblast ECM production and in resolving tissue repair through ECM degradation. We hypothesized that

macrophages act as sensors of the extracellular matrix within tissues, both to maintain ECM homeostasis under normal conditions and to monitor the progression of tissue repair to ensure an appropriate transition to resolution and avoid fibrosis.

In the studies presented in this dissertation, using *in vitro* hydrogel systems to mimic essential elements of tissue biology, we find that macrophages can sense changes in the extracellular matrix and that they respond by regulating a specific subset of their gene expression program involved in tissue repair. This program includes the protein FIZZ1, which drives fibroblast ECM deposition and, we find, is suppressed by increased ECM, suggesting that macrophages may be involved in a negative feedback loop to control tissue repair. We determine that macrophages sense, in particular, the mechanical properties of the extracellular matrix, and that they employ a novel, integrin-independent mechanosensing mechanism. Macrophage mechanosensing is mediated by intracellular changes in the dynamics of the actin cytoskeleton, which ultimately control chromatin availability and binding of the transcription factor C/EBPβ to specific genomic targets. Furthermore, we identify that the macrophage growth factor, macrophage colony stimulating factor (MCSF), converges on these cytoskeletal dynamics and downstream regulatory mechanisms to control the same gene expression program. Thus, we find that macrophages integrate mechanical and biochemical information about the tissue environment through changes in their actin cytoskeleton, in order to regulate their tissue repair program. In the final chapters, we present some of the implications of these findings, as well as broader perspectives on tissue biology, homeostasis, and inflammation.

Macrophage Mechanosensing of the Tissue Environment and Signal Integration Through the Cytoskeleton

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Matthew Lowell Meizlish

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professors, Dr. Marci Shore and Dr. Gilbert Joseph, were similarly influential and helped foster my love for deep analysis and discovery. I never met Dr. Frank Macfarlane Burnet, but he was one of the subjects of my dissertation in History and Philosophy of Science at Cambridge. Through his writings, his approach to science shaped the way that I think about biology. He had the audacity to stitch together disparate data into a unifying theory, all through the lens of ecology and evolution. His ideas repeatedly altered the history of immunology, and they continue to serve as an inspiration for me.

Beyond the intellectual sphere, I will be brief because there is too much to say. My greatest fortune is my family: my mom, Darcy Lowell; my dad, Jay Meizlish; and my brothers, Eric Meizlish and Jason Meizlish. I am the youngest of three boys and, as my dad has always told me, I have been defined by that birth order. I was raised by four people who loved me and supported me in a way that was complete and unconditional. Whenever I have leaped for anything in my life, I have been jumping from solid ground, with the confidence and security they have given me. From my dad, I learned the gratification of caring for individuals and the satisfaction of understanding the inner workings of biology. From my mom, I gained the belief that I could make a far-reaching impact and the conviction that, when something doesn't work as it should, the answer is to change the system. In the end, it would be impossible to enumerate the things that they taught me. Who I am is inseparable from them, and I know that I am incalculably lucky.

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Dedication

This dissertation is dedicated to my family and my friends, whose love and support have made this work possible and made the journey meaningful.

List of Tables and Figures

Chapter 1: Introduction

Biological vulnerabilities to disease

The studies described in this dissertation began with a pair of simple questions that arose in the first week of my PhD: 1) Why, in some cases, does tissue repair inappropriately persist, leading to fibrotic disease? and 2) How does tissue repair normally turn off? These ultimately led to a series of more fundamental questions and discoveries about the cellular mechanisms by which macrophages sense the tissue environment. Building from those first questions, though, I want to begin this dissertation with a broad framework for thinking about human disease, which has given shape to, and been shaped by, this work.

Human diseases are often described as malfunctions of human biology. However, they are not random malfunctions: the repetitive occurrence of certain discrete disease entities indicates that some pathways are especially vulnerable to disease and that they malfunction in particular, somewhat predictable ways. One very common type of malfunction takes place when we mobilize a response to a biological challenge but cannot turn off that response.

For instance, infection triggers mobilization of an immune response. If that inflammatory response cannot be appropriately shut off, it can lead acutely to pathologies like septic shock or acute respiratory distress syndrome, or chronically to autoinflammatory or autoimmune diseases. The response to endothelial injury is thrombosis. However, if this process is unrestrained, it can lead to a systemic, life-threatening cascade of clotting and bleeding known as disseminated intravascular coagulation.[1] The response to the appearance of old or damaged cells within tissues

includes cellular proliferation. However, cell proliferation without appropriate brakes leads to cancer. Finally, the response to tissue injury is tissue repair, including the deposition of extracellular matrix to rebuild the tissue. However, a failure to shut off the tissue repair process leads to sustained extracellular matrix deposition and fibrosis.[2]

Fibrosis, then, represents one example of a common biological theme: The adaptive responses that organisms mobilize to survive the immediate challenges that we face also represent our greatest vulnerabilities to disease.

Extracellular Matrix

The extracellular matrix (ECM) is an organized assembly of proteins and polysaccharides that comprises the architecture of every organ. It is produced by cells and in turn forms the physical environment in which cells reside. Together, diverse cell types and their surrounding extracellular matrix form units of organization known as tissues, which make up each organ and have a unique composition depending on their biological function (discussed in detail in Chapter 6). Extracellular matrix is an essential element of multicellular life. In the earliest multicellular organisms, its function was to provide structural support and organization for sheets of epithelial cells that formed a barrier between the external environment and the internal environment of the organism.[3–5] In contemporary organisms, the fundamental role of the ECM remains the structural organization of cells within the tissue and the provision of particular mechanical properties that are necessary for the proper function of each organ.

A typical tissue contains over 100 extracellular matrix components, including fibrillar proteins like collagens and elastin, glycoproteins like fibronectin and laminins, and proteoglycans (a subset of glycoproteins characterized by repeating glycosaminoglycan side chains) like those of the heparan sulfate family.[6,7] Each class of extracellular matrix proteins has characteristic mechanical functions: Fibrillar collagens, for example, lend the tissue tensile strength, the ability to resist pulling forces; elastin lends the tissue elasticity, the ability to recoil after being stretched; and proteoglycans sequester water with their negatively charged glycosaminoglycan side chains, hydrating the tissue and lending it compressive strength, the ability to resist compressive forces.[8,9] But the ECM is not simply a scaffold. ECM components also have essential signaling functions. Cellular sensing of ECM composition is known to dictate cellular fates during organ development[10] and stem cell differentiation[11,12] and to modulate cell proliferation,[13] survival,[14] migration,[15] and morphology.[16] ECM proteins often have several distinct structural domains, which mediate specific interactions with other extracellular matrix components, soluble proteins like growth factors, and cellular receptors, allowing for coordinated intracellular signaling of complex extracellular conditions.[7] The mechanical properties and biochemical signaling of ECM components are intimately connected. For instance, mechanical tension in the extracellular matrix exposes 'cryptic binding sites' on many ECM proteins that are otherwise masked, thus switching the set of available molecular interactions under distinct mechanical conditions.[17,18] The ECM is at once a structural meshwork and a complex signaling network whose language is just beginning to be understood.

In order to serve its core structural and mechanical purpose, the extracellular matrix must be tightly controlled. The repertoire of ECM components and their relative concentrations—which

we can call the ECM composition—together with the spatial arrangement of the ECM, give rise to its mechanical features. The required mechanics of each organ differ dramatically: The lung must be compliant and elastic to breathe, while the bone must be able to resist large forces in order to bear the body's weight, and their extracellular matrices differ accordingly. The composition and arrangement of the extracellular matrix can also undergo dramatic changes within a given organ, physiologically during tissue repair and pathologically in the setting of fibrosis, cancer, and degenerative diseases.[9] The state of the extracellular matrix, then, must be monitored and regulated, in order to maintain the ECM in its appropriate state during normal (homeostatic) conditions, or in order to alter the state of the ECM in the setting of repair.

Tissue homeostasis

The extracellular matrix is one of several crucial parameters that must be sensed and regulated within an organism's tissues. Other variables that must be tightly controlled to allow for normal organ function include cell number and organization, oxygen availability, and characteristics of the interstitial fluid, such as volume, pH, and osmolarity.[19–21] Control of most of these variables within the tissue is not well understood, but the concept of homeostasis can be usefully applied. Homeostatic circuits are set up to maintain a regulated variable at a particular set point (**Figure 1.1a**). In order to achieve that end, a sensor monitors the value of the regulated variable. In response to deviations of the variable from the set point, the sensor releases signals that act on an effector. The effector acts to modify the value of the regulated variable, returning it to the set point. If we think about temperature homeostasis within a room, variation in room temperature (regulated variable) is monitored by a thermostat (sensor), which sends signals to a furnace or air conditioner (effector), which blows hot or cold air to return the room temperature to its set point.

These types of homeostatic circuits are well appreciated in biology, predominantly on an organismal level, at which variables like glucose concentration and blood pressure are regulated, and on a cellular level, at which variables like metabolite concentrations are regulated.

This homeostatic framework, however, has not been widely applied to the study of tissue biology. Part of the reason for this may be a conceptual gap (which we try to help address in Chapter 6), but the study of tissue biology is also limited by experimental systems. Our biological tools are largely adapted for the study of individual cells (*in vitro*) and whole organisms (*in vivo*). The biology of tissues has been difficult to model in *in vitro* systems and difficult to manipulate within *in vivo* systems.

Macrophages, which reside within all tissues, are most famous for their role in sensing microbes. However, in recent years, we have begun to appreciate that macrophages are involved in sensing and regulating a number of other tissue-level variables, like oxygen tension and osmolarity, in order to support the functions of the tissues in which they reside.[21,22] We reasoned that macrophages are likely to sense the state of the extracellular matrix, as well (**Figure 1.1b**). It is well appreciated that macrophages play an essential role in altering the extracellular matrix, both through soluble signals to fibroblasts (such as TGFβ, PDGF, and Wnt family proteins) to regulate their synthesis of ECM and by directly degrading and remodeling the ECM through the production of proteases like matrix metalloproteases (MMPs) and cathepsins.[23–28] However, almost nothing is known about how these macrophage functions are regulated, or whether macrophages are responsive to variations in the extracellular matrix. We hypothesized that macrophages act as sensors of key parameters of the ECM, such as its composition and stiffness,

and respond to variations in the ECM either by signaling to fibroblasts—which serve as effectors that can deposit ECM components to restore the ECM to the appropriate state—or by acting as effectors themselves, directly modifying the ECM through the production of proteases (Fig. 1.1b).

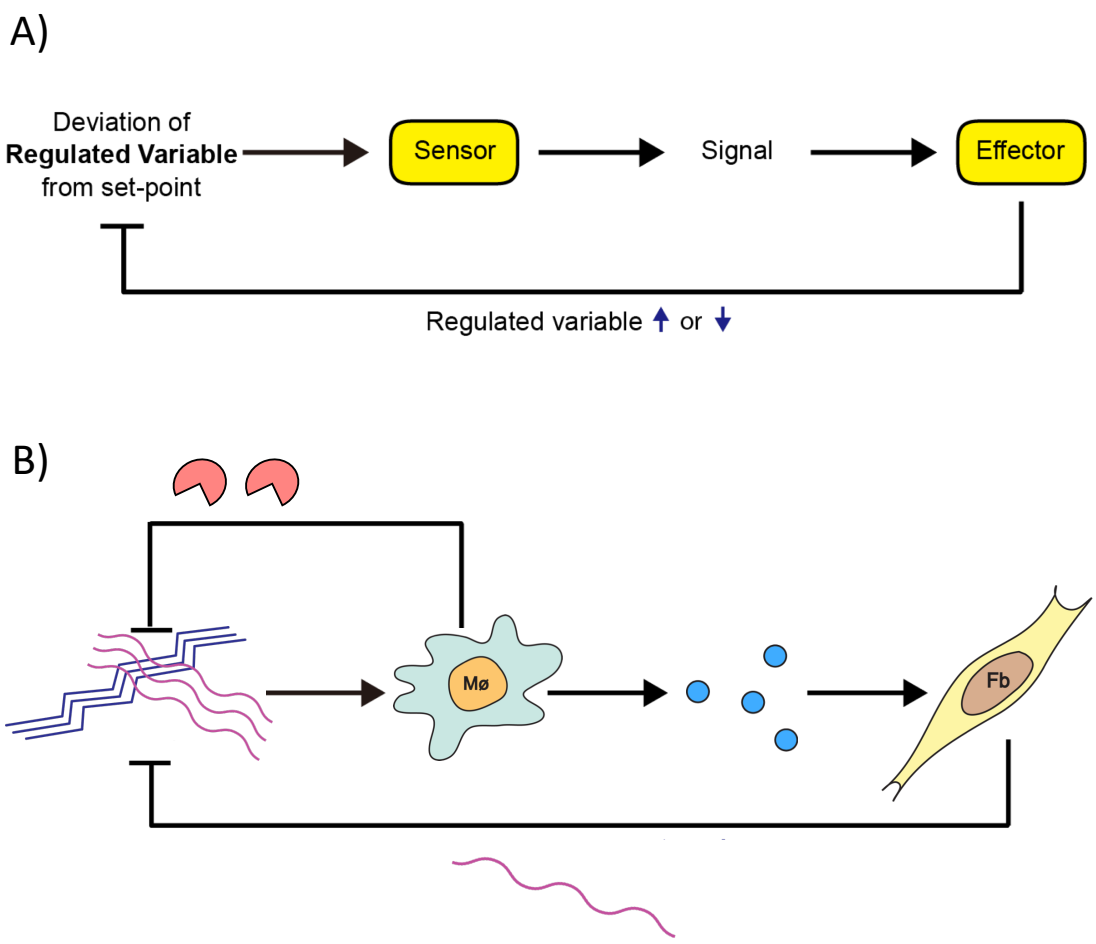


Figure 1.1. Hypothesized homeostatic circuit for extracellular matrix regulation

(A) In a homeostatic circuit, a regulated variable is maintained at a set point. A sensor monitors the regulated variable and, in response to deviations, sends a signal to an effector, which acts on the regulated variable to return it to the set point. **(B)** We hypothesized that the extracellular matrix within tissues is regulated by a homeostatic circuit, in which the composition or mechanical properties of the extracellular matrix (regulated variables) are monitored by macrophages (sensors), which respond to deviations in the ECM by secreting soluble factors (signals) to fibroblasts (effectors), which can modify the ECM to restore it to its set point. Alternatively, macrophages could act as both sensors and effectors, producing proteases that directly degrade the ECM.

Tissue repair and fibrosis

The importance of extracellular matrix regulation to human physiology is dramatically illustrated by the prevalence of fibrotic disease. Fibrosis is the accumulation of scar tissue—dense, stiff extracellular matrix—that replaces normal, functioning tissue, eventually leading to organ failure. Fibrotic diseases are ubiquitous and can occur in almost every organ. They include pulmonary fibrosis, chronic kidney disease, congestive heart failure, and liver cirrhosis. Fibrosis in all organs has been estimated to account for one third of all natural deaths worldwide, yet its underlying pathophysiology remains poorly understood, and few effective therapies have been developed that target the fibrotic process.[29]

Fundamentally, fibrosis is a disease of persistent tissue repair. After an injury, the organism normally mounts a sequence of responses: inflammation, repair, and resolution. First, during *inflammation*, an influx of neutrophils and macrophages serve to eliminate the source of injury and clear out dead cells and other debris. *Repair* follows: Parenchymal cells proliferate, and new extracellular matrix is laid down, in large part by cells called myofibroblasts, in order to reconstruct the architecture of the tissue. Importantly, the extracellular matrix is not simply returned to its original state; it typically becomes more abundant, more aligned, and stiffer during this phase, and it is characterized by particular extracellular matrix components, like matricellular proteins, that are absent or infrequent in normal tissue. As a result, a third phase of the tissue repair process is essential. In *resolution*, excess extracellular matrix is degraded and remodeled, and dedicated cells like myofibroblasts are eliminated, in order to return the tissue to a homeostatic state.[2,30,31] In fibrotic diseases, however, resolution does not take place. Instead, there is sustained repair, and ECM continues to accumulate. The transition from repair to

resolution, then, appears to be a key biological vulnerability that is prone to failure, leading to fibrosis. Yet, the signals that normally shift the tissue from a state of repair to a state of resolution remain unknown. We can hypothesize that cells within the tissue monitor the repair process in order to detect that repair is complete and trigger the transition to resolution.

Control of cell function during the tissue repair process likely emerges from a combination of soluble signals and instruction from the extracellular matrix.[32] Numerous secreted proteins have been consistently implicated in repair and fibrosis, including TGFβ,[33,34] PDGF proteins,[35] Wnt proteins,[36–38] CTGF,[39] IL-4,[40] and IL-13.[41,42] The importance of the extracellular matrix in shaping cell behavior has also been well described, especially with respect to fibroblasts. *In vitro* studies have demonstrated, for instance, that matrix extracted from patients with pulmonary fibrosis (compared to normal lung matrix) polarizes fibroblasts in culture toward a pro-fibrotic program.[43,44] Studies in mice have shown that pathways in fibroblasts that mediate ECM sensing are essential for repair and fibrosis.[45,46] The cells responsible for modifying the extracellular matrix must aggregate and interpret both soluble and matrix-derived information about their environment.

Macrophages in tissue repair

Based on mouse models in the lung,[26,47–49] heart,[50] liver,[27,51] kidney,[52] and skin,[28,53] macrophages appear to be essential in each phase of the response to tissue injury. They are well known for coordinating the inflammatory response; they drive ECM deposition during repair;[28,47–50,52,53] and they degrade and remodel ECM during resolution.[26,27,51,54–56] Though tissue-resident macrophages are present in all tissues under homeostatic conditions, tissue injury stimulates the

recruitment of bone-marrow derived monocytes from the blood into tissues, where they differentiate into macrophages and participate in tissue repair. Accumulating evidence suggests that it is these new arrivals that play the critical role in regulating repair.

The relative contribution of macrophage populations has been dissected, for instance, in models of tissue repair in the lung, which harbors multiple populations of tissue-resident macrophages. Alveolar macrophages are located within the lumen of the alveoli and have critical functions in microbial defense and surfactant homeostasis. They originate from the fetal liver and, barring tissue injury, self-renew throughout life with little replenishment from the blood. Interstitial macrophages are a heterogeneous population (subdivided by some authors) localized within the interstitial extracellular matrix of the lung. Their ontogeny and functions are poorly characterized, but they are good candidates to play a role in regulating tissue organization and ECM homeostasis, given their position along with fibroblasts within the interstitium adjacent to alveoli.[57–62] After injury, these populations undergo dramatic changes, as monocytes from the blood infiltrate the damaged tissue. In one study, following lung injury by intratracheal bleomycin, infiltrating monocytes were responsible for both the increase in number and functional contribution of macrophages to lung repair.[49] Once they entered the tissue, these monocytes could become tissue-resident macrophages, and over months (after repair) many gradually adopted the phenotype of alveolar macrophages.[49,63] Separately, another group determined that bone marrow-derived macrophages are required for optimal lung regeneration after pneumonectomy.[64] These analyses were focused on distinguishing between infiltrating monocytes and resident alveolar macrophages and do not rule out a role for tissue-resident interstitial macrophages (which may also be replenished from the bone marrow).[49,65] However,

these studies clarify that, in the context of repair and regeneration, infiltrating monocytes that originate from the bone marrow play a central role in regulating tissue growth and ECM deposition, and they indicate that the use of bone marrow-derived macrophages (BMDM) *in vitro* is appropriate for the study of these processes.

The type 2 cytokines IL-4 and IL-13, which signal through the shared IL4Rα, provide key signals that instruct macrophages to participate in tissue repair. In the pneumonectomy study described above, donor bone marrow required intact IL4Rα in order to facilitate lung regeneration.[64] In the intratracheal bleomycin model, knocking out both IL-4 and IL-13 almost completely abolishes collagen deposition.[66] After punch biopsy in the skin, IL4Rα signaling in myeloid cells is required to stimulate secretion of Found in inflammatory zone 1 (FIZZ1, also known as Resistin-like molecule alpha (RELMα), official gene symbol Retnla), which in turn induces fibroblast cross-linking of collagen at the telopeptides, allowing for organized collagen packing within the tissue and effective wound healing.[28] FIZZ1 is also dramatically induced in the lung following bleomycin injury and, like IL-4/IL-13, is required for effective ECM deposition.[66–68] *In vitro*, IL-4 or IL-13 have traditionally been used to polarize macrophages to an 'M2' or 'alternatively activated' state. Though this nomenclature does not capture the range of functional states that macrophages can assume in response to signals within the tissue environment,[22] it is useful as a well-defined polarization state associated with tissue repair. Bone marrow-derived macrophage stimulation with IL-4 or IL-13 *in vitro* induces a well-established set of genes, including Fizz1, Ym1, Arg1, and Mrc1, that also serve as markers of repair-associated macrophages *in vivo*. In an *in vivo* setting, other stimuli can almost certainly stimulate expression of these genes, which can be co-regulated or induced independently of one another.[69]

However, given the established role of IL-4/IL-13 in many models of tissue repair and the ability of these signals to induce a gene expression program in macrophages that is clearly involved in tissue repair, these proteins serve as a useful tool for the *ex vivo* study of elements of macrophage biology involved in tissue repair.

ECM receptors and mechanosensing

Transmembrane extracellular matrix receptors occupy the interface between cells and the extracellular matrix. The largest known family of ECM receptors is the integrin family, whose development predates the origin of multicellular animals. Though integrins are most famous for their functions in cellular adhesion and migration, the earliest known organisms with integrin receptors also had the requisite cytoplasmic machinery for downstream signaling, suggesting that integrins had a primordial function in reporting on the state of the extracellular environment.[70] Integrins have expanded dramatically and diversified their substrate specificity in metazoans, where they interact with the complex extracellular matrix of multicellular tissues. The integrin family in mammals is made up of 18 different α subunits and 8 different β subunits, which pair to form 24 known heterodimers that are expressed on the cell surface and that have distinct sets of ECM ligands.[71] The evolutionary and developmental importance of these interactions is evidenced by a triad of lethal mutations in mice: Loss-of-function mutations in fibronectin, a major ECM glycoprotein;[72] α5 integrin, a fibronectin receptor (when paired with β1 integrin);[73] or focal adhesion kinase (FAK), a kinase that signals downstream of integrin receptors,[74] all cause embryonic lethality.[75]

ECM receptors not only have molecular specificity; many also have the capacity to sense mechanical characteristics of the extracellular matrix. The ability of cells to convert mechanical stimuli into intracellular biochemical signals is called 'mechanosensing' or 'mechanotransduction'. In fibroblasts, integrins play a key role as mechanosensors. They are coupled intracellularly to large protein complexes called focal adhesions, through which they connect the extracellular matrix to the intracellular cytoskeleton. This allows for a bidirectional mechanical relationship between the extracellular matrix and the cell that is essential for mechanotransduction.[76,77] For example, for α5β1 integrin on fibroblasts to sense the stiffness of a fibronectin substrate, non-muscle myosin II inside the cell must generate force, pulling on the actin cytoskeleton, which is linked via scaffold proteins to the intracellular portion of the integrin receptor. That intracellular force causes α5β1 integrin to pull on its extracellular substrate, fibronectin. If the extracellular matrix is stiff, it exerts tension on the transmembrane α5β1 integrin receptor that switches the receptor from a 'relaxed' state, in which it binds the canonical RGD motif of fibronectin, to a 'tensioned' state in which it also binds a 'synergy site' in fibronectin. This interaction allows fibronectin to signal through α5β1 integrin and stimulate intracellular phosphorylation of FAK.[18] The extracellular mechanical state of the matrix is thus translated to an intracellular biochemical signaling pathway, allowing for downstream changes in cellular development and behavior.

Transcriptional coactivators Yes-associated protein (YAP) and WW-domain-containing transcription regulator 1 (WWTR1, or TAZ) have also been identified as key sensors (or, perhaps more accurately, transducers or mediators) of extracellular matrix stiffness. YAP and TAZ are best known for promoting cellular proliferation in epithelial cells by translocating from the

cytoplasm to the nucleus when the cell lack contacts with neighboring cells. In this case, the Hippo pathway regulates YAP and TAZ activity. In cultured mesenchymal cells and many epithelial cells, however, YAP and TAZ are even more potently activated by a stiff substrate, in a Hippo-independent fashion.[78] Lineage fate decisions in mesenchymal stem cells can be directed by substrate stiffness alone,[11] and YAP and TAZ are required for those responses.[78] Like integrin-mediated mechanosensing, YAP/TAZ-mediated mechanotransduction is dependent on tension in the cytoskeleton exerted by nonmuscle myosin II activity.[78] The literature on YAP and TAZ as mechanosensors has expanded dramatically in the last decade. Yet, interestingly, the upstream events regulating their activation and translocation to the nucleus remain poorly defined. In some models, integrins are required for YAP activation, but it is not clear whether there is a linear signaling pathway from integrins to YAP, or whether integrins are simply required because they are necessary for cellular adhesion to the extracellular matrix, allowing for cell spreading. Indeed, in these and other studies, cell morphology and actin dynamics control YAP activity.[12,79]

These findings suggest that YAP activity, in the context of mechanosensing, may be controlled directly by the state of the cytoskeleton. The best-defined example of this type of mechanism is the transcriptional coactivator myocardin-related transcription factor A (MRTF-A, or MRTF). MRTF binds to G-actin monomers, which sequester it in the cytoplasm. When G-actin polymerizes to F-actin, MRTF is released, allowing it to translocate to the nucleus and activate serum response factor (SRF).[80] Interestingly, this mechanism was defined not in the context of mechanosensing, but rather in investigating the response of fibroblasts to soluble factors in serum, which, surprisingly, depends upon these mechanical changes in the cytoskeleton.[81–83]

Later, it was discovered that MRTF also acts as a mechanosensitive factor in myofibroblasts, presumably through the same logic: stiff substrates cause increased cell spreading and actin polymerization, releasing MRTF to the nucleus.[45] Thus, in this case, cytoskeletal dynamics act as an integrator of soluble biochemical signals (serum) and mechanical signals (stiffness) from the environment, which are ultimately translated to changes in gene expression through the activity of MRTF. YAP activation, which integrates environmental information about both ECM mechanics and cell-cell contacts in a cytoskeleton-dependent manner, may follow a similar logic. Interestingly, at least one report suggests that YAP activity may be regulated by MRTF itself.[84]

Studies on the functional effects of mechanosensing in fibroblasts consistently identify a positive feedback loop that is mediated at least in part through YAP, TAZ, and MRTF: Fibroblast sensing of stiff extracellular matrix causes cellular activation, differentiation to myofibroblasts, and increased deposition of ECM and mechanical contraction of the matrix. Thus, fibroblast sensing of increased ECM stiffness causes further stiffening of the ECM.[45,85,86] This sort of positive feedback loop might be required for the accumulation of extracellular matrix during tissue repair, but its persistence would be expected to give rise to the dense, stiff extracellular matrix observed in fibrotic disease. Authors of these studies suggest that this may be an important mechanism underlying the development of fibrosis. This may well be true, but it obscures the important point that, normally, tissue repair does not lead to fibrosis. Part of the biological picture is missing here. The identification of this fibroblast-driven positive feedback loop with respect to ECM stiffness suggests the existence of a related negative feedback mechanism that, in response to increased ECM stiffness, serves to limit fibroblast activation and transition from repair to resolution. No such mechanism has been described.

Though research on extracellular matrix mechanosensing has focused on mesenchymal cells like fibroblasts, there is also intriguing evidence that macrophages may sense their mechanical environment and mount important functional responses. By forcing macrophages to adopt an elongated morphology through culture on micropatterned fibronectin substrates, partial induction of an M2-like polarization state can be observed.[87] This study suggests that macrophages, too, have the capacity for mechanosensing that can influence cell behavior. It remains to be learned whether macrophages sense properties of the extracellular matrix, how these signals are interpreted and relayed, and how that might influence macrophage polarization and function.

Hydrogels for ECM mimicry

As in many of the studies described above, engineered biomaterials have been used in recent years to mimic features of the extracellular matrix *in vitro* and, by systematically varying those features, gain unique insights into the biology of cellular interactions with the ECM. There is a rich array of biomimetic (biology-mimicking) materials that can be applied to fundamental questions in biology. Among the most useful tools are hydrogels, which are polymer networks that swell with water, creating a material with mechanical properties that are similar to those of mammalian tissues. Perhaps the most important difference between hydrogels and typical cell culture systems (like tissue culture plastic) is that the mechanical properties of hydrogels can be controlled. Tuning the gel's stiffness, in particular, can have far-reaching effects on cellular behavior.[11] The optimal hydrogel system for cell culture experiments depends on the experimental questions being asked.

Hydrogels can be two-dimensional (2D) or three-dimensional (3D) culture systems. Many of the seminal studies on the impact of substrate stiffness on stem cell differentiation were performed in 2D systems, with cells plated on top of ECM-coated gels.[11,78,88] Many assays, like imaging, are simpler and more reliable with a 2D hydrogel.[88] However, 3D culture systems, in which cells are encapsulated within the hydrogel, have the advantage of recapitulating the *in vivo* phenomenon of cell-ECM contact on all sides. This may be particularly important in the study of cells like macrophages and fibroblasts that often reside in the interstitium, surrounded by extracellular matrix, compared to epithelial or endothelial cells that typically form a monolayer bordering basement membrane ECM only on one side. The 3D environment captures regulatory dynamics between cell and ECM that are lost in two dimensions. For instance, Tang and colleagues demonstrated that differentiation of murine skeletal stem cells to osteoblasts within a 3D collagen gel depends on stem cell expression of MMP14, a collagen protease, whereas there was no such requirement on a 2D collagen substrate. Compellingly, the 3D defect phenocopied an *in vivo* defect in osteogenesis.[12]

Hydrogels can be composed of natural or synthetic materials. Hydrogels composed of type I collagen are an attractive natural option because collagens are the most abundant ECM protein in mammalian tissues.[89] Collagen gels provide a well-defined tissue environment that mimics important features of *in vivo* tissues but that lacks the complex interactions between many ECM components that are present in those tissues. *In vitro*, collagen self assembles into a solid gel when warmed and brought to a neutral pH. Cells can be encapsulated in a 3D collagen environment by mixing them with the liquid collagen substrate prior to gelation. The mechanical properties of the gel differ depending on the concentration of collagen that is used, and cells are

able to modify collagen gels through both mechanical contraction and protease activity.[90–93] Polyacrylamide (PA) and polyethylene glycol (PEG) are popular synthetic options because they are relatively inert, can be polymerized using a variety of chemical methods, and are highly modifiable. PA gels are typically used for 2D applications, while PEG gels can be used for the 3D cell culture. PA and PEG alone cannot support cell survival, so they must be modified with one or more cellular ligands, creating a hybrid natural-synthetic material.[88,94] One major advantage of this hybrid system is that ECM ligand concentration can be decoupled from the stiffness (or other mechanical properties) of the gel. Whereas in pure collagen gels the stiffness increases as a function of collagen concentration, in PA-collagen or PEG-collagen gels the stiffness can be controlled by varying the concentration or molecular weight of PA or PEG, while the collagen concentration can be varied independently.[95,96]

Polyacrylamide or polyethylene glycol can be chemically conjugated to whole proteins, like collagen or fibronectin, or to isolated peptides that are known to mediate binding of ECM proteins to cellular receptors. The RGD motif, which is found in several ECM proteins including fibronectin, and the GFOGER motif of collagen are often used.[94] These short peptides have the advantage of isolating specific ligand-receptor interactions, while whole proteins bind multiple cellular receptors and have the benefit of preserving a wider range of *in vivo* signaling properties.

Summary and study objectives

The extracellular matrix is a critical feature of all tissues, providing organization, structural support, and mechanical properties that allow for proper organ function. As a result, the ECM must be actively regulated by cells to maintain it in the appropriate state, both under homeostatic

conditions and in the context of tissue repair, which can lead to fibrosis if unchecked. We know that cells dynamically synthesize and degrade the extracellular matrix, but we do not know what parameters of the ECM are sensed in order to regulate these functions or what cells are responsible for sensing the ECM. We hypothesized that macrophages—which sense other features of the tissue environment and which are essential participants in tissue repair and ECM modification—act as sensors of the state of the ECM. In order to dissect this biology, we made use of hydrogel systems that served as simplified tissue environments and allowed for the manipulation of specific features of the ECM.

In this dissertation, I will explore the biology of ECM sensing and regulation by macrophages, as well as broader questions regarding cellular sensing and regulation of the tissue environment. In Chapter 2, I will show that macrophages are able to sense the mechanical properties of the extracellular matrix and that ECM sensing controls the macrophage gene expression program involved in tissue repair. In Chapter 3, I will present data indicating that macrophages sense ECM mechanics in an integrin-independent fashion that is distinct from established mechanisms of cellular mechanosensing, and that alterations in cytoskeletal dynamics control downstream changes in gene expression. In Chapter 4, I will discuss how macrophage cytoskeletal dynamics integrate this mechanical information with biochemical signaling from the growth factor macrophage colony stimulating factor (MCSF) and how this is ultimately translated to transcriptional regulation. In Chapter 5, I will discuss some of the conclusions and implications of this work, as well as the unanswered questions that it raises. Finally, in Chapter 6, I will present broader perspectives on homeostasis, inflammation, and tissue biology.

Chapter 2: Macrophages sense the mechanical properties of the extracellular matrix to regulate their tissue repair program

Introduction

In Chapter 1, I introduced the hypothesis that macrophages sense the state of the extracellular matrix, in order to maintain ECM homeostasis and regulate tissue repair. In this chapter, we employ *in vitro* hydrogel systems to first ask whether macrophages respond to differences in the extracellular matrix, and then to ask what specific properties of the extracellular matrix are monitored. We identify a specific transcriptional program involved in tissue repair that is regulated by extracellular matrix stiffness, and we explore the functional implications of this transcriptional program for regulation of fibroblast ECM deposition and tissue repair.

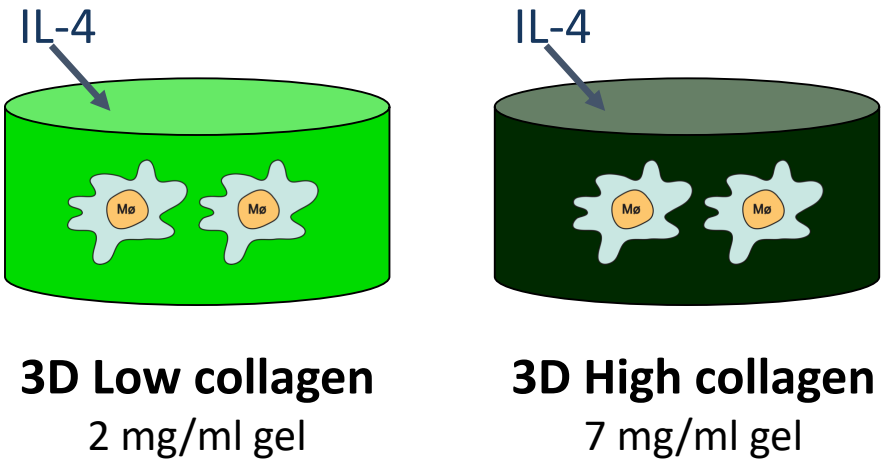
Results

Extracellular matrix regulates a specific subset of the IL-4 induced gene expression program in macrophages

In order to determine whether macrophages sense the extracellular matrix, we needed to set up an experimental system that retained key features of *in vivo* tissue environments, in which specific properties of the extracellular matrix could be manipulated, and in which we could study cellular responses to these changes. We made use of three-dimensional type I collagen gels, which met all of these criteria. When kept cold and at an acidic pH, type I collagen remains in a liquid phase. When it is warmed and brought to a neutral pH, type I collagen self-assembles into

a fibrillar collagen gel, resembling collagen architecture within tissues (although, interestingly, collagen assembly *in vivo* depends upon other ECM proteins).[97] By mixing collagen with different volumes of water, we are able to manipulate the concentration of collagen in the hydrogel, ranging from 2 mg/mL (low-collagen gels) to approximately 7 mg/mL (high-collagen gels) (**Figure 2.1a**), which also alters the stiffness of the gel (**Figure 2.1b**) and the pore size of the fibrillar network (**Figure 2.1c-d**). We mixed bone marrow-derived macrophages (BMDM) with the final collagen solution prior to gelation, in order to culture BMDM within a 3D collagen environment (Fig. 2.1a). To induce the tissue repair program in macrophages, we treated macrophages with IL-4 (Fig. 2.1a). This allowed us to ask whether macrophages integrate this soluble signal indicating the context of tissue repair with information about the tissue environment, in order to determine the appropriate response. We predicted that macrophages may alter their transcriptional response to IL-4, depending on the state of the extracellular matrix. We reasoned that, if we saw changes in gene expression in high-collagen compared to low-collagen gels, macrophages must be able to detect these differences in the extracellular matrix and relay that information in order to control gene expression.

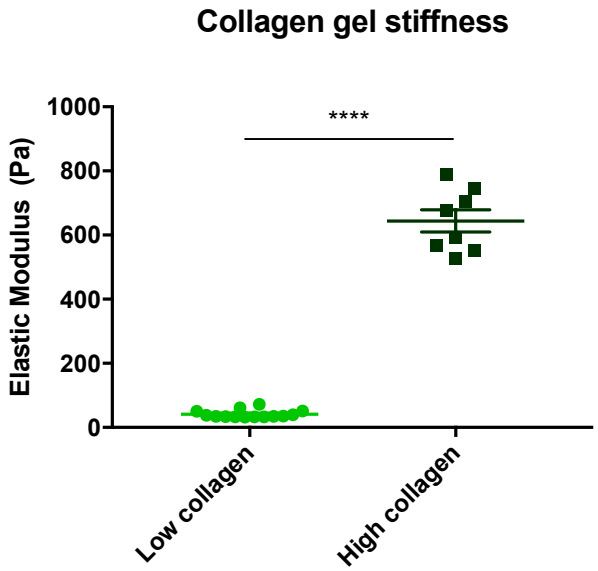
A)



3D Low collagen
2 mg/ml gel

3D High collagen
7 mg/ml gel

B)



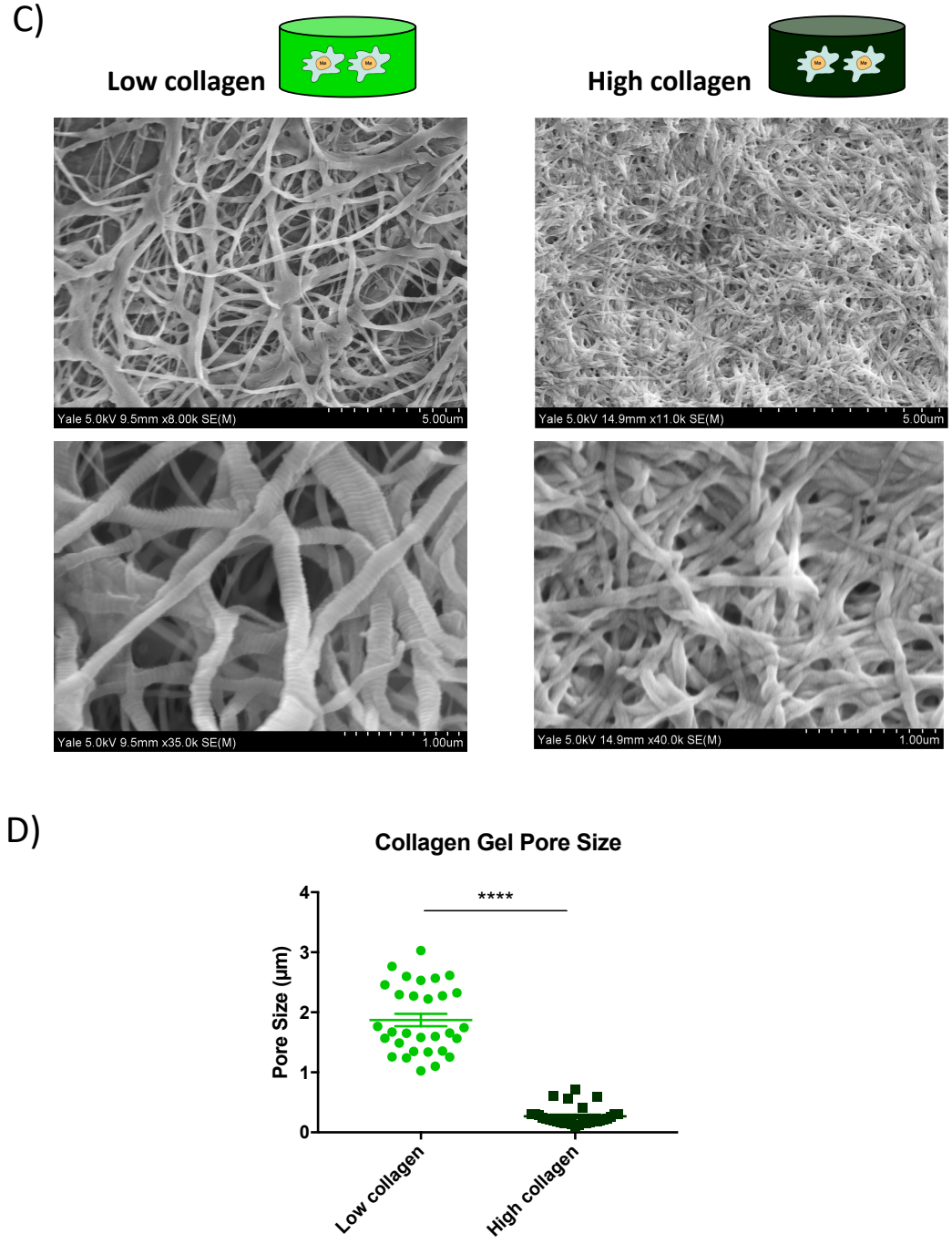
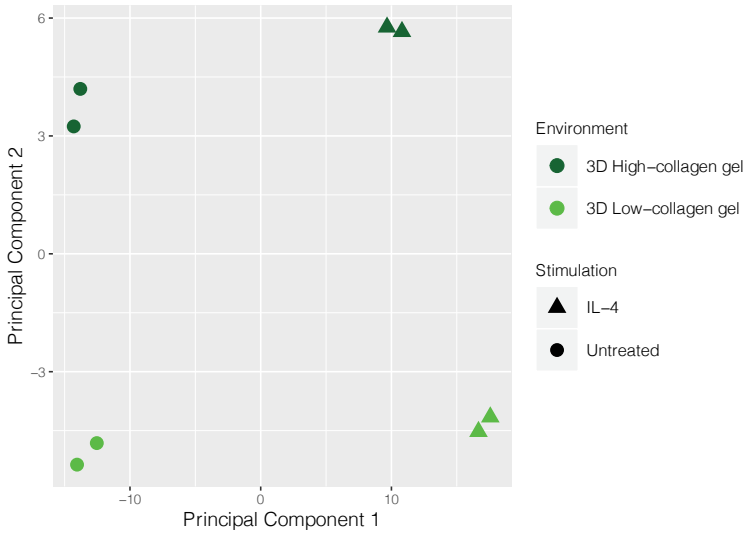


Figure 2.1. Three-dimensional collagen hydrogels allow for manipulation of extracellular matrix properties
(A) Bone marrow-derived macrophages are cultured within 3D collagen gels that vary from low (2 mg/mL) to high (7 mg/mL) collagen concentration and treated with or without IL-4. **(B)** Elastic modulus (stiffness) of collagen gels, measured by rheometry. **(C)** Scanning electron microscopy (SEM) of low- and high-collagen gels at lower (above) and higher (below) magnification. **(D)** Quantification of pore size in SEM images.

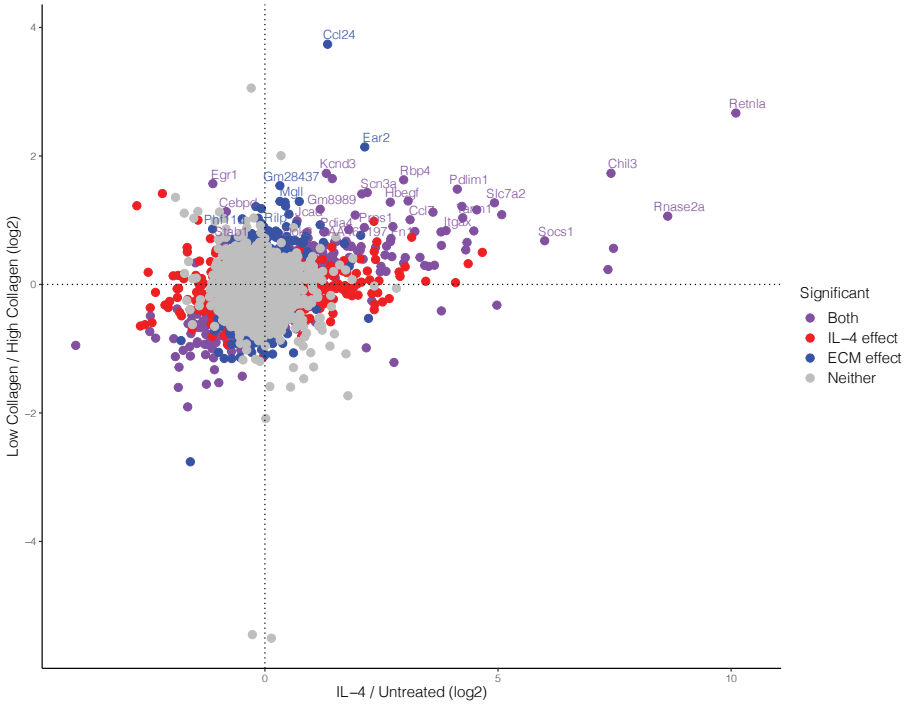
In order to capture the full gene expression program, we performed RNA sequencing (RNAseq) of macrophages cultured in low- or high-collagen gels, untreated or treated with IL-4. We performed principal component analysis (PCA) and found that samples segregated along Principal Component 1 (PC1) based on treatment with or without IL-4 and segregated along Principal Component 2 (PC2) based on whether they were cultured in low- or high-collagen gels (**Figure 2.2a**). Thus, altering the state of the extracellular matrix caused global transcriptional changes in macrophages.

Next, we investigated specific genes that were regulated by the extracellular matrix. We identified a cluster of genes that were induced by IL-4, whose expression was increased in low-collagen compared to high-collagen gels (**Figure 2.2b**). A group of these genes, including Fizz1, Rnase2a, Ear2, Ym1, Fn1, and Ccl24 were consistently upregulated in low- compared to high-collagen gels across dozens of experiments, while other genes that are also classically induced by IL-4, including Arg1 and Mrc1, were consistently unaffected by the state of the extracellular matrix (**Figure 2.2c**). Expression of ECM-sensitive genes, represented by Fizz1, showed a dose response to the concentration of collagen, while ECM-insensitive genes, represented by Arg1, did not (**Figure 2.2d**). We confirmed these findings on the protein level by performing flow cytometry with intracellular protein staining. We found that FIZZ1 protein levels were high in low-collagen gels and markedly suppressed in high-collagen gels, while ARG1 protein levels were unaffected (**Figure 2.2e**).

A)


Principal Component 2
Principal Component 1
6
3
0
−3
−10
0
10
Environment
3D High−collagen gel
3D Low−collagen gel
Stimulation
IL−4
Untreated

B)


Low Collagen / High Collagen (log2)
IL−4 / Untreated (log2)
Ccl24
Retnla
Ear2
Chil3
Egr1
Gm28437
Kcnd3
Rbp4
Pdlim1
Slc7a2
Rnase2a
Socs1
Significant
Both
IL−4 effect
ECM effect
Neither

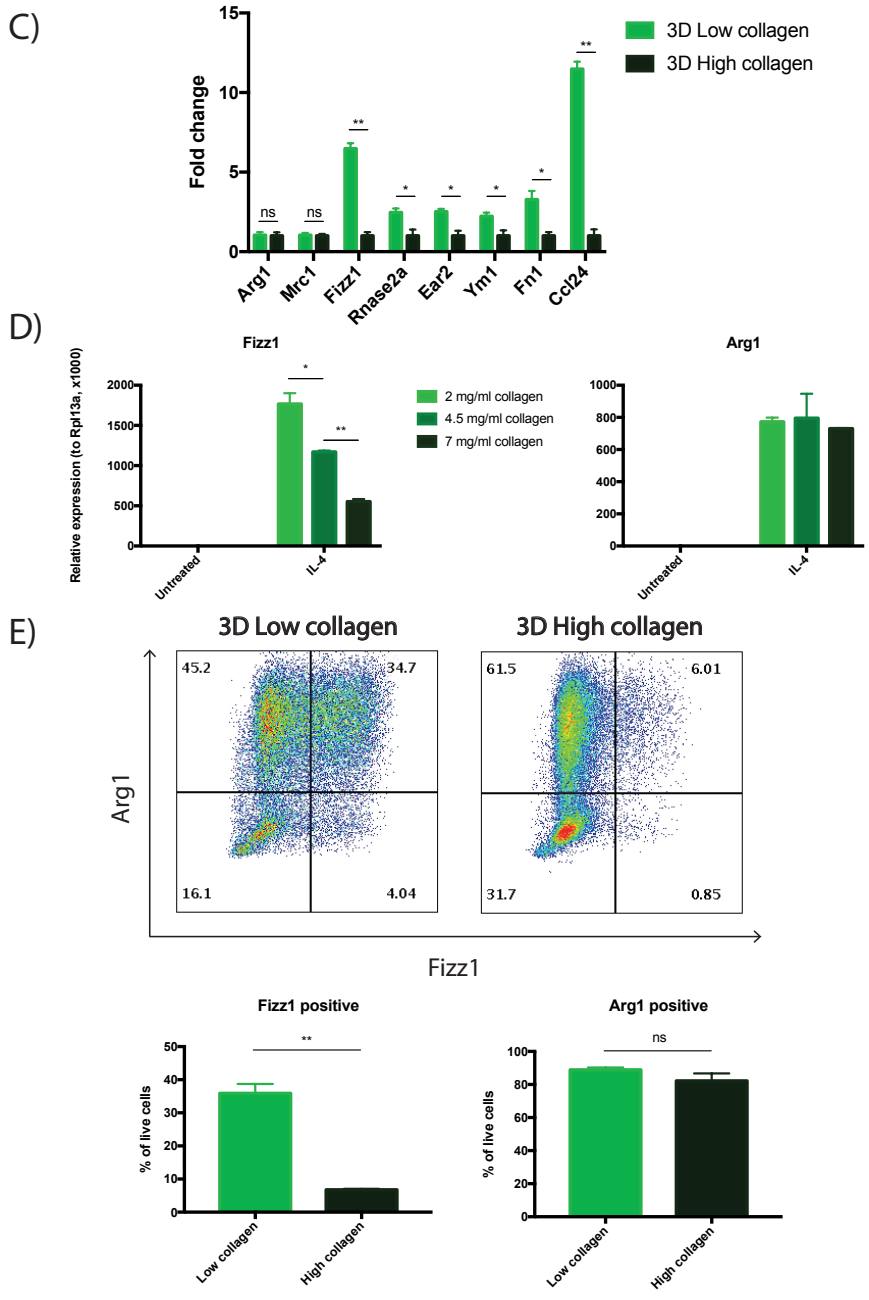


Figure 2.2. A specific IL-4-induced gene expression program is regulated by changes in the extracellular matrix

(A) Principal component analysis of RNAseq data from BMDM in low- or high-collagen gels, with or without IL-4 stimulation. **(B)** Depiction of all individual genes measured by RNAseq analysis, showing the effect of IL-4 (within high-collagen gels, x-axis) and the effect of low- vs. high-collagen gels (with IL-4 stimulation, y-axis) and colored according to the statistical significance of these effects (q-value < 0.2). Retnla = Fizz1, Chil3 = Ym1. **(C)** Expression of individual ECM-sensitive and -insensitive genes in the setting of IL-4 stimulation, measured by qPCR in a representative experiment. **(D)** Expression of Fizz1 and Arg1 mRNA, measured by qPCR, in a dose response to collagen hydrogel concentration. **(E)** FIZZ1 and ARG1 protein, detected by flow cytometry with intracellular protein staining and quantified by the percentage of cells expressing each protein (based on histogram plots).

This pattern of mRNA and protein expression, in which some IL-4 induced genes are regulated by the ECM and others are not, indicates that the observed effect is not simply a result of increased or decreased IL-4 signaling. To confirm this, we looked by Western blot at IL-4 signaling, which is mediated by STAT6. We found that phosphorylation of STAT6, representing STAT6 activation, was equivalent or even slightly lower in low- compared to high-collagen gels (**Figure 2.3a-b**), while FIZZ1 protein expression was markedly higher in low-collagen gels (**Figure 2.3a, c**). These data indicate that enhanced IL-4 signaling is not responsible for increased expression of the ECM-sensitive subset of genes, like Fizz1, in low-collagen gels. They suggest, instead, that there are at least two distinct sub-programs induced by IL-4. At least one of those programs, represented by Fizz1, is sensitive to changes in the tissue environment and in particular to the extracellular matrix and can be tuned according to those conditions (**Figure 2.3d**).

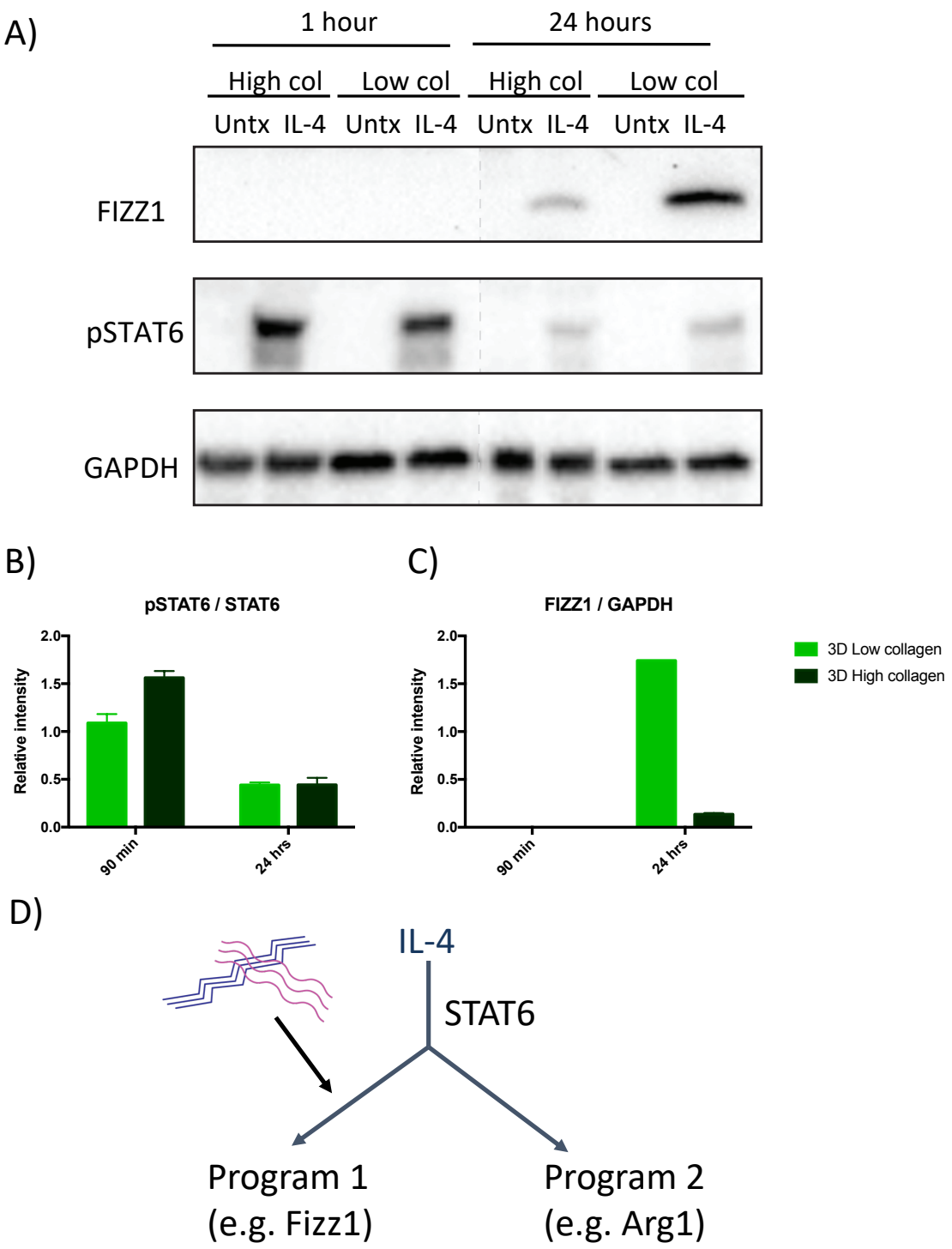


Figure 2.3. IL-4 signaling through STAT6 is not affected by the extracellular matrix
(A) Western blot showing phospho-STAT6 (pSTAT6) activation 1 hour and 24 hours after IL-4 stimulation (or untreated, "Untx") in high- or low-collagen gels. **(B)** Quantification of the relative intensity of pSTAT6 / total STAT6, and **(C)** of FIZZ1 / GAPDH (housekeeping gene) from a separate Western blot experiment with the same outcome. **(D)** Extracellular matrix regulates a sub-program of the IL-4 response.

Macrophages sense the mechanical properties of the extracellular matrix

Having established that macrophages regulate gene expression in response to changes in the extracellular matrix, we wanted to determine what properties of the extracellular matrix they sense. In principle, macrophages could sense the concentration of individual ECM components, or they could sense the mechanical properties of the ECM. There are several biological rationales for the latter hypothesis: 1) The mechanical properties of the ECM represent an emergent property of the assembly of individual ECM components, so measuring ECM mechanics would be an efficient way to assess the state of the extracellular matrix as a whole; 2) ECM function is largely determined by its mechanical properties, so ECM mechanics are likely to be actively regulated; and 3) the stiffness of the ECM changes dramatically during tissue repair, and increased ECM stiffness is a hallmark of fibrotic disease, so sensing changes in the ECM stiffness would be a way to detect the progression of tissue repair and monitor for the development of fibrotic changes.

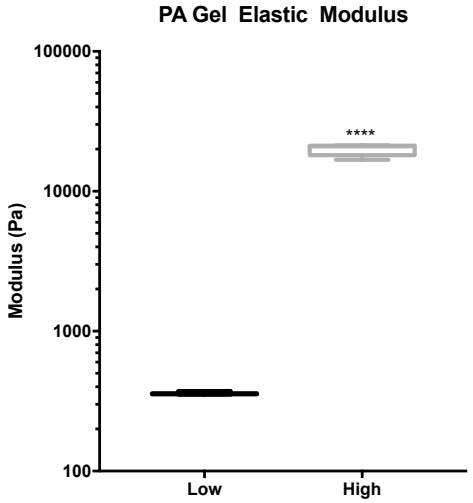
In collagen gels, the concentration of collagen is directly related to the mechanical properties of the gel, including its stiffness and pore size (Fig. 2.1). While this reflects the relationship that exists *in vivo*, it limits our ability to experimentally dissect the logic of ECM sensing. In order to decouple ECM concentration from ECM mechanics, we had to use a different experimental system. In collaboration with Rita Matta and Dr. Catherine Kim in the laboratory of Dr. Anjelica Gonzalez, we fabricated polyacrylamide (PA) gels conjugated to the major extracellular matrix protein fibronectin and cultured BMDM on these hydrogels (**Figure 2.4a**). In this system, the density of polyacrylamide (PA) crosslinking determines the stiffness of the gels, but PA is inert and not bound by cells. Cells bind instead to fibronectin on the surface of the PA gels, the

concentration of which is held constant. This system allowed us to manipulate the stiffness of the substrate (**Figure 2.4b**) without changing the concentration of the ECM ligand.

Morphologically, macrophages on high-stiffness polyacrylamide-fibronectin (PA-Fn) gels spread more than macrophages on low-stiffness PA-Fn gels, when visualized by light microscopy (**Figure 2.4c**) or by immunofluorescence after fixation and phalloidin staining of F-actin (**Figure 2.4d**). When mRNA expression was measured, the same pattern was observed as between low- and high-collagen gels. The ECM-sensitive gene expression program was elevated in macrophages on low-stiffness PA gels and suppressed in those on high-stiffness gels, while expression of Arg1 was unaffected by ECM stiffness (**Figure 2.4e**). These data indicate that macrophages are capable of sensing ECM stiffness and that changes in ECM stiffness are sufficient to regulate the ECM-sensitive gene expression program that we have identified. They provide persuasive evidence that mechanosensing is responsible for the changes in gene expression that we observe in the 3D collagen gel system. They also raise important questions about the mechanism by which macrophages sense the mechanical properties of their environment, which I will explore further in Chapters 3 and 4.

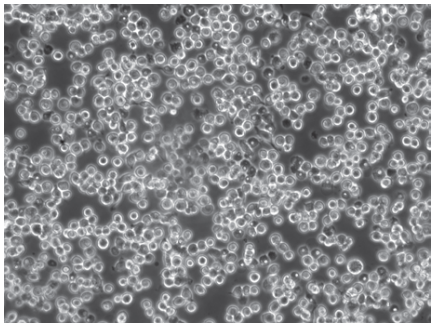
A)

B)

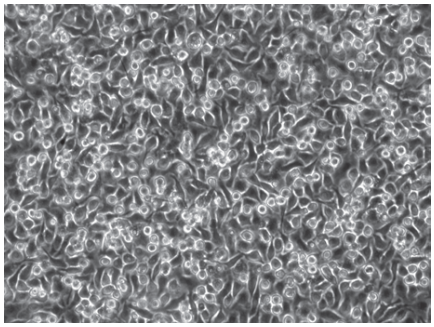


C)

Low-stiffness PA-Fn gel

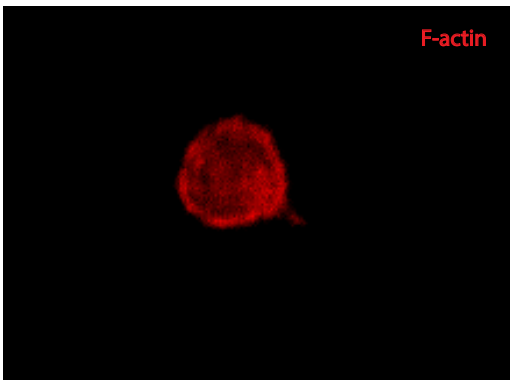


High-stiffness PA-Fn gel

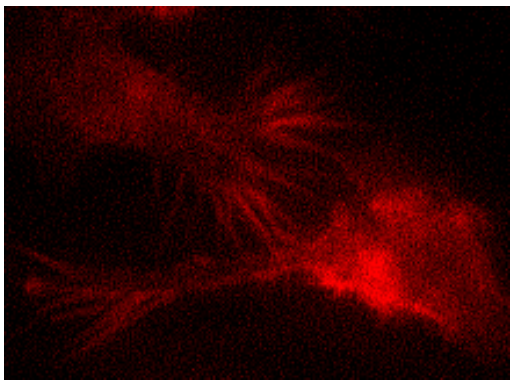


D)

Low-stiffness PA-Fn gel



High-stiffness PA-Fn gel



E)

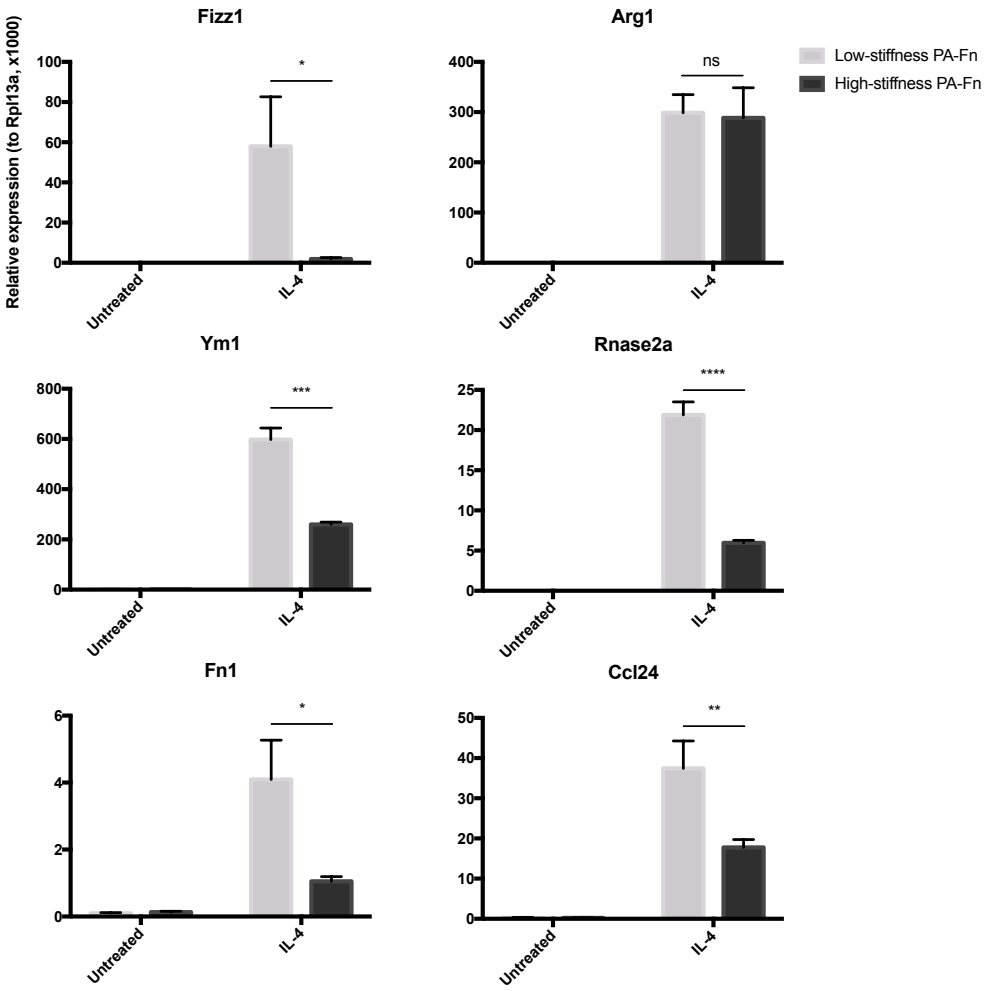


Figure 2.4. Extracellular matrix stiffness is sufficient to regulate the ECM-sensitive gene expression program

(A) Bone marrow-derived macrophages were cultured on 2D polyacrylamide (PA) gels of low or high stiffness conjugated to 0.2 mg/mL fibronectin. **(B)** Elastic modulus (stiffness) of PA gels. **(C)** Morphology of BMDM adhering to low- or high-stiffness PA-Fibronectin (PA-Fn) gels, by light microscopy and **(D)** by immunofluorescence with phalloidin staining after fixation. **(E)** mRNA expression of ECM-sensitive genes and Arg1 on low- or high-stiffness PA-Fn gels, with or without IL-4 treatment, measured by qPCR.

FIZZ1 acts on fibroblasts to regulate the extracellular matrix

We have shown that macrophage mechanosensing of the extracellular matrix regulates a subset of IL-4-induced genes. Fizz1 (Retnla) is one of the genes whose expression is most dramatically affected by changes in the ECM (Fig. 2.2b), and we confirmed by flow cytometry (Fig. 2.2e) and Western blot (Fig. 2.3a) that these changes are consistent on the protein level. Many of the genes induced in macrophages by IL-4 are also expressed by macrophages *in vivo* in the context of tissue repair and have therefore been implicated in the repair process. The function of many of these genes is unclear, however. In contrast, while many questions remain about the biology of FIZZ1, there is significant evidence that it plays a major role in driving effective tissue repair.

FIZZ1 protein is secreted by macrophages, but its receptor is unknown. Fizz1-knockout mice have impaired tissue repair in the lungs after bleomycin injury and in the skin after punch biopsy.[28,68] In both contexts, FIZZ1 was shown to activate fibroblasts, inducing expression in pulmonary fibroblasts of type I collagen and alpha-smooth muscle actin (αSMA, a marker of myofibroblast differentiation)[67] and expression in dermal fibroblasts of the collagen cross-linking enzyme lysyl hydroxylase 2 (LH2) and αSMA.[28] In the study of dermal tissue repair by Knipper and colleagues, IL-4 signaling in myeloid cells was required for effective wound healing after punch biopsy. Impaired IL-4 signaling led to a defect in collagen packing that was phenocopied in Fizz1-knockout mice and rescued by exogenous FIZZ1 and was traced to impaired collagen crosslinking by fibroblast LH2.[28] Thus, FIZZ1, secreted by macrophages, appears to play a key role in regulating fibroblast function during tissue repair.

Because the receptor for FIZZ1 and other features of its signaling mechanism are unknown, further examining its biological effects *in vitro* has been challenging. Fibroblasts often used for *in vitro* studies, like murine embryonic fibroblasts (MEFs), may not have the necessary receptors to respond to FIZZ1, and it appears likely that recombinant FIZZ1 produced in bacteria may not have the appropriate post-translational modifications to retain its signaling function. However, the dramatic regulation of Fizz1 that we observed, together with its still-mysterious biology, spawned a new project in the laboratory, led by Dr. Naomi Philip, to identify the receptor for FIZZ1 and better understand its biological functions. By expressing FIZZ1 in mammalian Expi293 cells and treating 3T3L1 fibroblast cells with conditioned media, we were able to confirm that FIZZ1 induces expression of Acta2 (αSMA) and Col1a1 (Collagen I) in fibroblasts (**Figure 2.5**). Gaining a better understanding of the biology of FIZZ1 is likely to continue to shed light on the significance of its regulation by extracellular matrix mechanics. The existing body of literature, however, suggests some important implications, which are discussed below.

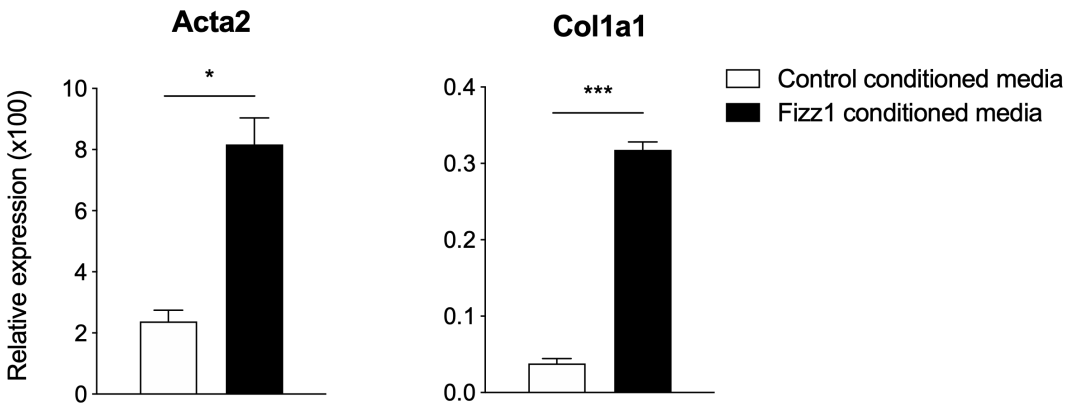


Figure 2.5. FIZZ1 induces αSMA and type I collagen expression in fibroblasts
Conditioned media from Expi293 cells transfected with a control vector or with Fizz1 was transferred onto 3T3L1 fibroblasts for 24 hours, after which cells were harvested and expression of Acta2 (αSMA) and Col1a1 (Collagen I) mRNA was measured by qPCR.

Discussion

In this chapter, we showed that macrophages sense the extracellular matrix in order to regulate their tissue repair program. We determined that ECM stiffness is the relevant property of the ECM that is monitored by macrophages. We also confirmed that this gene expression program, characterized by FIZZ1, has functional implications for ECM regulation by controlling gene expression in fibroblasts.

In vitro, IL-4 induces a global change in macrophage gene expression that is known as a polarization state. Macrophages polarized by IL-4 or IL-13 are often known as 'M2' or 'alternatively activated' macrophages, in contrast to 'M1' or 'classically activated' macrophages stimulated by LPS and IFNγ. This framework does not capture the plasticity of macrophages and their ability to adopt a wide range of polarization states in response to the functional demands of their environment.[22] However, when used to describe the *in vitro* states induced by specific cytokines, and when it is not understood as a full range of possible macrophage states, the M1/M2 language can be a useful shorthand. It has likely remained attractive to investigators in part because the genes induced by these cytokines *in vitro* map onto those identified in biological models *in vivo* and allow for translation between *in vivo* and *in vitro* contexts. When 'M2' macrophages are identified *in vivo*, however, they often express some but not all of the classic M2 marker genes. This disconnect is rarely investigated. One way to understand this finding is to recognize that macrophages respond not only to IL-4 (or another polarizing signal), but also to other signals in their environment that provide additional information, reporting on functional demand and tuning the macrophage polarization state to meet those demands.

Consistent with this model, we find that a subset of IL-4-induced genes is regulated by the extracellular matrix, while others are insensitive to changes in the ECM. This finding offers an example of separable programs within the 'M2' polarization state, which joins others in the literature showing that specific IL-4-induced genes are sensitive to lactic acid and apoptotic cells in the tissue environment.[69,98] The ECM-sensitive subprogram may also be sensitive to other environmental conditions, and in Chapter 4 we identify at least one other variable, growth factor availability, that converges on this gene expression program. The specificity of this gene expression program, together with the observation that STAT6 activation is unaffected by the ECM, also indicates that there must be a distinct mechanism of transcriptional regulation involved in controlling these sub-programs. This, too, will be further explored in Chapter 4.

In this chapter, we have identified Fizz1 as the hallmark of the mechanosensitive gene expression program in macrophages, and I have discussed the evidence that FIZZ1 drives myofibroblast differentiation, collagen synthesis, and collagen crosslinking during tissue repair, thus promoting ECM deposition and tissue stiffening. Our data indicate that Fizz1 is suppressed as ECM stiffness increases. This may be an excellent example of the type of homeostatic circuit (Fig. 1.1) that we predicted would regulate the extracellular matrix. FIZZ1 acts as a signal between macrophages (the sensor) and fibroblasts (the effector), reporting on the state of the extracellular matrix to control fibroblast behavior. In soft ECM, macrophages make more FIZZ1 to drive further ECM deposition and stiffening. When the ECM is stiffer, macrophages suppress FIZZ1 production to reduce fibroblast ECM production. Thus, this system is likely to function as a negative feedback loop. This may act as a check on the fibroblast-intrinsic positive feedback loop discussed in Chapter 1.

We can also see this biology through the lens of tissue repair. Early after injury, the tissue is soft, which will cause macrophages to make large amounts of Fizz1 and drive fibroblast ECM deposition. As ECM accumulates during the course of repair, macrophages will sense the increasing tissue stiffness and suppress Fizz1 expression, reducing fibroblast activation and helping to transition from repair to resolution. Notably, Ccl24, one of the other hallmarks of the mechanosensitive gene expression program, has also been shown to promote tissue repair and fibrosis in multiple organs and, we find, is suppressed by increasing tissue stiffness.[99–101] Thus, macrophage mechanosensing may serve as the kind of check on tissue repair that we sought to identify in this study, and it is possible that this checkpoint is impaired in the context of fibrosis. However, these potential conclusions await validation in animal models and patients. While Chapters 3 and 4 provide insights into the mechanisms of macrophage mechanosensing, translation of these results *in vivo* will require a suitable molecular target by which macrophage mechanosensing can be specifically disrupted.

Chapter 3: Macrophages sense ECM mechanics through integrin-independent changes in cytoskeletal dynamics

Introduction

In Chapter 2, we determined that macrophages sense the mechanical properties of the extracellular matrix in order to regulate their tissue repair program. Mechanosensing of the ECM is well-studied in cells like fibroblasts and epithelial cells, which are sessile within the tissue.[102] Immune cells like macrophages, meanwhile, can migrate rapidly within tissues, and their cellular mechanobiology and interactions with the ECM differ accordingly.[103] Mechanosensing in immune cells is just beginning to be investigated and may be governed by a distinct logic and set of biological mechanisms. In this chapter, we investigate the mechanisms of macrophage mechanosensing within 3D collagen gels. We discover that macrophages in this context sense ECM mechanics in a novel, integrin-independent fashion, mediated through changes in cytoskeletal dynamics.

Results

Macrophage mechanosensing is integrin-independent

Our understanding of cellular mechanosensing of the extracellular matrix has expanded dramatically in the last 15 years. In 2006, Engler and colleagues discovered, using a polyacrylamide gel system, that differentiation of mesenchymal stem cells (MSCs) could be controlled simply by tuning the stiffness of their substrate.[11] Dramatically, MSCs cultured on ECM approximating the stiffness of brain differentiated into neurons, while those cultured on

ECM approximating the stiffness of bone differentiated into osteoblasts. Since then, a paradigm has developed regarding the mechanism by which cells sense the stiffness of the ECM. Cells use transmembrane receptors known as integrins to bind to specific components of the extracellular matrix, such as fibronectin. Integrins are linked intracellularly to the actin cytoskeleton. When non-muscle myosin II contracts, it pulls on actin, which in turn pulls on the integrin receptor, which pulls extracellularly on the ECM ligand. The cell thus exerts tension on the extracellular matrix in order to probe its stiffness.[77] In the best worked out example, this tension on a stiff fibronectin substrate exposes a new binding site in fibronectin, which is bound by the integrin receptor and in turn causes intracellular signaling by the integrin receptor through focal adhesion kinase (FAK).[18] In most studies, however, this kind of linear signaling pathway is not identified. This is true, for example, when it comes to the function of YAP and TAZ as mechanosensors—they mediate mechano-signaling, leading to transcriptional regulation, but they are not cell surface receptors and are presumably not the upstream sensors of mechanical tension. Nonetheless, 1) cell adhesion to the extracellular matrix and 2) mechanical tension mediated by non-muscle myosin II are required for YAP/TAZ function.[78] The dominant assumption in the field has been that these elements—cell adhesion and mechanical tension—are essential for cellular mechanosensing of the extracellular matrix.[77]

Much of the biology of ECM mechanosensing has been learned from the study of fibroblasts. Therefore, before dissecting the mechanism of macrophage mechanosensing, we first determined whether murine embryonic fibroblasts (MEFs) in our 3D collagen gel system observe the expected biological rules based on the literature. Indeed, we found that fibroblasts upregulate the YAP target gene Ctgf and the myofibroblast marker Acta2 (αSMA) in high-collagen compared

to low-collagen gels. This mechanosensitive gene expression pattern was eliminated when cells were treated with blebbistatin, the non-muscle myosin II inhibitor (**Figure 3.1a**), and inhibited when collagen-binding integrins were blocked with an antibody targeting β1 integrin (**Figure 3.1b**). Thus, as expected, fibroblast mechanosensing in 3D collagen gels requires both integrin-mediated adhesion to the ECM and mechanical tension exerted by non-muscle myosin II.

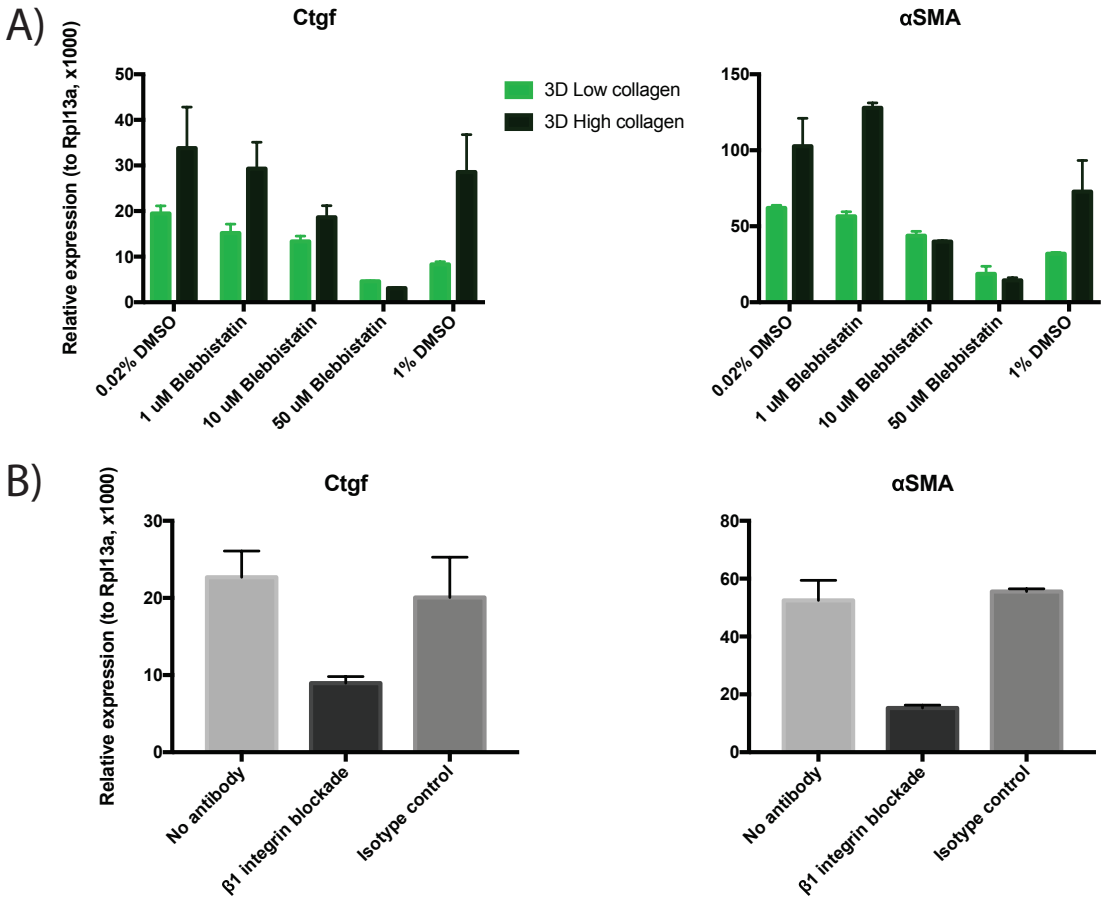


Figure 3.1. Fibroblast mechanosensing in 3D collagen gels is integrin- and myosin II-dependent **(A)** Murine embryonic fibroblasts (MEFs) were cultured in 3D low- and high-collagen gels and treated with the indicated doses of the non-muscle myosin II inhibitor blebbistatin, or with the vehicle DMSO at concentrations equivalent to the lowest (0.02%) and highest (1%) concentrations of blebbistatin. **(B)** MEFs in 3D collagen gels were incubated with a β1 integrin blocking antibody, compared to no antibody or an isotype control antibody.

One of the first suggestions that this model may not apply to macrophages was that macrophages do not appear to express any of the established collagen-binding integrins. Integrins are heterodimers consisting of an α chain and a β chain. The specific pair determines the specificity of the receptor. The known collagen-binding integrins all include β1 integrin, paired with α1, α2, α10, or α11 integrin. These α chains form an evolutionarily distinct cluster that appeared in vertebrates and include an "αI" domain that distinguishes them structurally from the other ECM-binding integrins.[71] None of these four α chains are expressed to any appreciable degree either in bone marrow-derived macrophages (with or without IL-4) based on our RNAseq data (**Figure 3.2a**) or in tissue-resident macrophages, based on re-analysis of RNAseq data published by Lavin and colleagues (**Figure 3.2b**).[63]

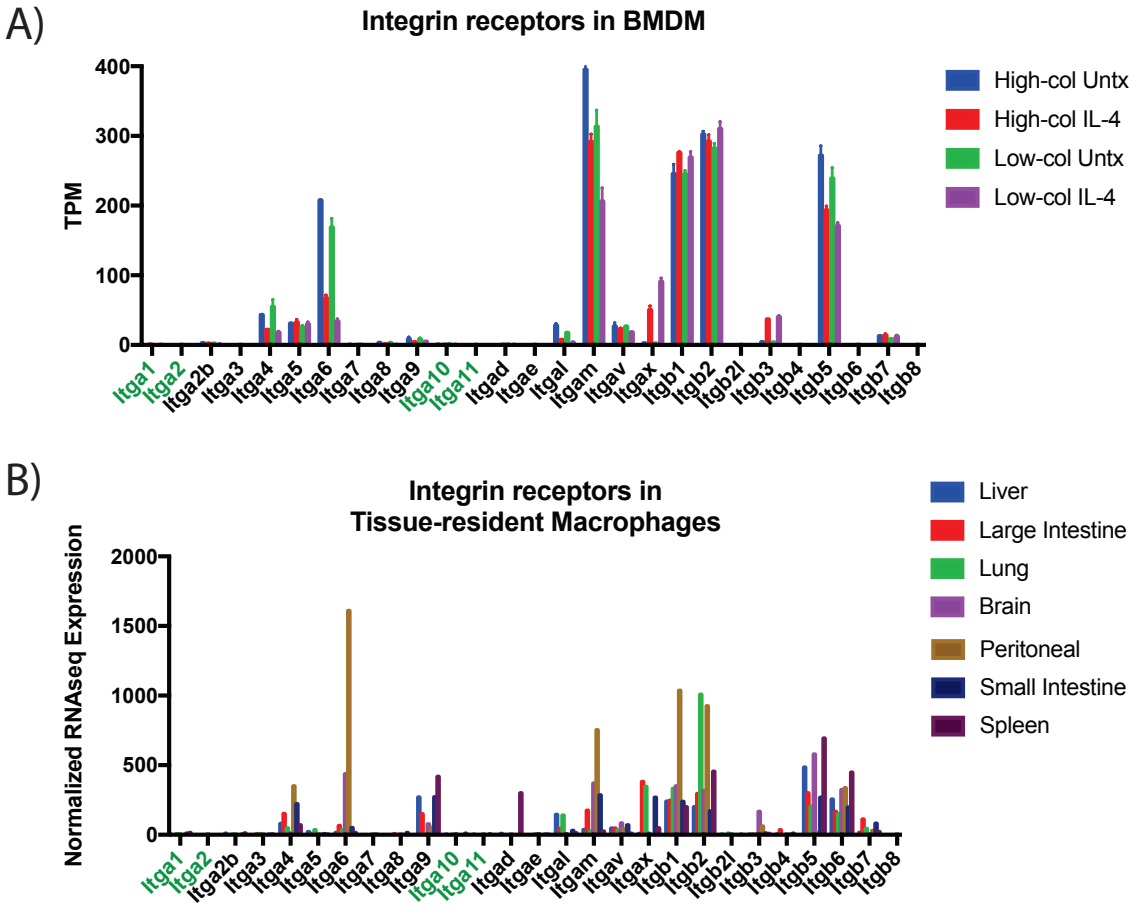
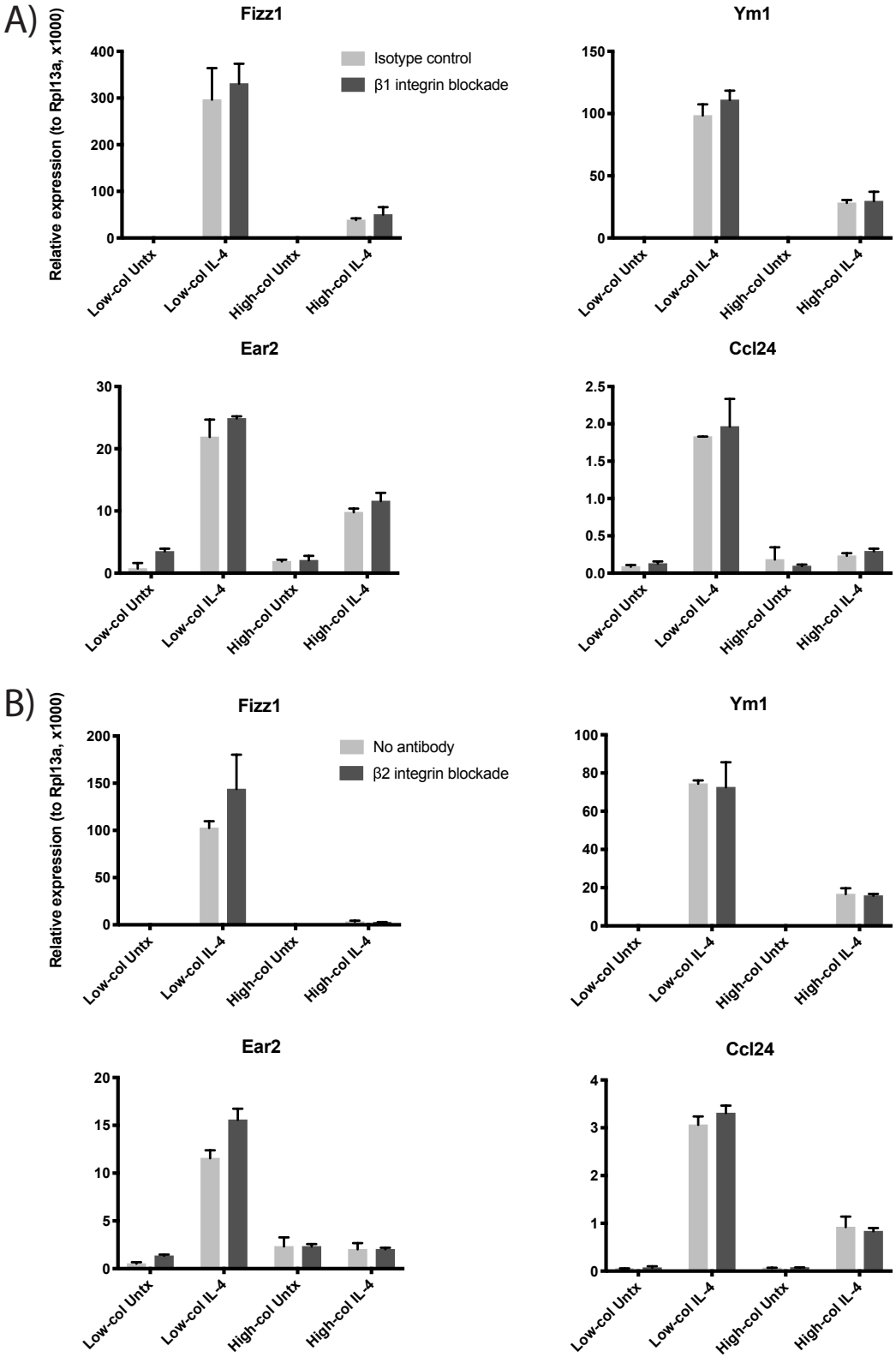


Figure 3.2. Macrophages show minimal expression of collagen-binding integrins
(A) Normalized expression levels (transcripts per million, TPM) of all integrin alpha and beta chains in bone marrow-derived macrophages (BMDM), from RNAseq analysis of BMDM cultured in low- or high-collagen gels, with or without IL-4 stimulation. **(B)** Normalized expression levels of all integrin alpha and beta chains in various tissue-resident macrophage populations, based on reanalysis of RNAseq data from Lavin et al (2014).[63] Alpha chains of collagen-binding integrins are highlighted in green (α1, Itga1; α2, Itga2; α10, Itga10; α11, Itga11).

In contrast to fibroblasts, when we blocked β1 integrin on macrophages, it had no effect on mechanosensitive gene expression (**Figure 3.3a**), indicating that the canonical collagen-binding integrins are not involved in macrophage mechanosensing in a 3D collagen environment. Blocking β1 integrin also had no apparent effect on macrophage morphology or migration within the collagen gels. Similarly, there was no effect on mechanosensitive gene expression after blocking β2 integrin, the β chain for all of the 'leukocyte integrins', which are classically involved in cell-cell interactions with the endothelium but some of which have been reported to interact with collagen (**Figure 3.3b**).[104–106] To test the involvement of all integrins, we targeted Talin1, an adapter protein that connects integrins to the cytoskeleton and is thought to be universally required for integrin activity. We made BMDM from $Tln1^{f/f}$ LysMCre (knockout) mice, compared to $Tln1^{f/f}$ (wildtype) mice. $Tln1^{f/f}$ LysMCre macrophages showed depletion of Talin1, but there was no defect in mechanosensing (**Figure 3.3c**), indicating that macrophage mechanosensing is integrin-independent.


A)
Fizz1
Ym1
Ear2
Ccl24
Relative expression (to Rpl13a, x1000)
Isotype control
β1 integrin blockade
Low-col Untx
Low-col IL-4
High-col Untx
High-col IL-4
B)
No antibody
β2 integrin blockade

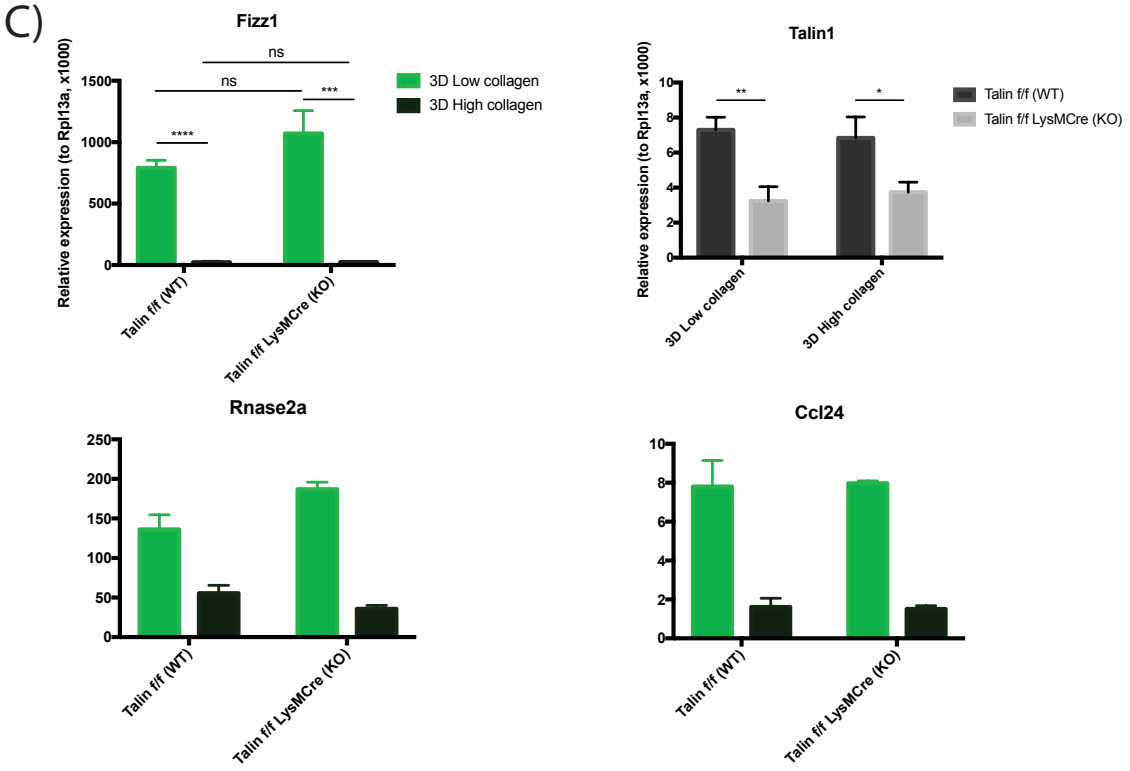


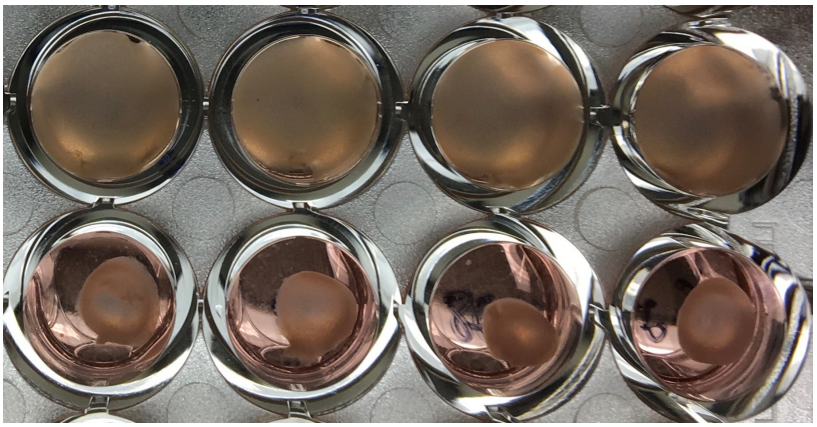
Figure 3.3. Macrophage mechanosensing in 3D collagen gels is integrin-independent
(A) mRNA expression in BMDM incubated with a β1 integrin blocking antibody or isotype control antibody. **(B)** mRNA expression in BMDM incubated with a β2 integrin blocking antibody or no antibody. **(C)** mRNA expression in BMDM from Talin-flox (wildtype, WT) and Talin-flox LysMCre (knockout, KO) mice.

Based on the canonical model of ECM mechanosensing, this result was very surprising. However, as we have discussed, this model was developed by studying cells like fibroblasts, mesenchymal stem cells, epithelial cells, endothelial cells, and smooth muscle cells. All of these cell types are typically stationary within the tissue and form firm adhesions to the extracellular matrix. Importantly, when they migrate, these cells also depend upon integrin-based adhesions to the extracellular matrix, in order to pull themselves forward. In contrast, leukocytes do not require integrin receptors in order to migrate within a three-dimensional tissue. Instead of attaching to the ECM and pulling themselves along ('crawling'), leukocyte migration is driven by actin protrusion at the leading edge of the cell and does not depend upon adhesion. This leukocyte mode of migration resembles 'swimming' and allows them to infiltrate a diverse range of tissues, migrate outside of prescribed paths, and achieve much higher speeds than is possible through the traditional, crawling mode of migration.[103,107,108] These biomechanical differences are plainly visible in the way that fibroblasts deform collagen gels, compared to macrophages. Fibroblasts cultured within 3D collagen gels physically contract the gels. In contrast, macrophages do not contract collagen gels because they do not exert pulling forces on the ECM (**Figure 3.4**). These differences between fibroblast and macrophage migration, and their general mode of interaction with the ECM, make sense of our experimental observations: Just as fibroblast interactions with the surrounding tissue are integrin-dependent and macrophage interactions are not, fibroblast mechanosensing is integrin-dependent, while macrophage mechanosensing of the ECM is integrin-independent.

A)

Macrophages

Fibroblasts



B)

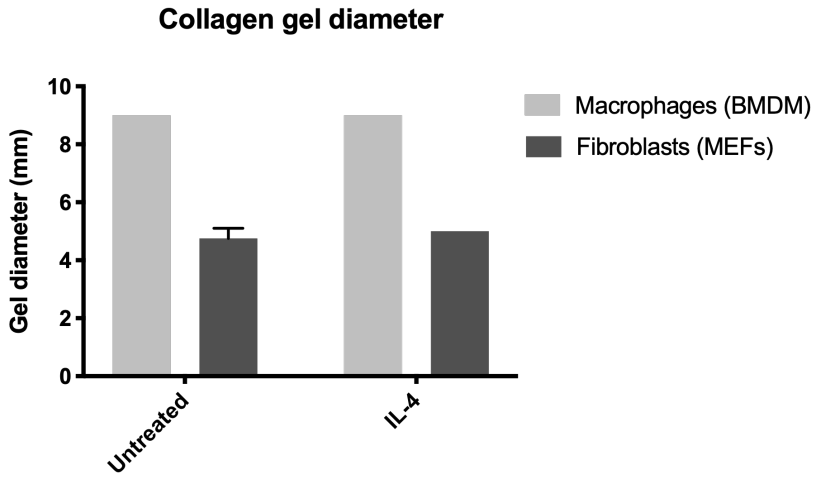


Figure 3.4. Fibroblasts contract collagen gels, while macrophages do not
(A) Photographs of collagen gels within which bone marrow-derived macrophages (top) or murine embryonic fibroblasts (bottom) have been cultured for 24 hours. **(B)** Measurements of the diameter of the collagen gels pictured in (A).

Macrophage mechanosensing is mediated through changes in cytoskeletal dynamics

Building on this link between migration and ECM sensing, I wondered whether observing macrophage migration within high- and low-collagen gels may offer further insights into the mechanism by which macrophages interpret these environments. Live cell imaging revealed that macrophages in low-collagen gels migrate much more rapidly than macrophages in high-collagen gels (**Figure 3.5a**). They also show marked differences in morphology: in low-collagen gels, macrophages were typically round or had short protrusions that rapidly turned over, while in high-collagen gels they were often dendritic, with numerous long protrusions that were also dynamic but turned over more slowly (**Figure 3.5b-c**). Other groups have similarly observed that macrophages can vary in morphology and migration speed, depending on matrix architecture.[91,93] And recent work has shown that dendritic cells and other leukocytes use dynamic membrane protrusions, like those we visualized, to mechanically sample and navigate 3D collagen gels and other complex pore networks.[109] I hypothesized that these cytoskeletal dynamics, dictated by the mechanics of the environment, serve as an intracellular measure of the extracellular environment and ultimately control the observed changes in gene expression.

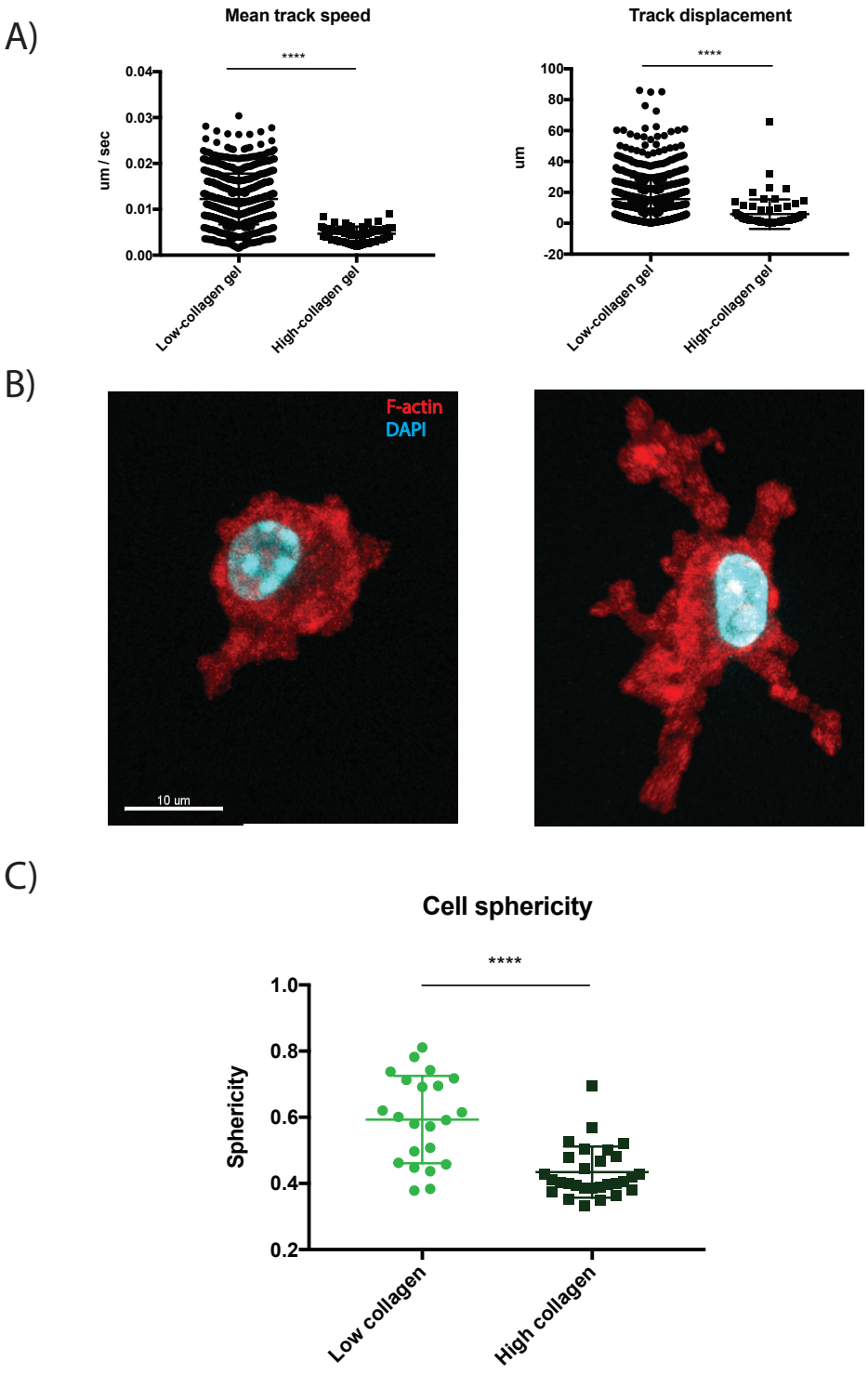
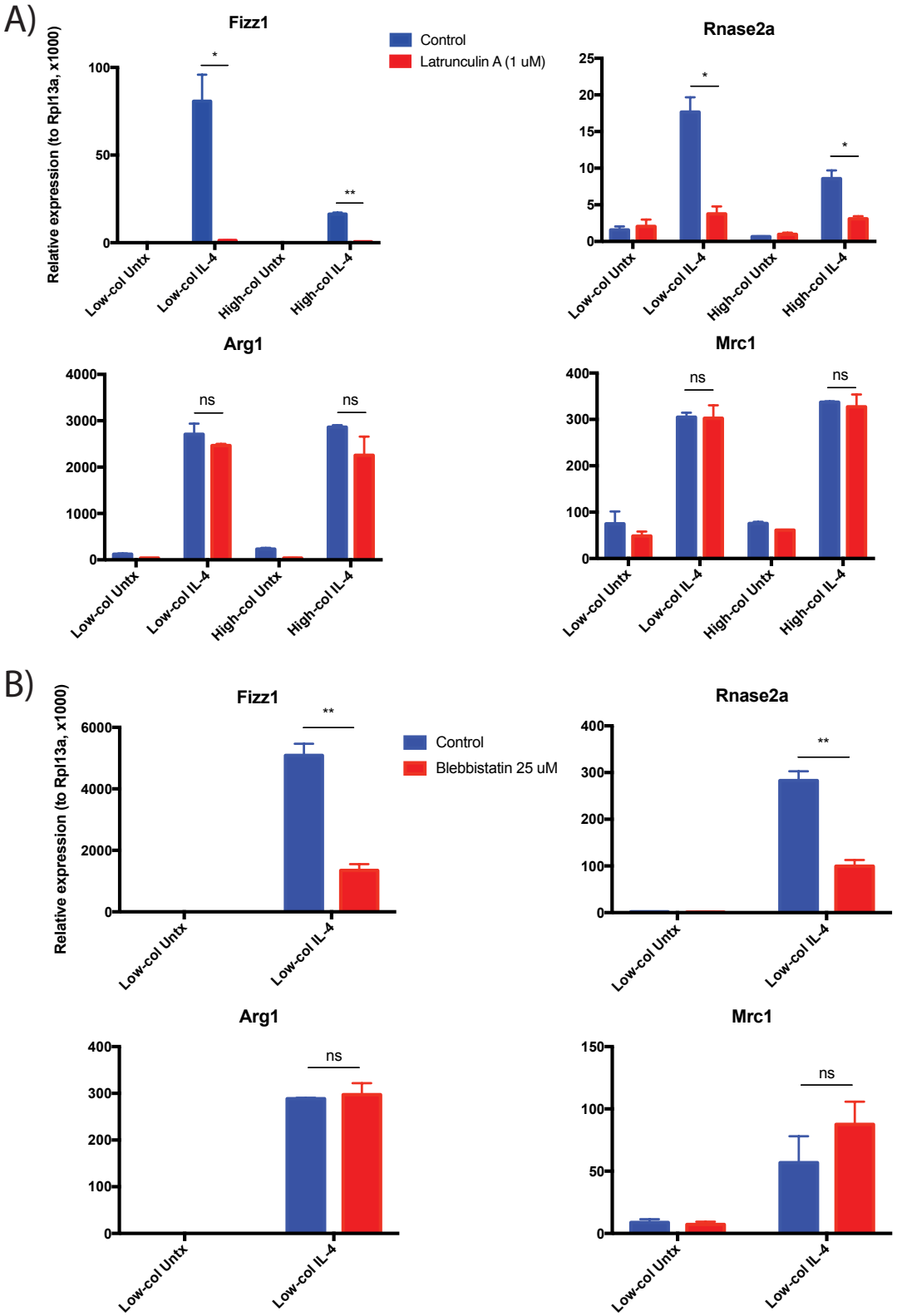


Figure 3.5. Macrophage mechanosensing is associated with changes in cytoskeletal dynamics **(A)** Quantification of macrophage migration speed and distance from live cell imaging of BMDM for approximately 20 hours in low- and high-collagen gels. **(B)** Characteristic confocal images of BMDM in low- and high-collagen gels, after fixation and staining with phalloidin (red) to visualize F-actin and DAPI (teal) to visualize the nucleus. **(C)** Quantification of cell sphericity of all cells captured in low- and high-collagen gels by confocal imaging.

To test this model, we directly manipulated macrophage cytoskeletal dynamics to determine whether this would control the mechanosensitive gene expression program. We blocked actin polymerization using the inhibitor latrunculin A and found that it profoundly suppressed Fizz1 and the rest of the mechanosensitive gene expression program, while it had no effect on Arg1 and Mrc1 (**Figure 3.6a**). We found a similar but subtler pattern when we used blebbistatin to block non-muscle myosin II (**Figure 3.6b**). In macrophages within 3D collagen gels, non-muscle myosin II likely does not exert tension on the ECM, but it does play a role in regulating actin dynamics, as evidenced by the highly dendritic morphology of cells treated with blebbistatin. Finally, we investigated the role of microtubules, which organize the actin cytoskeleton in macrophages and control leukocyte migration decisions within 3D collagen gels.[109,110] Perturbing microtubule dynamics with nocodazole similarly suppressed the mechanosensitive gene expression program, while in this case inducing Arg1 and Mrc1 (**Figure 3.6c**). Thus, within the broader response to IL-4, the mechanosensitive module of gene expression was specifically regulated by manipulating the cytoskeleton, independent of the rest of the IL-4 response. Taken together, these data support a model in which extracellular matrix mechanics act through their effects on the cytoskeleton to control macrophage gene expression programs and tune macrophage polarization.


A)
Fizz1
Control
Latrunculin A (1 uM)
Relative expression (to Rpl13a, x1000)
100
50
0
*
**
Low-col Untx
Low-col IL-4
High-col Untx
High-col IL-4
Rnase2a
25
20
15
10
5
0
*
*
Arg1
4000
3000
2000
1000
0
ns
ns
Mrc1
400
300
200
100
0
ns
ns
B)
Fizz1
Control
Blebbistatin 25 uM
Relative expression (to Rpl13a, x1000)
6000
4000
2000
0
**
Low-col Untx
Low-col IL-4
Rnase2a
400
300
200
100
0
**
Arg1
400
300
200
100
0
ns
Mrc1
150
100
50
0
ns

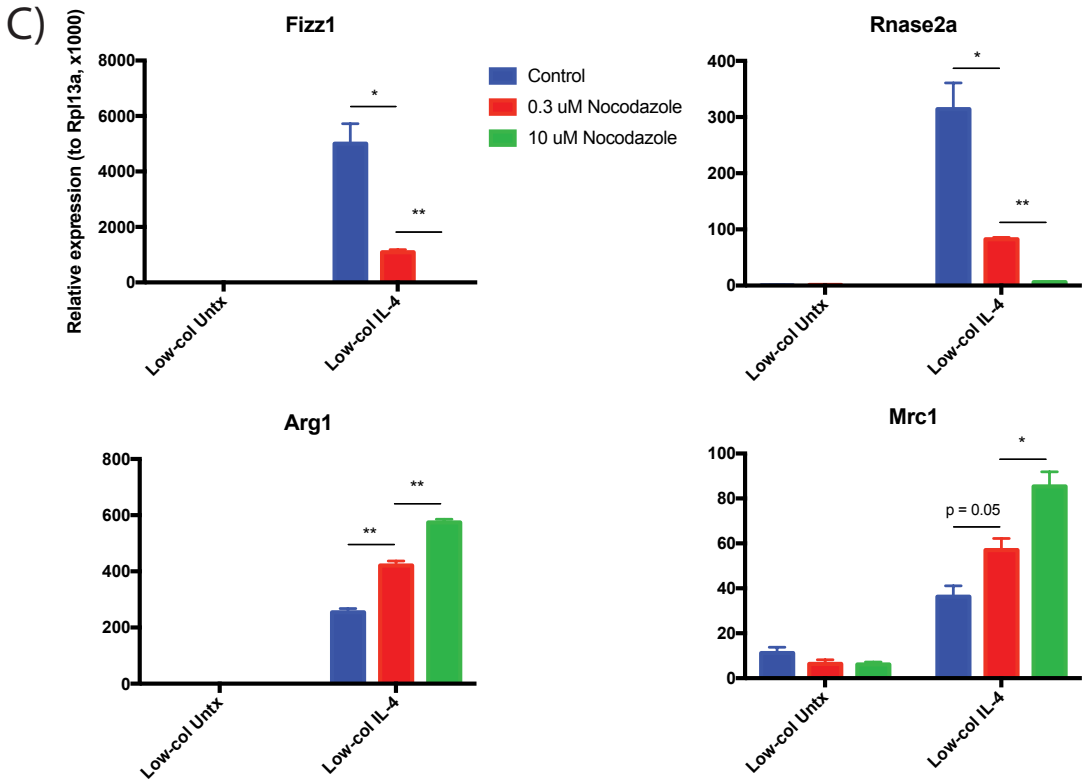


Figure 3.6. Cytoskeletal dynamics control the mechanosensitive gene expression program in macrophages

mRNA expression in BMDM treated with **(A)** the actin polymerization inhibitor latrunculin A, **(B)** the non-muscle myosin II inhibitor blebbistatin, and **(C)** the microtubule inhibitor nocodazole, compared to vehicle controls. Genes shown are representative members of the IL-4-induced mechanosensitive gene expression program (Fizz1, Rnase2a) and the IL-4-induced ECM-insensitive gene expression program (Arg1, Mrc1).

Discussion

In this chapter, we have dissected the cellular mechanism by which macrophages interpret the mechanical features of their environment within a 3D collagen gel. We found that macrophages do not employ the canonical integrin-dependent mode of mechanosensing that has been shown for many other cell types. Instead, they sense ECM mechanics in an integrin-independent fashion that is associated with changes in the cytoskeleton. We found that manipulating the cytoskeleton directly is sufficient to control the mechanosensitive gene expression program, indicating that these cytoskeletal changes relay the mechanical information that is sensed in the ECM. Thus, the mechanical properties of the extracellular matrix are translated into changes in the mechanical properties of the internal cytoskeleton, which must then result in biochemical changes to control gene expression. The latter point will be explored in Chapter 4.

This raises an important question that we can only speculate about for now and that I hope will provide the impetus for further studies: How do extracellular changes in ECM mechanics result in intracellular changes in the cytoskeleton, if this effect is not mediated through receptor-based adhesion to the ECM? My intuitions regarding the mechanisms of macrophage mechanosensing have been very substantially influenced by the progress that has been made in understanding the mechanisms of leukocyte migration. Here, I owe a debt of gratitude in particular to the Sixt group, whose elegant studies helped me understand both the apparent integrin-independence of macrophage interactions with the ECM in our system and the actin cytoskeletal dynamics and mechanical forces involved in leukocyte migration.[103,107,109,111,112] After discovering that leukocytes in 3D environments do not require integrins to migrate,[103] the Sixt group went on to establish, using inert substrates, that leukocytes can migrate without adhering at all to the

external environment (though non-specific friction forces are likely necessary).[113] They proposed that this migratory mechanism is driven by actin polymerization at the leading edge of the cell causing deformation of the cell body, rather than the classic model in which the retrograde force of actin flow is coupled to extracellular adhesion at transmembrane receptors.[111]

Related work by the Paluch group emphasized the key conceptual point that adhesion-free migration has the opposite force distribution of adhesion-based migration: in adhesion-free migration, the net cellular forces point outward, causing the cell membrane to push against the environment, while in adhesion-based migration, much larger net cellular forces point inward, as cell-surface receptors pull against the environment.[108] Thus, macrophages engaging in integrin-independent migration in a 3D collagen gel may be considered "pushers" while fibroblasts engaging in adhesive migration would be considered "pullers" (evidenced by their contraction of the collagen gel).

How might these pushing forces be translated into changes in actin cytoskeletal dynamics? In 2017, in a different line of work, the Sixt group found that actin filaments pushing against different mechanical loads at the cell membrane organized into distinct configurations. This process did not require any signaling pathways, instead emerging from the geometry of branched actin and limited access of capping proteins to the actin filaments near the cell membrane.[112] Thus, the actin network has an intrinsic response to extracellular mechanics that does not require any receptor-mediated binding to extracellular proteins.

The problem of how cytoskeletal dynamics in leukocytes respond to their physical environment is not only a question that is important for understanding mechanosensing, but also a fundamental question in cell migration. In 2019, the Sixt group showed that leukocytes are able to discriminate between larger and smaller pores, in order to navigate a complex 3D environment. They argue that, as leukocytes extend actin protrusions into pores to sample their environment, the bulky nucleus follows and acts as a mechanosensor to detect ECM resistance. In response, the microtubule organizing center coordinates actin dynamics to redirect the cell along the path of least resistance.[109] This helps to explain the dendritic morphology and slow migration that we observe in high-collagen gels, compared to the more amoeboid morphology and faster migration in low-collagen gels. These cytoskeletal dynamics are a direct response to the mechanical environment: Macrophages in high-collagen gels encounter smaller pores and a stiffer extracellular matrix and thus greater resistance from their environment, which forces them to extend membrane protrusions to explore a wider variety of paths. Macrophages in low-collagen gels navigate a matrix with larger pores and softer ECM and thus encounter less resistance, allowing them to extend only short protrusions and to migrate more rapidly. How exactly the cell translates that mechanical resistance into changes in actin dynamics is an extremely complex mechanobiological question and is an area of active study. What we can conclude, though, from this body of literature together with our data, is that the mechanical environment clearly shapes the dynamics of the actin cytoskeleton, and that these dynamics are not only directly useful for cell migration, but also contain information about the external environment and can therefore be measured by the cell in order to inform other cellular functions, such as gene expression.

Several additional predictions and questions arise from the discussion above. First, whereas fibroblasts and other cells sense tension forces as they pull on the extracellular matrix, I predict that macrophages sense compression forces as they migrate and push against the surrounding extracellular matrix. This is consistent with the idea that macrophages are "pushers" rather than "pullers", exerting net outward forces against their environment, and it makes sense that sensing compressive forces from the environment would not require any specific adhesions to the extracellular matrix. Second, macrophages may sense these compression forces at the cell membrane, perhaps causing direct rearrangement of the actin cytoskeleton,[112] or at the nucleus, perhaps leading to microtubule-directed control of the actin cytoskeleton,[109] or a combination of these and other mechanisms. More traditional signaling pathways that regulate actin dynamics, like the Rho-GTPases,[114,115] may also play a role, but we found that chemical inhibitors targeting these pathways did not specifically modulate the mechanosensitive gene expression program. Third, we do not know the specific mechanical parameter that is sensed in 3D collagen gels. On 2D polyacrylamide gels, we specifically manipulate the stiffness of the substrate and can say with some certainty that macrophages are sensing this difference in stiffness. Within a more complex 3D environment, though, multiple mechanical properties vary together. We have shown that high-collagen gels are not only stiffer, but also have smaller pore sizes than low-collagen gels, and we have discussed evidence from the literature that leukocytes can discriminate between smaller and larger pores.[109] As I have indicated, I suspect that the relevant property of the ECM is the resistance or compressive force that macrophages encounter, and multiple features of the environment, including stiffness and pore size, may contribute to this net effect. Importantly, these features are also likely to co-vary *in vivo*, as increased ECM deposition would lead to both increased stiffness and reduced pore size.

Finally, it is important to note that we used a 2D polyacrylamide-fibronectin system (Chapter 2) to formally demonstrate that the ECM-sensitive gene expression program is in fact a mechanosensitive gene expression program. Unlike 3D collagen gels, this 2D system is adhesion-dependent. In fact, we used fibronectin rather than collagen because macrophages do not adhere to collagen (presumably due to the absence of collagen-binding integrins, as we have shown), but do adhere to fibronectin. This is consistent with the literature: the same paper that demonstrates integrin-independent leukocyte migration in 3D environments (in 3D collagen gels *in vitro* and within tissues *in vivo*) also demonstrates that integrins are absolutely required for migration on 2D substrates *in vitro* and for transmigration from vasculature into tissues *in vivo*. Surprisingly, β1 integrin blockade (which would block the fibronectin receptor α5β1 integrin) did not impair adhesion or mechanosensing on PA-Fn gels. However, other fibronectin receptors, such as αVβ3, may be involved, and we assume at present that macrophage mechanosensing on 2D PA-Fn gels is integrin-dependent. If that is right, it is very interesting that integrin-independent mechanosensing (likely sensing compressive forces) and integrin-dependent mechanosensing (likely sensing tension forces) converge on the same gene expression program. Macrophages showed increased cell spreading on stiff compared to soft PA-Fn gels, and we can imagine that these cytoskeletal changes may be similar to those that take place in 3D high-collagen compared to low-collagen gels, ultimately communicating the same information intracellularly and leading to the same events in the regulation of gene expression.

Chapter 4: The cytoskeleton integrates mechanical and biochemical signals in the tissue environment to regulate transcription

Introduction

In Chapter 2, we identified that macrophages sense extracellular matrix mechanics to regulate gene expression, and in Chapter 3, we determined that this mechanosensing was mediated through changes in the actin cytoskeleton. In this chapter, we show that signaling by macrophage colony stimulating factor (MCSF), the lineage-restricted growth factor for macrophages, can cause similar changes in actin dynamics and regulate the same gene expression program. These cytoskeletal effects are mediated by MCSF signaling through PI3K and GSK3 and are required for the MCSF effect on the mechanosensitive gene expression program but not other genes. Both MCSF stimulation and direct perturbation of the cytoskeleton regulate chromatin accessibility and C/EBPβ binding at the Fizz1 but not the Arg1 locus. Thus, we show in this chapter that the cytoskeleton acts as an integrator of mechanical and biochemical signals to regulate specific transcriptional targets.

Results

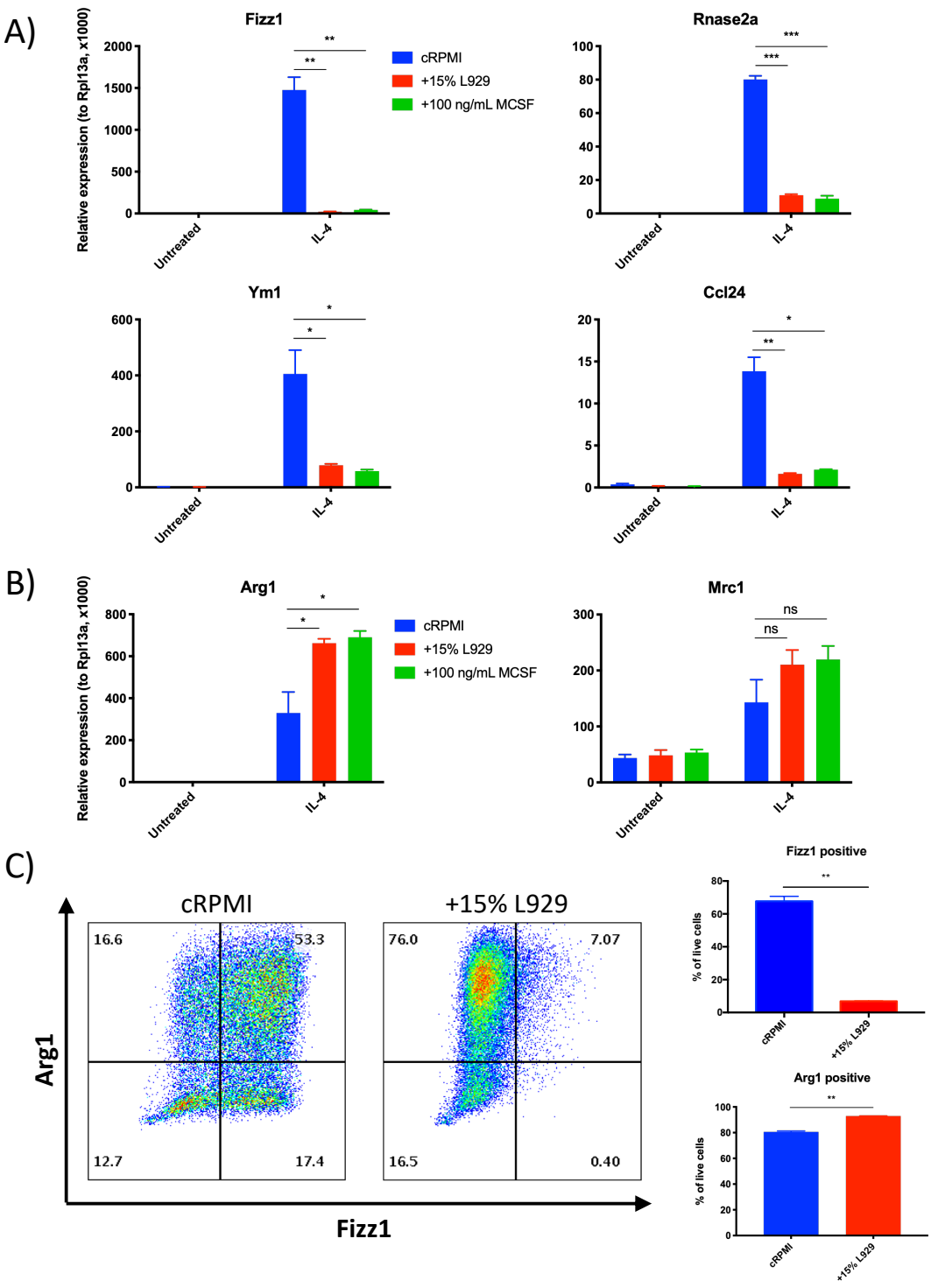
MCSF controls cytoskeletal dynamics to regulate the mechanosensitive gene expression program

MCSF induces macrophage differentiation, proliferation, and survival. To grow bone marrow-derived macrophages, we use conditioned media from a fibroblast cell line called L929 that makes large quantities of MCSF, which induces the differentiation of hematopoietic stem cells obtained from the bone marrow into mature macrophages. Several years ago, Dr. Scott Pope in

our lab made the observation that, after macrophage differentiation, removing L929 conditioned media had significant effects on macrophage gene expression in response to the polarizing signals LPS and IL-4. Fizz1 was among the genes that was most highly regulated. Since our initial experiments were performed in L929-conditioned media, we began to perform experiments to make sure that the phenotype we observed was not a consequence of the relative availability of MCSF in low- and high-collagen environments. We first confirmed that macrophage responses to ECM are intact in the absence of L929 conditioned media or other sources of MCSF (and we then continued to use MCSF-free conditions in most of the experiments presented throughout Chapters 2 and 3). In these experiments, we also made the discovery that L929 conditioned media or recombinant MCSF specifically suppressed the same gene expression program as high-stiffness ECM, including Fizz1, Ym1, Rnase2a, and Ccl24 (**Figure 4.1a**). In contrast, MCSF either increased or had no significant effect on the expression of the ECM-insensitive IL-4-induced genes Arg1 and Mrc1 (**Figure 4.1b**). We confirmed these findings on the protein level using flow cytometry with intracellular protein staining for FIZZ1 and ARG1 (**Figure 4.1c**).

MCSF also had dramatic morphologic effects on macrophages. On cell culture dishes, the addition of MCSF caused macrophages to spread and extend long protrusions of their cell membranes (**Figure 4.1d**) Similarly, in live cell imaging in 3D collagen gels, macrophages treated with MCSF migrated more slowly and became more dendritic, extending long membrane protrusions, closely resembling the cytoskeletal dynamics in high-collagen compared to low-collagen gels. Thus, MCSF and high-stiffness ECM appear to converge on similar effects on both the cytoskeleton and gene expression. To determine whether the effects on gene expression

are mediated through the effects on the cytoskeleton, we treated macrophages with MCSF under normal conditions or after inhibiting actin polymerization using latrunculin A. While MCSF potently suppressed Fizz1 when the cytoskeleton was intact, treatment with latrunculin A strongly attenuated the effects of MCSF on Fizz1 expression. In contrast, the effects of MCSF on Arg1 were unaffected by the state of the cytoskeleton (**Figure 4.1e**). Control of the mechanosensitive gene expression program by MCSF, then, appears also to be mediated by MCSF remodeling of the cytoskeleton.


A)
Fizz1
Rnase2a
Ym1
Ccl24
Relative expression (to Rpl13a, x1000)
cRPMI
+15% L929
+100 ng/mL MCSF
Untreated
IL-4
B)
Arg1
Mrc1
ns
C)
cRPMI
+15% L929
16.6
53.3
12.7
17.4
76.0
7.07
16.5
0.40
Arg1
Fizz1
Fizz1 positive
Arg1 positive
% of live cells

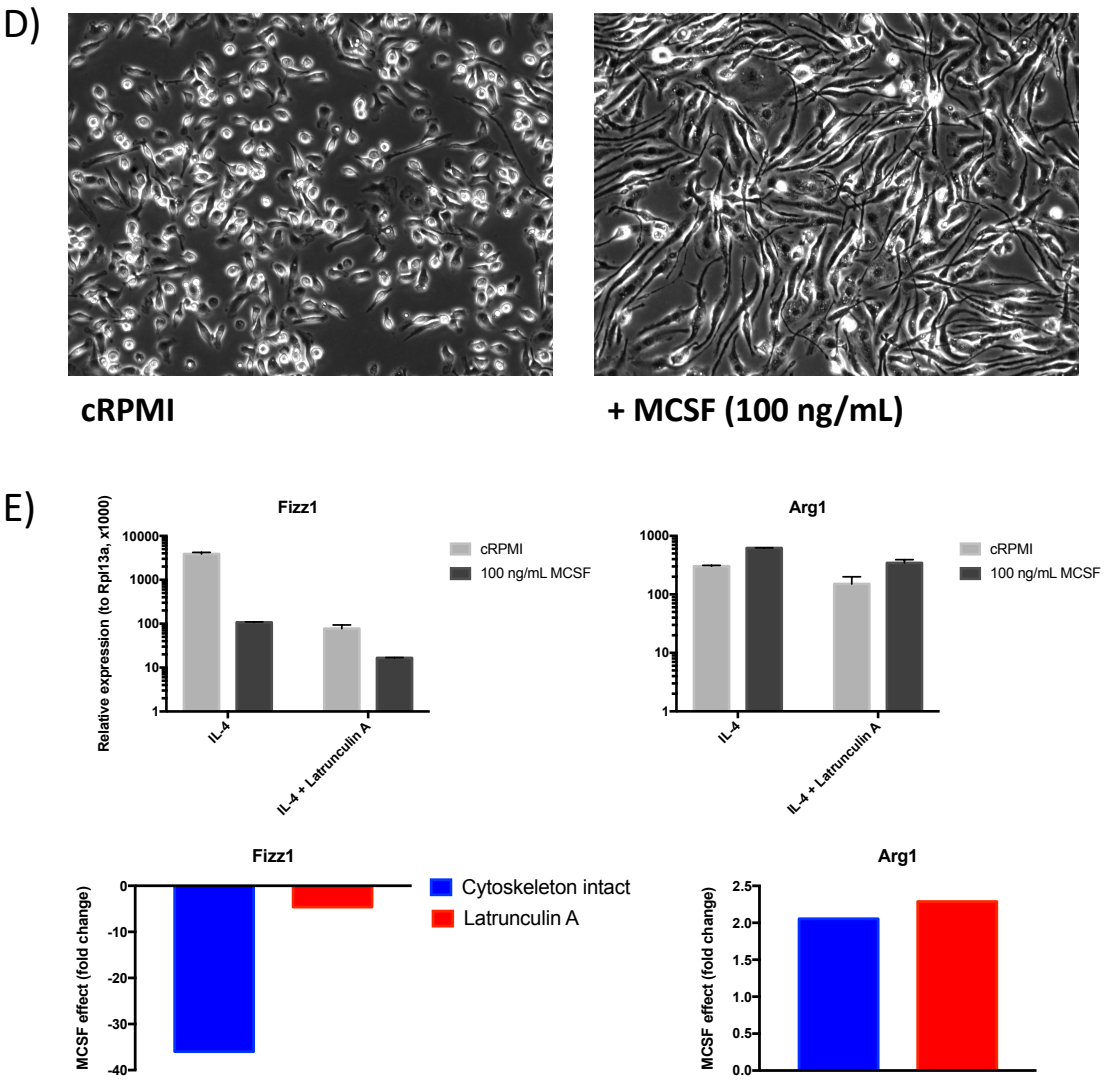


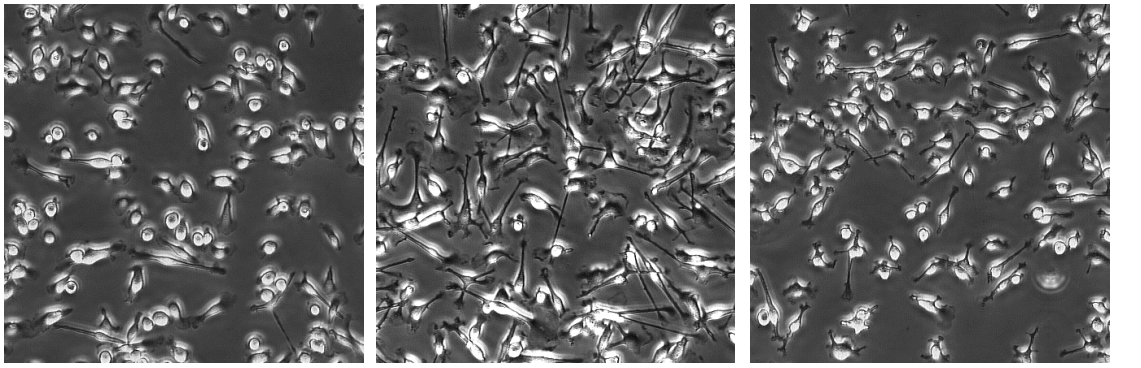
Figure 4.1. MCSF-induced cytoskeletal remodeling regulates the mechanosensitive gene expression program

(A) mRNA expression of mechanosensitive genes in BMDM cultured in standard complete RPMI (cRPMI) media or with the addition of 15% conditioned media from L929 cells, which produce large quantities of MCSF (+15% L929), or 100 ng/mL recombinant MCSF protein (+100 ng/mL MCSF). **(B)** mRNA expression of IL-4-induced ECM-insensitive genes from the same experiment. **(C)** FIZZ1 and ARG1 protein in BMDM cultured in cRPMI or 15% L929-conditioned media, detected by flow cytometry with intracellular protein staining and quantified by the percentage of cells expressing each protein (based on histogram plots). **(D)** Representative morphology of BMDM on tissue culture plates, cultured in standard cRPMI media or stimulated with 100 ng/mL recombinant MCSF. **(E)** mRNA expression (above) of Fizz1 and Arg1 after BMDM stimulation with IL-4, with or without latrunculin A treatment and with or without MCSF stimulation. Quantification (below) of the relative effect of MCSF on gene expression without latrunculin A treatment (cytoskeleton intact) and with latrunculin A treatment, which inhibits actin polymerization.

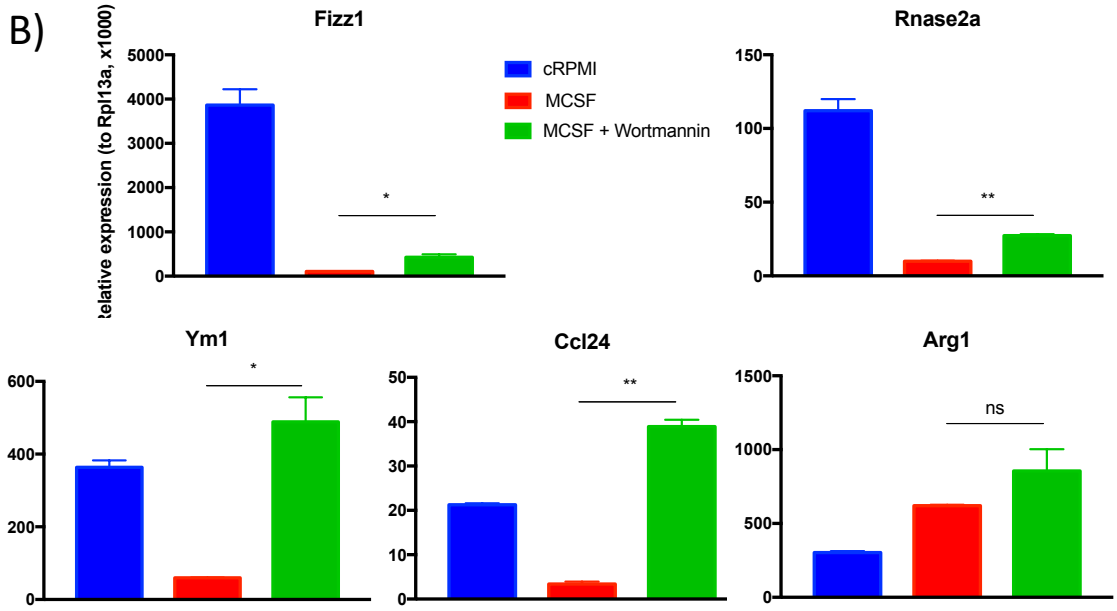
Next, we dissected what signaling pathways are responsible for the effects of MCSF on the macrophage cytoskeleton and downstream gene expression program. The fact that MCSF induces actin remodeling in macrophages is well established in the literature, and these effects are thought to be initiated by activation of PI3K by the MCSF receptor CSF1R.[116–118] When we treated macrophages with MCSF-containing media and blocked PI3K signaling using the inhibitor wortmannin, both the cytoskeletal effects (**Figure 4.2a**) and the downstream gene expression effects (**Figure 4.2b**) of MCSF were attenuated, whereas there was no effect on the expression of Arg1, which we have found to be cytoskeleton-independent and is presumably regulated by MCSF by a distinct signaling pathway.

One of the effects of the PI3K signaling pathway is to phosphorylate and thereby inhibit the kinases GSK3α and GSK3β. When we inhibited these kinases pharmacologically using the inhibitor SB216763, this recapitulated the morphologic effects of MCSF (**Figure 4.2c**) and inhibited Fizz1 without affecting Arg1 expression (**Figure 4.2d**). These data suggest that MCSF signaling through a PI3K-GSK3 pathway may be responsible for the cytoskeletal remodeling and the cytoskeleton-dependent gene expression effects of MCSF.

A)


cRPMI
+ L929 (MCSF)
+ L929 (MCSF) + Wortmannin

B)


Fizz1
Rnase2a
Ym1
Ccl24
Arg1
cRPMI
MCSF
MCSF + Wortmannin
elative expression (to Rpl13a, x1000)
5000
4000
3000
2000
1000
0
150
100
50
600
400
200
40
30
20
10
1500
1000
500
*
**
ns

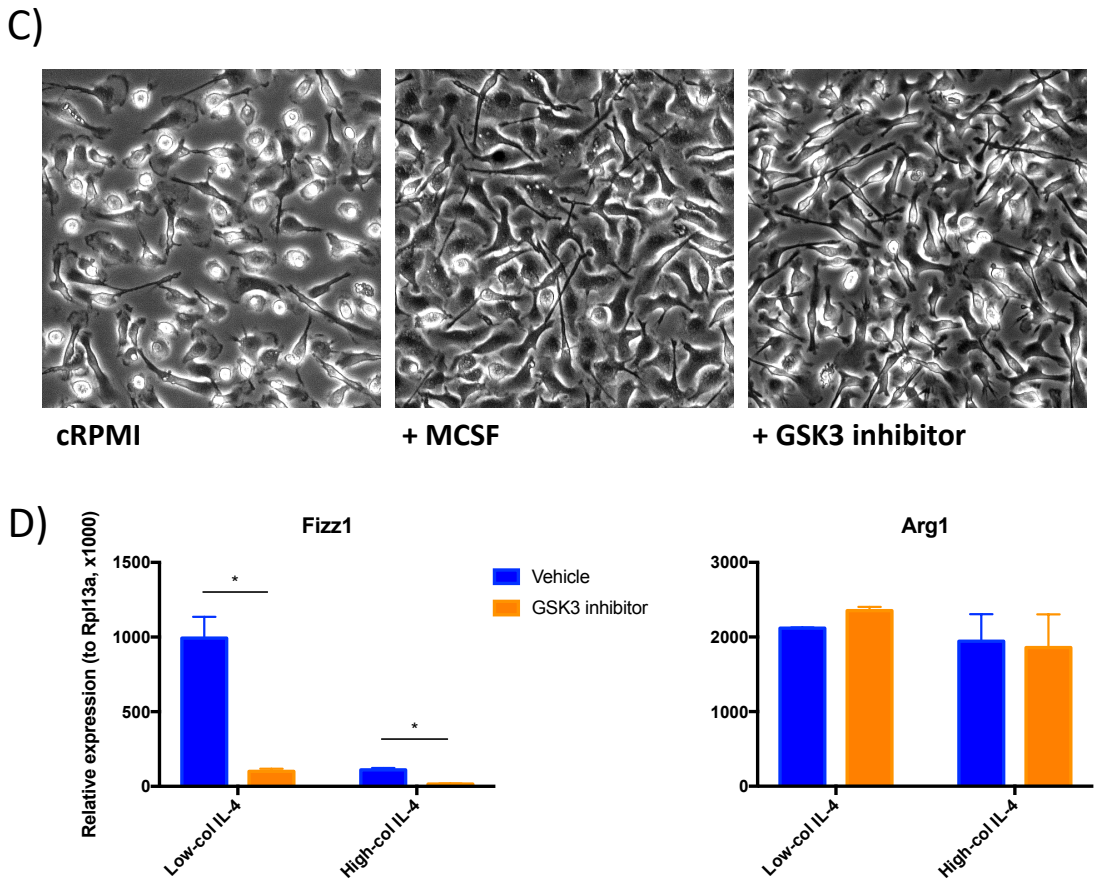


Figure 4.2. MCSF signals through a PI3K-GSK3 pathway to regulate cytoskeletal dynamics and gene expression

(A) Characteristic morphology of BMDM cultured on tissue culture plates in complete RPMI media (cRPMI), with 30% conditioned media from MCSF-producing L929 cells (+ L929), or with 30% L929-conditioned media and the PI3K inhibitor wortmannin (5 uM) (+ L929 + wortmannin). **(B)** mRNA expression in BMDM cultured in cRPMI media without stimulation, treated with 100 ng/mL MCSF, or treated with 100 ng/mL MCSF and 5 uM wortmannin. **(C)** Characteristic morphology of BMDM on tissue culture plates in cRPMI media, treated with 100 ng/mL MCSF, or treated with the GSK3 inhibitor SB216763 (10 uM). **(D)** mRNA expression in BMDM treated with vehicle control (0.05% DMSO) or the GSK3 inhibitor SB216763 (10 uM).

The actin cytoskeleton controls chromatin availability and C/EBPβ binding at specific genomic loci

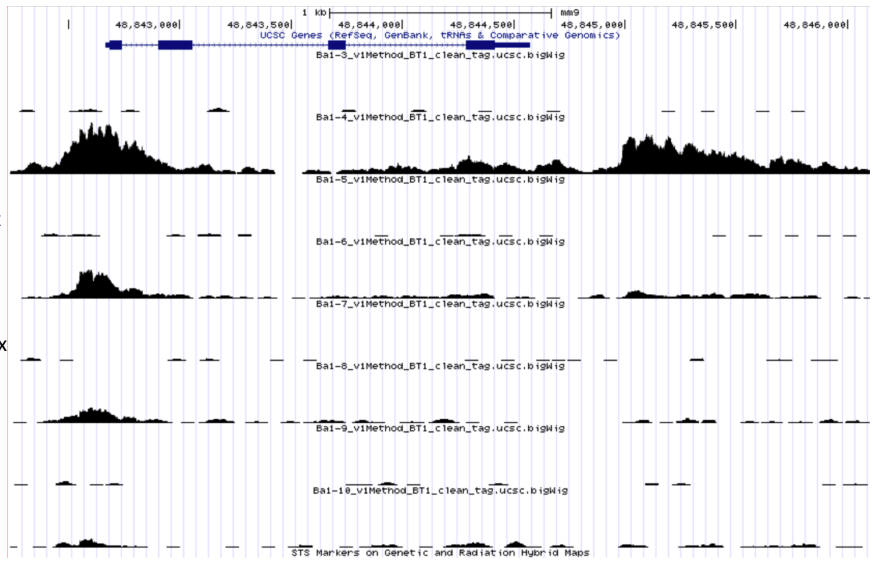
Having determined that ECM mechanosensing and MCSF signaling converge on actin remodeling in macrophages in order to regulate a specific gene expression program, we next asked how these changes in cytoskeletal dynamics are translated to control of gene expression. To investigate whether actin remodeling in macrophages results in changes in chromatin accessibility, we performed ATACseq in BMDM treated with and without IL-4 and with or without latrunculin A, SB216763 (GSK3 inhibitor), and MCSF, all of which control macrophage cytoskeletal dynamics and suppress expression of Fizz1. We found that IL-4 induced chromatin opening at the Fizz1 promoter (immediately upstream of the gene, containing the functional STAT6 binding site)[119] and that this chromatin opening was suppressed by latrunculin A, Gsk3 inhibition, and MCSF treatment (**Figure 4.3a**). In contrast, the Arg1 locus did not show marked changes in chromatin accessibility, including at the enhancer where STAT6 binding is required for induction (**Figure 4.3b**).[120,121] These data suggest that actin remodeling controls chromatin accessibility, in order to regulate gene expression.

To gain insight into the transcription factors that may be involved, we took a global view of the ATACseq data and asked which transcription factor motifs were enriched in regions of chromatin that were made significantly less accessible by the cytoskeletal perturbations (corresponding to the suppressed expression of mechanosensitive genes like Fizz1). In regions of chromatin suppressed by latrunculin A, SB216763, or MCSF, C/EBP binding sites were among the most enriched transcription factor binding motifs, in the setting of IL-4 (**Figure 4.3c**) and similarly without IL-4 treatment. This pattern was particularly interesting because Fizz1 is

known to be regulated by C/EBPβ, which binds the Fizz1 promoter adjacent to STAT6.[119] Several genes in the mechanosensitive gene expression program also have consensus C/EBP binding sites in their promoters, including Ear2 and Rnase2a. However, Arg1 is also known to be regulated by C/EBPβ, which binds the Arg1 enhancer adjacent to STAT6, similar to its regulation of Fizz1. If C/EBPβ is involved in regulating the transcription of Fizz1 and other mechanosensitive genes downstream of cytoskeletal control, it was not immediately clear how this could result in the pattern that we have consistently observed, in which regulation of Fizz1 is decoupled from that of Arg1.

To test whether C/EBPβ activity is regulated by the cytoskeleton, we performed chromatin immunoprecipitation (ChIP) for C/EBPβ without IL-4 stimulation, with IL-4 stimulation, with IL-4 plus L929-conditioned (MCSF-rich) media, and with IL-4 plus latrunculin A. We found that IL-4 stimulation potently induced C/EBPβ binding to the Fizz1 promoter and that additional treatment with L929 or latrunculin A completely abrogated that binding. In contrast, C/EBPβ binding to the Arg1 enhancer was present at baseline, increased with IL-4 stimulation, and was essentially unaffected by the cytoskeletal perturbations (**Figure 4.3d**). These patterns of C/EBPβ transcription factor activity closely mirrored the gene expression patterns of Fizz1 and Arg1, suggesting that regulation of C/EBPβ is likely to be a key biochemical mechanism by which changes in cytoskeletal dynamics are translated to control of gene expression.

A) **Fizz1**



Control Untx

Control IL-4

Latrunculin A Untx

Latrunculin A IL-4

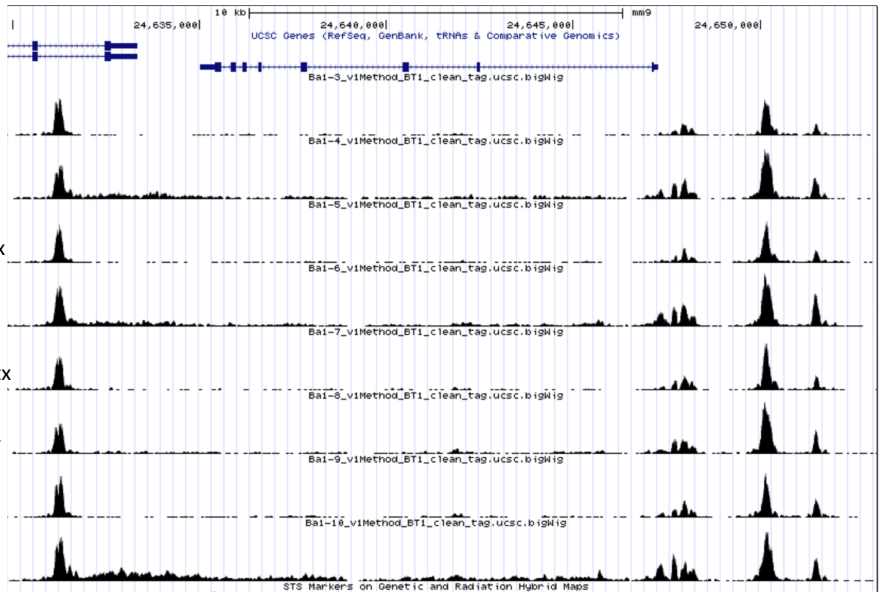
Gsk3 inhibitor Untx

Gsk3 inhibitor IL-4

MCSF Untx

MCSF IL-4

B) **Arg1**



Control Untx

Control IL-4

Latrunculin A Untx

Latrunculin A IL-4

Gsk3 inhibitor Untx

Gsk3 inhibitor IL-4

MCSF Untx

MCSF IL-4

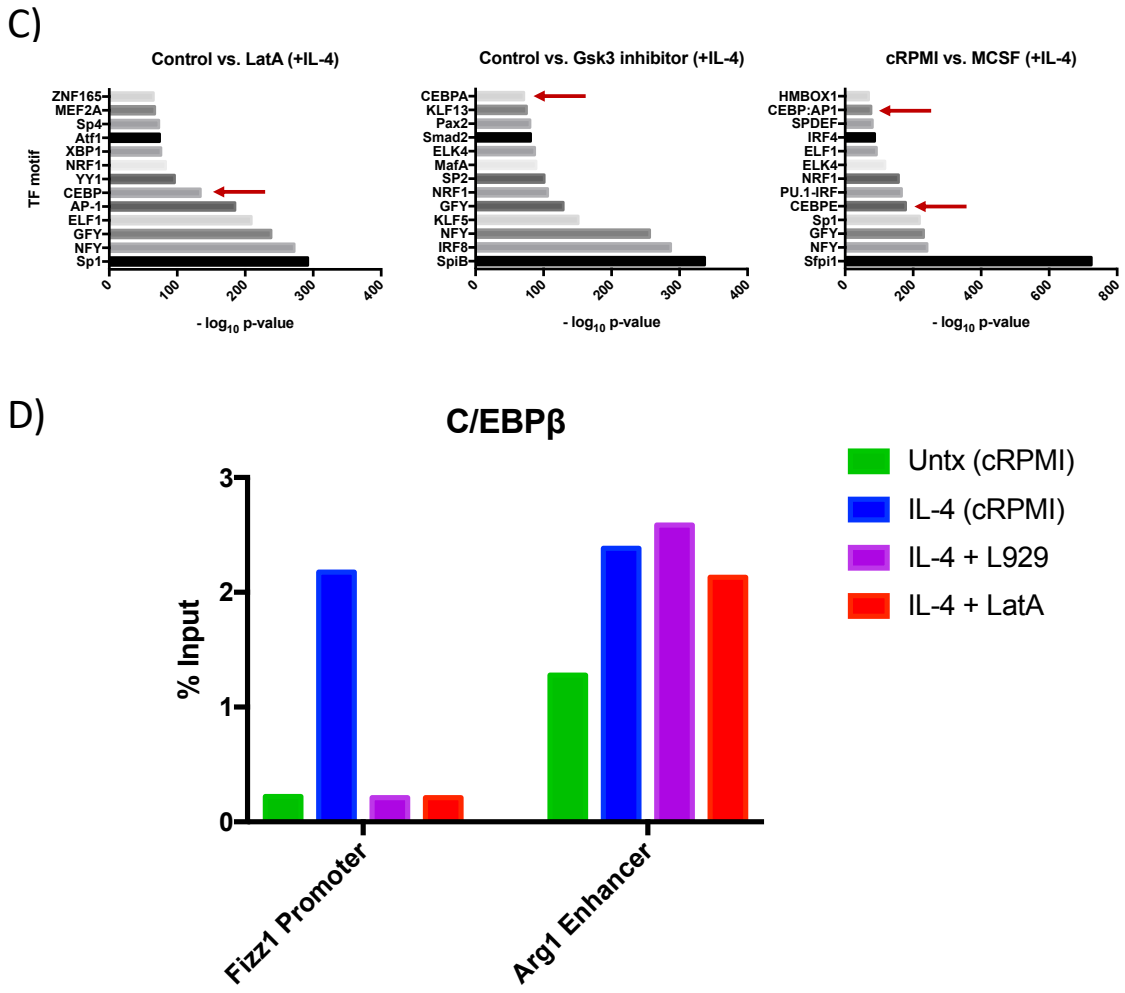


Figure 4.3. Cytoskeletal dynamics control chromatin availability and C/EBPβ activity in a targeted fashion

(A-B) Tracks representing the chromatin state of the genomic regions surrounding the Fizz1 (A) and Arg1 (B) genes, based on ATACseq analysis of BMDM treated with or without IL-4 and with vehicle control (0.025% DMSO), latrunculin A (1 uM), SB216763 (GSK3 inhibitor, 5 uM), or recombinant MCSF (100 ng/mL). **(C)** Transcription factors motifs (and corresponding p-values) that are most enriched in regions of open chromatin in IL-4 stimulated BMDM cultured under control conditions compared to BMDM treated with latrunculin A, SB216763 (GSK3 inhibitor), or MCSF. **(D)** Binding of the transcription factor C/EBPβ to the Fizz1 promoter and Arg1 enhancer regions of DNA in BMDM cultured in complete RPMI (cRPMI) media, treated with IL-4, treated with IL-4 and L929-conditioned media, or treated with IL-4 and latrunculin A (LatA), based on chromatin immunoprecipitation.

Discussion

In this chapter, we found that stimulation of macrophages with the growth factor MCSF regulates the same gene expression program as mechanosensing of the ECM. We found that MCSF induces similar changes in the cytoskeleton as those observed in response to high-stiffness ECM and that these changes are required for MCSF control of the mechanosensitive gene expression program. We identified a PI3K-GSK3 mediated signaling pathway that is required for both the cytoskeletal and downstream gene expression effects of MCSF. Finally, we interrogated the mechanisms by which actin dynamics may be translated to changes in gene expression. We determined that alteration of the actin cytoskeleton controls chromatin accessibility and binding of the transcription factor C/EBPβ at the genomic locus for regulation of Fizz1 but not Arg1, which could explain why a specific module of the IL-4 response is regulated by mechanosensing and growth factor signaling, while other elements of the IL-4 response are unaffected.

Among the most interesting implications of the findings presented in this chapter is that MCSF must remodel the actin cytoskeleton in order to regulate some of its genetic targets. In the case of mechanosensing, it is somewhat intuitive that extracellular mechanical information (contained in the ECM) would be translated to intracellular mechanical information (contained in the cytoskeleton), which would ultimately be translated to a biochemical signal to regulate gene expression. In the case of growth factor signaling, however, it is not intuitive why the biological system would be structured in this way: Extracellular biochemical information (in the form of MCSF) is converted to intracellular mechanical information (in the effects on the cytoskeleton), which ultimately is translated back to biochemical information to control gene expression. The

intermediate mechanical step seems to be an unnecessary complication in the transmission of biochemical information. Yet, this model appears elsewhere in biology. The best example is the story of serum response factor, mentioned in Chapter 1. It was known that fetal calf serum activates a transcriptional program in fibroblasts (and smooth, skeletal, and cardiac muscle cells) controlled by the transcription factor serum response factor (SRF), but the link between the growth factors present in the serum and the activation of SRF in the nucleus was unknown. The key to this puzzle was that treatment of fibroblasts with serum also causes morphologic changes in the cells, indicating remodeling of the cytoskeleton. The Treisman group discovered initially that these changes in actin dynamics are required for SRF activation of many of its transcriptional targets.[81] They later determined the mechanistic basis by which cytoskeletal changes are translated to control of gene expression: The transcriptional co-factor MRTF is sequestered in the cytoplasm by G-actin monomers and released to the nucleus when these monomers polymerize into F-actin.[82] A greater degree of complexity in these regulatory mechanisms, including a role for nuclear actin, is now appreciated,[80] but the conceptual implications are the same: The growth factors present in serum signal not through a linear biochemical mechanism, but rather by remodeling the cell's cytoskeleton, the state of which regulates the biochemical activity of a transcription factor. This is highly reminiscent of our discovery presented in this chapter, that a subset of MCSF's gene expression effects are mediated through its remodeling of the macrophage cytoskeleton, which is likely translated to transcriptional regulation in part through the activity of the transcription factor C/EBPβ.

While the advantages of this signaling architecture are not immediately obvious, we can speculate: One possibility is that the cytoskeleton acts as a critical signal integrator. I will

explore this theme—that cellular morphology may be a key indicator and regulator of the cellular state—more in the following chapter. For now, I will note that MCSF acting through a cytoskeleton-dependent mechanism allows it to converge on the same transcriptional program as that regulated by ECM stiffness. Thus, distinct types of information are effectively integrated in their effects on the cytoskeleton. The same is true for the MRTF-SRF mechanism in fibroblasts: ECM stiffness, through its effects on the fibroblast cytoskeleton, converges on the same gene expression program as serum stimulation.[45,122]

However, the cytoskeleton-dependent targets of MCSF signaling, like Fizz1, are separable from other targets of MCSF regulation, such as Arg1, which are likely regulated by a more traditional biochemical signaling pathway and are, accordingly, also unaffected by ECM stiffness. The separation between these modules may have to do with the functional roles of the genetic targets. This suggests a second, related reason that cells may use a cytoskeleton-dependent mechanism of transcriptional regulation: target genes may be involved in cellular processes that are consistently associated with morphologic change, such as migration, adhesion, and interaction with other cells.

A third potential rationale for biochemical signals to remodel the cytoskeleton in order to control gene expression is that these cytoskeletal dynamics allow for a distinct mechanism of transcriptional regulation. For instance, the actin cytoskeleton may exert tension on the nuclear membrane, which could mechanically alter the availability of local chromatin. In order to test a version of this idea, we performed experiments with BMDM deficient in Sun2, a member of the LINC complex that connects the nuclear membrane to the actin cytoskeleton.[123,124] Interestingly,

we did see a partial attenuation of the effects of MCSF on Fizz1 and especially Rnase2a expression, but we did not further pursue this hypothesis because Sun2 deficiency had a negligible effect on expression of these genes in response to ECM mechanosensing. A related idea is that nuclear actin may play a key role in chromatin remodeling and transcriptional regulation.[125–127] We have taken initial steps to collaborate with Dr. Shangqin Guo's group to test a version of this hypothesis by expressing actin with a nuclear localization signal (NLS) (or actin with a NLS that is constitutively in the G-actin or F-actin form) and testing whether this alters regulation of the mechanosensitive gene expression program, chromatin availability, and activity of C/EBPβ. In support of this general model, in which the cytoskeleton is involved in chromatin remodeling in macrophages, we observed a global increase in chromatin availability by ATACseq when actin polymerization was inhibited with latrunculin A.

In this chapter, we determined that binding of the transcription factor C/EBPβ to the regulatory region of Fizz1 but not Arg1 is regulated by the actin cytoskeleton. I originally identified C/EBP family members as interesting candidates because of a study published by Stutz and colleagues in 2003 identifying a C/EBP binding site, adjacent to the STAT6 binding site in the Fizz1 promoter, that is required for full induction of Fizz1 by IL-4 or IL-13.[119] There is also some precedent for C/EBP family members, and C/EBPβ in particular, to act as mechanosensitive transcription factors in other contexts, for instance in osteoblasts in response to shear stress and in aortic smooth muscle cells in response to cyclic stretch.[128,129] C/EBP binding motifs are also present in the promoters of several of the mechanosensitive genes in macrophages. The fact that Arg1 expression, which we find to be regulated independently of Fizz1, was also known to be regulated by C/EBPβ did not initially fit with this hypothesis, however. This puzzle was

satisfyingly resolved by the results of the ChIP experiment, which showed that C/EBPβ indeed binds both the Fizz1 promoter and the Arg1 enhancer, but its binding at the Fizz1 promoter is dramatically regulated by the actin cytoskeleton, which is not the case at the Arg1 enhancer. Thus, what distinguishes regulation of Fizz1 from that of Arg1 may not be the transcription factor that is responsible, but rather an upstream mechanism that controls that transcription factor's activity in a site-specific manner.

What is responsible, then, for the differential control of C/EBPβ activity at the Fizz1 compared to the Arg1 locus? Our data show that the actin cytoskeleton regulates C/EBPβ binding to the Fizz1 promoter, but it is not clear whether this effect is direct or indirect or what type of mechanism is involved. One possibility is that actin dynamics regulate the availability of chromatin for transcription factor binding, perhaps through mechanical effects on the nucleus or through the effects of nuclear actin, as discussed above. Our ATACseq data indicate that chromatin openness at the Fizz1 (but not the Arg1) locus is regulated by actin dynamics, providing general support for a model in which cytoskeletal dynamics directly control chromatin remodeling, which in turn opens up Fizz1 and other mechanosensitive loci for C/EBPβ and other transcription factor binding. However, these changes in chromatin openness could also be a secondary effect of C/EBPβ binding, which could remodel the local chromatin landscape to allow for active transcription.

It is entirely possible that the cytoskeleton regulates C/EBPβ activity directly, by a mechanism that remains to be defined. We investigated the possibility that C/EBPβ localization is regulated by actin dynamics, but we did not observe a clear signal of nuclear translocation in response to

MCSF or latrunculin A, by immunofluorescence or cell fractionation. Under all conditions, C/EBPβ was predominantly nuclear. A more likely scenario is that actin dynamics regulate C/EBPβ activity within the nucleus.

Another alternative is that GSK3 acts as the direct regulator of C/EBPβ activity. We have observed that GSK3 inhibition causes cytoskeletal remodeling, and we have suggested that these cytoskeletal changes mediate the effect of this pathway on gene expression (in part because of data indicating that the full effect of GSK3 inhibition requires an intact cytoskeleton). It is also possible, however, that MCSF- and ECM-induced cytoskeletal dynamics instead control GSK3 activity. I initially became interested in GSK3β because of evidence in the literature that it can act on C/EBPβ to control its DNA-binding activity. When 3T3-L1 preadipocytes are induced to differentiate into adipocytes, GSK3β undergoes a delayed translocation from the cytoplasm to the nucleus, where it phosphorylates C/EBPβ to activate its DNA-binding activity, allowing it to drive the adipocyte differentiation program.[130,131] Intriguingly, expression of Fizz1 is also induced in adipocytes upon differentiation. Furthermore, GSK3β only phosphorylates C/EBPβ once it has been "primed" by phosphorylation at another site. That initial phosphorylation event, which is performed by MAPK, also occurs in the late stages of monocyte differentiation to macrophages and appears to be critical for macrophage identity.[132] MCSF is the growth factor that drives monocyte differentiation into macrophages, and it activates MAPK, so it is likely that MCSF (perhaps cytoskeleton-independent) signaling is responsible for the initial phosphorylation of C/EBPβ by MAPK, which induces C/EBPβ activation of the genes that are responsible for macrophage identity, as well as its basal binding activity to the Arg1 locus, which we observe in our ChIP data. In contrast, DNA binding to other targets, such as Fizz1, may

require the secondary phosphorylation event by Gsk3β. Unfortunately, we were not able test this hypothesis directly because, to our knowledge, there are no published antibodies against the GSK3β-mediated phosphorylation site on C/EBPβ, and we did not have the available tools, such as mass spectrometry and mutated versions of C/EBPβ, to further dissect this biology. We were not able to detect clear evidence that cytoskeletal perturbations control either GSK3β translocation from the cytoplasm to the nucleus or inhibition of GSK3β through phosphorylation, though these events are not strictly necessary for this model. Thus, while our experimental evidence to date best supports a model in which MCSF-induced activation of GSK3β acts upstream of cytoskeletal remodeling, there is intriguing circumstantial evidence from the literature that suggests an alternative model, in which cytoskeletal remodeling controls GSK3β activity, which in turn phosphorylates C/EBPβ and controls site-specific DNA-binding to targets in the mechanosensitive gene expression program.

Finally, I must note that the data we have presented shows cytoskeletal regulation of C/EBPβ binding to the Fizz1 promoter, independent of its binding to the Arg1 enhancer, but we do not establish that this event is necessary for regulation of Fizz1 gene expression. Our efforts to test this requirement by developmental knockout and transient suppression of C/EBPβ have thus far yielded ambiguous results. It is notable that, to our knowledge, a requirement for C/EBPβ for specific gene induction has not been demonstrated in other contexts, including in its well-established role in the expression of IL-12 in macrophages in response to LPS. This observation has been attributed to a high degree of redundancy or compensation by other C/EBP family members, which may limit our ability to formally demonstrate the requirement of C/EBPβ for control of mechanosensitive gene expression.[133] Alternatively, C/EBPβ binding may not be the

driver of gene expression, but rather an indication of other changes, such as chromatin opening in response to actin dynamics.

Chapter 5: Conclusions, implications, and future directions

Conclusions

In the studies presented in this dissertation, we set out to understand whether, and if so how, macrophages sense the extracellular matrix in order to regulate tissue homeostasis and tissue repair. We established that macrophages sense changes in the mechanical properties of the extracellular matrix and that ECM mechanosensing regulates a specific subset of the macrophage gene expression program involved in tissue repair. We then determined that macrophages sense ECM mechanics in a novel, integrin-independent fashion that is mediated through cytoskeletal responses to the mechanical environment. We learned that macrophage growth factor signaling converges on these same cytoskeletal dynamics to regulate the same gene expression program. Finally, we discovered that these changes in the actin cytoskeleton are translated to changes in chromatin accessibility and site-specific binding of the transcription factor C/EBPβ to regulatory elements of target genes (**Figure 5.1**).

These studies have revealed several novel elements of macrophage biology and its interaction with the tissue environment. They also have broader conceptual implications and raise new questions that I hope will spark future fruitful studies by other investigators. In this chapter, I discuss these implications and promising areas of future investigation.

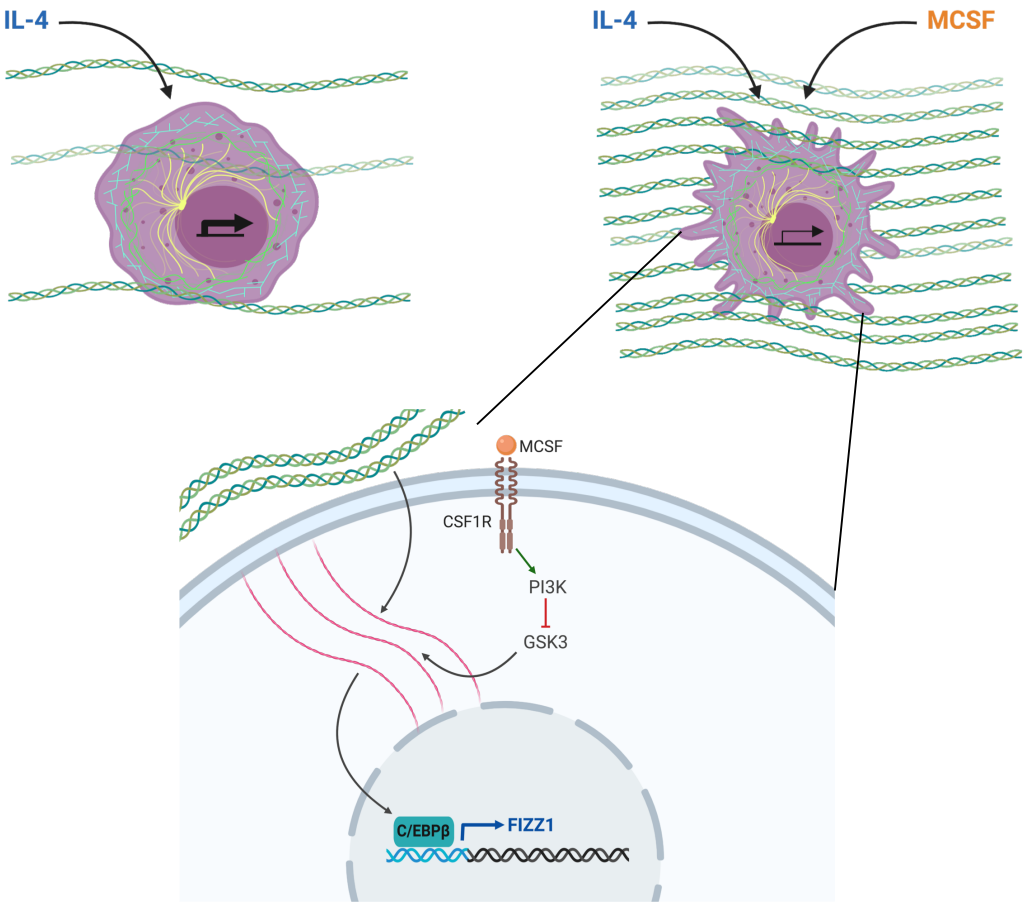


Figure 5.1. Model of cytoskeletal integration of ECM mechanics and MCSF signaling in macrophages

Implications and future directions

Identity and tissue specificity of macrophages

During the last decade, one of the major areas of interest and progress in the field of macrophage biology has been how tissue-resident macrophages acquire their tissue-specific identities.[57,63,134–138] These identities arise from a combination of ontogeny (the developmental origins of the macrophage in the yolk sac, fetal liver, or bone marrow), together with specific signals that macrophages receive within the tissue. Lavin and colleagues demonstrated that the chromatin landscape controlling tissue-specific macrophage gene expression programs is predominantly dictated by the local tissue environment and could be induced in donor bone marrow-derived cells or even reprogrammed in tissue-resident macrophages transplanted from one tissue into another tissue.[63] Most of the local signals involved in this specification of tissue-resident macrophage identity remain unidentified, and to date, those that have been identified are all soluble signals, such as retinoic acid in the specification of peritoneal macrophages and heme in the specification of red pulp macrophages in the spleen.[135,139]

However, the tissue architecture, ECM composition, and mechanical properties of different organs also vary widely, and we can predict that these features of the ECM might provide critical information about the local environment that may guide macrophages' tissue-specific identities. In this study, we have established that macrophage mechanosensing of the ECM can control chromatin availability and gene expression programs. Extending this principal to the question of macrophage tissue specification would be a natural next step. It would also be an extension of the work by Engler and colleagues showing that mesenchymal stem cell differentiation can be specified by mimicking the mechanical stiffness of different tissues.[11]

We can imagine that the mechanical properties of tissues may not only provide information about the specific type of tissue that the macrophage is entering, but may also play an essential role in driving the differentiation step from monocytes to macrophages in general. Monocytes are produced in the bone marrow and circulate in the blood. They become macrophages when they enter a tissue, which coincides with a fundamental change in their mechanical environment, from fluid (with flow) in the bloodstream, across an endothelial barrier (which requires integrin-mediated attachment),[103] into a three-dimensional tissue with extracellular matrix surrounding the cell. I hypothesize that the cytoskeletal changes that happen in this process may provide key signals to induce macrophage differentiation. These signals from the ECM may converge with growth factor signaling, similar to what we have found in the present studies. MCSF drives macrophage differentiation, and we have established here that a subset of its effects on gene expression are mediated through its remodeling of the cytoskeleton. Intriguingly, macrophage differentiation can be achieved *in vitro* from monocytes without MCSF, and these methods, such as lysophosphatidic acid (LPA) and phorbol 12-mystate 13-acetate (PMA), also induce cytoskeletal remodeling in macrophages and may require C/EBPβ.[140,141] C/EBPβ has been identified as a pioneer transcription factor, working together with PU.1 to shape the chromatin landscape to establish and maintain macrophage identity.[132,142] These hypotheses are experimentally tractable and could begin with differentiating macrophages from either bone marrow precursors or monocytes on plastic dishes (as usual) compared to collagen gels or polyacrylamide gels of different stiffnesses and comparing the effects on macrophage transcriptional and chromatin landscapes by RNAseq, ATACseq, and ChIPseq.

Tissue repair and fibrosis

This study began with the question of why tissue repair fails to turn off in fibrotic diseases. We ultimately focused on the fundamental question of how macrophages sense the tissue environment, rather than the specific pathologic context of fibrosis, but our results reveal biological mechanisms that may have important implications for the biology of fibrosis.

The gene that showed the most dramatic and consistent regulation by the ECM is Fizz1. As discussed in Chapter 2, macrophage-derived, IL-4-induced Fizz1 has been shown to be critical for tissue repair in the skin, due to its effect on fibroblast collagen deposition.[28] Fizz1 likely plays a similar role in the lung, where it is also potently induced by IL-4 upon tissue injury and required for effective ECM deposition.[66–68] We confirmed that FIZZ1-conditioned media stimulates collagen and αSMA expression in fibroblasts, suggesting the induction of a myofibroblast-like phenotype, cells that produce and contract ECM and drive tissue repair and fibrosis. These data suggest that macrophages may respond to increasing ECM stiffness by suppressing further ECM deposition and perhaps inducing a pro-resolution program. (Interestingly, we found that the matrix metalloprotease Mmp12 was generally regulated in an inverse fashion to Fizz1 by tissue stiffness and cytoskeletal perturbations, though there were some exceptions to this pattern.) This would position macrophages to exert negative feedback on the repair process and to prevent unrestrained ECM deposition that would otherwise lead to fibrosis. It could put a check on the positive feedback loop that has been described in fibroblasts, wherein increasing ECM stiffness induces further ECM production and matrix stiffening.

These considerations return us to the key question of whether there is a common vulnerability to the development of fibrotic diseases. If fibroblasts, left to their own devices, would perpetuate a positive feedback loop of ECM accumulation, perhaps fibrosis arises when the negative feedback provided by macrophages or other cells fails. Regulation of Fizz1 may be one example of a broader repertoire of mechanisms that have not yet been identified. Indeed, studies in the lung and liver have shown that macrophages are required for effective resolution of tissue repair and have identified mechanisms of ECM remodeling that may contribute to this function.[26,27,47,51] Defects in these effector responses, or in macrophage sensing of the ECM to regulate these responses, could be central to the development of fibrosis.

One of the challenges, however, for establishing this and many other hypotheses regarding the pathogenesis of fibrotic diseases, is that we don't have the tools to identify these defects in the context of human disease, and most models of fibrotic disease, such as the intratracheal bleomycin model, are better understood as tissue repair models than fibrosis models. Because the mice are normal, they respond normally to the tissue injury caused by bleomycin. They have an acute inflammatory phase (roughly 0-7 days), then progress to a phase of dense ECM deposition (around 14-21 days), which is what is typically labeled "fibrosis", but then ultimately progress to a resolution phase (around 28-35 days) characterized by degradation and remodeling of the ECM. This type of model is very useful for studying the normal progression of tissue repair, and we used it in this capacity, but it does not tell us anything directly about why some individuals instead develop persistent ECM deposition and fibrotic disease.

In our studies, we found fortuitously that MCSF converges on the same gene expression program characterized by Fizz1. This finding raises the question of why MCSF should suppress a pro-fibrotic program in macrophages. MCSF in tissues can be produced by multiple cell types, but fibroblasts are a major source of MCSF and form a regulatory circuit with macrophages to control cell numbers within the tissue.[20] One possibility, then, is that the concentration of MCSF acts as a proxy for the number of fibroblasts in the local tissue environment. The accumulation of fibroblasts, like the accumulation of the extracellular matrix they produce, could serve as a signal to macrophages to exert negative feedback on the repair process. In support of this model, we found that co-culture of BMDM with increasing numbers of L929 fibroblasts effectively suppressed Fizz1 and induced Arg1, just like increasing concentrations of MCSF.

Mechanosensing

The finding that macrophages can sense the mechanical properties of the extracellular matrix was an important step in our understanding of macrophage biology, but not a particularly surprising one, given our knowledge that many other cell types are mechanosensitive. Recently, other groups working in parallel have published related findings, including that macrophages can sense cyclic stretch and spatial confinement and that both macrophages and T cells can sense extracellular matrix stiffness to regulate inflammatory responses.[143–146] The latter studies on ECM mechanosensing, however, propose that these immune cells use the same mode of mechanosensing—based on adhesion to the ECM and regulation of YAP translocation—as mesenchymal cells like fibroblasts. Perhaps the most provocative finding in our studies is that macrophages can employ an entirely different mode of ECM mechanosensing that is not based on integrin-based adhesions.

The discovery in 2008 that leukocytes can migrate in three-dimensional tissues without adhering to the ECM opened up a new way of thinking about cell migration.[103] It was made possible by studying migration within 3D model systems, in particular collagen gels, that resemble the tissue environment, because on two-dimensional surfaces leukocytes follow the same rules of cell migration as mesenchymal cells. As I discussed at length in Chapter 3, we believe that macrophage mechanosensing in 3D environments is similarly adhesion-independent and that, as a general rule, cell migration and mechanosensing are closely linked biological processes that employ common mechanisms. As in the discovery of integrin-independent migration, using a 3D model system was essential for our identification of this distinct mode of mechanosensing.

This discovery raises at least two fundamental questions for further exploration. First, what are the relevant mechanical properties that are sensed by macrophages in a three-dimensional context? On a 2D surface, we know that macrophages can sense changes in substrate stiffness alone. However, in a 3D context, manipulating the stiffness of the environment also meant changing the architecture of the extracellular matrix, such as its pore size. Macrophages may sense stiffness per se, or they may sense another parameter like physical confinement. As I discussed in Chapter 3, I predict that adhesion-independent mechanosensing operates in a manner that is essentially the opposite of adhesion-based mechanosensing. Instead of pulling on the extracellular matrix, I suspect that macrophages interpret their environment by pushing against it as they migrate. Instead of tension being the relevant force, I hypothesize that compression is the relevant type of force. This mode of mechanosensing is consistent with what we understand about the mechanobiology of adhesion-independent migration, it would not

require receptor-mediated interaction with the ECM, and it would allow cells to integrate properties of their environment like stiffness and confinement. It would also fit with a consistent theme of our studies, which is that, when it comes to the extracellular matrix and the cytoskeleton, macrophage biology follows a distinct set of rules from those defined in adhesive cell types like fibroblasts. The critical mechanical experiments to determine whether macrophages indeed sense compressive forces remain to be done, and I hope that they will be taken up by future investigators.

Second, if we extend the analogy with cell migration, we know that diverse types of leukocytes employ a common set of mechanisms to achieve rapid migration through tissues.[103] Thus, we can hypothesize that they might also employ common mechanisms of mechanosensing. I would predict that dendritic cells, B cells, T cells, and other leukocytes might similarly sense mechanical compression in a receptor-independent fashion mediated through cytoskeletal remodeling, in order to control their transcriptional programs and tissue-specific functions.

The cytoskeleton

Perhaps the most far-reaching implications of these studies involve the role of the cytoskeleton in cell biology. Throughout our experiments, our data consistently pointed us toward a surprisingly central role of the cytoskeleton in the biological processes we were studying. By observing cells microscopically across many dozens of experiments, I began to notice that the morphology of macrophages closely tracked with their gene expression profiles. This became even more clear when I began performing live cell microscopy and was able to watch their dynamic patterns of migration and extension and retraction of membrane protrusions. I realized that I could observe

the effects of a given stimulation or inhibitor on macrophage morphology and migration and reliably predict whether it was going to affect the gene expression program characterized by Fizz1 (which I would then assay, often in the same experiment, by qPCR). The effect of MCSF on both the cytoskeleton and gene expression was the most striking example of this pattern and helped drive my intuition that the cytoskeleton was at the crux of the biology we were investigating.

Our findings directly raise questions about the macrophage cytoskeletal dynamics involved in mechanosensing and growth factor signaling, and they suggest broader conceptual questions about the cytoskeleton as a cellular signaling hub. In our studies, we were not able to determine what exactly is measured about the actin cytoskeleton to relay information about the tissue environment. In the case of MRTF, the best-studied example of a transcription factor that reports on the state of the cytoskeleton (discussed in Chapters 1 and 4), G-actin binds to MRTF to sequester it in the cytoplasm, so the concentration of G-actin is thought to be the critical parameter of the cytoskeleton that is sensed. In our studies, we found that the MRTF inhibitor CCG-203971 dramatically increased macrophage membrane protrusions, but it potently suppressed not only the specific mechanosensitive gene expression program characterized by Fizz1, but also other IL-4 induced genes like Arg1. Interestingly, this inhibitor has not been shown to act on MRTF directly and may act instead through its effects on the cytoskeleton. It is also notable that, based on conversations with other investigators, it inhibits not only MRTF/SRF target genes, but also other unrelated genes in fibroblasts, suggesting that it may be rather non-specific, which could explain its effect on both Fizz1 and Arg1. More compelling evidence that our gene expression program is likely not MRTF-dependent came from immunofluorescence and

cell fractionation experiments that did not show a clear translocation of MRTF from the nucleus to the cytoplasm when cells were treated with latrunculin A (which causes almost all actin to be in the monomeric G-actin state). These data suggest that MRTF may follow different sets of rules in macrophages than mesenchymal cells, similar to other differences in cytoskeletal and mechanobiology that we have observed. Interestingly, however, one group has implicated MRTF/SRF in macrophage sensing of physical confinement.[144]

Whether or not MRTF plays a role in macrophage mechanosensing, it seems unlikely that a static parameter like the concentration of G-actin or F-actin/G-actin ratio is the relevant property of the cytoskeleton that controls the downstream gene expression program that we have identified. While most stimuli that suppressed Fizz1 also induced dendritic macrophage morphology, with long F-actin protrusions, latrunculin A was an interesting exception to this rule. It caused macrophages to become round, with very little F-actin (confirmed by immunofluorescence with phalloidin, which binds F-actin polymers). If the amount of F-actin or G-actin, or some other static feature of cell morphology, were the relevant parameter, then latrunculin A would be expected to cause the opposite effect on gene expression from stimuli like MCSF, blebbistatin, and GSK3 inhibition that induce dendritic morphology. Instead, we hypothesize that a dynamic feature of the actin cytoskeleton, such as the polymerization or depolymerization rate of actin filaments (or the force of actin retrograde flow involved in this process), is sensed. In low-collagen gels and other high-Fizz1 states, cells migrate quickly and form shorter protrusions that appear to turn over at a rapid rate. In low-Fizz1 states, like high-collagen gels or with MCSF or blebbistatin treatment, cells have decreased migration and form long membrane protrusions that turn over at a slower rate. Similarly, after treatment with latrunculin A, cell migration and actin

turnover are potently inhibited, and Fizz1 expression is dramatically suppressed. (Interestingly, with nocodazole treatment, which inhibits microtubule polymerization, macrophages migrate but fail to retain cellular integrity because the microtubule network regulates the actin cytoskeleton. As a result, the actin protrusions in different portions of the cell appear to go rogue, losing their central coordination and ultimately causing cell fragmentation. The reason for Fizz1 suppression, in this case, may have to do with an inability to relay the properties of actin dynamics in the cytoplasm to the nucleus, either because of cell fragmentation or because the microtubule network plays an essential role in that process, as well.) Altogether, our data suggest that a dynamic property of the actin cytoskeleton is measured by the cell in order to regulate downstream signaling, chromatin availability, or transcription factor activity. Our work has revealed a fascinating puzzle of macrophage cytoskeletal biology and its control of gene expression that I believe will yield many more important insights in the future.

While I have emphasized the differences between macrophage and fibroblast biology throughout this dissertation, it is also likely that what we have learned here about macrophage mechanosensing and cytoskeletal biology has lessons for the biology of non-hematopoietic cell types. For instance, while we generally think of fibroblasts as sensing ECM mechanics through integrins, which then signal downstream through mediators like focal adhesion kinase, this kind of linear signaling pathway may be the exception rather than the rule. In studies on YAP as a mechanosensor, the upstream sensors and signaling pathways leading to YAP activation are generally not identified. Integrins are likely required, but there is not a clear connection between them and YAP. A different way of thinking about this biology is that it is governed by the state of the cytoskeleton. Integrins are required for fibroblast adhesion to their environment, and

binding to and exerting tension on a stiff substrate causes reciprocal changes in the fibroblast cytoskeleton that are visible as increased cell spreading. I suspect that it is these changes in the cytoskeleton itself that are directly or indirectly sensed by YAP. There has recently been very interesting progress in this direction,[147] though as in macrophages, it remains to be determined what specific cytoskeletal parameters are monitored and how this is translated to YAP localization and activity.

Finally, our studies raise a deep conceptual question: Why does the cytoskeleton act as an integrator of diverse types of signals? In Chapter 4, I proposed several specific reasons why it may be advantageous for biochemical signals to act through effects on the cytoskeleton. Here, I will take a broader view that the cytoskeleton may act as a central hub of cell biology.

The cytoskeleton is one of the most ancient features of eukaryotic cells. It enables fundamental cellular activities such as cell movement and feeding in unicellular amoebae and, in multicellular organisms, coordinates similar functions of cell migration and phagocytosis, as well as everything from the uptake and release of cellular contents for cell-cell communication to cell proliferation to the cellular organization that allows for the creation of intact layers of epithelial and endothelial cells.[148] Much of cell biology has evolved around these core cellular functions and, since evolution builds upon what is already present, signaling pathways have likely been layered onto the existing cytoskeletal dynamics involved in those cellular processes. We can imagine that the cytoskeleton may have become a signaling hub over the course of evolution. I would also suggest that the state of the cytoskeleton offers a key indicator of the state of the cell—whether migrating or stationary, fixed in or detached from a sheet of cells, proliferating or

quiescent, as well as far more subtle changes in cell state. In order for the cell to perform almost any process, the cytoskeleton likely has to be in a compatible state. Thus, it makes sense that there should be close coordination between the dynamics of the cytoskeleton and the signaling that controls gene expression and other cellular functions. This role for the cytoskeleton is, I propose, dramatically under-appreciated at present, in large part because of the difficulty of studying the dynamics of the cytoskeleton and the very non-linear logic involved in the regulation of cytoskeletal biology. These features do not lend themselves to neat biological stories, but I would posit that there is an enormous amount of fundamental biology to be unlocked by understanding how the cytoskeleton communicates biological information and coordinates biological processes that have, up to now, appeared entirely independent from it.

To highlight this point, I will make two final observations. First, there has been a major trend in recent years toward the field of immunometabolism. Metabolism had until recently been an area of biochemistry that appeared to have little bearing on immunology. But it now appears to play a role in nearly every immunologic process. The explanation for this, I believe, is similar to what we have described about the role of the cytoskeleton in cell biology: Metabolic processes are so ancient and fundamental to cellular function—and are both a critical indicator of cell state and an essential element of most cellular processes—that they have become a hub for the regulation of a wide diversity of cellular functions. I would suggest that, if we begin to look, we may find that the cytoskeleton plays a similarly ubiquitous role, for very similar reasons.

Second, I would point out that cell morphology—an easily accessible, if crude, indicator of the state of the cytoskeleton—is reliably altered by signals that activate cells and induce

transcriptional changes and other cellular responses. IL-4 and LPS, for instance, each induce distinct, stereotypical morphologic changes in macrophages, the purpose of which has never been defined. In our experiments, TGFβ, Wnt3a, PDGFβ, and Oncostatin M treatment of fibroblasts all induced both distinct morphologic and distinct transcriptional responses. Turning to the clinical realm, we have appreciated for decades that cancer cells can be reliably identified histologically, in large part based on their morphology. This is a remarkable fact, given the diversity of cancer drivers, and suggests that the cytoskeleton must be in a particular state to permit oncogenic transformation or to facilitate cancer cell growth or survival. Thus, it seems to be a general rule that changes in cell state are accompanied by changes in the cytoskeleton that can be detected by cell morphology. Perhaps, in the coming years, we will begin to attend to and to understand the significance of this intimate relationship.

Chapter 6: Perspectives on homeostasis, inflammation, and tissue biology

A version of this chapter has been published as a review article:

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Abstract

There is a growing interest in understanding tissue organization, homeostasis, and inflammation. However, despite an abundance of data, the organizing principles of tissue biology remain poorly defined. Here, we present a perspective on tissue organization based on the relationships between cell types and the functions that they perform. We provide a formal definition of tissue homeostasis as a collection of circuits that regulate specific variables within the tissue environment, and we describe how the functional organization of tissues allows for the maintenance of both tissue and organismal homeostasis. This leads to a natural definition of inflammation as a response to deviations from homeostasis that cannot be reversed by homeostatic mechanisms alone. We describe how inflammatory signals act on the same cellular functions involved in normal tissue organization and homeostasis, in order to coordinate emergency responses to perturbations and ultimately return the system to a homeostatic state. Finally, we consider the hierarchy of homeostatic and inflammatory circuits and the implications for the development of inflammatory diseases.

Introduction

Homeostasis and inflammation are conventionally described as opposing states of biological systems, typically associated with health and disease, respectively. This understanding has deep historical roots that can be traced to a debate between Rudolf Virchow and Elie Metchnikoff, two founding figures in the study of inflammation. Virchow viewed inflammation as a pathological phenomenon, which gave rise to the perspective that prevails to this day. Metchnikoff, on the other hand, had the insight that the vascular changes responsible for the cardinal signs of inflammation were not a pathological accident of biology but rather were induced on purpose, in order to deliver phagocytes to the site of infection.[149] Moreover, Metchnikoff conceived of a spectrum of biological states, from homeostasis to physiological inflammation to—only at the extreme—pathological inflammation and immunity.[150] To refer to homeostasis, Metchnikoff used the term harmony-disharmony balance; the term homeostasis was not coined until decades later, in 1929, when Walter Cannon published his seminal paper defining homeostasis and its mechanisms.[151] However, Metchnikoff's insights, identifying a spectrum from homeostasis to inflammation, did not gain much traction. (Perhaps his disagreement on this issue with the undeniable authority of Virchow did not help.) One unfortunate consequence was that for the next century inflammation was studied primarily in the context of pathology, largely disconnected from physiology.

The concept of homeostasis plays a central role in our understanding of mammalian physiology. Many aspects of systemic homeostasis are now understood in great detail. The picture is much less clear when it comes to homeostasis at the tissue level, where a lack of formal definitions has led to ambiguity and obscured important biological mechanisms. This problem is amplified by

the largely descriptive knowledge of tissue organization. In this review, we discuss a functional perspective on tissue organization and its relationship to tissue homeostasis and inflammation. Based on that framework, we revisit Metchnikoff's idea of a homeostasis-inflammation spectrum and discuss the hierarchy of cellular, tissue, and organismal levels of homeostasis and inflammation.

Basic principles of tissue organization

At first glance, mammalian tissues appear very different from one another. The skin, lungs, liver, and bone have distinct gross anatomy, cellular composition, and organization. Yet, each of these tissues is organized according to the same principles. We can understand this fundamental organization by considering the primordial design of multicellular tissues. The earliest metazoan tissues, like those of our common ancestors with Ctenophora and Cnidaria, consisted of epithelial and mesenchymal cells. The layer of epithelial cells created a barrier that separated the internal environment of the organism from the external environment, in order to defend against external threats and maintain internal homeostasis. This was the primary function of these primordial tissues. The mesenchymal cells provided the tissue with structural integrity and organization, through the production of extracellular matrix (ECM) and soluble signals to the epithelial cells. These were supportive functions that allowed the epithelial cells to perform the primary barrier function.[3–5] This primordial epithelial-mesenchymal tissue unit illustrates an important design principle that is conserved in modern multicellular organisms: Cells within a given tissue fall into two functional categories, (*a*) primary cells, responsible for performing the primary function of the tissue, and (*b*) supportive cells, responsible for performing supportive functions, which facilitate the performance of the primary function.

As numerous specialized cell and tissue types appeared over the course of evolution, the basic tissue design composed of primary and supportive cell types was preserved and elaborated. Each tissue or organ is specialized to perform one or more essential functions for the organism. The skin, for instance, serves primarily as a barrier, the lung functions to exchange oxygen and carbon dioxide, the liver regulates systemic metabolism, and bone provides structural integrity and organization for the body. As in primordial tissues, each of these tissues contains a cell type dedicated to performing the primary function of the organ. These include specialized epithelial cells in the skin, lung, intestines, kidney, and liver; neurons in the brain; and cardiomyocytes in the heart. All of the other cells within the tissue are supportive cells, which serve to optimize the performance of the primary function by creating the appropriate conditions within the tissue. Examples of these supportive components include endothelial cells, pericytes, and smooth muscle cells, which form vasculature to deliver oxygen and nutrients to the primary cell; fibroblasts, which (as in primordial tissues) produce growth factors and ECM to position the primary cells and provide the necessary mechanical properties to facilitate the particular function of the organ; stem cells, which replenish the primary cell type; neurons innervating the tissue, which transmit critical information to and from other parts of the organism; and tissue resident macrophages, which sense and respond to changing conditions within the tissue to maintain an optimal environment (**Table 6.1**).[22] While these cell types play supportive roles in most organs, they can also serve the primary function in other, specialized organs. For instance, endothelial cells are the primary cell type in large blood vessels like the aorta, osteoblasts (a specialized type of fibroblast) are the primary cell type in bone and cartilage, hematopoietic stem cells are the primary cell type in the bone marrow, and neurons are the primary cell type in the central

nervous system. We can think of these organs as elaborations of the supportive functions that those cells normally serve. In these cases, the supportive functions (such as nutrient transport) are outsourced to whole organs (such as the aorta), in order to adequately supply multiple tissues or the organism as a whole.

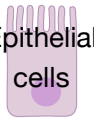
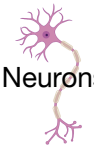
CELL TYPE	CORE FUNCTIONS	CHARACTERISTIC EXAMPLES
 Epithelial cells	Barrier function	Skin epithelial cells: External environment Intestinal epithelial cells: Intestinal contents
	Transport: Absorption	Small intestinal epithelial cells: Nutrients Type 1 alveolar epithelial cells: Gases Kidney epithelial cells: Electrolytes
	Transport: Secretion	Goblet cells: Mucus Hepatocytes: Plasma proteins Paneth cells: Antimicrobial peptides Type 2 alveolar epithelial cells: Surfactants
	Sensing	Enterochromaffin cells: Metabolites & noxious stimuli
 Stromal cells	ECM production	Osteoblasts: Bone matrix
	Growth factor production	Niche cells: Stem cell survival factors
 Endothelial cells	Barrier	Vascular endothelium: Fluids, plasma proteins, & solutes
	Transport	Pulmonary capillaries: Gases
 Smooth muscle cells	Contraction and Relaxation	Vascular smooth muscle: Blood flow Intestinal smooth muscle: Peristalsis
 Macrophages	Phagocytosis	All macrophages: Microbes & apoptotic cells Alveolar macrophages: Surfactants Microglia: Cellular debris & unnecessary neuronal structures Splenic red pulp macrophages: Red blood cells
	Sensing	Intestinal macrophages: Microbes Splenic red pulp macrophages: Heme
 Adipocytes	Lipid storage and release	White adipocytes
	Thermogenesis	Brown adipocytes
 Neurons	Sensing	Sensory neurons
	Computation	Interneurons
	Control of target tissues	Motor neurons
 Stem cells	Self-renewal and Differentiation	Hematopoietic stem cells Intestinal stem cells

Table 6.1. Cell types, their core functions, and characteristic examples within specific tissues

These examples highlight an important principle: The distinction between primary and supportive cell types does not describe intrinsic characteristics of cells but rather the relationship between cells (**Figure 6.1**). To illustrate this point, we can consider a blood vessel within an organ, such as the gut. In the blood vessel, endothelial cells serve the primary function, creating a barrier between the systemic circulation and the tissue and regulating the transport of specific contents. Smooth muscle cells and pericytes are supportive cells that optimize those functions. If we zoom out to the whole tissue, though, those same vascular endothelial cells are playing a supportive role, delivering oxygen to the intestinal epithelial cells, which serve the primary function of nutrient absorption. If we further zoom out to the organismal level, intestinal epithelial cells are playing a supportive role, supplying nutrients for the organism as a whole. We can see from this example that the terms primary and supportive are relational rather than absolute and also that the relationship of supportive to primary components exists along a hierarchical axis. Each biological component supports the functions of a higher-order unit (**Figure 6.1**). This hierarchy of functions is not unique to biological systems and can be seen, for instance, in the structure of large companies. Each department has supportive personnel that optimize the performance of that department, and the department in turn supports the overall function of the company. The departments are analogous to tissues, and the company is analogous to an organism. This hierarchical support structure allows for the multiscale organization of biological systems, beginning from subcellular units and building up to cells, tissues, organ systems, and organisms.

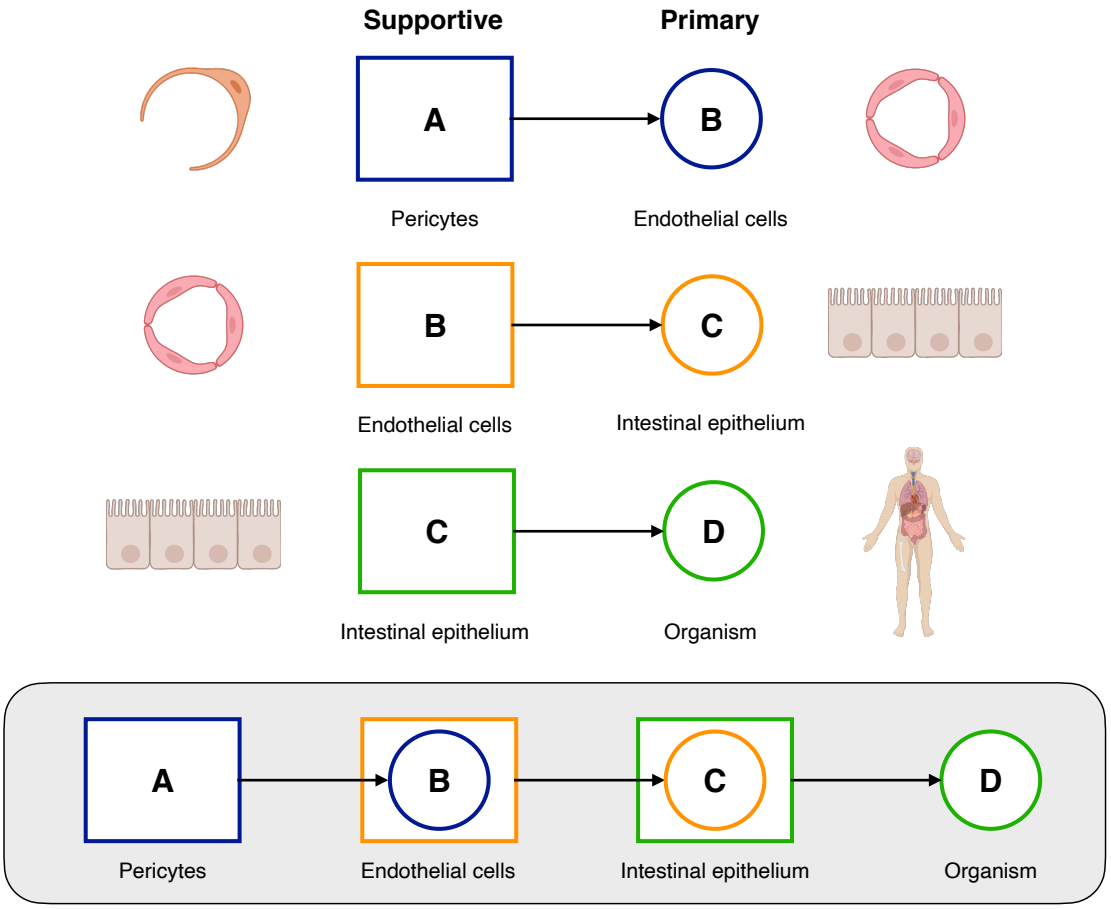


Figure 6.1. Hierarchy of supportive and primary cellular functions
Using the hierarchy of blood vessels to intestine to organism, the relationships between supportive and primary functions are illustrated. Within the blood vessel, pericytes (A) are supportive for the primary barrier and transport functions performed by endothelial cells (B). At the level of the intestine, endothelial cells play a supportive role for the primary function of nutrient absorption performed by intestinal epithelial cells (C). At the organismal level, the intestinal epithelium supports primary functions of the organism as a whole (D). Rectangles represent supportive functions, and circles represent primary functions. Colors denote primary to supportive relationships.

Core functionalities of cell types

Most tissues in complex metazoans are composed of multiple cell types, each specialized on one or more core functionalities (**Table 6.1**). For instance, the core functionalities of epithelial cells are barrier, transport (absorption and/or secretion), and environmental sensing, while the core functionalities of smooth muscle cells are contraction and relaxation. The diverse array of vertebrate cell types, with their distinct core functions, provides a biological tool kit for building tissues. Each tissue uses a unique complement of these same fundamental building blocks to achieve the particular function of that organ and to optimize its performance.

Comparing two barrier tissues, those of the lung and the gut, exemplifies how these same basic cellular units are utilized across different tissues, as well as the variations in how they can be deployed. The primary functions of both the lung and the gut are transport and barrier functions. However, they perform these functions in different contexts and for different purposes, which has driven specialization of the basic epithelial unit for each organ in several ways. First, both epithelia are specialized for transport, but of entirely different kinds of substances. Intestinal epithelial cells must absorb nutrients, for which they express specific enzymes and transporters.[152] Alveolar epithelial cells must exchange gases (oxygen and carbon dioxide), so they have a flattened morphology to minimize the distance for diffusion.[153] Second, both epithelia are adapted for a barrier function, separating the internal from the external environment and defending against pathogens. This function is supported by further specialized subsets of epithelial cells that have a dedicated secretory function: Goblet cells in both organs secrete mucus that lines the epithelial barrier and helps prevent the penetration of microbes. In the lung, type 2 alveolar epithelial cells solve the lung-specific problem of surface tension by secreting

surfactants, and in the gut, Paneth cells secrete antimicrobial peptides to deal with ubiquitous exposure to microbes.[154,155] Third, both epithelia have distinct, specialized sensory cells. For example, the intestinal epithelium contains enteroendocrine, enterochromaffin, and tuft cells that detect nutrients and noxious substances and produce hormones, neurotransmitters, and cytokines to coordinate appropriate responses to the luminal contents of the gut.[156,157]

Supportive cells are also deployed in each tissue to facilitate those unique primary functions and to help address the particular challenges faced by each organ. To help regulate the large microbial community in the intestines, there is an extensive and highly organized population of gut-resident immune cells.[155] In the lung, alveolar macrophages support the critical surfactant biology described above by sensing surfactant levels and appropriately clearing surfactant from the alveoli, balancing surfactant production by epithelial cells.[158] The fibroblasts in each tissue produce ECM that confers distinct mechanical properties, optimized for cycles of inspiration and expiration in the lung and distention and motility in the gut.[159–161] Endothelial cells supply oxygen and nutrients from blood to both tissues, but in the lung they also have the special function of absorbing oxygen from the air and off-loading carbon dioxide. To achieve this, the endothelial cells of pulmonary capillaries are closely apposed to the alveolar epithelial cells, sharing a single basement membrane to minimize the distance for gas diffusion.[162]

Both organs pair smooth muscle with endothelium (with the exception of capillaries and postcapillary venules) to regulate the diameter of blood vessels. However, this partnership follows tissue-specific rules. In the gut, like elsewhere in the body, hypoxia stimulates smooth muscle relaxation and thus vasodilation to increase blood flow and oxygen delivery to the tissue,

correcting the hypoxia.[163] In contrast, in the lung, hypoxia stimulates smooth muscle contraction and thus vasoconstriction to prevent blood flow to regions of the lung that are not being well-ventilated. The failure to match oxygenated air with blood flow in the lung is called ventilation/perfusion mismatch and is a major cause of hypoxemia.[164] Each organ also deploys smooth muscle in other contexts. The lung pairs smooth muscle with bronchial epithelial cells to regulate the caliber of the airways, similar to the role of smooth muscle in blood vessels. Smooth muscle in the gut uses the same core functionalities of contraction and relaxation to a very different end, generating coordinated waves of peristalsis that cause directional movement of the intestinal contents. Neurons in both organs, in turn, have the function of controlling smooth muscle contraction. They also relay local information, from sensory enteroendocrine cells in the gut, for instance, to other parts of the organism.[165,166] Finally, each tissue has stem cells that are located within the organ's basic units of organization, the villi in the intestine and the alveoli in the lungs, which allows them to regenerate regularly and in response to damage.[155,167]

Like the tissues that make up the lung and gut, each tissue in an organism can be described as an assembly of complementary core functions. We elaborate on some additional examples below, as we detail how these core functions are modified in the context of homeostasis and inflammation. For now, we can make two important generalizations based on the discussion above. First, there is intimate coordination between the primary and supportive components of tissues because the supportive functions exist to facilitate and optimize the primary functions. As a consequence, certain supportive and primary cell types are typically paired together. Cell types specialized in transport (like epithelium or endothelium) are typically paired with smooth muscle to control the flow of luminal contents, as well as neurons to control the contraction of the smooth muscle.

Pericytes also regulate endothelial transport, in some cases together with other stromal cells, such as astrocytes in the brain to maintain the blood-brain barrier and podocytes in the kidney to control the filtration of blood into urine.[168–170] Epithelial cells that serve a barrier function are paired with immune cells to prevent infection. Primary cells specialized in sensing are often paired with afferent neurons to integrate and communicate that information to other parts of the organism. Second, the role of the supportive cells within a tissue is often to maintain the internal environment of the tissue—such as the composition of ECM and the concentrations of oxygen, nutrients, and waste products—in an appropriate and stable state. In a similar fashion, primary cells of each organ regulate internal conditions of the whole organism, like blood pressure and the concentrations of ions and metabolites. Thus, supportive cells are responsible for maintaining homeostasis on the tissue level, while primary cells are responsible for maintaining homeostasis on the organismal level.

Tissue homeostasis

Tissue homeostasis is a term that is often used to describe a normal, steady-state, or uninflamed condition of a tissue. However, this loose definition obscures important features of tissue biology. To arrive at a more precise definition of tissue homeostasis, we first discuss the components of a homeostatic circuit and examples of systemic and cellular homeostasis, which are better understood, and then examine these concepts on the tissue level.

Homeostasis describes the active maintenance of certain quantitative characteristics of the system, known as regulated variables, within a desired range. The homeostatic circuit is structured to maintain them at a stable level, close to a target value known as the set point. In

order to be maintained, the values of regulated variables have to be monitored by specialized sensors and corrected by effectors. Sensors must be able to communicate with effectors through dedicated signals that report on changes in the regulated variable. Together, regulated variables, sensors, signals, and effectors make up a homeostatic circuit (**Figure 6.2a**). In the case of systemic homeostasis, the components of homeostatic circuits are typically well-defined. For instance, to regulate blood glucose (regulated variable), pancreatic alpha and beta cells serve as sensors; glucagon and insulin are signals that reflect the glucose concentration; and liver, skeletal muscle, and adipose are effectors that can correct any deviations of blood glucose levels from a set point value.[171]

Cellular homeostasis is not yet understood as completely as systemic homeostasis, since most of the knowledge comes from studies of cellular stress responses (discussed below). The known regulated variables of cellular homeostasis include concentrations of various metabolites and macromolecules, like oxygen, ATP, and proteins, as well as membrane potential and the number and size of various organelles, like mitochondria, lysosomes, and endoplasmic reticulum (ER).[172] The known sensors of these variables are HIF-1a (for oxygen), AMPK (for ATP), HSF1 and IRE1 (for cytosolic and ER proteins), mTOR and GCN2 (for amino acids), and TFEB (for lysosomes).[173–178] Each of these sensors activates a set of effectors that can correct deviations in the regulated variables. AMPK activates catabolic metabolism to increase ATP production, the IRE1-XBP1 pathway regulates ER size (in part through control of lipid synthesis), and TFEB controls lysosome size and number by inducing expression of lysosome-resident proteins. The signals that connect sensors and effectors in cellular homeostasis are either signaling pathways that control activity of effectors or transcription factors that control their expression.

Tissue homeostasis should be similarly defined in terms of regulated variables, sensors, signals, and effectors.[22] Regulated variables at the tissue level include local concentrations of oxygen and nutrients, ECM density and stiffness, osmolarity and pH of interstitial fluid, and cell numbers and composition.[19] The sensors for these variables include tissue-resident macrophages and sensory neurons.[179,180] Sensing mechanisms of some of the regulated variables are well understood, particularly when the molecular sensors are the same as in cellular homeostasis, such as HIF-1a for oxygen and NFAT5 for osmolarity.[181,182] In tissue homeostasis, however, activation of these sensors results in the production of paracrine signals that engage effectors on the tissue level: HIF-1a stabilization leads to VEGFA production to induce angiogenesis, which increases oxygen delivery,[183] while NFAT5 activation leads to VEGFC production to induce lymphangiogenesis and lymphatic drainage, which reduces osmolarity.[184] Interstitial fluid pH can be sensed by GPR4, GPR65, and GPR68 expressed by endothelial cells, macrophages, and sensory neurons.[185] The mechanisms for sensing many other tissue homeostatic variables, such as cell number and ECM composition and stiffness, are unknown. It can be predicted, however, that sensing a deviation in any of these variables will result in production of a paracrine signal that acts on the appropriate effector cell types to correct the deviation (except when a single cell type serves as both the sensor and effector for the same variable). In most cases, the specific homeostatic circuits that control tissue-level variables remain to be defined.

It is important to note that the sensors and effectors in tissue homeostatic circuits are the supportive cells within tissues. In fact, their supportive functions are largely defined by their roles in tissue homeostasis. These core functions can be quantitatively dialed up and down by the

homeostatic signals that communicate between sensors and effectors, allowing supportive cells that serve as effectors to respond dynamically to the needs of the tissue. The cell types that perform primary functions within tissues, on the other hand, are the sensors and effectors of systemic homeostasis. Thus, pancreatic alpha and beta cells have the primary function of sensing blood glucose, a systemic homeostatic variable, and skeletal muscle, hepatocytes, and adipocytes are primary cells in their respective organs that function as effectors to correct blood glucose concentration. This example illustrates a principle that connects tissue organization with homeostatic circuits: Systemic homeostasis is maintained by primary cells, while tissue homeostasis is maintained by supportive cells within tissues (**Figure 6.2b**).

Stress responses

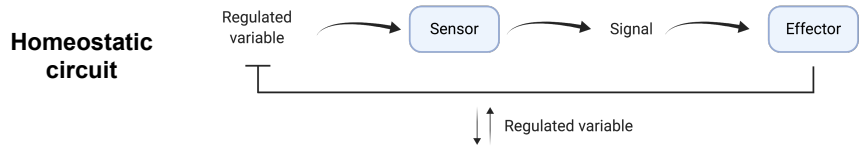
Each homeostatic variable is characterized by a normal range of variation—the maximum deviation from the set point that can be tolerated. When values of regulated variables change within that range, homeostatic mechanisms correct them through negative feedback, as discussed above. Deviations that approach the limits of this homeostatic range put more strain on the system, resulting in what is commonly referred to as a stress response.[186,187] These larger changes in regulated variables are detected and corrected by the same homeostatic sensors and effectors. In fact, what we know about cellular homeostasis was learned primarily by studying extreme perturbations to regulated variables, such as hyperosmolarity, hypoxia, and nutrient deprivation, or by manipulations that indirectly affect regulated variables, such as treatment with tunicamycin (glycosylation inhibitor), thapsigargin (calcium ATPase inhibitor), or proteasome inhibitors. Accordingly, the pathways involved in correcting these cellular perturbations are traditionally referred to as stress pathways, rather than homeostatic pathways.[186] It is important to emphasize,

however, that cellular stress is just a large deviation of a homeostatic variable nearing the limits of what can be addressed through homeostatic effector mechanisms. Stress responses are part of a continuum of homeostatic responses. Yet, the outcomes of stress responses can be qualitatively different from those of homeostatic responses. In addition to engaging homeostatic effectors to correct the deviations in regulated variables, stress responses may also suppress processes that either contribute to these deviations or are incompatible with correcting the deviations. For example, most cell stress responses inhibit cell proliferation, while homeostatic responses to normal variations in the regulated variable do not.

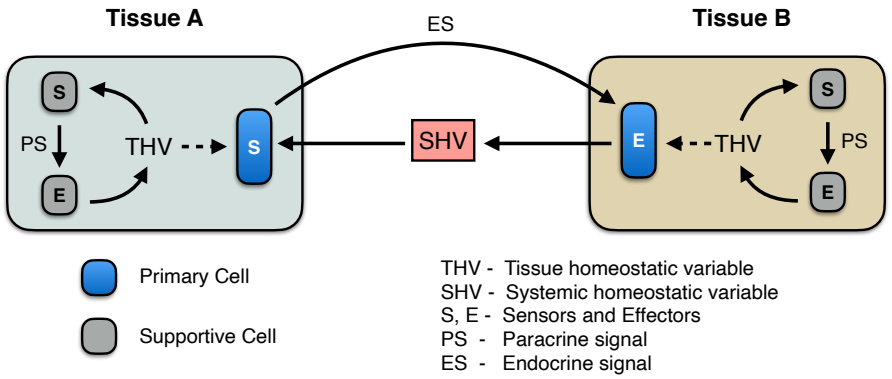
Consistent with the hierarchy of homeostatic circuits, stress responses can be engaged at the levels of the cell, tissue, and organism. All individual cells can detect and respond to stress. These cell-autonomous stress responses allow individual cells to adapt and survive in changing environments. For example, the cellular response to hypoxia is initiated by the hypoxia sensor HIF-1α, which activates transcription of the genes for the glucose transporter GLUT1 and glycolytic enzymes to promote anaerobic glycolysis (**Figure 6.2c**).[188] This response occurs in all nucleated cells and is an example of the cellular stress response. However, cell-intrinsic adaptations to stress provide only short-term solutions, as they do not correct the problem itself. In this example, the cellular stress response does not eliminate local hypoxia. In multicellular organisms, cells can also coordinate with one another to perform tissue-level stress responses. Although all cells can sense stressors such as hypoxia, tissue-level responses generally rely on cells that have specialized sensory functions, such as tissue-resident myeloid cells and sensory neurons. In the case of hypoxia, tissue-resident macrophages detect low oxygen levels and secrete vascular endothelial growth factors and other angiogenic signals to endothelial cells,

which act as effectors (**Figure 6.2d**).[189] The outcome of this tissue-level response is local angiogenesis and increased oxygenation of the tissue, thus correcting the low oxygen levels directly. This response, however, may not be adequate when blood oxygen levels are low (i.e., systemic hypoxia or hypoxemia), which requires a stress response on the level of the whole organism. The sensors of systemic hypoxia are peritubular interstitial fibroblasts in the kidney, as well as hepatocytes, which detect hypoxia through HIF-1α and HIF-2α and induce expression of erythropoietin (EPO).[190,191] EPO acts on erythroid progenitors in the bone marrow to promote erythropoiesis and increase oxygen delivery by red blood cells (**Figure 6.2e**). The example of the stress response to hypoxia illustrates two points: First, even for the same homeostatic variable, sensors, signals, and effectors can differ at the cellular, tissue, and organismal levels. Second, it highlights again that supportive cell types in tissues serve as sensors and effectors to maintain tissue homeostasis, while the primary cells of tissues function as sensors and effectors to maintain organismal homeostasis.

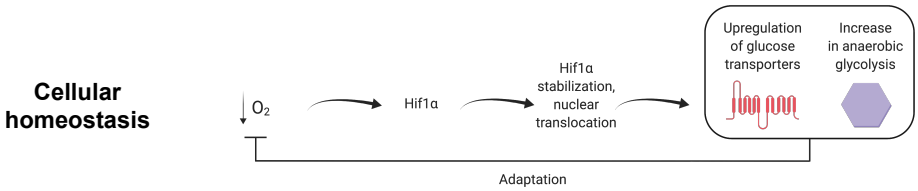
a


Homeostatic circuit
Regulated variable
Sensor
Signal
Effector
Regulated variable

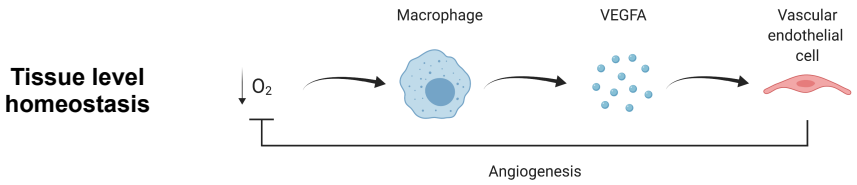
b


Tissue A
ES
Tissue B
S
PS
THV
E
S
SHV
E
THV
S
PS
E
Primary Cell
Supportive Cell
THV - Tissue homeostatic variable
SHV - Systemic homeostatic variable
S, E - Sensors and Effectors
PS - Paracrine signal
ES - Endocrine signal

c


Cellular homeostasis
O2
Hif1α
Hif1α stabilization, nuclear translocation
Upregulation of glucose transporters
Increase in anaerobic glycolysis
Adaptation

d


Tissue level homeostasis
Macrophage
VEGFA
Vascular endothelial cell
O2
Angiogenesis

e

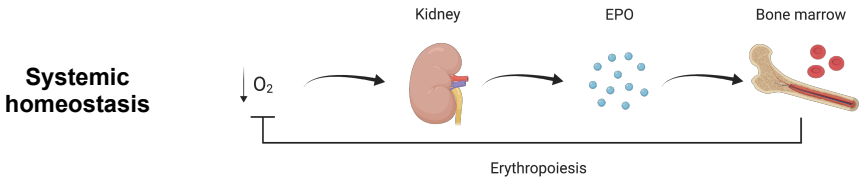

Systemic homeostasis
Kidney
EPO
Bone marrow
O2
Erythropoiesis

Figure 6.2. Homeostasis at the levels of the cell, tissue, and organism
(A) The homeostatic circuit illustrates how regulated variables are maintained within an appropriate physiological range. Deviations in regulated variables are detected by sensors, which then signal to effectors to correct the deviation. **(B)** The interplay between tissue-level and systemic homeostatic circuits is shown. Supportive cell types within tissues are the sensors and effectors for tissue-level homeostatic variables. Maintaining these variables in the proper range allows for effective function of the primary cells within the tissue. Thus, tissue homeostasis is maintained by supportive cells. Conversely, at the systemic level, primary cells are the sensors and effectors for systemic homeostatic variables. Therefore, organismal-level homeostasis is maintained by primary cells. **(C-E)** The regulated variable oxygen is sensed and maintained at the cellular, tissue, and organismal levels. These pathways are depicted using the logic of the homeostatic circuit. Abbreviations: E, effector; EPO, erythropoietin; ES, endocrine signal; PS, paracrine signal; S, sensor; SHV, systemic homeostatic variable; THV, tissue homeostatic variable; VEGFA, vascular endothelial growth factor A.

Tissue microenvironment

The framework for tissue homeostasis described above allows us to better define the tissue microenvironment. Individual cells within tissues are surrounded by a milieu that includes nutrients, oxygen, metabolic waste products, and the composition and mechanical properties of neighboring cells and ECM. From the perspective of the individual cell, these are features of their environment. From a tissue-level perspective, however, these are regulated variables: They are actively monitored by homeostatic sensors and corrected by effectors. When these variables deviate to the edges of the homeostatic range, stress responses are mobilized at the cellular and tissue levels. When these variables are within a normal range, close to the homeostatic set point, individual cells enjoy an optimal environment.

The notion of a tissue microenvironment became particularly popular in reference to tumors, which have characteristically altered homeostatic variables. Unrestrained proliferation of cancer cells within tumors results in oxygen and nutrient depletion and alteration of cell composition and tissue architecture.[192,193] These changes tend to limit proliferation of cells. The deviations in these regulated variables are sensed by tissue homeostatic sensors, such as tissue-resident macrophages (in this context referred to as tumor-associated macrophages).[194] Various effectors, like endothelial cells and stromal cells, attempt to correct them, just as they would in normal tissues. By maintaining tissue homeostasis, the supportive cell types (macrophages, endothelial cells, and stromal fibroblasts) enable tumor growth, which makes them essential components of most solid tumors. However, in fast-growing tumors, the homeostatic capacity is eventually overwhelmed by uncontrolled proliferation of cancer cells, leading to the formation of a necrotic core at the center of the tumors, where homeostatic alterations are most severe.[195,196] The

extreme deviations and ultimately the loss of tissue homeostasis observed in tumors can also occur in normal tissues in response to severe perturbations of homeostatic variables that exceed homeostatic capacity. This can result in cell death and the loss of normal tissue architecture and function. When this happens, tissues engage in the next line of defense—the inflammatory response.

Tissue inflammation

Inflammation is usually defined as a response to infection or injury. While this view is certainly correct, it does not capture the essence of the inflammatory response or explain its role in a wide range of physiological and pathological conditions. Infection and injury are extreme perturbations. As a result, they cause inflammatory responses of a magnitude that is readily observable. However, it is now well appreciated that inflammation can occur without infection or overt tissue damage. A common theme of conditions that initiate inflammation is the disruption of cellular and tissue homeostasis.[197–200] There are several well-known examples of this: 1) Cells with disrupted homeostasis that undergo senescence can release inflammatory mediators known as the senescence-associated secretory phenotype (SASP).[201] 2) Excessive cell stress, such as ER, mitochondrial, or osmotic stress, that cannot be handled by effector mechanisms within the homeostatic regime activates the NLRP3 inflammasome.[187] This leads to production of IL-1 family cytokines,[198,202,203] as well as ligand-independent activation of tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL) receptors.[204] In obesity, lipid overload in adipocytes and hepatocytes causes ER stress and production of inflammatory signals.[199] This type of inflammation, called metaflammation,[205] is caused by a disruption of metabolic homeostasis.

The pathological roles of these inflammatory responses are well appreciated, as exemplified by the contribution of ER stress–induced inflammation to metabolic disease and the contribution of SASP to tumor progression and aging.[206–208] However, they also have important physiological roles that are becoming clearer. For instance, endurance exercise increases production of proinflammatory cytokines, such as IL-13 and IL-6. IL-13 induces the expression of mitochondrial and fatty acid oxidation genes in myocytes, which leads to a transition to slow-twitch oxidative muscle fibers and enhanced mitochondrial biogenesis, in order to optimize energy utilization and adapt to high-endurance exercise.[209] IL-6 is produced by myocytes based on the duration and intensity of physical activity and acts on liver and adipose tissues to regulate glucose and lipid metabolism, in order to meet long-lasting energy demands.[210,211] IL-6 is also produced by brown adipocytes under conditions of acute psychological stress, in response to adrenergic stimulation by the sympathetic nervous system, and promotes hepatic gluconeogenesis to support fight-or-flight responses.[212] In these cases, inflammatory cytokines regulate both tissue-level and systemic adaptations to anticipated physical activity that exceeds homeostatic demands.

The examples above illustrate the common principle that extreme perturbations of tissue homeostasis induce inflammation. While deviations of regulated variables within a normal range are corrected by the homeostatic circuit (including stress responses), extreme deviations of regulated variables beyond the homeostatic range trigger the inflammatory response (**Figure 6.3a**). On this view, homeostatic, stress, and inflammatory responses are activated based on the degree of deviation in the regulated variable, rather than qualitatively different types of challenges. Thus, homeostasis, stress, and inflammation represent a continuum of responses to

tissue perturbations. The cellular sensors that trigger these different responses can either be the same, such as IRE1, which coordinates the cell-intrinsic ER stress response and can also induce inflammatory signals such as IL-6 and TNF—or distinct, as in the case of the NLRP3 inflammasome, which responds to the most extreme deviations to activate inflammation.[186,187,213]

Similarly, although the homeostatic and inflammatory signals released by these sensors are traditionally thought to be distinct, accumulating evidence indicates that many inflammatory mediators can also function as homeostatic signals. For instance, TNF promotes epithelial cell proliferation, and IL-6 maintains self-renewal of hematopoietic, mesenchymal and epithelial stem cells.[214–218] Prostaglandins have both inflammatory and homeostatic functions, including their role in maintaining epithelial barrier integrity.[219] Histamine is a potent inflammatory mediator produced by mast cells and basophils but also plays an important role in controlling intestinal peristalsis and gastric acid secretion and functions as a neurotransmitter in the brain.[220–223] Whether these signals perform homeostatic or inflammatory functions may depend on their expression range and the source and target cell types.

In addition to sensing extreme perturbations in homeostatic variables, cells can sense the loss of tissue homeostasis retrospectively by monitoring its consequences, such as cell death. Cells undergoing several forms of unscheduled cell death, including necrosis, necroptosis, pyroptosis, and ferroptosis, are known to release signals that initiate inflammatory responses.[224–229] These signals include ATP, HMGB1, histones, some amino acid–tRNA synthetases, succinate, and the IL-1 family members.[198,230–234] Finally, the inflammatory response can be induced prospectively when inflammatory sensors detect stimuli that are anticipated to result in the loss of tissue

homeostasis.[172] These stimuli include microbial agents and foreign bodies.[200] Their detection initiates the inflammatory response even before there is any loss of tissue homeostasis. For instance, detection of LPS by TLR4 on macrophages leads to an inflammatory response not because LPS causes disruption of homeostasis, but because it is associated with bacterial pathogens that do. The inflammatory response to LPS is therefore induced preemptively, in anticipation of the loss of homeostasis. The sensors involved in initiating preemptive inflammatory responses are various pattern recognition receptors, typically on innate immune cells, and the resulting signals are well-known inflammatory mediators, including cytokines and chemokines.[235]

In summary, inflammatory responses are induced either as a result or in anticipation of the loss of tissue homeostasis. In the former case, inflammation is induced either by extreme deviations of homeostatic variables or by tissue damage that results from the loss of homeostasis. In the latter case, inflammation is induced when conserved microbial products (pathogen-associated molecular patterns) or allergens are detected, before tissue damage takes place.[172,220] In all cases, the inflammatory response follows the same general design as the homeostatic circuit, with the same four universal components (**Figure 6.3b**). Inflammatory triggers are monitored by a sensor that produces inflammatory signals that act on various effectors, either locally within the tissue or systemically. The ultimate goal of the inflammatory response is to restore the system to a homeostatic state and its regulated variables to their set points.

Effect of inflammation on core functionalities in tissues

In previous sections, we described how primary and supportive cells within tissues act as the effectors within homeostatic circuits. Homeostatic and stress signals tune the core functions of those cell types in order to maintain regulated variables at the appropriate levels, both systemically and within the tissue environment. Similarly, inflammatory signals increase or decrease those same core cellular functions in order to achieve their effects. To understand how inflammation alters the cellular functions within tissues, we can consider the well-studied example of microbial infection. Sensing of microbes within tissues triggers the production of inflammatory cytokines and chemokines that cause vasodilation and increase vascular permeability, allowing for the recruitment of neutrophils into the tissue. At the infection site, neutrophils become activated and release neutrophil extracellular traps (NETs), proteases, and other antimicrobial agents to contain the pathogens and prevent them from spreading systemically.[236,237] In the short term, these inflammatory responses alter cellular functions (like endothelial permeability) and tissue composition (like neutrophil recruitment) in the service of inflammation. However, in the long term, this is necessary to prevent more severe damage by the spread of microbes and ultimately to restore tissue homeostasis.

To achieve these ends, the inflammatory response follows three important principles: First, inflammatory signals alter tissues by making quantitative changes to the performance of their core functionalities. Second, inflammatory functions take priority over homeostatic functions: The inflammatory signals must override incompatible homeostatic signals to change the performance of cells' core functions. Third, similar to the hierarchy that exists in tissue-level and systemic homeostasis, changes in the core functionalities of supportive cell types enable

inflammation at the tissue level while changes in the core functionalities of primary cell types support inflammation at the systemic level. In this section, we discuss each of these generalizations and their implications.

Most commonly, inflammatory signals act directly on a given cell type to enhance or suppress certain core functions that it performs (**Figure 6.3c**). For example, core functions of fibroblasts are production of ECM and production of growth factors. These functions can be modulated by inflammatory cytokines such as TNF and IL-13.[238,239] The core functions of adipocytes are lipid uptake from circulation for storage and lipid release when there is a demand for fatty acids in other organs. Inflammatory cytokines (TNF, IL-1, and IL-6) suppress lipid storage by inhibiting lipoprotein lipase and at the same time promote lipolysis to increase fatty acid release.[240] A core function of endothelial cells is to form an internal barrier to separate the tissue environment from the systemic environment while allowing selective exchange of oxygen and metabolites.[241] They are often the first target of inflammatory signals and provide a good example of how these signals change core functionalities. Endothelial barrier function is modified to either increase or decrease endothelial permeability. Histamines released from mast cells during allergic inflammation or prostaglandins produced during microbial infections can activate specific G protein–coupled receptors (GPCRs) on endothelial cells, leading to an increase in intracellular calcium and activation of Rho signaling through the Gαq subunit.[242–244] Elevated calcium activates myosin light chain (MLC) kinase, and Rho signaling inhibits the MLC phosphatase. Synergistically, these two signaling pathways increase phosphorylated MLC, which initiates actin filament contraction to increase permeability.[245] In contrast, activation of GPCRs through the Gαs subunit increases cyclic AMP synthesis, which strengthens tight junctions, reduces

actomyosin contraction, and decreases barrier permeability.[246,247] Ligands such as adenosine, when signaling through adenosine A2 receptors, activate Gαs proteins to change endothelial permeability in the opposite direction of inflammatory signals.[248] These GPCR-mediated inflammatory and anti-inflammatory effects take place within minutes of stimulation.[249] In addition to GPCR signaling, cytokines such as TNF and IL-1β activate downstream transcription factors NF-κb and AP-1 in endothelial cells and drive vascular permeability and leukocyte adhesiveness in a manner that is dependent on new protein synthesis.[244–250] This process takes a few hours and thus follows GPCR activation to regulate permeability on a longer timescale. Although different types of inflammation elicit different inflammatory mediators, these signals generally converge to dial up or down the core functions of particular cell types.

To enable inflammatory responses within tissues, inflammatory signals must act on multiple cell types and change their core functionalities in a coordinated fashion. For example, the core function of vascular smooth muscle is to control blood flow through contraction and relaxation, and the core functions of vascular endothelial cells include barrier and transport functions. During inflammation, cytokines, prostaglandins, and nitric oxide promote relaxation of vascular smooth muscle, causing increased blood flow to the site of inflammation.[251,252] This is coupled with the activation of endothelial cells, which secrete chemokines, increase surface expression of adhesion molecules (ICAM-1, VCAM-1, E-selectin, P-selectin) for circulating neutrophils, and increase barrier permeability for fluids rich in plasma proteins.[244] Extensive signaling between endothelial and smooth muscle cells ensures coordinated actions to recruit immune cells from circulation into the tissues (an extension of the transport function of the endothelium).

In the examples cited above, the inflammatory signals change the core functions by acting directly on the cell types that perform these functions. However, in some cases, the effects of inflammatory signals can be indirect, through processes that contribute to or control the core functions (**Figure 6.3d**). These indirect effects are often mediated through control of differentiation of cells performing a given function, particularly when these cell types are short-lived. For example, IL-13 indirectly promotes epithelial barrier function by stimulating differentiation of goblet cells, which in turn secrete mucus to reinforce the epithelial barrier.[253] Similarly, TNF and granulocyte colony-stimulating factor (G-CSF) produced during bacterial or fungal infection act on hematopoietic progenitors to increase neutrophil differentiation, while IL-5 produced during helminth infection promotes generation and migration of eosinophils.[254,255] This theme applies not only to inflammatory signals but also to the homeostatic signals discussed above, which can have their effects on core functions either directly or indirectly (**Figure 6.3d**). EPO acts indirectly, for example, because it increases the differentiation of red blood cells to increase oxygen-carrying capacity, rather than directly enhancing their oxygen-carrying function.[256] Finally, inflammatory signals can also modulate core functionalities through their effects on homeostatic signals (**Figure 6.3c,d**).

This leads us to the second generalization, which is that inflammatory signals take precedence over homeostatic signals, in order to coordinate emergency functions. This can occur in a few ways. Inflammatory signals can act on the same effector that participates in the homeostatic circuit, overriding the effect of the homeostatic signal and changing the quantitative performance of the effector's core functionalities. Alternatively, inflammatory signals can act on the homeostatic sensor, changing its 'gain' (input-output function) (**Figure 6.3b-d**). To illustrate

this, consider the bone, the primary function of which is to provide mechanical support for the organism. To perform this function, osteoblasts (primary cells) and osteoclasts (supportive cells) work as a complementary unit. The core function of osteoblasts is to deposit ECM, and the core function of osteoclasts is to degrade or resorb ECM. (These are essentially specialized fibroblasts and macrophages, respectively, which also regulate ECM in their roles as supportive cells in other tissues.) Homeostatic control of this system operates through the production of growth factors for each cell type (**Table 6.2**). The main growth factors for osteoclasts are macrophage colony-stimulating factor (MCSF) and receptor activator of NF-κB ligand (RANKL), both of which are produced by osteoblasts. Under normal conditions, therefore, osteoblasts regulate the numbers of the supportive osteoclasts to maintain control of bone homeostasis.[257] Inflammatory signals like TNF, however, override this homeostatic circuit in one of two ways: (*a*) They act directly on osteoclasts to promote osteoclast differentiation and activity or (*b*) they act on osteoblasts to increase the level of RANKL, which in turn controls osteoclast numbers. Similarly, the cytokine IL-4 can oppose the activity of TNF by preventing both its direct effects on osteoclasts and its effect on osteoblast expression of RANKL.[258,259]

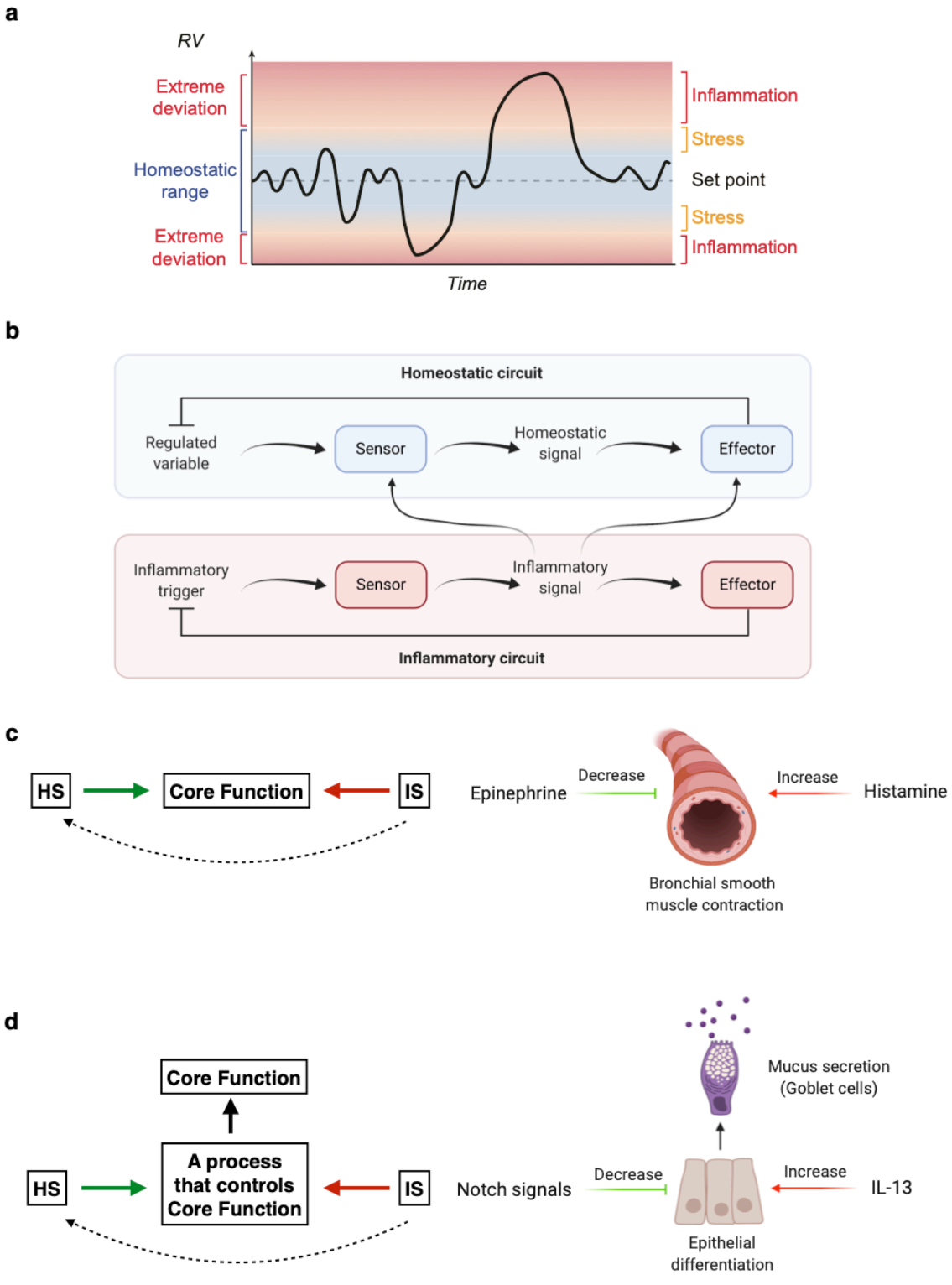

a
RV
Extreme deviation
Homeostatic range
Extreme deviation
Inflammation
Stress
Set point
Stress
Inflammation
Time
b
Homeostatic circuit
Regulated variable
Sensor
Homeostatic signal
Effector
Inflammatory trigger
Sensor
Inflammatory signal
Effector
Inflammatory circuit
c
HS
Core Function
IS
Epinephrine
Decrease
Increase
Histamine
Bronchial smooth muscle contraction
d
Core Function
HS
A process that controls Core Function
IS
Notch signals
Decrease
Increase
IL-13
Mucus secretion (Goblet cells)
Epithelial differentiation

Figure 6.3. Regulation of core functionalities by homeostatic and inflammatory signals
(A) The value of a regulated variable (RV) varies as a function of time, but homeostatic mechanisms maintain it within a homeostatic range around a set point. When the RV experiences more significant deviations within the homeostatic range, the stress response acts to restore the RV to the set point. When the RV deviates beyond the homeostatic range, this extreme deviation triggers an inflammatory response to return the RV to the homeostatic range. **(B)** The inflammatory circuit has a parallel structure to that of the homeostatic circuit, consisting of an inflammatory trigger, sensor, signal, and effector. The effectors of inflammatory responses eliminate inflammatory triggers (e.g. bacteria) or resolve the tissue disruption caused by the inflammatory triggers. In addition to regulating effector functions specific to inflammation, inflammatory signals also act on sensors and effectors of homeostatic circuits to control homeostatic effector functions. **(C)** Homeostatic signals and inflammatory signals can directly control the core functionalities of cells. Inflammatory signals can also operate by changing the levels of homeostatic signals (*dashed arrow*). These signals quantitatively increase or decrease the core functions and can operate in the same or opposite directions. For example, epinephrine decreases bronchial smooth muscle contraction (or causes relaxation) by acting on β-adrenergic receptors. During allergic responses, histamine released by mast cells and basophils increases smooth muscle contraction, antagonizing homeostatic control to cause bronchial constriction. **(D)** Homeostatic signals and inflammatory signals can act indirectly by regulating a cellular process, such as cell differentiation or migration, that in turn controls the core function of cells. For example, Notch signals inhibit goblet cell differentiation, which limits the quantity of mucus secretion by goblet cells during homeostasis. Parasitic infection increases IL-13 production, which increases goblet cell differentiation and enhances mucus production. Abbreviations: HS, homeostatic signal; IS, inflammatory signal; RV, regulated variable.

Table 6.2.

Tissue	Cell type	Core function	Homeostatic signals	Inflammatory signals
Vasculature	**Smooth muscle cells**	Contraction	↑ Endothelin-1, thromboxanes,[260] norepinephrine (α receptors)[261]	↑ leukotrienes,[262] histamine (H_1 receptor)[263]
		Relaxation	↑ NO,[251] norepinephrine (β_2 receptor)[264]	↑ TNF, IL-1β,[265] histamine (H_2 receptor)[263]
	Endothelial cells	Vessel permeability	↑ NO and PG [241]	↑ TNF and IL-1β,[244] histamine [266]
Bone	**Osteoblasts**	Matrix deposition	↑ BMPs, IGF-1, FGFs [257]	↑ IL-22,[259] IL-23 [267]
	Osteoclasts	Matrix resorption	↑ M-CSF,[268] RANKL [269] ↓ OPG [270]	↑ TNF, IL-1, IL-6, IL-17,[259] histamine [271] ↓ IL-4,[272] IL-12, IL-18, IL-33,[259] IFN-β [273]
Adipose	**Adipocytes**	Lipid storage	↑ Insulin [274]	↑ IL-6 [275]
		Lipid release	↑ catecholamines [274]	↑ TNF,[276] IL-1,[277] IL-6 [206]
Small intestine	**Enterocytes**	Nutrient and mineral absorption	?	↓ IL-22 [278,279]
	Goblet cells	Mucus secretion	↑ acetylcholine [280] ↓ Notch [281]	↑ IL-13 [253]
	Smooth muscle cells	Peristalsis	↑ acetylcholine, serotonin[a], histamine[a] [165,221]	↑ serotonin[a], histamine[a] [221]
Hematopoietic system	**Stem cells**	Self-renewal and differentiation	↑ Wnt,[282] PG,[283] IL-7, EPO,[256] M-CSF, GM-CSF [284]	↑ TNF, IL-1β, IL-6 (neutrophils, monocytes),[254] IL-5 (eosinophils),[255] TSLP (basophils)[285]

Effects of homeostatic and inflammatory signals on core functionalities of cell types within tissues

Abbreviations: BMP, bone morphogenetic protein; FGF, fibroblast growth factor; GM-CSF, granulocyte-macrophage colony-stimulating factor; IGF-1, insulin-like growth factor 1; M-CSF, macrophage colony-stimulating factor; NO, nitric oxide; OPG, osteoprotegrin; PG, prostaglandin; RANKL, receptor activator of NF-κB ligand; TNF, tumor necrosis factor; TSLP, thymic stromal lymphopoietin.

[a]These signals can be both homeostatic and inflammatory. In these contexts, the quantity of the signal determines whether it has homeostatic or inflammatory functions.

Most of the examples that we discuss above describe changes in supportive functions that control tissue-level biology during inflammation. However, recall that, under homeostatic conditions, primary cell types within tissues perform supportive functions at the organismal level to maintain regulated variables of the entire organism. Similarly, in the context of inflammation, we can make the final generalization that changing core functionalities of primary cells supports inflammatory responses at the organismal level (**Figure 6.4**). In the case of systemic infections, for instance, adjustments in the primary function of different organs are coordinated to enable systemic inflammatory responses against pathogens and promote tissue-level tolerance to those responses.[286,287] In systemic inflammation, cytokines TNF, IL-1, and IL-6 act on many primary cell types to tune their core functionalities. For example, as noted earlier, these cytokines act on adipocytes to suppress lipid uptake from the circulation and promote lipolysis,[206,276,277] on skeletal muscle cells to release amino acids,[288] and on hepatocytes in the liver to optimize the production of ketone bodies.[289] These signals also act on pancreatic beta cells to inhibit insulin production and on hepatocytes, skeletal muscle cells, and adipocytes to reduce responsiveness to insulin.[290,291] They dramatically increase the secretory function of hepatocytes, inducing secretion of large quantities of acute phase proteins into circulation, which in turn further propagate the inflammatory response.[292] In addition, they change the activity of hypothalamic neurons to induce fever and anorexia.[293,294] These changes both support the metabolic demands of the immune response and render the host more tolerant to the damage caused by inflammation.[286,295–297] The primary functions of different tissues are thus adjusted and coordinated by systemic inflammatory signals to optimize the systemic inflammatory response.

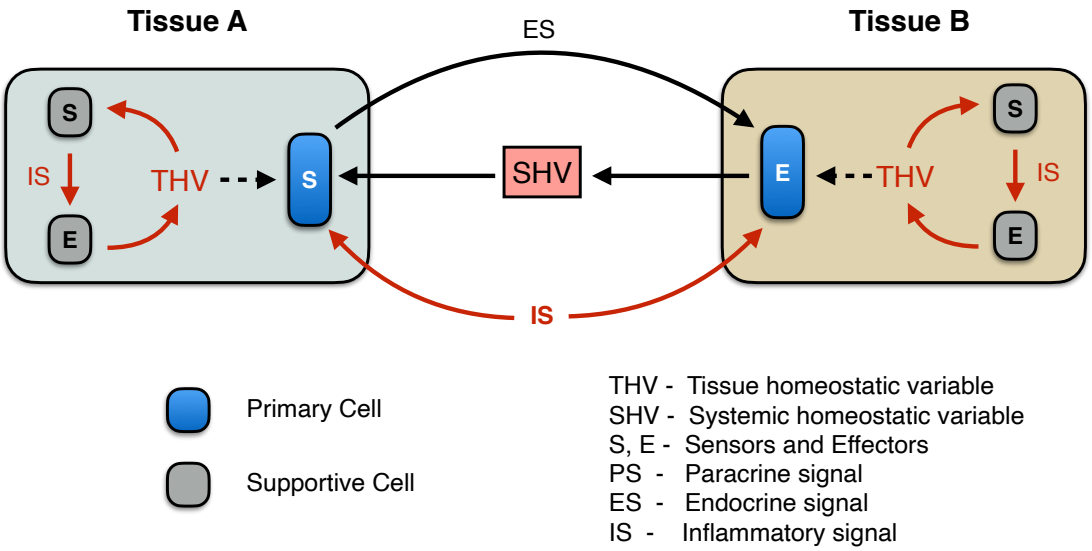


Figure 6.4. Local and systemic effects of inflammatory signals
At the tissue level, supportive cell types act as inflammatory sensors and effectors. Inflammatory signals act on effectors to change tissue homeostatic variables, such as cell composition and endothelial permeability. These changes are often required to facilitate inflammatory responses at the tissue level. At the systemic level, inflammatory signals act on primary cell types to change systemic homeostatic variables and coordinate the systemic inflammatory response. Abbreviations: E, effector; ES, endocrine signal; IS, inflammatory signal; S, sensor; SHV, systemic homeostatic variable; THV, tissue homeostatic variable.

Inflammatory diseases

The goal of the inflammatory response is ultimately to restore homeostasis. This is obvious in the case of infection-induced inflammation, where a successful inflammatory response results in elimination of the pathogen. However, in the process of achieving that goal, the inflammatory response leads to temporary disruption of homeostasis. This occurs in part because collateral tissue damage is an unavoidable consequence of antimicrobial immune responses, but also because inflammation deliberately alters many aspects of tissue homeostasis. Recruitment of monocytes and granulocytes changes cell numbers and composition within tissue compartments, and the inflammatory exudate changes the interstitial fluid volume and protein concentration.[200] Proteases produced by neutrophils degrade ECM proteins, and the functions of most cell types within an inflamed tissue are altered by inflammatory cytokines.[237] Similarly, inflammation alters many aspects of systemic homeostasis, such as body temperature, metabolism, and endocrine functions.[286] These changes, which are clearly a departure from a homeostatic state, are necessary to eliminate the inflammatory trigger (such as a pathogen) and ultimately restore homeostasis.

As described in the previous section, a general theme of inflammation is that inflammatory signals override homeostatic pathways that are incompatible with inflammatory processes. For example, maintaining stable cell composition within the tissue compartment is clearly incompatible with recruitment of inflammatory cells necessary to combat an infection.[298] Therefore, the mechanisms that maintain cell numbers within tissues have to be overridden by inflammatory signals to allow for neutrophil and monocyte recruitment. Inflammation has higher biological priority than many homeostatic functions because the benefits of the inflammatory

response generally outweigh the costs of temporary loss of homeostasis. This very property of inflammatory signals, however, also creates a vulnerability: Homeostasis can be persistently overridden and disrupted by dysregulated or chronic inflammation, leading to disease. To illustrate this, we can examine how the inflammatory signals that regulate core functions in bone can also drive clinical pathology. In rheumatoid arthritis, which is characterized by erosion of bone tissue within joints, sustained type 1 inflammation (including TNF, IL-1, and IL-6) drives increased RANKL expression in osteoblasts, which in turn causes increased osteoclast activity.[259] Another set of chronic inflammatory disorders known as spondyloarthropathies (such as ankylosing spondylitis and psoriatic arthritis) are characterized by the opposite process—new bone formation at specific anatomical sites. These diseases are driven by a distinct set of cytokines that have the opposite effect on core cellular functions: IL-23 (together with IL-22 and IL-17) acts to increase osteoblast deposition of ECM, again both directly and by increasing levels of normal, homeostatic growth factors.[259,267] The same principle applies to type 2 (allergic) inflammation. For instance, patients with chronic asthma often have prominent infiltrates of type 2 inflammatory cells in the airways, such as eosinophils, mast cells, basophils, type 2 T helper cells, and group 2 innate lymphoid cells.[299] These cells produce type 2 cytokines, like IL-13, which are responsible for changing the core functions of local cell types, including increasing goblet cell differentiation and mucus production, fibroblast ECM deposition, and bronchial smooth muscle contractility. These changes, when they are persistent, lead to pathologic airway remodeling in asthma.[299,300] Sepsis is a common and often fatal example of sustained inflammatory signaling. One of the hallmarks and dangers of sepsis is hypotension. As described above, inflammatory cytokines (like TNF, IL-1, and IFN-γ) and prostaglandins induce relaxation of vascular smooth muscle, both directly and through induction of nitric oxide production by

macrophages and smooth muscle itself.[251,265,301] This inflammatory regulation of smooth muscle function is typically adaptive, allowing for increased blood flow to the site of inflammation. But, because these signals override normal homeostatic regulation, it makes the system susceptible to disease and even death in the case of sustained inflammatory signaling, which can cause systemic hypotension and decreased blood flow to vital organs.[301]

Concluding remarks

Here we present a perspective on tissue homeostasis and inflammation based on a framework for understanding the functional organization of tissues. We describe how the core functions of supportive and primary cell types are assembled into tissues that are optimized to perform particular functions for the organism, how these core functionalities are tuned up and down to maintain tissue and organismal homeostasis, and how inflammation overrides homeostatic control to coordinate emergency functions and ultimately defend homeostasis.

Homeostasis is a powerful concept that, if carefully defined, can be applied for understanding tissue biology and inflammation. The homeostatic and inflammatory circuits are composed of the same components, including sensors, which monitor variables of interest, and effectors, which can change the values of these variables in the desired direction. Inflammation is induced by one of three possible indicators of the loss of homeostasis. First, inflammation can be induced by extreme deviations of regulated variables, beyond their normal range—a direct indication of the loss of homeostasis. This form of inflammation relates to what Metchnikoff called physiological inflammation and, if the inflammatory response is successful, likely does not manifest in clinical symptoms. Second, inflammation can be induced retrospectively by the consequences of the loss

of homeostasis, such as nonapoptotic cell death or the disruption of tissue architecture. This type of inflammation may fall into Metchnikoff's pathological inflammation category (where pathological refers to the cause rather than the consequence). Finally, inflammation can be induced prospectively, when inflammatory sensors detect pathogens or allergens, as indicators of forthcoming loss of homeostasis. The ensuing inflammatory response would match what Metchnikoff referred to as immunity—the highest end of the inflammatory spectrum. While we can now appreciate that Metchnikoff had a deep intuition about the homeostasis-inflammation spectrum, more work will be required to fully elucidate its underlying principles and mechanisms.

Chapter 7: Materials and Methods

Cell culture

All cells were cultured at 37˚ Celsius (C), 5% carbon dioxide (CO2), and atmospheric oxygen. Experimental techniques were performed in BSL2 biosafety cabinets under sterile conditions. Cells were monitored by microscopy using a Zeiss Axio Vert.A1 microscope, which was also used to photograph live cells to document cell morphology. Photographs were saved as .tiff files.

Bone marrow derived macrophages

Bone marrow derived macrophages (BMDM) were differentiated from mouse bone marrow cells for at least 7 days in MCSF-rich macrophage growth media (MGM) composed of 30% L929-cell conditioned media and 70% complete RPMI media (cRPMI), which consisted of RPMI-1640 (Corning) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Gibco), 2 mM L-glutamine, 200 U/mL penicillin/streptomycin, 1 mM sodium pyruvate, and 10 mM HEPES. Bone marrow was prepared by crushing mouse femurs and tibias to release marrow, followed by ACK (ammonium-chloride-potassium) lysis of red blood cells and passage through a 70-μm cell strainer. Bone marrow was plated on day of isolation (day 0) at 5×10^6 cells in 20 mL of MGM in a 15-cm petri, or non-tissue culture (non-TC), dish. Every three days, cells were supplemented with 10 mL of MGM until they reached confluence. After day 6, adherent cells were lifted with cold phosphate-buffered saline (PBS) containing 5 mM EDTA. Cells were then centrifuged at 1350 rpm at room temperature for 5 minutes, resuspended in 3 mL cRPMI, counted at a 1:1 ratio with Trypan blue, and then diluted to the appropriate concentration (or centrifuged again and

resuspended at the appropriate concentration) in cRPMI and used for experiments. Experiments on tissue culture (TC) or non-TC dishes were typically performed at 0.33×10^6 cells per well of a 12-well dish. Experiments in collagen gels or on polyacrylamide gels are described below.

Murine embryonic fibroblasts

Murine embryonic fibroblasts (MEFs) were harvested from male and female E13.5-E14.5 embryos and sorted for purity. Embryos were removed from a pregnant female by removing the uterus and separating each embryo from its amniotic sac. The head and ‘‘red tissue,’’ including fetal liver, were removed and discarded. The remaining portion of each embryo was minced using razor blades in 0.05% trypsin + EDTA and placed in a 37˚C incubator for 30 minutes. After digestion, the tissue was transferred into a conical tube, washed with complete DMEM (DMEM + 10% FBS, 2 mM L-glutamine, 1 mM sodium pyruvate, 10 mM HEPES, 200 U/mL penicillin/streptomycin) and resuspended in complete DMEM in 15 cm tissue culture plates overnight. The following day, cells and undigested tissue debris were lifted from the plates using 0.05% trypsin + EDTA, spun down, resuspended, and filtered over a 70-μm cell strainer. These cells were expanded for 1-2 passages and then sorted for CD45-, CD11b-, and F4/80-negativity to exclude contaminating macrophages. The sorted MEFs were split once after sorting to allow for recovery and used for experiments between passage 3 and passage 7.

Materials

Recombinant murine IL-4 (Peprotech, 214-14) was used at 20 ng/mL, and recombinant murine MCSF (Cell Signaling Technologies, 5228) at 100 ng/mL, for stimulation of BMDM unless otherwise specified. Chemical inhibitors used in cell culture included latrunculin A (Cayman

10010630, Sigma 428021), (-)-blebbistatin (Sigma B0560), nocodazole (Sigma M1404), wortmannin (Millipore 681675), and SB216763 (Cayman 10010246).

For antibody blockade of cell-surface receptors, cells were pre-incubated in a small volume of media with 160 ug/mL of antibody for 15 minutes at room temperature, followed by dilution to a final concentration of 20 ug/mL for the duration of the experiment. β1 integrin blockade was performed with Purified NA/LE Hamster Anti-Rat CD29 (Clone Ha2/5, BD Pharmingen, 555002) and the isotype control antibody Purified NA/LE Hamster IgM, λ1 Isotype Control (Clone G235-1, BD Pharmingen, 553957). β2 integrin blockade was performed with Purified NA/LE Rat Anti-Mouse CD18 (Clone GAME-46, BD Pharmingen, 555280).

Talin-flox (mice originally produced by Dr. David Critchley) and Talin-flox LysMCre bone marrow were generously provided by the laboratory of Dr. Gwendalyn Randolph (with the help of Dr. Nan Zhang), with permission of the University of Leicester, Oklahoma Medical Research Foundation, and Washington University-St. Louis, as specified in a Material Transfer Agreement.

Three-dimensional collagen gels

Preparation of 3D collagen gels with embedded cells for cell culture

Three-dimensional collagen gels with embedded BMDM or MEFs were synthesized using high-concentration rat tail collagen I (Corning, 354249), using a protocol adapted from Corning's "alternate gelation procedure" provided with the product.[302] A detailed protocol is provided in Appendix 1. Excel spreadsheets to facilitate calculations of reagent quantities and cell numbers

could not be embedded but are available upon request to matt.meizlish@gmail.com, and the approach is summarized here.

Briefly, collagen is obtained from Corning in a 0.02 N acetic acid solution and remains in a liquid state when kept cold and at an acidic pH. Until gelation is desired, collagen solution should be kept on ice as much as possible. Collagen is mixed with 10x PBS and sterile H2O to achieve the desired collagen concentration. 1 N NaOH is added to neutralize the acetic acid in the collagen solution, titrating up and checking the pH (after gently vortexing the collagen solution to mix it) by pipetting 10 uL of solution onto a pH strip until a pH of 7-7.5 is achieved. For subsequent experiments, NaOH volume is conservatively estimated based on previous experiments, scaling up or down according to the volume of collagen being used (because this is the source of acid) and again checking with pH strips. After the collagen solution is at a neutral pH, cells are added such that they make up 1/8 of the volume of the gel and are mixed into the collagen solution with a pipette (not vortexed). Gels were typically plated at 200 uL in 48-well non-TC plates. BMDM were added to the collagen solution at 20-40 million cells/mL, for a total of 0.5-1 million cells per gel. MEFs were typically added to the collagen solution at 8 million cells/mL, for a total of 0.2 million cells per gel. Notably, the high-concentration collagen solution is viscous, and there is some loss of volume during this process, so excess collagen solution must be made to have enough for plating. Typically, scaling up by a factor of 1.6 for high-collagen gels and 1.3 for low-collagen gels is appropriate. When multiple genotypes or other conditions (such as antibody blockade) required splitting the collagen solution into two batches prior to adding cells, a master stock was made for each collagen concentration and then split into batches. An additional scaling factor of 1.3-1.4 is required for the loss that occurs

during this splitting, but it ensures that the batches are cultured in equivalent gel conditions. After collagen-cell solutions are plated, they are incubated for 45 minutes to 1.5 hours at 37˚C and 5% CO2 to achieve gelation. A small pipette tip is used to score around the edge of the gels to detach them from the walls of the wells and ensure that they float (to avoid cells interacting with the stiff bottom of the plate). Media (400 or 500 uL) is then added with any stimulation or inhibitors, and the gels are scored once again before placing them back in the incubator for the duration of the experiment.

Harvesting RNA for qPCR from cells in 3D collagen gels

Collagen gels were removed from the cell culture incubator 16-28 hours after stimulation and brought to a chemical fume hood. Gels were transferred with curved, blunt, serrated forceps to a pre-labeled 24-well non-TC plate, rinsing forceps between each gel. RNA-Bee (Tel-Test, CS-501B) 1 mL was then added to each gel and allowed to sit for approximately 5 minutes or until gels began to fragment. RNA-Bee and gel were then pipetted up and down with a P1000 repeatedly until the gel disintegrated and the solution was homogenous. This solution was then transferred to labeled 1.5 mL Eppendorf tubes on ice. When all samples were collected, Eppendorf tubes were stored at -80˚C until RNA isolation.

Harvesting protein for Western blot from cells in 3D collagen gels

2x SDS-PAGE sample buffer with β-mercaptoethanol and protease/phosphatase inhibitor was pre-heated at approximately 105˚C for approximately 10 minutes, in labeled Eppendorf tubes containing 200 uL of sample buffer. Gels (200 uL each) were then transferred to prewarmed 2x sample buffer using a curved, blunt, serrated forceps (effectively diluting the sample buffer to

1x). Samples were then heated at 105˚C for 15 minutes, visualized to ensure homogenization, and transferred to -80˚C for storage until continuing with the Western blot procedure.

Harvesting cells from 3D collagen gels for flow cytometry

Collagenase Type IV (Worthington, LS004189) was added at 18 mg/mL, 100 uL per well, to 500 uL media in which cells were cultured, for a final concentration of 3 mg/mL. Gels were diced with clean scissors directly in the wells. Plates were taped in an orbital incubator shaker (Barnstead MaxQ4000) and rotated at 205 rpm for 20 minutes. Samples were then transferred (with partially homogenized gels) to labeled 50 mL conical tubes (kept on ice) through a cell strainer, using the top of a plunger to push the contents through the strainer, followed by 2 washes with PBS (using the plunger in between). Samples were then centrifuged in a large table-top centrifuge at 1350 rpm (385 x g) at 4˚C for 5 minutes and resuspended in 150 uL cold PBS before being transferred to a FACS plate for staining.

Measurement of collagen gel diameter

Collagen gel diameter was measured by holding a ruler against the bottom of the cell culture plate and measuring the diameter at the widest portion of the gel. Photographs were also obtained using an iPhone camera.

Confocal microscopy of cells in 3D collagen gels

In preparation for confocal microscopy, gels were prepared in chambers with coverglass well-bottoms (Lab-Tek Chambered #1.0 Borosilicate Coverglass System, 8-chamber, #155411). Collagen gels were 200 uL per well as usual but were not scored (they should sit on the bottom

of the well to be as close as possible to the microscope objective), and 300 uL of media was added after gelation. At the end of the experiment, media was removed with a pipette, gels were washed gently with PBS, and gels were fixed in 300 uL 4% paraformaldehyde (PFA) for 1 hour at room temperature on a bench-top rotator (secured with tape). Gels were washed three times with 400 uL PBS and then permeabilized with 0.5% Triton X-100 for 1 hour at room temperature on the rotator. Gels were washed three times with PBS and incubated with Phalloidin-Texas Red (ThermoFisher Scientific, T7471) at 1:100 in PBS/5%BSA (or 5% donkey serum) for 1 hour in the dark at room temperature on the rotator. Gels were then counterstained with DAPI 1:10,000-1:40,000 for 5 minutes. Gels were washed three more times with PBS, this time rotating for 5 minutes in the dark during each wash. Gels were kept in PBS and imaged by confocal microscopy, scanning for cells above the bottom of the gel and obtaining Z stacks to capture several whole cells in each image, which were then used for downstream analyses. Cellular morphology was quantified in Imaris Software (Oxford Instruments) for each cell whose borders were fully captured in the acquired confocal images. “Surfaces” were created automatically for cells using the default setting of 0.359 um for Surfaces Detail (to avoid bias) and were then filtered manually to include only genuine cells. The software was then used to calculate the morphology of surfaces, including cell sphericity.

Polyacrylamide-fibronectin gels

Polyacrylamide gel fabrication

Hydrogels were generated by polymerizing various ratios of solutions of acrylamide (Bio-Rad, 3% and 10% for low- and high-stiffness, respectively) and bis-acrylamide (Bio-Rad, 0.3% and 0.225% for low- and high-stiffness, respectively) with ammonium persulfate (Sigma) and

tetramethylethylenediamine (Bio-Rad) sandwiched between two glass coverslips. One coverslip was activated with 3-aminopropyltriethoxysilane using 0.1 M NaOH (Macron Fine Chemicals) and 0.05% glutaraldehyde (Polysciences Inc.) and the other coated with dichloromethylsilane (Sigma). Fibronectin was conjugated to the polymerized hydrogels by succinimide chemistry. Briefly, 0.2 mg/mL Sulfa-SANPAH (ThermoFisher Scientific) in 50 mM HEPES (Sigma) were placed under UV (365 nm, 10 mW/cm), washed thoroughly with HEPES, and incubated overnight with 0.2 mg/mL fibronectin (Millipore) at 37ºC. After incubation, the hydrogels were washed and kept at 4ºC until cell seeding.

Cell culture on Polyacrylamide-fibronectin gels

Gels (attached to coverslips) were transferred to 6-well non-TC plates using sterile, curved, pointed forceps. BMDM (0.85 million cells per gel in 250 uL cRPMI) were gently plated at the center of each gel such that the media formed a meniscus and did not spread beyond the border of the gel. Cells were left to incubate undisturbed for 30 minutes to 1 hour, after which adhesion was confirmed by microscopy. An additional 1.75 mL cRPMI was then added to each gel by gently pipetting 0.875 mL along the back wall of the well and 0.875 mL along the front wall of the well, such that the new media merged with the media on the gel, without disrupting the cells. Cell culture plates were then returned to the incubator for 3 hours, after which photographs of cells were obtained and stimulation with IL-4 (20 ng/mL) was performed. Cells were returned to the incubator for an additional 16 hours.

RNA isolation from Polyacrylamide-fibronectin gels

Gels were transferred to new 6-well non-TC plates in the cell culture hood, taking care to cause minimal mechanical disruption. RNeasy RLT buffer (QIAGEN) with 1:100 β-mercaptoethanol was added to the center of the gel at 350 uL per gel. Plates were allowed to sit for several minutes and then brought to the laboratory bench, where RLT buffer on each gel was gently pipetted up and down and then transferred to Eppendorf tubes. The plate was then tipped forward to collect the remainder of the RLT buffer, which was transferred to the same Eppendorf tubes. Samples were then stored at -80˚C until RNA isolation, which was performed with the RNeasy Micro Kit (QIAGEN, 74004) according to manufacturer instructions.

Immunofluorescence imaging on Polyacrylamide-fibronectin gels

For immunofluorescence (IF) experiments, BMDM were plated at a lower cell density (0.4 million cells per gel) in 250 uL cRPMI. Cells were allowed to adhere for 2 hours (without addition of more media), after which gels were transferred to new wells, washed once with 400 uL PBS, and fixed with 400 uL 4% PFA for 10 minutes at room temperature. Gels were then washed three times with ice cold PBS and permeabilized with 0.1% Triton X-100 in PBS for 10 minutes at room temperature. Gels were again washed three times with PBS and then stained with 400 uL Phalloidin-Texas Red (ThermoFisher Scientific, T7471) at 1:100 in PBS/1% BSA for 1 hour in the dark. DAPI solution was added for the last 10 minutes of staining in 100 uL at 1:2,000 for a final concentration of 1:10,000. Gels were washed three times with PBS (incubating for a few minutes in the dark with each wash). A dot of Prolong Diamond Antifade Mountant (ThermoFisher Scientific, P36961) was then placed in the center of the gel, and a coverslip of the same size as that to which the gel was attached (Fisher Microscopic Cover Glass, 12-546-2, 25CIR-2) was placed on top of the gel to spread the mounting media. The mounting

media was allowed to cure in the dark overnight, and nail polish was then used to seal the edges of the coverslips. Cells were imaged at 20x magnification on a Leica DMI6000 B microscope.

RNA isolation, cDNA synthesis, and quantitative PCR

After cell collection in RNA-Bee, RNA was isolated by chloroform extraction and isopropanol precipitation, except when isolating RNA from PA gels, as indicated, or in preparation for RNAseq experiments, for which the aqueous phase after chloroform extraction was mixed 1:1 with 70% ethanol and further processed with QIAGEN RNeasy Mini columns (74106), with on-column DNAse treatment, according to manufacturer instructions. To synthesize, cDNA, RNA was annealed to oligo-dT6 primers, and cDNA was reverse-transcribed with MMLV reverse transcriptase (Clontech). Quantitative reverse-transcriptase polymerase chain reaction (qPCR) was performed on a CFX96 or CFX384 Real-Time System (Bio-Rad) using PerfeCTa SYBR Green SuperMix (Quanta Biosciences). Relative expression units were typically calculated as transcript levels of target genes relative to Rpl13a *1000. Primers used for qPCR are listed in Appendix 2.

Live cell imaging

Image acquisition began shortly after the addition of media to collagen gels and was performed using a Leica AF6000 Modular System with stage-top incubator INUBTFP-WSKM-F1 (Tokai Hit) maintained at 37 ˚C and 5% CO2. Multiple images were acquired from each sample every 3-5 minutes for 16-20 hours, and videos were assembled from serial images at a single position. Imaris Software (Oxford Instruments) was used to track individual cells throughout the video, in order to quantify the mean track speed and track displacement of each cell.

Flow cytometry

BMDM were collected for flow cytometry from collagen gels as described above or from non-TC dishes with cold PBS with 5 mM EDTA. All staining steps and washes were performed in FACS buffer (PBS with 2% FBS, 0.01% sodium azide) unless otherwise indicated. Cells were Fc blocked with anti-CD16/CD32 (clone 93, eBioscience) at 1:500 and stained with Zombie Yellow at 1:200 for 10 minutes at room temperature in the dark. Cells were washed twice with FACS buffer and then fixed and permeabilized with BD Cytofix/Cytoperm buffer (51-2090KZ) for 15-20 minutes. Cells were then washed twice with BD Perm/Wash buffer (554723), and intracellular staining was performed in BD Perm/Wash buffer for 45 minutes at room temperature or at 4˚C overnight. Antibodies included anti–RELMα–PE at 1:200 (clone DS8Relm, eBioscience, catalog no. 12-5441-80), anti–Arginase1-APC at 1:200 (clone AlexF5, eBioscience, catalog no. 17-3697-82), and anti-Cleaved Caspase-3 (Asp175) at 1:400 (Cell Signaling Technologies, catalog no. 9661). After intracellular staining, cells were washed twice with BD Perm/Wash buffer and then incubated with secondary antibodies, including Goat anti-rabbit Alexa Fluor 488 at 1:400 (Invitrogen A11034). Cells were washed twice more with BD Perm/Wash buffer and filtered into FACS tubes. Samples were run on a BD LSR II Green, followed by analysis with FlowJo software. Histograms were used to determine the percentages of cells expressing a given protein.

Western blot

Samples were run on Bio-Rad Mini-PROTEAN TGX Stain-Free Gels, 4-15%, typically with a 10- or 12-well comb (4568085) in Tris/Glycine/SDS buffer. Protein was transferred onto

activated PVDF membranes using the Bio-Rad Trans-Blot Turbo system according to manufacturer instructions. Membranes were blocked with TBST (20 mM Tris, 150 mM NaCl, 0.05% Tween 20)/5% BSA for 1 hour and then incubated with primary antibodies in TBST/5% BSA with sodium azide at 4˚C overnight. Primary antibodies included rabbit anti-phospho-STAT6 at 1:1000 (Cell Signaling Technologies, 56554), rabbit anti-STAT6 at 1:1000 (Cell Signaling Technologies, 9326), rabbit anti-RELMα (Peprotech, 500-P214) at 1:1000, and mouse anti-GAPDH (Santa Cruz, sc-32233) at 1:2000. Membranes were washed three times with TBST, then incubated with secondary antibodies (anti-rabbit or anti-mouse) at 1:10,000 dilution for one hour at room temperature. Membranes were washed three more times and then developed using Pierce ECL Western Blotting Substrate (ThermoFisher Scientific, 32209) or SuperSignal West Pico Chemiluminescent Substrate (ThermoFisher Scientific, 34080). Protein was visualized using the Bio-Rad Image Lab system, and the optical density of bands was quantified based on pixel intensity within boxes of consistent size encompassing each band, normalized to the background intensity of the membrane.

Animals

All use of animals was performed in accordance with institutional regulations after protocol review and approval by Yale University's Institutional Animal Care and Use Committee. Bone marrow was obtained from adult (≥8-week-old) C57BL/6J mice.

Synthesis of recombinant FIZZ1 (RELMα) and supernatant transfer

Expi293 cells were subcultured at 2-3 million cells/mL on day -1 in Expi293 medium, shaking at 8% CO2. On day 0, cells were diluted back to 2-3 million cells/mL in 25mL Expi293 media, and

25ug His-FLAG-RELMα (cloned into a derivative of pD2610-v1 from Atum Bio, kindly provided by Dr. Aaron Ring's lab) was transfected with ExpiFectamine as per manufacturer's instructions. On day 1, enhancer 1 and 2 were added, and conditioned media was harvested on day 4. Cells were spun at 500 x g, and supernatant was collected, spun again at 500 x g, and collected prior to filtering through a 0.22 um filter to obtain the final FIZZ1-conditioned media, which was used to stimulate 3T3L1 fibroblast cells.

RNA sequencing and analysis

Sequencing libraries were constructed following Illumina Tru-seq stranded mRNA protocol. Paired-end sequencing was performed with Next-seq 500 with paired end reads of 38 base pairs. Illumina fastq files were downloaded from Illumina Basespace and were aligned with Kallisto v0.46.1 using the "-b 100 and -t 20" options to obtain transcript abundances in transcripts per million (TPM) and estimated counts.[303] The kallisto index used during transcript quantification was built from the Mus musculus transcriptome GRCm38 downloaded as a fasta file from Ensembl (ftp://ftp.ensembl.org/pub/release-90/fasta/ mus_musculus/cdna/s). Transcripts were annotated using the Bioconductor package biomaRt v2.40.5.[304] Significant differences in gene expression between conditions were calculated, with correction for multiple comparisons, using Sleuth in R v3.5.1.[305]

ATACseq

ATACseq was performed according to the protocol detailed by Buenrostro and colleagues.[306] UCSC Genome Browser (https://genome.ucsc.edu/) was used for data visualization. HOMER was used to analyze transcription factor motif enrichment (http://homer.ucsd.edu/homer/).

Chromatin immunoprecipitation

Approximately 45 million BMDM per condition (15 million cells/plate on 15 cm TC plates) were crosslinked with 1% paraformaldehyde directly in TC plates for 10 minutes with gentle shaking at room temperature. The reaction was quenched by adding glycine for a final concentration of 125 mM for another 5 minutes with shaking at room temperature. The cells were washed 3 times with cold PBS and scraped with 10 mL of PBS into 50 ml conical tubes (one per condition). The cells were centrifuged for 5 minutes at 475 x g at 4 ˚C and supernatant was removed by decanting. Pellets were resuspended in remaining PBS and transferred to Eppendorf tubes. Samples were centrifuged at 450 x g at 4 ˚C, supernatant was aspirated with glass pipettes, and pellets were frozen at -80 ˚C. The next day, pellets were thawed on ice, resuspended in 1 mL nuclear lysis buffer (10 mM EDTA, 50 mM Tris-Cl (pH 8.0), 1% SDS, 1x protease inhibitors (ThermoFisher Halt)), and incubated at room temperature for 15 minutes with mechanical disruption after 7 minutes. Samples were transferred to a 1.5 ml Bioruptor Plus TPX microtube (Diagenode). The nuclear lysate was sonicated for 3 rounds of 10 cycles of 30 seconds on/30 seconds off on high power (Diagenode Biorupter plus). After sonication the chromatin was centrifuged for 15 minutes at max speed at 4 ˚C. Chromatin was diluted with Chip dilution buffer (16.7 mM Tris pH 8.0, 1.1% Triton X-100, 167 mM NaCl, 1.2 mM EDTA, 0.01% SDS, 1x protease inhibitor (ThermoFisher Halt)). The diluted chromatin was split into low binding tubes (one tube per antibody), antibody was added, and tubes were rotated overnight at 4 ˚C. Approximately 100ug of chromatin (measured by nano-drop) were used for ChIP with rabbit polyclonal anti-C/EBPβ IgG antibody (Santa Cruz, clone C-19, sc-150x) and IgG control (Abcam). The next day, protein G Dynabeads (ThermoFisher 10004D) were washed 3 times with

PBS + 0.5% BSA. A Dynal magnet (Invitrogen) was used for Dynabeads washing and eluting steps. After washing, 50 uL of Dynabeads in PBS+BSA were added to each overnight tube containing the chromatin and antibody and rotated at 4 ˚C for 2 hours. IgG samples were placed on a magnet and 5% was removed for input. Samples were then washed in the following sequence: 1× with 1 mL of low-salt wash buffer [0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM tris-HCl (pH 8.1), and 150 mM NaCl], 1× with 1 ml of high-salt wash buffer [0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM tris-HCl (pH 8.1), and 500 mM NaCl], 1× with LiCl1 wash buffer [0.25 M LiCl, 1% IGEPAL CA-630, 1% deoxycholic acid (sodium salt), 1 mM EDTA, 10 mM tris (pH 8.1)], and 2× with 1-ml tris-EDTA (TE) (pH 8.0). DNA was eluted by resuspending the beads with 250 uL of nuclear lysis buffer supplemented with 3 ul of 20 mg/ml Proteinase K (Roche) and rotating the beads for 10 minutes at room temperature. The samples were digested and crosslinks reversed by incubating at 55 ˚C for 2 hours and 65 ˚C overnight. DNA was purified using the Qiagen MinElute PCR Purification Kit, and DNA was eluted in 200 uL EB buffer. Quantitative PCR was performed using the following ChIP primers: *Fizz1* promoter, AGTCTCTTGAACCACACCTCTTC (forward) and AGCACTTTCAGTACATTTTGGCC (reverse); *Arg1* enhancer, TTAGCCCAGCACCCTCAAC (forward) and GTGAGGCATTGTTCAGACTTCC (reverse).

Rheology

A PA or collagen hydrogel was cast between the rheometer base plate and 25 mm diameter parallel plate. Gels were swollen overnight and kept hydrated during testing. The shear modulus of the gel was measured using a strain amplitude sweep of 0.1-10% strain at a constant frequency rate (1 rad/s). The measured shear modulus remained constant over the specified strain range.

The elastic modulus was calculated using the shear modulus values, assuming that the gels were incompressible (Poisson ratio = 0.5).

Scanning Electron Microscopy (SEM)

Samples were fixed after swelling overnight in a 24 well plate. Hydrogels were then snap-frozen in liquid nitrogen and lyophilized overnight. Freeze-dried samples were sputter coated with palladium and imaged via SEM (Hitachi SU-70). Images were taken at 10 kV for pore quantification. The width of individual pores on the surface was analyzed from SEM images using ImageJ, quantifying the pore size as the distance between fibers in three dimensional space.[307]

Statistical analyses

We used unpaired t tests to determine statistical significance between groups ($p < 0.05$ was considered significantly different) using Prism. All data points are presented as Mean ± SD, unless specified. *$p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$. Statistical analyses for RNAseq data, including principal component analysis, were performed in R and are described in greater detail above.

Figures

Figures and schematics were generated using Microsoft PowerPoint, Adobe Illustrator, Apple Keynote, and Biorender (biorender.com).

Appendix 1: Protocol for culturing BMDM within 3D collagen gels

Gelation approach is adapted from Corning's "Alternate gelation procedure" that comes with their Type I collagen

Materials:

On ice:

1. Corning® Collagen I, High Concentration, Rat Tail (Product Number 354249)
2. Sterile 10x PBS
3. Sterile H2O
4. Sterile 1 N NaOH
5. 14 mL polystyrene round bottom tubes (label these and put them on ice before starting)

Other:

6. pH strips (EMD Millipore MColorpHast pH 5.0 – 10.0)
7. 48-well Non-TC plates
8. cRPMI (RPMI-1640 with supplements and 10% FBS)
9. Bone marrow derived macrophages (BMDM)

Protocol:

1. Calculate quantities of each reagent needed to make your stock of gel. If you have one type of BMDM, use the spreadsheet "Collagen gel calculations". If you have two types of BMDM, use the spreadsheet "Collagen gel calculations – WT vs. KO".
2. Lift BMDM with PBS/EDTA (at 4˚C or room temp), followed by PBS wash of plate to retrieve remaining macrophages.
3. Spin down BMDM in 50 mL conical.
4. Aspirate media and resuspend in 5 mL cRPMI.
5. Count BMDM (if you're doing this with 2 batches of cells, e.g. WT vs. KO, increase accuracy by using multiple aliquots to count because cell # may affect phenotypes; if you're using one batch of cells, exact cell # is less important).
6. Take desired cell number (see calculations) and spin again; resuspend in desired volume for final concentration e.g. 40 million cells/mL. (Or spin all BMDM and resuspend in appropriate volume for 40 million cells/mL). Place cells on ice.
7. Make gels in 14 mL polystyrene round bottom tubes, following protocol in "Collagen gel calculations" (including notes in italics). Remember to keep them on ice as much as possible.
8. After suspending cells in gels, use P1000 to plate 200 uL gel per well. Pipette slowly to avoid bubbles. First pipette up and down once in your tube to get rid of bubble at the tip. Then slowly distribute gel to wells, only going to the first stop to push gel out and going back into tube before lifting plunger again. I do low-collagen first, then high-collagen, so that high-collagen doesn't begin to solidify before I put it in the incubator.
9. Incubate gels for 45 min. (up to 1.5 hours is okay) at 37˚C, 5% CO2.

10. Score in a circle around edge of gels, 1 time per gel, using a P2 pipette. (Change tips as appropriate for switching between samples.)
11. Add 400 uL or 500 uL cRPMI, containing all relevant inhibitors and stimulations (e.g. 20 ng/mL IL-4, 100 ng/mL MCSF). Don't add stimulations after adding media because the gel changes its mechanical properties and I believe that penetration of proteins from the media decreases.
12. Score around edge of each gel one more time to ensure that gels are floating and not attached to the bottom of the plate, which can change the mechanical properties of the environment that cells are exposed to. (Change tips as appropriate for switching between samples.)
13. Place gels back in incubator, generally for 16-24 hours before harvesting (or other timing applicable for your experiment).

Appendix 2: qPCR primers

gene name	primer name	Sequence (5'->3')
Arg1	Arg1_F	CTGGTGTGGTGGCAGAGG
	Arg1_R	TGGCCAGAGATGCTTCCAAC
Mrc1	Mrc1_F	AAAGGGACGTTTCGGTGGAC
	Mrc1_R	CACTCCGGTTTTCATGGCAAC
Fizz1	Fizz1_F	GATGAAGACTACAACTTGTTCC
	Fizz1_R	AGGGATAGTTAGCTGGATTG
Rnase2a	Rnase2a_F	TCCACGGGAGCCACAAAG
	Rnase2a_R	GAGGCAAGCATTAGGACATGTC
Ear2	Ear2_F	TCCACGGGAGCCACAAAG
	Ear2_R	GAGGCAAGCATTAGGACAAGTC
Ym1	Ym1_F	CCCTACAATTAGTACTGGCCCAC
	Ym1_R	CCTCAGTGGCTCCTTCATTCAG
Fn1	Fn1_F	CAACCTCTGCAGACCTACCC
	Fn1_R	ACTGGATGGGGTGGGAATTG
Ccl24	Ccl24_F	AGCATCTGTCCCAAGGCAG
	Ccl24_R	TGTATGTGCCTCTGAACCCAC
Ctgf	Ctgf_F	AGGGCCTCTTCTGCGATTTC
	Ctgf_R	GACCCACCGAAGACACAGG
Acta2	Acta2_F	ATCACCATTGGAAACGAACGC
	Acta2_R	TAGGTGGTTTCGTGGATGCC
Col1a1	Col1a1_F	ACGAGATGGCATCCCTGGA
	Col1a1_R	GCCATAGGACATCTGGGAAGC
Talin1	Tln1_F	TCTACCATGGTGTACGACGC
	Tln1_R	GACAGAAAGAGCCCAAAGTCG
Rpl13a	Rpl13a.M_F	GAAGGAAAAGGCCAAGATGCAC
	Rpl13a.M_R	TGAGGACCTCTGTGAACTTGC

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