Molecular regulation of TLR signaling in health and disease: mechano-regulation of macrophages and TLR signaling

Erika J Gruber* and Cynthia A Leifer

Abstract

Immune cells encounter tissues with vastly different biochemical and physical characteristics. Much of the research emphasis has focused on the role of cytokines and chemokines in regulating immune cell function, but the role of the physical microenvironment has received considerably less attention. The tissue mechanics, or stiffness, of healthy tissues varies dramatically from soft adipose tissue and brain to stiff cartilage and bone. Tissue mechanics also change due to fibrosis and with diseases such as atherosclerosis or cancer. The process by which cells sense and respond to their physical microenvironment is called mechanotransduction. Here we review mechanotransduction in immunologically important diseases and how physical characteristics of tissues regulate immune cell function, with a specific emphasis on mechanoregulation of macrophages and TLR signaling.

Keywords

Innate immunity, macrophage, mechanosensing, mechanotransduction, stiffness, TLR signaling

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Introduction

Immune cells continuously patrol the body to rapidly detect and respond to endogenous and exogenous insults. Compared with other cell types, immune cells are extremely motile, able to undergo dramatic morphological changes during tissue transit, and capable of dynamic cell–cell interactions for Ag presentation and transmigration under static and circulatory flow conditions. A direct consequence of their motility is that immune cells encounter microenvironments that vary tremendously in terms of both physical and biochemical properties (Table 1). For over 40 yr, immunologists have studied biochemical signals, such as cytokines, to understand intercellular communication networks. Initial work in the 1970s and the cloning of IL-1 in 1984 paved the way for the subsequent development of numerous knockout mouse lines deficient in cytokines or their receptors that advanced our detailed understanding of these biochemical networks. For at least a decade, we have also known that the physical microenvironment can drive differentiation of mesenchymal stem cells, yet these studies on biophysical signaling have only recently been described for immune cells, and our understanding of the mechanisms and molecular players in immune mechanobiology are not well described.

Physical signals are converted into biochemical signals inside the cell and can directly induce the formation of focal adhesions, receptor microclustering, changes in the cytoskeleton, and alterations in gene expression and other signaling pathways. This review will introduce the concept of mechanobiology and the types of physical signals immune cells can receive, review what is known about mechanoregulation of macrophages, and focus specifically on more recent studies on mechanoregulation of Toll-like receptor (TLR) signaling.

*Erika J Gruber is currently affiliated to Department of Population Health and Pathobiology, North Carolina State University, Raleigh, NC, USA

Corresponding author:

Cynthia Leifer, Veterinary Medical Center, 930 Campus Road, Ithaca, NY 14853, USA. Email: Cynthia.leifer@cornell.edu
Variability in tissue mechanics and cellular mechanotransduction

Tissue stiffness is a well-studied mechanical signal. Expressed in pascals (Pa), stiffness is defined as the normal stress divided by the linear strain. Healthy tissues vary in stiffness from very soft adipose tissue and brain (a few hundred pascals) to stiff or rigid tissues, with bone representing the highest stiffness (>1 GPa). Even within one tissue, regional stiffness can vary dramatically. For example, detailed in vivo mapping of the biomechanical properties in mouse brain revealed that regional stiffness varies from <1 kPa to ∼3 kPa. Importantly, the mechanical properties of tissues constantly change; infection, chronic inflammation, damage, and remodeling can all increase or decrease stiffness (Table 2). Although largely determined by the biochemical composition and the physical structure and organization of the extracellular matrix (ECM), stiffness is also affected by a number of physical factors including interstitial fluid flow and hydrostatic pressure.

Cells survey their physical environment through mechanosensors that form attachments with the ECM or other cells and transmit signals to the cytoskeleton, which provides the shape and mechanical strength to cells. Actin and microtubule polymerization and depolymerization impart traction forces on a cell’s surroundings. During the process of mechanotransduction, tensional forces between the ECM and cytoskeleton are transformed into biochemical signaling pathways. For example, force applied across integrins that link ECM and cytoskeleton via focal adhesion complexes (FACs) opens the folded FAC protein, talin, to reveal cryptic binding sites for vinculin. Vinculin binding to talin, in turn, assembles a signaling complex that initiates phosphorylation signaling cascades, directs cytoskeletal changes, or activates transcriptional regulatory networks to modulate the transcriptional, and thus functional, profile of cells.

Table 1. Characteristics of tissue microenvironments that change and modulate immune cell function.

| Biochemical                  | Physical          |
|------------------------------|-------------------|
| Cytokines/chemokines         | Temperature       |
| Metabolites                  | Topology          |
| Microbial PAMPs              | Shear stress      |
| ECM composition              | Stiffness         |

Table 2. Examples of some systems and diseases associated with tissue stiffness changes.

| System          | Example                 |
|-----------------|-------------------------|
| Vasculature     | Atherosclerosis         |
| Lung            | Asthma                  |
| Global          | COPD                    |
| Skin            | Cancer                  |
| Adipose         | Scleroderma             |
| Immune system   | Hypertrophic scars      |
|                 | Obesity                 |
|                 | Virus infection          |
|                 | Bacterial phagocytosis   |
|                 | Autoimmune disease (SLE)|
|                 | Foreign body response    |

Tissue mechanics and macrophages in disease

During the natural inflammatory process in response to pathogens and non-infectious tissue damage, the ECM undergoes profound changes, a process that continues with remodeling during the reparative phase. ECM remodeling alters its biophysical properties (e.g. topology, stiffness, and structure) and biochemical composition (e.g. collagen, glycosaminoglycans, associated cytokines, and growth factors). Excessive changes to the ECM have direct effects on immune cells that can compromise tissue function in diseases such as atherosclerosis or cancer, response to foreign bodies, autoimmunity, and fibrosis.

Atherosclerosis is characterized by remodeling of the arterial wall ECM and accumulation of cholesterol-rich low-density lipoprotein (LDL). Arterial remodeling increases the physical stiffness of the artery, contributes to development of hypertension, and is a risk factor for cardiovascular disease. Macrophages contribute to this pathologic remodeling of the arterial wall, critical for LDL accumulation, and their presence and accumulation as foam cells are required for plaque formation in ApoE−/− mice on a high fat diet. In vitro modeling shows that increased sub-stratum stiffness reduces endothelial cell tight junction activity, suggesting stiffness-mediated increase in endothelial permeability as a possible mechanism for increased cholesterol entry to the vessel wall intima during plaque development. We observed that substratum mechanics regulate macrophage inflammatory potential as well as oxidized and acetylated LDL accumulation (unpublished observation, Gruber and Leifer). These in vitro observations are likely important in vivo since in the murine ApoE−/− model of atherosclerosis, arterial stiffness increases with age and arterial stiffening precedes development of hypertension in C57BL/6 mice high fat diet to induce obesity. Importantly, modulating tissue stiffness has been...
shown to be cardioprotective. For example, in hypercholesterolemic ApoE−/− mice, inhibition of lysyl oxidase, an enzyme that crosslinks collagen fibers to increase tensile strength and tissue stiffness, reduces arterial stiffening and plaque development, with no effect on serum cholesterol. While these studies show that biomechanics play a key role in disease pathogenesis of atherosclerosis and are a target for therapeutic development, we are just starting to understand the interplay between arterial mechanics and immune cells.

Changes in tissue mechanics are important during cancer development and metastasis. Indeed, breast cancer and other solid tumor cancers are frequently identified by detecting the difference in the mechanics of the tumor compared with the surrounding normal tissue. Tumors initiate dramatic remodeling of the interstitial ECM through a process known as the desmoplasic response, or desmoplasia. In breast cancer, tumor-associated ECM is enriched in collagen type I and is of increased stiffness compared with ECM from healthy breast tissue (160 kPa versus 3–5 kPa). Birefringence microscopy analysis of human breast cancer show that the invasive edges of breast cancer are notably more stiff. These areas also contained the highest number of macrophages. Importantly, studies in mice demonstrate that the increased stiffness drives development and pathogenesis of cancer. Breast cancer cells instruct cancer-associated fibroblasts to assemble the dense matrix and increase production of pro-angiogenic and tumor proliferative signals, which can be sequestered within the dense matrix material. Breast cancer cells also directly induce polarization of macrophages to an M2-like phenotype, which suggests there is a complex interplay between ECM changes, breast cancer cells, fibroblasts, and macrophages. As in atherosclerosis, modulating tissue remodeling enzymes to decrease stiffness reduces tumor growth and metastasis.

Implants induce foreign body immune responses that lead to fibrosis and increased tissue stiffness, and macrophages are essential to this process. The mechanical properties of the implants can influence the extent of these effects. For example, Moshayedi et al. fabricated composite gels that were stiff (30 kPa) on one end and soft (0.1 kPa) on the other end. Gels were implanted into rat brains, and after 3 wk, the gels and surrounding tissue were isolated and analyzed to measure cell association and markers of inflammation. There was a significant increase in the number of microglial cells (a brain specific macrophage) associated with the stiff side of the gel compared with the soft side of the gel. Astrocyte numbers and IL-1 cytokine were also increased near the stiff side of the gel. We have also shown that macrophages and dendritic cells are important in the foreign body response to biomaterials used for tissue regeneration. These studies demonstrate that stiffness directly correlates with foreign body inflammatory responses.

In idiopathic pulmonary fibrosis (IPF), chronic inflammatory responses increase tissue stiffness and progressively impair lung expansion and air exchange, which lead to death. Decellularized lung ECM from human patients with IPF is of higher stiffness (10 kPa versus 1 kPa), and contains increased glycosaminoglycan, latent TGF-β, collagen III, and collagen VI compared with decellularized ECM from healthy lung. Intratracheal instillation of bleomycin in mice is a preclinical model that recapitulates the key features of human IPF. In this model, inhibition of lysyl oxidase reduces both lung stiffening and disease severity. Macrophages drive the fibrotic response in IPF, thus are a potential therapeutic target to slow or halt progression of disease. Recent studies, outlined in the next section, show that the biochemical and physical changes in ECM regulate macrophage responses.

Mechano-regulation of macrophages

Macrophages are important for tissue inflammation and repair. Macrophages drive the ECM remodeling events that result in increased tissue stiffness, but there is a growing body of literature demonstrating that macrophages also respond to changes in tissue stiffness (Figure 1). From cell adhesion and morphology to migration to functional polarization and signal-\text{ing}, stiffness regulates macrophages.

Macrophages are known for their adhesiveness and “fried egg” morphology. Yet, this characteristic morphology is not observed in macrophages adhered to gels approximating physiologic stiffness. Unlike the spread morphology when attached to glass (>1 MPa),

![Figure 1. Insults leading to, and outcomes of, mechanoregulation of macrophages. A number of insults lead to changes in tissue stiffness, which in turn regulate aspects of macrophage biology. This figure shows many of the processes that are influenced by macrophage mechanosensing.](image-url)
rat alveolar macrophages have reduced spreading and cell area, increased height, and reduced adhesion when attached to 40 kPa polyacrylamide (PA) gels. Growth surface mechanics also regulate murine and human macrophage cell line (RAW264.7 and THP-1) attachment, cell spreading, and overall morphology. Macrophages on gels of 150 kPa, which mimics the stiffness of fibrotic tissues, are significantly larger and less round than those on softer gels mimicking normal muscle tissue. The differences in attachment and morphology likely contribute to the observation that human monocyte-derived macrophages migrate faster on stiff gels (280 kPa) compared with soft gels (1–5 kPa). For some cell types, migration velocity and distance are higher on stiff areas of gradient gels where the stiffness approximates diseased tissues, compared with softer areas of the same gradient gels. It is important to note that the stiffness of glass (>1 MPa) and even plastic is several orders of magnitude greater than physiologic stiffness (<150 kPa), so the observations made on these stiff surfaces likely do not reflect adhesive, morphologic, and migratory characteristics of in vivo macrophages.

Phagocytosis and cell migration depend on the ability of a cell to engage a physical surface and exert force. Therefore, stiffness of the cytoskeleton and plasma membrane are crucial for these processes. During formation of the phagocytic cup, the membrane spreads, which increases membrane tension. LPS stimulation of macrophages also increases membrane rigidity, and the importance of rigidity in macrophage plasma membrane for response to LPS was postulated over 30 yr ago when Vogel et al. noted that a number of LPS-sensitizing agents were associated with changes in the properties of macrophage plasma and lysosomal membranes. Plasma membrane stiffness is determined by the composition of the lipid bilayer, cytoskeleton stiffness, and interactions of the transmembrane proteins linking the cytoskeleton to the ECM. There are conflicting reports on whether macrophages on high stiffness surfaces, thus those with increased cytoskeletal stiffness, have enhanced or similar phagocytic capacity as those on physiologic stiffness surfaces. Patel et al. found that macrophages on high stiffness surfaces exhibited increased phagocytosis of IgG opsonized and unopsonized latex beads by both murine macrophages and human alveolar macrophages, and of bacteria by murine macrophages. In contrast, two additional groups reported no difference in particle or Escherichia coli uptake. We also observed no difference in phagocytosis of beads, Gram-positive or Gram-negative bacteria, or zymosan particles in unstimulated murine macrophages attached to different stiffness substrata (unpublished observation, Gruber and Leifer). Both IFN-γ and LPS stimulation increase macrophage membrane stiffness, and cells stimulated with these cytokines demonstrate enhanced particle and bacterial phagocytosis. Although the full mechanism underlying mechanoregulation of macrophage phagocytosis remains unclear, both contractile forces and the calcium channel transient receptor potential (TRP) vanillin 4 (TRPV4) have been implicated in LPS response and LPS-induced phagocytosis.

In three-dimensional (3D) culture, stiffness of the ECM-based gels determined the mechanism of macrophage migration. Dense Matrigel or collagen 3D gels encouraged mesenchymal mode migration while loose fibrillar collagen gels that contain larger interfiber spaces promoted amoeboid mode migration. Care should be taken when interpreting studies conducted in three dimensions since there are many physical characteristics that vary, including porosity, that directly regulate macrophage functional polarization independently of stiffness. Interestingly, similar gel and tissue ECM architecture also regulates T cell migratory modes, suggesting that ECM mechanics likely play a major role in immune cell migration. Human monocyte-derived macrophages plated in 3D matrix accumulated podosome markers such as talin, vinculin, and actin, as well as proteolytic enzymes at protrusions that enabled the cells to migrate through the collagen matrix. In two-dimensional (2D) cultures, macrophage attachment to high stiffness surfaces increased internal cytoskeletal stiffness, suggesting that tissue and growth microenvironment mechanics control these processes.

Surface mechanics and topology regulate other macrophage functions such as polarization. Murine bone marrow-derived macrophages induced to an M1-like phenotype adopt an amorphous shape while those induced to an M2-like phenotype are elongated. Furthermore, placing unpolarized macrophages into elongated micropatterned trenches induced an M2-like phenotype. THP-1 cell line macrophages had lower inflammatory M1-like activity (lower TNF-α) and adopted a more M2-like phenotype (higher CD206 expression) on softer gels. This physical topology-driven macrophage polarization was dependent on actin cytoskeleton contractility since inhibition of ROCK and MLCK eliminated the shape-induced phenotypic changes. Similarly, Chen et al. showed that RAW264.7 macrophages on growth surfaces imprinted with parallel gratings 250 nm–2 μm wide were more elongated than those on planar controls. Macrophages on these substrates were slightly less inflammatory, with decreased secretion of TNF-α and VEGF compared with controls. These data suggest that cell shape and growth substrate stiffness play key roles in macrophage functional activity.
Other physical stimuli that influence macrophage activity include flow and temperature. Interstitial flow (~3 μm/s) placed across 3D collagen type I gels induced mouse bone marrow-derived macrophages to adopt a more M2-like phenotype. The macrophages up-regulated Arg1, TGFβ, CD206, CD163, and transglutaminase 2 (TGM2), but expression of the M1 markers CD86, TNFα, and iNOS were not affected. Migration speed and directionality through 3D culture were also enhanced under flow via a β1 integrin- and Stat6-dependent pathway. Housing BALB/c mice at elevated external temperature enhanced TNF-α response to LPS challenge. Furthermore, those mice then exhibited reduced LPS tolerance upon second challenge. Higher ambient temperature for housing mice that better represented wild mouse external environmental temperature led to reduced monocye trafficking and reduced atherosclerosis in a model of disease. In humans, seasonal temperature variation correlated with the number of circulating monocytes— but not neutrophils or lymphocytes. Importantly, macrophages are not the only immune cells regulated by microenvironmental mechanics. For more information on mechanobiology of specific immune cells the reader is referred to other reviews on T cells and B cells, dendritic cells, and on neutrophils. Further on mechanobiology of specific immune cells the reader is referred to other reviews on T cells and B cells.

**Mechanoregulation of TLRs**

Inflammation is initiated by innate immune receptors such as TLRs, yet there is a paucity of data on the mechanoregulation of TLR signaling. Here we will review the regulation of TLRs and discuss what is known about mechanoregulation of TLRs.

The general regulatory mechanisms governing TLRs are well-described. For example, localization and trafficking of both the receptor and ligand are key steps in regulating signaling. TLR9 is primarily localized in the endoplasmic reticulum (ER), traffics from the ER to the Golgi compartment, and is sorted to endosomes with the help of chaperone-like proteins, such as UNC93B1 and gp96. Once in endosomes, TLR9 is proteolytically processed in multiple independent ways. A C-terminally tagged TLR9 is proteolyzed in the unstructured hinge region separating leucine rich repeats (LRRs) 1–14 and LRRs 15–29. This proteolytic event results in a fragment (LRR15 to the C-terminus, termed p80, or the mature form) proposed to be the functional form of the receptor plus an N-terminal fragment of approximately 68 kDa (N-ter).

The reported differences in mechanoregulation of TLR9 signaling appear to be due to a number of factors. Several groups report using different growth surface stiffnesses for their studies, which could influence interpretations. In one case, TNF-α production by RAW264.7 was lower on intermediate stiffness (20 kPa) compared with soft (1 kPa) PA gels. In contrast to the previous study’s findings with TLR4 stimulation, we found that IL-10 secretion in response to LPS is largely the same when equilibrated to stiffnesses ranging from 0.3 kPa to 1 kPa and lower when equilibrated to gels of 8 kPa and 25 kPa. In contrast, they showed secretion of anti-inflammatory IL-10 is maximal in macrophages on 25 kPa gels compared with 8 kPa or 1 kPa gels. We recently reported that activation of TLR4 by LPS and TLR9 by CpG DNA in murine bone marrow-derived macrophages induces secretion of TNF-α that is lower when equilibrated to stiff (20 kPa and 150 kPa) compared with soft (1 kPa) PA gels. In contrast to the previous study’s findings with TLR4 stimulation, we found that IL-10 secretion in response to stimulation of TLR9 is similar to that of TNF-α. Previtera et al. reported that, in bone marrow derived macrophages, TLR4 stimulation in response to LPS is largely the same when equilibrated to stiffnesses ranging from 0.3 to 120 kPa. In contrast to the findings from other groups, these investigators observed that secretion ofTNF-α, IL-6, IL-1β, and NO are all increased on the stiffest substrate, 230 kPa.

If one does not account for these differences, artificially

signaling and able to inhibit signaling through the full-length receptor. We identified an independent proteolysis site near the transmembrane domain (between AA7 24 and 735) of *endogenously* expressed TLR9 that generates a soluble, negative regulatory, form of the ecto-domain. Other nucleic acid-sensing TLRs are similarly proteolytically cleaved. Furthermore, some pathogens secrete proteases capable of cleaving TLRs. Thus, proteolytic cleavage occurs and is important, but the full extent of the role in health and disease of the immune system remains unclear.
high cytokine production could be measured from macrophages on stiffer surfaces simply because there are more cells attached. Further studies are needed to clarify the role of mechano-regulation in TLR signaling.

The mechanisms of mechano-regulation of TLR signaling are also not fully understood. FAC proteins and downstream signaling molecules, including integrins and rho associated kinases (ROCK and cdc42), have been implicated in the regulation of TLR signaling. In astrocytes, rho proteins negatively regulate TLR2-3, TLR3-, and TLR4-induced expression of IL-1β, IL-6, and TNF-α. Murine bone marrow-derived macrophages and RAW264.7 macrophages plated on stiff glass express more ROCK1 and have more phosphorylated ROCK1 than macrophages on gels. Furthermore, inhibition of ROCK1/2 increases LPS-induced TNF-α production, which is due, in part, to enhanced magnitude and duration of p38, ERK, and NFκB phosphorylation. The regulatory role of ROCK1/2 on TLR4-mediated cytokine secretion depends on the strength of the TLR4 stimulus. At lower concentrations of LPS, inhibition of ROCK1/2 does not augment response. Supraphysiologic doses of LPS (10 μg/mL) in corneal epithelial cells requires ROCK1/2 for maximal TNF-α release. A different study using human alveolar macrophages and RAW264.7 macrophages showed that cells on high stiffness (150 kPa) gels have more polymerized actin and that attachment of macrophages to these substrates leads to an early increase in cdc42 activity that returns to baseline by 3 h. Inhibition of actin using cytochalasin D or latrunculin A, or inhibition of WASP with wiskostatin, augments LPS-induced TNF-α production.

Integrins are major mechanosensors, yet the data on whether TLR signaling is inhibited or augmented by integrin activation are not conclusive. Integrins are heterodimeric transmembrane proteins composed of an α and β subunit that physically link the actin cytoskeleton to other cells or to the ECM, change conformation in response to tension and endogenous signals, and transduce signals through proteins such as focal adhesion kinase (FAK) and ROCK. Vertebrates encode 18 integrin complexes. Several studies have shown that integrins negatively regulate TLR signaling, suggesting that mechanotransduction inhibits TLR signaling. For example, in the absence of all β2 integrins, macrophages were hyper-responsive to CpG DNA (TLR9), Pam3Cyk4 (TLR2), and LPS (TLR4) due to enhanced NF-κB activation. Absence of the β2 binding partner αM integrin, also known as CD11b, in mice exacerbated response to LPS, poly(I:C) (TLR3), and CpG DNA for production of multiple inflammatory cytokines. Acute engagement of αM integrin activated Syk and Src kinases, which in turn promoted degradation of MyD88 and TRIF and reduced TLR signaling. Another study demonstrated an integrin-mediated, IL-10-dependent, indirect regulation of TLR signaling through up-regulation of several negative regulatory proteins such as A20 and SOCS3.

In contrast, other studies have shown that integrins are required for TLR signaling. For example, αM integrin-deficient macrophages had reduced IL-6 production in response to LPS, suggesting that integrin signaling was required for TLR4 signaling. Integrons were also required for TLR2 signaling through direct interaction with TLR2. FAK, a key downstream mediator in integrin signaling, was necessary for optimal TLR2-, TLR3-, TLR4-, and TLR9-induced motility in RAW264.7 cells.

These studies all investigated β2 integrins, which are restricted to hematopoietic cells and primarily mediate cell-cell interactions, but are less important for the cell-ECM interactions. In the absence of cell-ECM integrins such as β1 and β3, TLR signaling in B cells was generally reduced. TLR9 signaling was the exception, and was actually enhanced in the absence of αvβ3. Important, because global or cell-specific deficiency of one integrin leads to compensatory increased expression of other integrins, all of these experiments must be interpreted with caution. Further studies are needed to determine whether integrins are major mediators of mechano-regulation of TLR signaling.

Several other mechanosensors, including mechano-sensitive members of the TRP family, may be important for TLR regulation. Scheraga et al. showed that peritoneal macrophages from TRPV4-deficient mice had a significantly lower response to LPS than wild type macrophages. In contrast, Alpizar et al. reported that in airway epithelial cells from TRPV4-deficient mice, mRNA expression of IL-6 and the chemokine CXCL-1 in response to LPS was increased compared with wild type controls. This correlated with increased neutrophil and macrophage infiltration in the airways of TRPV4-deficient mice. Another mechano-sensitive member of the TRP family, TRPM7, has recently been implicated in regulating TLR4 signaling and internalization/trafficking. Macrophages deficient in TRPM7 had reduced IL-1β secretion, reduced induction of genes in response to LPS, and prolonged retention of TLR4 at the cell surface. This led to an overall reduction in activation of both NF-κB and IRF3, and protection from LPS challenge in vivo.

Regardless of whether macrophage mechano-signals are transduced via integrins or other mechano-sensors, they play an important role in various disease processes and are potential therapeutic targets. For example, as discussed above, atherosclerosis and
Conclusions and future directions

The recent advances in bioengineering and materials science have provided the basis to study the role of mechanotransduction in the regulation of cell function. We now know that mechanotransduction regulates function of numerous immune cells, including macrophages, as well as key biochemical signaling pathways, including TLR signaling. This newfound recognition may have numerous implications for our understanding of immunity in many chronic diseases, including atherosclerosis and cancer. Yet, considerable gaps in our understanding still exist. For instance, how exactly does the composition and function of focal adhesions in the highly motile immune cells differ from those of the better-characterized FACs of sessile fibroblasts and endothelial cells? What is the precise mechanism by which Rho/ROCK and cdc42 signaling pathways regulate TLR signaling? What role do the other hundreds of focal adhesion proteins play in regulating TLR signaling or immune cell function, in general? Do immune cells integrate mechanical signals from multiple forces (i.e. tension, compression, shear, flow), and what are the underlying mechanisms? Are macrophage functions altered by differences in physical properties of the tissue in vivo? Can we chemically augment or inhibit mechanotransduction pathways to modulate immune cell function therapeutically?

Studies in mechanoimmunology also raise questions about the usefulness of conducting experiments in 2D cultures on tissue culture plastic or glass with stiffnesses that several orders of magnitude higher than those experienced by cells in vivo. We have gained tremendous insight into biochemical signaling pathways through these traditional techniques; yet it is now known that mechanotransduction pathways regulate multiple biochemical signaling pathways and functional phenotype in immune cells. We must consider the potential impact that mechanical properties of traditional cell cultures have had on our conclusions. It is likely that many of the observed differences in findings between in vitro and in vivo studies are at least partly due to the vastly different physical environmental conditions and subsequent mechanosignaling. Advanced techniques in tissue culture that more closely mimic the biomechanical properties of tissues are important to bridge this gap.

In vivo studies have been vital to our understanding of the complexity of biochemical signaling pathways, and it is tempting to look toward these models to study biomechanical signaling. At this time, significant challenges exist to using in vivo approaches. First, we have only a cursory understanding of the major molecular mechanotransduction players in immune cells. Considerable effort must first be made to identify candidate proteins and pathways prior to attempting in vivo experimentation. An additional consideration is that, given the importance of these mechanotransduction pathways during development, generation of full knockout models has been hindered by embryonic lethality (FAK, ROCK2). Attempts to change the stiffness of tissue in wild type mice (e.g. bleomycin-induced mouse model of pulmonary fibrosis) inevitably alter the biochemical composition of the ECM and induce an intense inflammatory response, thus making it impossible to specifically isolate the effects of mechanical cues from biochemical cues. Inhibition of collagen crosslinking by lysyl oxidase with the drug, β-aminopropionitrile, has offered some clues to the role of stiffness in the pathogenesis of disease (e.g. atherosclerosis); however, treatment alters tissue stiffness diffusely throughout the body, complicating interpretation. To our knowledge, no in vivo models have been developed that allow investigators to reliably and reproducibly isolate stiffness as a single controllable variable. Thus, the careful interrogation of potential pathways using a variety of in vitro approaches will be critical to developing a comprehensive understanding of the major players prior to moving to in vivo work.

In conclusion, after decades of careful investigation of the biochemical basis of immune cell function, we are now at the precipice of an entirely new avenue of discovery as we begin to interrogate the role of mechanical signals in regulating immunity. Detailed investigations into mechanotransduction pathways may offer novel approaches to target and modulate immune cell function in vivo. These studies could have implications in management of auto-immune diseases, infectious disease, or chronic diseases such as atherosclerosis or cancer.

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ORCID iD
Cynthia A Leifer http://orcid.org/0000-0003-0987-9747

References
1. Auron PE, Webb AC, Rosenwasser LJ, et al. Nucleotide sequence of human monocyte interleukin 1 precursor cDNA. Proc Natl Acad Sci USA 1984; 81: 7907–7911.
2. Engler AJ, Sen S, Sweeney HL, et al. Matrix elasticity directs stem cell lineage specification. Cell 2006; 126: 677–689.
3. Janmey PA and Miller RT. Mechanisms of mechanical signaling in development and disease. J Cell Sci 2011; 124: 9–18.
4. Rho JY, Ashman RB and Turner CH. Young’s modulus of trabecular and cortical bone material: ultrasonic and microtensile measurements. J Biomech 1993; 26: 111–119.
5. Antonovaite N, Beekmans SV, Hol EM, et al. Regional variations in stiffness in live mouse brain tissue determined by depth-controlled indentation mapping. Sci Rep 2018; 8: 12517.
6. Huynh J, Nishimura N, Rana K, et al. Age-related intimal stiffening enhances endothelial permeability and leukocyte transmigration. Sci Transl Med 2011; 3: 112ra122.
7. Wells RG. Tissue mechanics and fibrosis. Biochim Biophys Acta Mol Basis Dis 2013; 1832: 884–890.
8. Chen Y, Ju L, Rushdi M, et al. Activation of a vinculin-binding site in the talin rod involves rearrangement of a five-helix bundle. EMBO J 2004; 23: 2942–2951.
9. Del Rio A, Perez-Jimenez R, Liu R, et al. Stretching single talin rod molecules activates vinculin binding. Science 2009; 323: 638–641.
10. Carisey A and Ballestrem C. Vinculin, an adapter protein in control of cell adhesion signalling. Eur J Cell Biol 2011; 90: 157–163.
11. Cox TR and Erler JT. Remodeling and homeostasis of the extracellular matrix: implications for fibrotic diseases and cancer. Dis Model Mech 2011; 4: 165–178.
12. Chaudhuri O, Koshy ST, Branco da Cunha C, et al. Extracellular matrix stiffness and composition jointly regulate the induction of malignant phenotypes in mammary epithelium. Nat Mater 2014; 13: 970–978.
13. Ueha S, Shand FHW and Matsushima K. Cellular and molecular mechanisms of chronic inflammation-associated organ fibrosis. Front Immunol 2012; 3: 71.
14. Gotschy A, Bauer E, Schrotz C, et al. Local arterial stiffening assessed by MRI precedes atherosclerotic plaque formation. Circ Cardiovasc Imaging 2013; 6: 916–923.
15. Weisbrod RM, Shiang T, Al Sayah L, et al. Arterial stiffening precedes systolic hypertension in diet-induced obesity. Hypertension 2013; 62: 1105–1110.
16. Felton CV, Crook D, Davies MJ, et al. Relation of plaque lipid composition and morphology to the stability of human aortic plaques. Arterioscler Thromb Vasc Biol 1997; 17: 1337–1345.
17. Rajavashisth T, Qiao JH, Tripathi S, et al. Heterozygous osteopetrotic (op) mutation reduces atherosclerosis in LDL-receptor-deficient mice. J Clin Invest 1998; 101: 2702–2710.
18. Smith JD, Trogan E, Ginsberg M, et al. Decreased atherosclerosis in mice deficient in both macrophage colony-stimulating factor (op) and apolipoprotein E. Proc Natl Acad Sci USA 1995; 92: 8264–8268.
19. Lusis AJ. Atherosclerosis. Nature 2000; 407: 233–241.
20. Gordon S and Mantovani A. Diversity and plasticity of mononuclear phagocytes. Eur J Immunol 2011; 41: 2470–2472.
21. Woolard KJ and Geissmann F. Monocytes in atherosclerosis: subsets and functions. Nat Rev Cardiol 2010; 7: 77–86.
22. Kothapalli D, Liu SL, Bae YH, et al. Cardiovascular protection by ApoE and ApoE-HDL linked to suppression of ECM gene expression and arterial stiffening. Cell Rep 2012; 2: 1259–1271.
23. Robertson C. The extracellular matrix in breast cancer predicts prognosis through composition, splicing, and crosslinking. Exp Cell Res 2016; 343: 73–81.
24. McCormack VA and dos Santos Silva I. Breast density and parenchymal patterns as markers of breast cancer risk: a meta-analysis. Cancer Epidemiol Biomarkers Prev 2006; 15: 1159–1169.
25. Lu P, Weaver VM and Werb Z. The extracellular matrix: a dynamic niche in cancer progression. J Cell Biol 2012; 196: 395–406.
26. Levental KR, Yu H, Kass L, et al. Matrix crosslinking forces tumor progression by enhancing integrin signaling. Cell 2009; 139: 891–906.
27. McKnight AL, Kugel JL, Rossman PJ, et al. MR elastography of breast cancer: preliminary results. J Am Roentgenol 2002; 178: 1411–1417.
28. Acerbi I, Cassereau L, Dean I, et al. Human breast cancer invasion and aggression correlates with ECM stiffness and immune cell infiltration. Integr Biol 2015; 7: 1120–1134.
29. Paszek MJ, Zahir N, Johnson KR, et al. Tensional stress induces fibronectin-collagen interactions. Matrix Biol 2017; 60: 241–254.
30. Wang K, Wu F, Seo BR, et al. Breast cancer cells alter the dynamics of stromal fibronectin-collagen interactions. Matrix Biol 2017; 60: 86–95.
31. Hollmen M, Roudniicky F, Karaman S, et al. Characterization of macrophage-cancer cell crosstalk
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in estrogen receptor positive and triple-negative breast cancer. Sci Rep 2015; 5: 9188.
34. Butcher DT, Alliston T and Weaver VM. A tense situation: forcing tumour progression. Nat Rev Cancer 2009; 9: 108–122.
35. Paszek MJ and Weaver VM. The tension mounts: mechanics meets morphogenesis and malignancy. J Mammary Gland Biol Neoplasia 2004; 9: 325–342.
36. Anderson JM, Rodriguez A and Chang DT. Foreign body reaction to biomaterials. Semin Immunol 2008; 20: 86–100.
37. Doloff JC, Veiseh O, Vegas AJ, et al. Colony stimulating factor-1 receptor is a central component of the foreign body response to biomaterial implants in rodents and non-human primates. Nat Mater 2017; 16: 671–680.
38. Moshayedi P, Ng G, Kwok JC, et al. The relationship between gliad cell mechanosensitivity and foreign body reactions in the central nervous system. Biomaterials 2014; 35: 3919–3925.
39. Londono R, Dziki JL, Haljasmaa E, et al. The effect of cell debris within biologic scaffolds upon the macrophage response. J Biomed Mater Res A 2017; 105: 2109–2118.
40. Potuck AN, Weed BL, Leifer CA, et al. Electrostatically self-assembled biodegradable microparticles from pseudoproteins and polysaccharide: fabrication, characterization, and biological properties. Biomacromolecules 2015; 16: 564–577.
41. Leifer CA. Dendritic cells in host response to biologic scaffolds. Semin Immunol 2017; 29: 41–48.
42. Tilbury K, Hocker J, Wen BL, et al. Second harmonic generation microscopy analysis of extracellular matrix changes in human idiopathic pulmonary fibrosis. J Biomed Opt 2014; 19: 086014.
43. Th annickal VJ, Henke CA, Horowitz JC, et al. Matrix biology of idiopathic pulmonary fibrosis: a workshop report of the national heart, lung, and blood institute. Am J Pathol 2014; 184: 1643–1651.
44. Jenkins RG, Simpson JK, Saini G, et al. Longitudinal change in collagen degradation biomarkers in idiopathic pulmonary fibrosis: an analysis from the prospective, multicentre PRO File study. Lancet Respir Med 2015; 3: 462–472.
45. Booth AJ, Hadley R, Cornett AM, et al. Acellular normal and fibrotic human lung matrices as a culture system for in vitro investigation. Am J Respir Crit Care Med 2012; 186: 866–876.
46. Adamson IY and Bowden DH. The pathogenesis of bleomycin-induced pulmonary fibrosis in mice. Am J Pathol 1974; 77: 185–197.
47. Liu T, De Los Santos FG and Phan SH. The bleomycin model of pulmonary fibrosis. Methods Mol Biol 2017; 1627: 27–42.
48. Cheng T, Liu Q, Zhang R, et al. Lysyl oxidase promotes bleomycin-induced lung fibrosis through modulating inflammation. J Mol Cell Biol 2014; 6: 506–515.
49. Adlerz KM, Aranda-Espinoza H and Hayenga HN. Substrate elasticity regulates the behavior of human monocyte-derived macrophages. Eur Biophys J 2016; 45: 301–309.
50. Blakney AK, Swartzlander MD and Bryant SJ. The effects of substrate stiffness on the in vitro activation of macrophages and in vivo host response to poly(ethylene glycol)-based hydrogels. J Biomater Res A 2012; 100: 1375–1386.
51. Fereol S, Fodil R, Labat B, et al. Sensitivity of alveolar macrophages to substrate mechanical and adhesive properties. Cell Motil Cytoskeleton 2006; 63: 321–340.
52. Irwin EF, Saha K, Rosenbluth M, et al. Modulus-dependent macrophage adhesion and behavior. J Biomat Sci Polym Ed 2008; 19: 1363–1382.
53. Leporatti S, Gerth A, Kohler G, et al. Elasticity and adhesion of resting and lipopolysaccharide-stimulated macrophages. FEBS Lett 2006; 580: 450–454.
54. Okamoto T, Takagi Y, Kawamoto E, et al. Reduced substrate stiffness promotes M2-like macrophage activation and enhances peroxisome proliferator-activated receptor gamma expression. Exp Cell Res 2018; 367: 264–273.
55. Patel NR, Bole M, Chen C, et al. Cell elasticity determines macrophage function. PLoS One 2012; 7: e41024.
56. Scheraga RG, Abraham S, Niese KA, et al. TRPV4 mechanosensitive ion channel regulates lipopolysaccharide-stimulated macrophage phagocytosis. J Immunol 2016; 196: 428–436.
57. Fereol S, Fodil R, Laurent VM, et al. Prestress and adhesion site dynamics control cell sensitivity to extracellular stiffness. Biophys J 2009; 96: 2009–2022.
58. Lee HS, Stachelek SJ, Tomczyk N, et al. Correlating macrophage morphology and cytokine production resulting from biomaterial contact. J Biomed Mater Res A 2013; 101: 203–212.
59. Vincent LG, Choi YS, Alonso-Latorre B, et al. Mesenchymal stem cell durotaxis depends on substrate stiffness gradient strength. Biotechnol J 2013; 8: 472–484.
60. Lachowski D, Cortes E, Pink D, et al. Substrate rigidity controls activation and durotaxis in pancreatic stellate cells. Sci Rep 2017; 7: 2506.
61. Masters TA, Pontes B, Viasnoff V, et al. Plasma membrane tension orchestrates membrane trafficking, cytoskeletal remodeling, and biochemical signaling during phagocytosis. Proc Natl Acad Sci USA 2013; 110: 11875–11880.
62. Vogel SN, English KE and O’Brien AD. Silica enhancement of murine endotoxin sensitivity. Infect Immun 1982; 38: 681–685.
63. Souza ST, Agra LC, Santos CE, et al. Macrophage adhesion on fibronectin evokes an increase in the elastic property of the cell membrane and cytoskeleton: an atomic force microscopy study. Eur Biophys J 2014; 43: 573–579.
64. Van Goethem E, Poincloux R, Gauffre F, et al. Matrix architecture dictates three-dimensional migration modes of human macrophages: differential involvement of proteases and podosome-like structures. J Immunol 2010; 184: 1049–1061.
65. Van Goethem E, Guiet R, Balor S, et al. Macrophage podosomes go 3D. *Eur J Cell Biol* 2011; 90: 224–236.

66. Garg K, Pullen NA, Oskeritzian CA, et al. Macrophage functional polarization (M1/M2) in response to varying fiber and pore dimensions of electrospun scaffolds. *Biomaterials* 2013; 34: 4439–4451.

67. Overstreet MG, Gaylo A, Angermann BR, et al. Inflammation-induced interstitial migration of effector CD4(+) T cells is dependent on integrin alphaV. *Nat Immunol* 2013; 14: 949–958.

68. McWhorter FY, Wang T, Nguyen P, et al. Modulation of macrophage phenotype by cell shape. *Proc Natl Acad Sci USA* 2013; 110: 17253–17258.

69. Chen S, Jones JA, Xu Y, et al. Characterization of topographical effects on macrophage behavior in a foreign body response model. *Biomaterials* 2010; 31: 3479–3491.

70. Li R, Serrano JC, Xing H, et al. Interstitial flow promotes macrophage polarization toward an M2 phenotype. *Mol Biol Cell* 2018; 29: 1927–1940.

71. Lee CT, Zhong L, Mace TA, et al. Elevation in body temperature to fever range enhances and prolongs subsequent responsiveness of macrophages to endotoxin challenge. *PloS One* 2012; 7: e30077.

72. Williams JW, Elvington A, Ivanov S, et al. Thermoneutrality but not UCP1 deficiency suppresses monocyte mobilization into blood. *Circ Res* 2017; 121: 662–676.

73. Upadhyaya A. Mechanosensing in the immune response. *Semin Cell Dev Biol* 2017; 71: 137–145.

74. Huse M. Mechanical forces in the immune system. *Nat Rev Immunol* 2017; 17: 679–690.

75. Mennens SFB, Bolomini-Vittori M, Weiden J, et al. Substrate stiffness influences phenotype and function of human antigen-presenting dendritic cells. *Sci Rep* 2017; 17: 15711.

76. Ekpenyong AE, Toepfner N, Chilvers ER, et al. Mechanotransduction in neutrophil activation and deactivation. *Biochim Biophys Acta* 2015; 1853: 3105–3116.

77. Leifer CA and Medvedev AE. Molecular mechanisms of regulation of Toll-like receptor signaling. *J Leukoc Biol* 2016; 100: 927–941.

78. Leifer CA, Rose WA, 2nd and Botelho F. Traditional biochemical assays for studying Toll-like receptor 9. *J Immunossay Immunochrom* 2013; 34: 1–15.

79. Barton GM and Kagan JC. A cell biological view of Toll-like receptor function: regulation through compartmentalization. *Nat Rev Immunol* 2009; 9: 535–542.

80. Leifer CA, Brooks JC, Hoelzer K, et al. Cytoplasmic targeting motifs control localization of Toll-like receptor 9. *J Biol Chem* 2006; 281: 35585–35592.

81. Brooks JC, Sun W, Chiosis G, et al. Heat shock protein gp96 regulates Toll-like receptor 9 proteolytic processing and conformational stability. *Biochem Biophys Res Commun* 2012; 421: 780–784.

82. Chockalingam A, Rose WA 2nd, Hasan M, et al. Cutting edge: a TLR9 cytoplasmic tyrosine motif is selectively required for proinflammatory cytokine production. *J Immunol* 2012; 188: 527–530.

83. Latz E, Schoenemeyer A, Visintin A, et al. TLR9 signals after translocating from the ER to CpG DNA in the lysosome. *Nat Immunol* 2004; 5: 190–198.

84. Leifer CA, Kennedy MN, Mazzoni A, et al. TLR9 is localized in the endoplasmic reticulum prior to stimulation. *J Immunol* 2004; 173: 1179–1183.

85. Chockalingam A, Brooks JC, Cameron JL, et al. TLR9 traffics through the Golgi complex to localize to endolysosomes and respond to CpG DNA. *Immunol Cell Biol* 2009; 87: 209–217.

86. Barton GM, Kagan JC and Medzhitov R. Intracellular localization of Toll-like receptor 9 prevents recognition of self DNA but facilitates access to viral DNA. *Nat Immunol* 2006; 7: 49–56.

87. Brinkmann MM, Spooner E, Hoebe K, et al. The interaction between the ER membrane protein UNC93B and TLR3, 7, and 9 is crucial for TLR signaling. *J Cell Biol* 2007; 177: 265–275.

88. Kim YM, Brinkmann MM, Paquet ME, et al. UNC93B1 delivers nucleotide-sensing toll-like receptors to endolysosomes. *Nature* 2008; 452: 234–238.

89. Ewald SE, Engel A, Lee J, et al. Nucleic acid recognition by Toll-like receptors is coupled to stepwise processing by cathepsins and asparagine endopeptidase. *J Exp Med* 2011; 208: 643–651.

90. Ewald SE, Lee BL, Lau L, et al. The ectodomain of Toll-like receptor 9 is cleaved to generate a functional receptor. *Nature* 2008; 456: 658–662.

91. Park B, Brinkmann MM, Spooner E, et al. Proteolytic cleavage in an endolysosomal compartment is required for activation of Toll-like receptor 9. *Nat Immunol* 2008; 9: 1407–1414.

92. Fukui R, Yamamoto C, Matsumoto F, et al. Cleavage of Toll-like receptor 9 ectodomain is required for in vivo responses to single strand DNA. *Front Immunol* 2018; 9: 1491.

93. Sepulveda FE, Maschalidi S, Colisson R, et al. Critical role for asparagine endopeptidase in endocytic Toll-like receptor signaling in dendritic cells. *Immunity* 2009; 31: 737–748.

94. Sinha SS, Cameron J, Brooks JC, et al. Complex negative regulation of Toll-like receptor 9 ectodomain is required for in vivo responsiveness to single strand DNA. *J Immunol* 2016; 197: 1343–1352.

95. Onji M, Kanno A, Saitho S, et al. An essential role for the N-terminal fragment of Toll-like receptor 9 in DNA sensing. *Nat Commun* 2013; 4: 1949.

96. Lee S, Kang D, Ra EA, et al. Negative self-regulation of TLR9 signaling by its N-terminal proteolytic cleavage product. *J Immunol* 2014; 193: 3726–3735.

97. Chockalingam A, Cameron JL, Brooks JC, et al. Negative regulation of signaling by a soluble form of Toll-like receptor 9. *Eur J Immunol* 2011; 41: 2176–2184.

98. de Zoete MR, Bouwman LI, Keestra AM, et al. Cleavage and activation of a Toll-like receptor by microbial proteases. *Proc Natl Acad Sci USA* 2011; 108: 4968–4973.
TLR4 activity in macrophages. *PLoS One* 2015; 10: e0145813.

100. Borysiewicz E, Fil D and Konat GW. Rho proteins are negative regulators of TLR2, TLR3, and TLR4 signaling in astrocytes. *J Neurosci Res* 2009; 87: 1565–1572.

101. Gong J, Guan L, Tian P, et al. Rho kinase type 1 (ROCK1) promotes lipopolysaccharide-induced inflammation in corneal epithelial cells by activating Toll-like receptor 4 (TLR4)-mediated signaling. *Med Sci Monitor* 2018; 24: 3514–3523.

102. Campbell ID and Humphries MJ. Integrin structure, activation, and interactions. *Cold Spring Harbor Perspect Biol* 2011; 3: a004994.

103. Yee NK and Hamerman JA. β2 integrins inhibit TLR responses by regulating NF-κB pathway and p38 MAPK activation. *Eur J Immunol* 2013; 43: 779–792.

104. Han C, Jin J, Xu S, et al. Integrin CD11b negatively regulates TLR-triggered inflammatory responses by activating Syk and promoting degradation of MyD88 and TRIF via Cbl-b. *Nat Immunol* 2010; 11: 734–742.

105. Wang L, Gordon RA, Huynh L, et al. Indirect inhibition of Toll-like receptor and type I interferon responses by ITAM-coupled receptors and integrins. *Immunity* 2010; 32: 518–530.

106. Ling GS, Bennett J, Woollard KJ, et al. Integrin CD11b positively regulates TLR4-induced signalling pathways in dendritic cells but not in macrophages. *Nat Commun* 2014; 5: 3039.

107. Gerold G, Abu Ajaj K, Bienert M, et al. A Toll-like receptor 2-integrin beta3 complex senses bacterial lipopeptides via vitronectin. *Nat Immunol* 2008; 9: 761–768.

108. Gianni T and Campadelli-Fiume G. The epithelial alphavbeta3-integrin boosts the MYD88-dependent TLR2 signaling in response to viral and bacterial components. *PLoS Pathog* 2014; 10: e1004477.

109. Maa MC, Chang MY, Li J, et al. The iNOS/Src/FAK axis is critical in Toll-like receptor-mediated cell motility in macrophages. *Biochim Biophys Acta* 2011; 1813: 136–147.

110. Acharya M, Sokolovska A, Tam JM, et al. αx Integrins combine with LC3 and atg5 to regulate Toll-like receptor signalling in B cells. *Nat Commun* 2016; 7: 10917.

111. Lerman YV, Lim K, Hyun YM, et al. Sepsis lethality via exacerbated tissue infiltration and TLR-induced cytokine production by neutrophils is integrin alpha3-beta1-dependent. *Blood* 2014; 124: 3515–3523.

112. Alpizar YA, Boonen B, Sanchez A, et al. TRPV4 activation triggers protective responses to bacterial lipopolysaccharides in airway epithelial cells. *Nat Commun* 2017; 8: 1059.

113. Liu YS, Liu YA, Huang CJ, et al. Mechanosensitive TRPM7 mediates shear stress and modulates osteogenic differentiation of mesenchymal stromal cells through Osterix pathway. *Sci Rep* 2015; 5: 16522.

114. Numata T, Shimizu T and Okada Y. Direct mechanostress sensitivity of TRPM7 channel. *Cell Physiol Biochem* 2007; 19: 1–8.

115. Schilling T, Miralles F and Eder C. TRPM7 regulates proliferation and polarisation of macrophages. *J Cell Sci* 2014; 127: 4561–4566.

116. Schappe MS, Szteyn K, Stremiska ME, et al. Chanzyme TRPM7 mediates the Ca2+ influx essential for lipopolysaccharide-induced Toll-like receptor 4 endocytosis and macrophage activation. *Immunity* 2018; 48: 59–74.

117. Huang L, Xu H and Peng G. TLR-mediated metabolic reprogramming in the tumor microenvironment: potential novel strategies for cancer immunotherapy. *Cell Mol Immunol* 2018; 15: 428–437.

118. Karampitsakos T, Woolard T, Bouras D, et al. Toll-like receptors in the pathogenesis of pulmonary fibrosis. *Eur J Pharmacol* 2017; 808: 35–43.

119. Li K, Qu S, Chen X, et al. Promising targets for cancer immunotherapy: TLRs, RLRs, and STING-mediated innate immune pathways. *Int J Mol Sci* 2017; 18: 404.

120. Roshan MH, Tambo A and Pace NP. The role of TLR2, TLR4, and TLR9 in the pathogenesis of atherosclerosis. *Int J Inflam* 2016; 2016: 1532832.

121. Thumkeo D, Keel J, Ishizaki T, et al. Targeted disruption of the mouse rho-associated kinase 2 gene results in intrauterine growth retardation and fetal death. *Mol Cell Biol* 2003; 23: 5043–5055. 2003/07/02.

122. Moeller A, Ask K, Warburton D, et al. The bleomycin animal model: a useful tool to investigate treatment options for idiopathic pulmonary fibrosis? *Int J Biochem Cell Biol* 2008; 40: 362–382.