Matrix-enabled mechanobiological modulation of osteoimmunology

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Summary

Modulation of osteoimmunology is central to recovering the functions of defective and diseased bone. Here we highlight the materials-enabled matrix roles played in regulating the osteoimmunological phenomena, such as inflammation, bone hemostasis, and regeneration. We give an overview of the concept of osteoimmunology and matrix-induced mechanobiology, and detail the effort to exploit materials with tunable parameters (ligands, stiffness, viscoelasticity, nano-/micro-topology, and three-dimensionality) to instruct those events, and discuss the mechanotransduction process and mechanisms underlying the matrix-related interactions between immune/inflammatory cells and bone forming/resorbing cells. The current communication will help understand the matrix-related mechanobiological modulation of osteoimmunology and develop materials with tunable matrix properties for bone healing and regeneration.

Keywords: Matrix properties; Mechanobiology; Osteoimmunology; Cell-matrix interactions; Bone

Progress and Potential

Osteoimmunology has become a key agenda in bone healing and regeneration. Extracellular matrix regulates the communication between immune/inflammatory cells and bone forming/resorbing cells. Materials implanted in defective bone also play decisive roles in the repair process, by modulating the cellular biophysical interactions. Our discussion here highlights the importance of matrix roles played in such inter-cellular events in a mechanobiological context, thus helping researchers to understand the matrix-induced mechanisms in more detial, and guide how to develop materials with tunable matrix properties for bone regeneration. The concepts and strategies disseminated here are not limited to bone but can be applicable to other tissues, where immune/inflammation and homeostasis/regeneration events are critically involved and central to their repair.

Introduction

Bone injuries and diseases, including traumatic fractures, tumor-dissected tissue loss, osteomyelitis and osteoporosis, constitute a challenging medical area, particularly in the elderly.[1–4] For example, seven million bone fractures are reported in United States and approximately 10% of the fractures fail to heal properly, imposing significant healthcare burdens.[5] Bone healing involves highly intricate events such as immune/inflammation, angiogenesis, tissue differentiation and remodeling, whereby diverse cell types interplay[6]. Of note, immune and inflammatory responses prevail in many traumatic bone injuries and in elderly patients who suffer from osteoporosis or diabetes.[7–9]

Materials have taken a central part in replacing or augmenting such dysfunctional bone tissues. Implants for hip and tooth replacement or 3-dimensional (3D) scaffolds for bone grafting are representative examples that have been clinically available and highly successful over the last decades.[10] Viscoelastic hydrogels and nanostructured 3D scaffolds have more recently been designed to fine-tune the responses of cells in regeneration events.[2,11] The use of those materials is thus considered to offer appropriate extracellular matrix (ECM) conditions and ultimately to recapitulate the microenvironment suitable for the regenerative cascade of cells. Therefore, the physico-mechano-chemical properties of materials can be key players in dictating the regenerating events of cells and the clinical outcomes.

Indeed, a wealth of evidence has shown the important role of artificial or native matrices in dictating diverse behaviors of cells, such as adhesion, migration and differentiation. For example, nano-grooved matrices activates the adhesion of osteoblasts and guides their elongation and migration[12] , the viscoelastic mechanical property of hydrogels influences the osteogenic differentiation of mesenchymal stem cells (MSCs)[2], and the surface-tethered ligands alter the secretome profile of MSCs[13]. Although the significance of matrices in cell fate control has been examined, the studies on matrix-related phenomena have mainly focused on bone forming events related with MSCs, osteoblasts, or osteocytes.

Bone regeneration, however, is a process mediated through dynamic interactions of bone forming cells with immune/inflammatory cells[6], i.e., the interplay between immune and skeletal systems regulates bone healing and homeostasis. As such, the biological roles of matrix should be interpreted in this immune-skeletal axis. Understanding this can further guide how to develop materials, i.e., offering design principles to coordinate both immune and skeletal cells toward successful bone regeneration while minimizing detrimental inflammatory pathways that would lead to bone impairment. Recent findings reveal discrepancies between *in vitro* osteogenesis in biomaterials and *in vivo* bone regeneration outcomes, implying the essential role of immune/inflammatory cells in the materials-activated osteogenesis[14].

Materials properties (*e.g.,* stiffness, nanotopology, chemistry) have recently been identified and shown to shape the responses of immune/inflammatory cells, such as T cells, B cells, neutrophils, and macrophages of different origins[15–18]. When considering bone and its surrounding microenvironment (i.e., bone marrow and blood), the physico-mechanical properties vary dynamically depending on the anatomical site. For instance, stiffness scales over a wide range; blood (fluid-like), bone marrow (0.1~10 kPa), pre-mineralized collagen (10~30 kPa), and mineralized bone (10~30 GPa)[3]. Thus, immune/inflammatory cells taking part in bone repair can sense the stiffness dynamics during the processes of attachment, dissemination, migration, and differentiation, whereby they transduce the extracellular physical sensation to intracellular biochemical signalings, so called ‘mechanotransduction’ [19].

Thus, our motivation here is to understand the matrix-enabled bone healing process within the context of osteoimmunology, which will be key to identify the mechanisms underlying the healing events and then to develop materials helpful for bone regeneration through modulating immune/inflammation (as outlined in **Fig. 1**). We start with an overview of the concept of osteoimmunology and matrix-induced mechanobiology, then detail the *in vitro* and *in vivo* phenomena of matrix-modulated immune/inflammation process, and then discuss the implications in bone repair with a view to future perspectives.

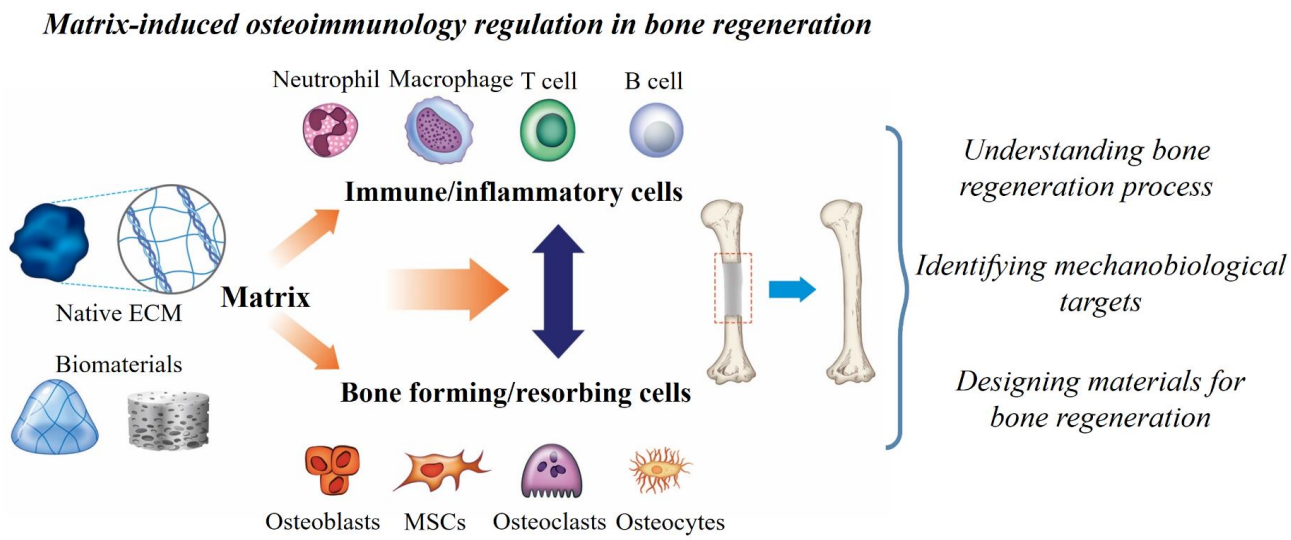

Fig. 1. Matrix-modulation of osteoimmunology in bone regeneration. Native ECM or engineered biomaterials are key to determine the interactions between immune/inflammatory cells and bone forming/resorbing cells. This helps to understand the matrix-enabled bone regeneration process, to identify the mechanobiological mechanisms and therapeutic targets underlying the events, and to guide how to design materials for bone regeneration by immune/inflammation modulation.

Overview: osteoimmunology and matrix-induced mechanobiology

Osteoimmunology: communication between immune/inflammatory cells and bone forming/absorbing cells in bone damage and regeneration

Bone matrix is composed of highly mineralized collagenous proteins produced by bone forming osteoblasts and is continuously remodeled by the counteraction of bone resorbing osteoclasts[20]. Bone marrow, a semi-solid tissue enclosed inside the bone, is the primary production site of many stem cells, such as MSCs and haematopoietic stem cells (HSCs), and immune cells, whereby MSCs turn into osteoblasts and a portion of monocytes from haematopoietic origin differentiates into osteoclasts. Other groups of HSCs have potential to become components of blood (red blood cells, platelets), or a wide variety of immune cells, including neutrophils, monocytes, T cells, and B cells.

Due to their anatomical proximity, the immune cells and bone cells interplay by secreting paracrine molecules (as illustrated in **Fig. 2**). Discovered as early as in 1972 was interleukin-1 (IL-1), which is secreted from immune cells and can activate osteoclasts[21]. Since then, a number of regulatory molecules, including cytokines, receptors, and transcription factors have been identified from immune cells that regulate osteoblast or osteoclast behavior. Exemplar findings are receptor activator of nuclear factor-κB (NF-κB) ligand (RANKL) as a key ligand for inducing differentiation of osteoclasts, not only expressed by osteoblasts but also by activated T cells[22], and the identification of key cytokines released by activated T-cells that are promoting (IL-6 and TNF-α)[23–25] or inhibiting (IFN-γ, IL-4, IL-10, GM-CSF, IL-12, and IL-18) osteoclasts bone resorbing functions[21,22,26–37]. Moreover, disruption of immunomodulatory molecules, such as SHP1 and NFATc1 results in mitigation of bone volume by osteoblasts deactivation[38]. **Table 1** lists the secretory molecules which mediate the interplay between immune cells and bone forming/resorbing cells. As evidenced, the cells from immune and skeletal systems crosstalk mutually, highlighting the importance of understanding osteoimmunological phenomena in bone regeneration.

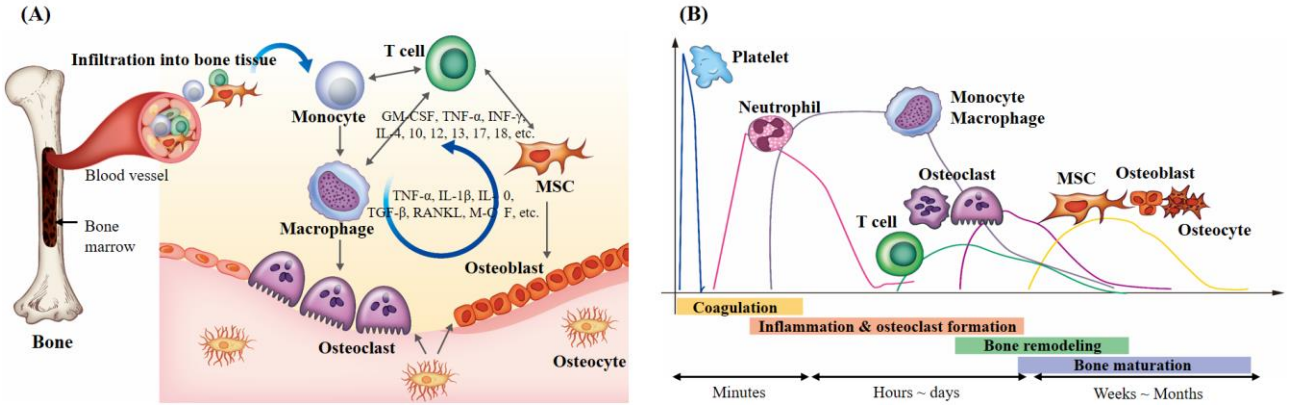

Fig. 2. Crosstalk between immune cells and bone forming/resorbing cells during bone healing and the regeneration process. (A) Key immune cells and bone forming/resorbing cells involved in bone healing and regeneration process. Stem/progenitor cells from bone marrow are infiltrated to bone tissue, which differentiate and are highly interactive by secreting a number of paracrine molecules. (B) These cells are activated in the sequential events (coagulation, inflammation and osteoclast formation, and bone remodeling and maturation) in bone healing and regeneration process. Temporal regulation of such serial osteoimmunological events would be key to determining the success of bone regeneration.

Table 1. Summarizing the secretory molecules that mediate the interplay between immune cells and bone cells.

Cytokine	Originates from	Target cells	Effect on OC/OB	Reference
IL-1	leukocytes	osteoclast precursors, dendritic cells	OC inhibitory	Horton *et al.*[21], Guo *et al.*[39], Hengartner *et al.*[40]
		osteoblasts	OB inhibitory	
RANKL	activated T cells, osteoblasts	osteoclast precursors, dendritic cells	OC activating	Anderson *et al.*[26], Lacey *et al.*[27], Wong *et al.*[28], Yasuda *et al.*[29]
		osteoblasts	OB activating	
IL-6	activated T cells (Th2), DCs	T cells, osteoclasts	OC activating	De La Mata *et al.*[23], Peruzzi *et al.*[41]
		osteoblasts	ambiguous on OB	
TNF-a	activated T cells (Th1), macrophages	osteoclast precursors, mesenchymal stem cells	OC inhibitory	Cenci *et al.*[24], Pfeilschifter *et al.*[25], Gilbert *et al.*[42]
		osteoblasts	OB activating	
IFN-g	activated T cells (Th1), NK cells, osteoblasts	osteoclast precursor, macrophages, NK cells	OC activating	Takayanagi *et al.*[22], Maruhashi *et al.*[43]
		osteoblasts	OB inhibitory	

IL-4	activated T cells (Th2), NK cells	osteoclast precursor, T cells, B cells	OC inhibitory	Kasono *et al.*[30], Abu-Amer *et al.*[31], Riancho *et al.*[44]
		osteoblasts	OB inhibitory	
IL-10	activated T cells (Th2)	osteoclast precursors	OC activating	Hong *et al*.[32], Chen *et al.*[45], Dresner-Pollack *et al.*[46]
		osteoblasts	OB inhibitory	
GM-CSF	activated T cells (Th1)	osteoclast precursors	inhibitory	Miyamoto *et al*.[33]
IL-12	macrophages	T cells	OC inhibitory	Horwood *et al.*[34], Yamada *et al*.[35], Zhang et al.[47]
		osteoblasts	OB inhibitory	
IL-17	activated T cells (Th17), memory T cells	osteoclast precursors, primary osteoblasts	OC activating	Kotake *et al.*[36], Croes *et al.*[48], HJ Kim *et al.*[49], YG Kim *et al*[50].
		osteoblasts	ambiguous on OB	
IL-18	macrophages and DCs	T cells	activating	Udagawa *et al*.[37], Cornish *et al.*[51]
		osteoblasts	OB inhibitory	

RANKL, receptor activator of nuclear factor-κB ligand; TNF-α, tumor necrosis factor-α; IFN, interferon; GM-CSF, granulocyte-macrophage colony stimulating factor; DCs, dendritic cells; NK cells, natural killer cells; OC, Osteoclast or osteoclast precurosr; OG, osteogenic cells including msenchymal stem cells and osteoblast

Matrix-cues and the cellular mechanotransduction: From extracellular space to nucleus

Native ECM and engineered materials have a wide spectrum of physico-chemico-mechanical properties (as described in **Fig. 3A**). Among others, the surface biochemistry (e.g., ligand type and density), stiffness (or rigidity), and nano/microscale topography, have proven relatively well understood matrix cues; i) type and density of surface ligand can define the spatial site for cellular mechano-recognition, ii) stiffness, as a typical tissue-derived ECM property, confers mechano-adaptation forces to cells, and iii) nano-/micro-topography regulates molecular-level mechano-perception by cell receptors. More recently, dynamic properties of ECM, involving the change in mechanics and chemistry over time, such as viscoelasticity (e.g., stress relaxation) and degradation, are considered important[2,52–54].

Cells sense these cues provided by ECM and materials, through membrane receptors, such as integrins and stretch-activated ion channels[19]. Such mechanosensed extracellular cues are then transmitted to intracellular space through mechanosensitive mechanisms, such as focal adhesion molecules (e.g., talin, vinculin, paxillin, etc.) and actin filaments, and the signals are further propagated into the nucleus through a linker of the nucleoskeleton and cytoskeleton (LINC) complex and lamin A/C[19] (as illustrated in **Fig. 3B,C**). In this way, the matrix cues can shape the expression profile of genes. Such a process that cells convert extracellular physico-chemical cues to intracellular biochemical signalings is called ‘mechanotransduction’, which underlines the importance of matrix properties in dictating diverse cell behaviors, such as adhesion, spreading, migration,

and differentiation.

Details on the cellular mechanotransduction can be referenced from some key review articles[19,55–57]. Central to this are the representative mechanosensitive machineries and the biochemical signaling processes mediated mainly by kinases, such as Rho-associated kinases (ROCK), Myosin light chain kinase (MLCK), focal adhesion kinases (FAK), mitogen activated protein kinases (MAPK)/extracellular signal-regulated kinase (ERK). These mechanosensitive mechanisms and signaling pathways identified in many different types of cells are also heavily involved in the cells related with bone repair and regeneration, such as osteoblasts, osteoclasts, osteocytes, MSCs, and immune/inflammatory cells.

Instead of detailing the mechanosensitive machineries and the related signalings, here we briefly comment on some emerging aspects in this field. Among others, how the matrix properties can regulate the expression of genes within the nucleus that (de)activate various cell responses would be worth discussing. Recent findings underscore the epigenetic modifications of the chromatin structure by the matrix cues[58,59], i.e., matrix cues increase the chromatin accessibility of target genes by epigenetic modifications, such as histone acetylation or (de)methylation. This is considered possible either by i) the biophysical forces transmitted directly through the actin-myosin and LINC molecules, or ii) the biochemical signaling molecules which are nuclear-transported through nuclear pores; the observations of less condensed chromatin structure (thus a higher number of genes activated) under extracellular forces, possibly demonstrate the former physical contribution to nuclear mechanics[59,60], while for the latter case, one recent study evidenced the opening of nuclear pores under conditions of a stiffer matrix where cells flatten more, which allows higher nuclear transport of key mechanosensitive transcription factor YAP that possibly activates target genes[61] (as illustrated in **Fig. 3D**).

Despite the importance of extracellular effects on nuclear mechanics, studies have centered primarily on 2D matrix conditions and with limited extracellular cues (stiffness or external forces), thus, studies on 3D environments and with other matrix cues, particularly dynamically changing matrix properties, such as stress relaxation and degradation over time (as shown in Fig. 3A) need to be explored in the future. For instance, the MSCs encapsulated in highly stress-relaxing 3D gels experience more mechanically-dynamic responses, such as actomyosin contractility, leading to enhanced osteogenesis, and it is of note that this could override the effect of initial stiffness of gels[62]. Moreover, the MSC-mediated degradation of the matrix alters the composition, rebuilding the ligand type over time, which ultimately influences the spatiotemporal cell behavior, including osteogenic differentiation[63,64].

Overall, investigating how these dynamic matrix cues influence the cellular nuclear mechanics in 3D conditions will help us to interpret the *in vivo* phenomena more accurately. Furthermore, such studies need to be conducted with immune/inflammatory cells, in consideration of their interactions with bone cells, which will deepen our understanding of the matrix-related bone regeneration mechanisms. Given that the effects of matrix cues on bone forming cells (osteoblasts and MSCs) were relatively well studied and reviewed[65–67], we discuss in the following sections with a focus on the matrix-induced immune/inflammatory cell responses and their interplay with bone forming cells, and the implications in bone healing and regeneration. While our discussion is mainly based on *in vitro* cell behavior, a particular emphasis is also given to some of the recent *in vivo* findings.

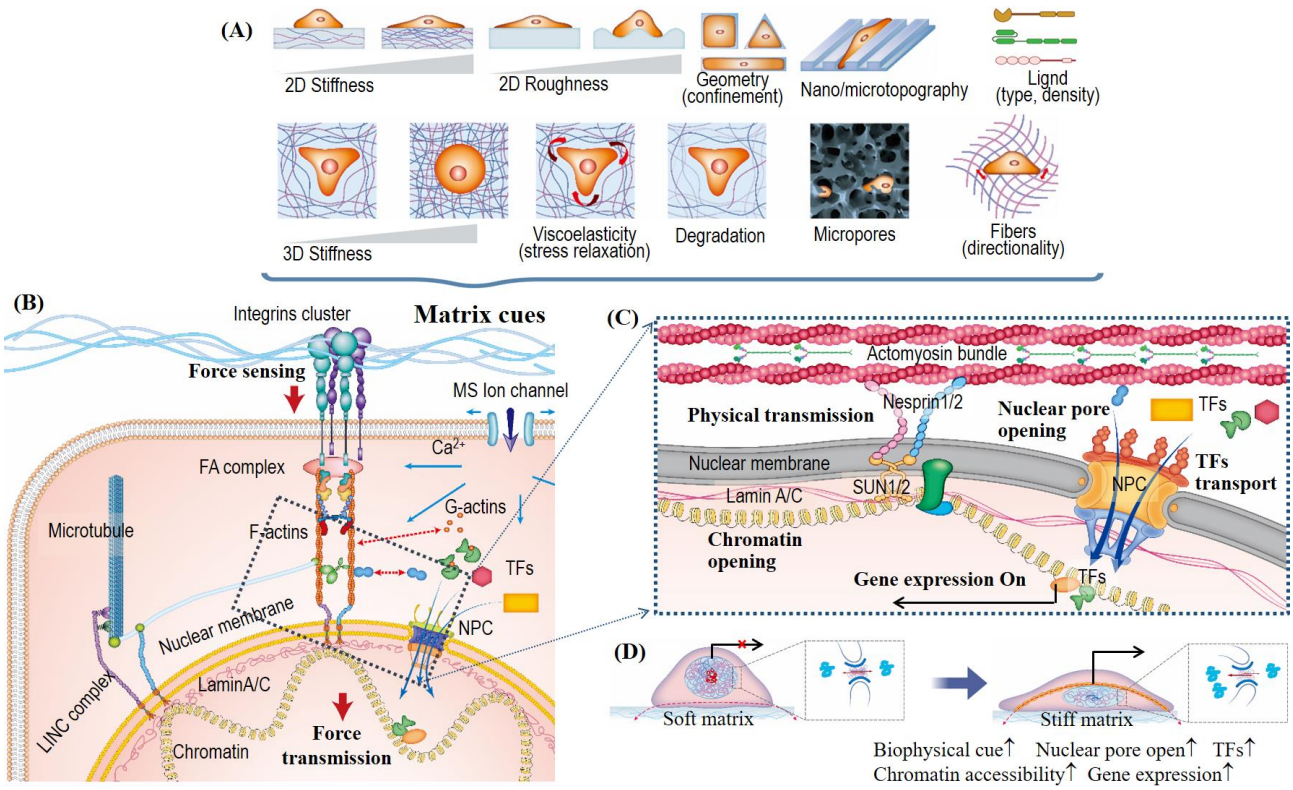

Fig. 3. Matrix cues and the cellular mechanosensing and intracellular signalings. (A) Representative matrix cues are indicated, such as 2D stiffness, roughness, confinement, nano/microtopology, and ligand type and density, and the 3D matrix dynamic properties, such as viscoelasticity (stress relaxation) and degradation, and 3D structures, such as micropores and fiber dimensionality, are also highlighted. (B) Cellular mechanotransduction process, whereby cells sense the extracellular physico-chemical cues, and then convert them to intracellular biochemical signals through various mechanosensitive machineries (e.g., integrins, mechano-activated (MS) ion channels, focal adhesion (FA) complex, actin-myosin, LINC complex, and lamin A/C), and the inset is highlighted in (C) to show mechano-signalings from cytosol to nucleus. Mechano-signalings to nucleus are possible by either biophysical force transmission directly through the actomyosin bundle to LINC complex (nesproin1/2, SUN1/2) to LaminA/C, or nuclear transport of biochemical transcription factors (TFs) through nuclear pores. These mechano-signalings alter the chromatin structure and activate gene expressions. (D) Typical example of mechano-signaling through a nuclear pore opening process. Matrix stiffening increases TFs transport to nucleus, enhancing chromatin accessibility and target gene expressions. Illustration in (D) is recreated from reference[61].

Effects of matrix cues on the behaviors of immune/inflammatory cells

Matrix stiffness

Stiffness (or rigidity) of a solid matrix defines the degree of stress-to-strain, thus the matrix with higher stiffness is more difficult to deform. Cells involved in osteoimmunology experience a wide range of stiffness values across the tissues of bone and its surrounding microenvironment (bone marrow and blood); blood (fluid), bone marrow (0.1~100 kPa, depending on region), pre-mineralized collagen (10~30 kPa), cartilage (~1500 kPa), and mineralized bone (10~30 MPa) (as presented in **Fig. 4**). For example, upon injury and inflammation in bone tissue, different types of immune/inflammatory cells in a blood stream escape the blood vessels to migrate toward the injured site, during which cells experience diverse tissues, such as blood vessels, basement membrane, stromal connective tissue, and calcified bone, and thus the corresponding various stiffness levels. Furthermore, the migrated cells (or their differentiated ones) experience altered ECM stiffness of implanted biomaterials with various types (from soft polymer gels to hard ceramic/metallic implants). For these reasons, investigations of the stiffness effects on immune/inflammatory cells are highly relevant to clinical situations.

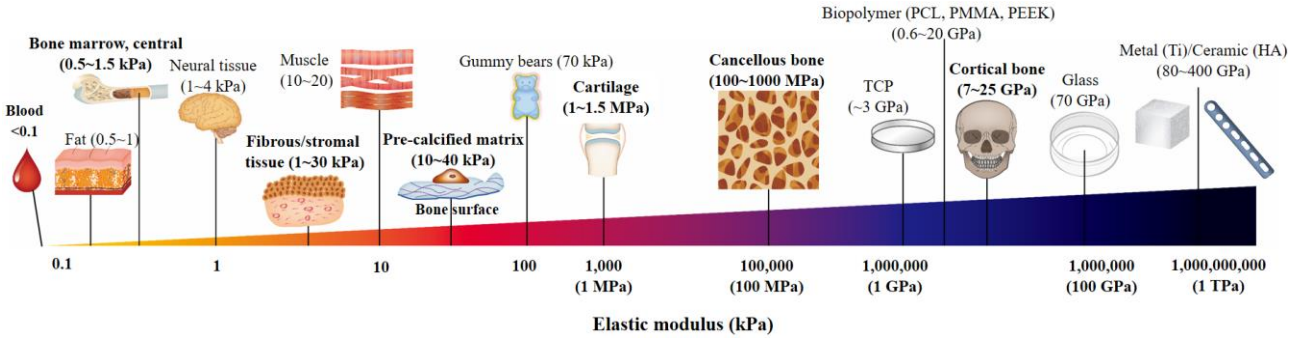

Fig. 4. Stiffness of tissues related with osteoimmunology. Bone marrow (~1.5 kPa), pre-mineralized collagen (~40 kPa), cartilage (~1500 kPa), and mineralized bone (~1GPa). Elastic modulus is a physical parameter representing how stiff the matrix is. Values of other tissues and bone implantable materials are also shown for comparison. Polystyrene or glass substrate typically used for cell culture are extremely stiff (1-100 GPa).

As such, the effects of matrix stiffness have been extensively studied *in vitro* using 2D model biomaterials, such as polymer gels and micropillars. Polymer gels, such as polyacrylamide (PAA) and polydimethylsiloxane (PDMS) gels, have been extensively used to provide a wide range of stiffness levels; for instance, from highly soft (~0.1-1 kPa) to rigid (~100-1000 kPa) matrix, which was mainly tuned by the matrix density and crosslinking degree [refs]. Due to their lack of cell binding motifs, the gel surfaces can be modified with RGD peptide, gelatin or collagen. On the other hand, the micropillars, typically made of PDMS, are flexible 2D microarray of pillars, upon which cells sense the underlying matrix stiffness, thus the stiffness is controlled by the intrinsic modulus or the height of pillars [refs]. Although these 2D *in vitro* platforms have been extensively used to interpret the mechanics of diverse types of cells [refs], studies on immune/inflammatory cells, such as neutrophils, macrophages, and T cells, have more recently been carried out (as outlined in **Fig. 5**).

Neutrophils, as the first line of defense in acute inflammation, were proven to be stiffness-sensitive. Similar to other anchorage-dependent cells, they express integrins (mainly β2 subtype) to adhere to the matrix surface[68] and have a unique actin-related pathogen trapping system, called neutrophil-extracellular-trap-osis (NETosis), which kills extracellular pathogens while minimizing damage to host cells[69]. During this process, actins dynamically polymerize and depolymerize to shrink the nucleus and release DNA[70,71]. Observations with various 2D gel matrices, such PAA and PDMS gels, revealed that an increase in stiffness activated this NETosis behavior[15,72] and the release of pro-inflammatory cytokines, where phosphatidylinositol 3-kinase (PI3K) signaling is heavily involved[73].

Macrophages, as a key player in inflamed tissues, were also shown to be highly activated upon stiffer matrices. Soft gels reduced pro-inflammatory signs in macrophages, such as a decrease of TNF-α secretion and NF-kB signalling, while concurrently increasing anti-inflammatory IL-10 secretion when compared with stiff glass, whereby mechano-activation, including actin polymerization, actomyosin contractility, and YAP nuclear-shuttling, were significantly reduced[16]. As a result, the macrophages cultured on soft gels polarized less toward M1, indicating less pro-inflammatory phenotype change with softening of the matrix. The stiffness-dependent polarization (M1 or M2) of macrophages was also correlated with the mechanosensitive ion channel Piezo1[74]; upon encountering a stiff matrix, macrophages polarized more to M1 with higher Piezo1 expression and intracellular Ca^{2+} level.

T cells, as one of the most characteristic cell types in adaptive immunity, are actively fluxed towards injured and diseased tissues. Given their importance in immunotherapies, researchers paid special attention to their *ex vivo* culture and expansion[75]. The matrix stiffness is thus considered important to find optimal T cell culture conditions. In fact, T cells can sense the matrix stiffness through not only conventional integrin receptors but also immune receptors, i.e., T cell receptors (TCRs), and are activated distinctively on different levels of matrix stiffness[17]. A couple of studies have recently reported the activation of T cells was altered when expanded upon engineered gels with varying stiffness[76,77]. However, the outcomes seem to be inconsistent across the

reports. For example, with increasing stiffness of PDMS gels (within a range of 100~2300 kPa) coated with antigens (CD3 and CD28), the primary human CD4+ and CD8+ T cell functions, such as proliferation, metabolism, and cytokine secretion, were decreased[78]; whereas, in another report of using PAA gels with the same antigen coatings, a stiff gel (100 kPa) enhanced the T cell migration and spreading, and the production of cytokines, such as TNF-α and IFN-γ, compared with soft gels (0.5 or 6.4 kPa)[79]; likewise, increasing stiffness of PAA gels (in a range of 7.1, 9.3, and 50.5 kPa) enhanced IL-2 secretion[80]. In some cases, the stiffness response was biphasic, i.e., T cell proliferation and IL-2 secretion were found to peak at an intermediate stiffness (25 kPa) rather than at 5 or 110 kPa[18]. Such a discrepancy in stiffness-dependent and T cell activation might be attributed to the different density of TCR activating antibodies or ligand composition, which needs further investigation to clarify. Clearly, T cells sense the matrix cues, especially stiffness, and adopt them in their immunological synapse by TCR[81,82], through activated mechanosensitive machineries, such as actomyosin-mediated traction forces and YAP nuclear-translocation[83]. The effects of matrix stiffness on the *in vitro* behaviors of various types of cells involved in osteoimmunology (except bone cells) are summarized in **Table 2**.

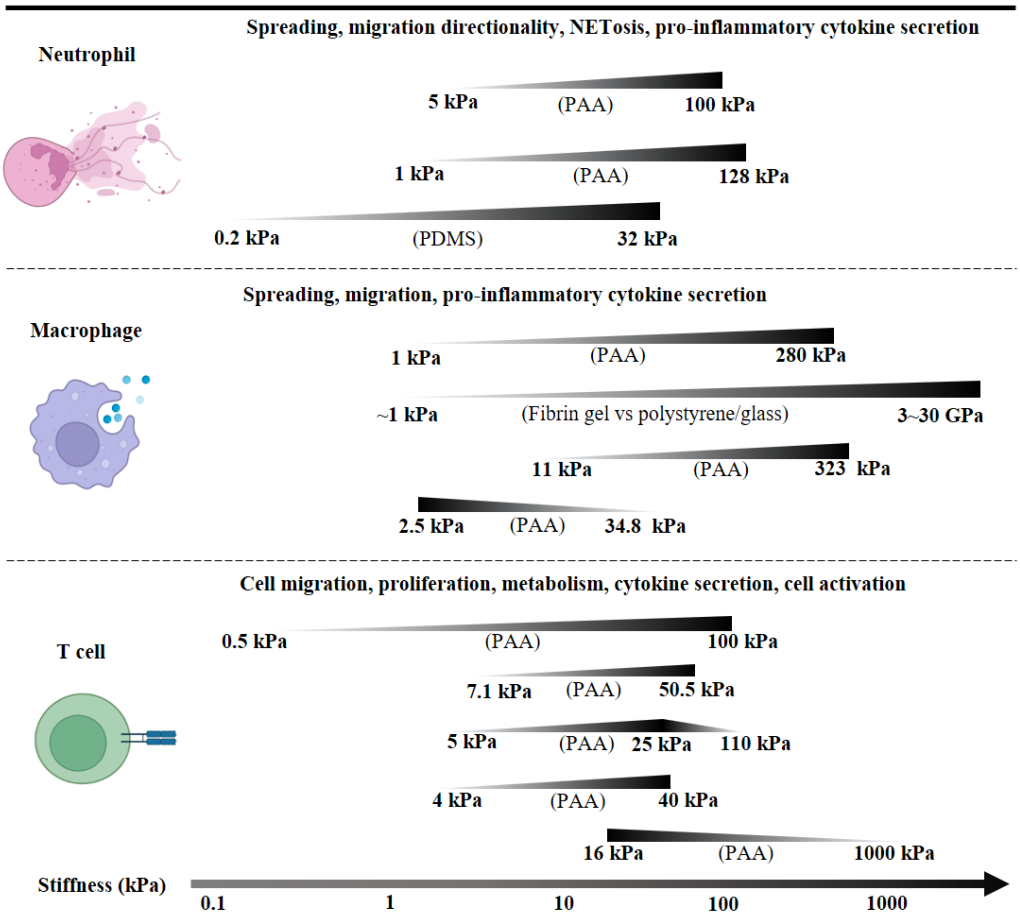

Fig. 5. 2D matrix stiffness-dependent immune/inflammatory cell behaviours. Representative immune/inflammatory cell behaviours on the engineered 2D matrix in terms of stiffness; neutrophils[15,72,73], macrophages[16,74,84,85], and T cells[18,76,79,80,83] cultured on either polyacrylamide (PAA), polydimethylsiloxane (PDMS), or fibrin gel.

Matrix topography and geometry (confinement)

The topography and geometry of matrix are important parameters for cellular mechanosensing. During migration and invasion, immune/inflammatory cells experience a wide range of topographies and geometries, including cell-based differing geometrical shapes (e.g., myofibers, adipocytes, and vessel curvatures) and sharp patterns or fissures found between cells or tissue layers[86]. In addition, bone implant materials have

different length scales of surface roughness and texture, providing altered biophysical cues for cellular mechanosensing.

Earlier studies on immune/inflammatory cells have also been investigated using 2D-modelled nano/micro-topographical surfaces, as with other cells. Neutrophils were shown to sense the different surface roughness of Ti (e.g., roughness level of Sa = 0.61 μm 'smooth' and 3.22 μm 'rough')[87]. In particular, the rough surface was fabricated by a sandblasting and acid-etching method, a technique commercially available for dental implants. Neutrophils on the rough surface displayed reduced NETosis and secretion of pro-inflammatory cytokines and chemokines (IL-1β, IL-6, IL-12, TNF-α, IL-17, CCL series, CXCL-10, MCP-1, MPO, etc.) but activated regenerative cytokines (IL4, IL10, TGF-β), when compared with those on smooth surfaces. In addition, the secretome from the neutrophils affected the macrophage polarization with less M1 polarization on the rough surface. In fact, many studies reported the positive effects of rough surfaces on bone growth to Ti implants, and reasoned the outcome mainly to the role of bone forming cells, however, such early inflammatory events by neutrophils were largely underestimated. Given the importance of initial inflammatory responses that might be influential in late-stage bone growth to implant surfaces, more in-depth studies on the interplay between inflammatory responses and bone formation are needed to be explored.

Studies on the surface topography were more extensively carried out with macrophages. An earlier study showed the effects of micropatterned surface (20-μm or 50-μm width patterns, and flat control, PDMS with fibronectin coating) on macrophage phenotype change. Macrophages cultured on 20-μm-wide micropatterned surface were highly elongated, and expressed more M2 phenotype marker (Arg1), which was even without the use of M2-inducing exogenous cytokines (IL-4 and IL-13), suggesting a possible effect of topographical cues overriding biochemical signals. On a flat control surface, however, macrophages with a less elongated shape showed more M1 phenotype marker (iNOS) even they had a cell spreading area similar to those on micropatterned surface[88], implying the importance of cell shape being altered by topographical cues. Such behaviors were then correlated with the mechanosensing machinery actomyosin contractility of cells. The results highlight that the cell shape, mainly elongated morphology, which was incurred by sensing of the underlying surface micro-topography, is determinant to the macrophage phenotype change.

A more recent work underpinned the phenomena of surface micro-topographical sensing by macrophages in the context of confinement effects. Macrophages were confined on relatively small circular fibronectin-coated substrates with an adhesive area of no more than 200 μm^2, which is quite restrictive for cell spreading when compared with the high cell spreading area of ~1750 μm^2 on a normal surface. When confined, the inflammatory genes, especially those relatively late-responsive (IL-6, CXCL9, IL-1β, and iNOS), were all significantly lowered compared with those cultured on normal surfaces[89]. The confined cells exhibited reduced actin polymerization and nuclear translocation of mechanosensitive transcription factor MRTF-A, which was also related with the nuclear mechanics, i.e., enhanced chromatin compaction with increasing levels of histone deacetylation (HDAC3) and methylation (H3K36) down-regulated the LPS-induced genes.

While the microscale topographies correspond to cell size, thus confining or limiting cell shape directly, nanoscale topographies (less than a micrometer) govern the spatiotemporal interactions of cell surface receptors, such as integrins. Although studies on the effects of surface nano-topographies on MSCs and bone forming cells have been conducted extensively, those with immune/inflammatory cells have just emerged. Different scales of honeycomb-like topographies (90~5000 nm) made of TiO_2 were engineered on a Ti substrate[90]. Effects seen were that a 90-nm-scale surface facilitated filopodia formation of macrophages with up-regulation of mechanosensitive RhoA/Rho-associated signals, which led to M2 polarization of macrophages. Although this study did not detail the adhesion-related cellular mechanics, the macrophages would sense the tens-to-hundreds of nanometer scale topographies though different integrin assemblies and dynamics, as deduced from the integrin-mediated mechano-sensing nature of macrophages[91,92] and the responses of other cells to similar nano-topographies[93,94]. The effects of matrix topography and geometry on the *in vitro* behavior of various types of cells involved in osteoimmunology are summarized in **Table 2**.

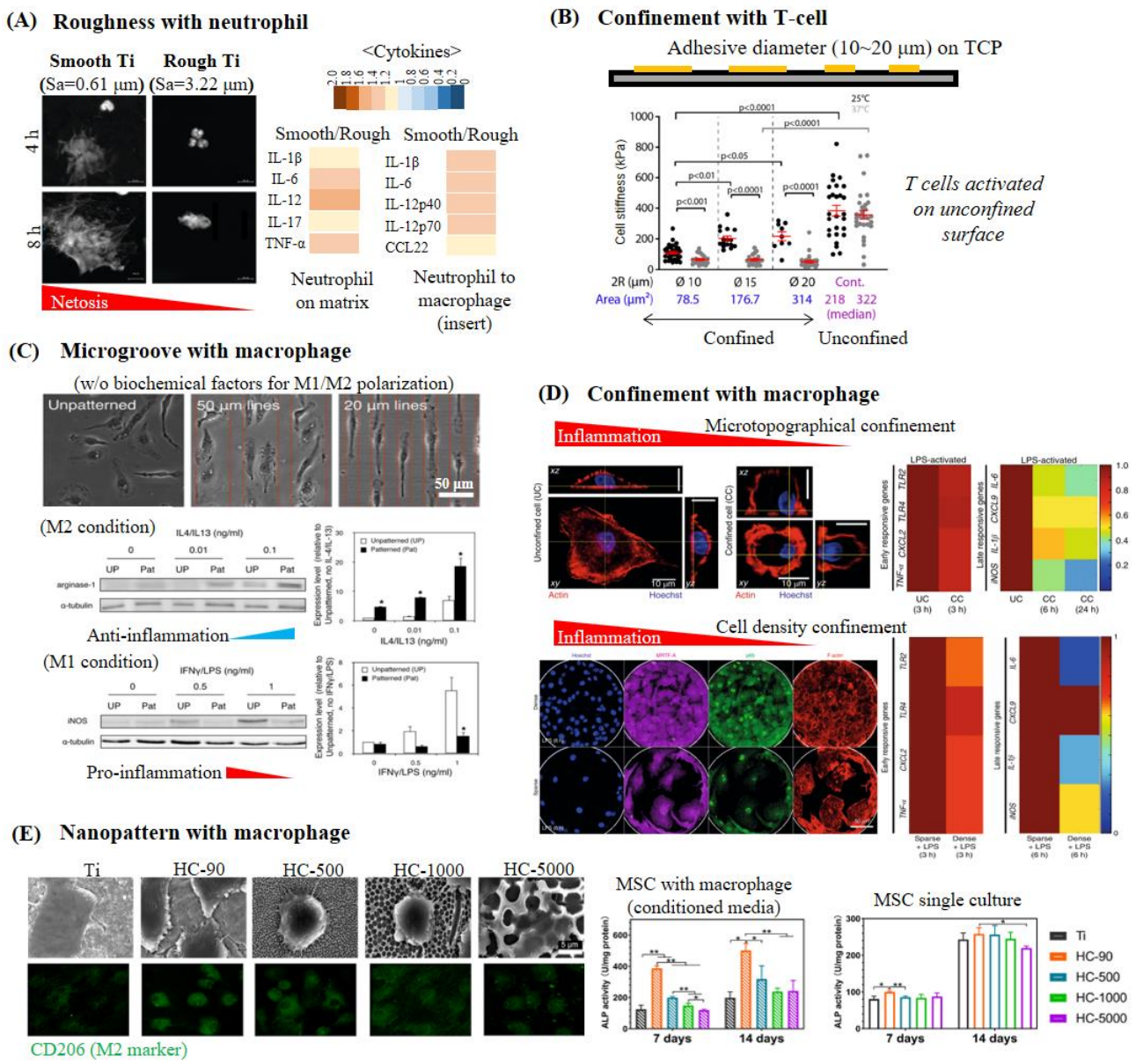

Fig. 6. Effects of matrix topography and geometry on immune/inflammatory cellular activation. (A) High roughness Ti down-regulates NETosis of neutrophils and the release of inflammatory cytokines which affects macrophages secretion of inflammatory cytokines. Neutrophil was cultured on different roughness values of Ti surface while macrophage was co-cultured using insert. (B) Confined adhesive area (10-20 μm) with activating molecules (anti CD45) reduces T-cell activation (measured in terms of T-cell stiffness). (C) Microgrooved surface (20 μm width) elongated macrophages which presented enhanced M2 polarization (upon IL4/13 induction) but reduced M1 polarization (upon LPS/INFγ induction), compared to flat unpatterned surface. (D) Confinement of macrophages either by micropatterning or cell density control down-sizes the expression of late-responsive genes (iNOS, IL1b, CXCL9, and IL-6) upon LPS stimulation (while little change in early-responsive genes, TNF-a, CXCL2, TLR4, and TLR2). (E) Different scales of patterned nano-topographies (90~5000 nm) made of TiO_2 on Ti regulate macrophages M2 polarization which further influences MSCs osteogenesis (by ALP assay). Adapted from Ref. [87] (Abaricia et al. in Biomaterials Science, 2020) for (A), Ref. [95] (Sadoun et al. in Scientific Reports, 2021) for (B), Ref. [88] (McWhorter et al. in PNAS, 2013) for (C), Ref. [89] (Jain et al. in Nat Materials, 2018) for (D), and Ref. [90] (Zhu et al. in Nat Comm, 2021) for (E).

Three-dimensionality and anisotropy in 3D gels and scaffolds

Recent studies have demonstrated that cell behavior in 3D is different from that in 2D, underscoring 'three-dimensionality' as a key matrix parameter in interpreting cell behaviors[63]. For example, neutrophils were shown to behave differently in 2D and 3D microfluidic devices, in terms of the role of integrin regulatory proteins in cell polarity and directed migration[96]. In addition, cells encapsulated within 3D gels could not spread as much as they did on 2D gels[97]. For the 3D cell studies, biocompatible gels that can encapsulate and expand cells have been engineered; among the synthetic and natural polymeric systems, PEG-, collagen-, alginate-, and hyaluronic acid-based gels were well designed for the model studies of mechanobiological behaviors [76,98–100]. Compared with 2D gels, 3D gels generally need more adhesive ligands, and should be softer to allow cells to survive and proliferate. More importantly, 3D gels need to be designed to decouple chemical and physical properties., i.e., ligand density, pore (mesh) size, and stiffness.

Effects of 3D matrix environment on the regulation of neutrophil migration were examined using 3D collagen matrices[98]. At different concentrations (0.25-2 mg/mL) of collagen, the matrices were tuned to have varying pore sizes, mimicking the environment that neutrophils encounter during migration as they have to pass through narrow pores of the extravascular space. Neutrophils were shown to mechanically interact with the collagen networks, turning and passing through to find a migratory path across chemotactic gradient. They even deformed the matrix substantially when the concentration increased (thus pore size decreased). Although the work centered on the pore size effect of 3D matrices on neutrophil migration, the stiffness of collagen also varies coupled with pore size as the concentration changes. For this reason, other 3D systems that can decouple the porosity and stiffness were prepared to study 3D mechanics of immune cells. Alginate gels were thus designed to have a range of stiffness levels (4, 25 and 40 kPa) with a similar pore size (~130 μm) by crosslinking with Ca^{2+} ions (10~40 mM), which was then modified with an RGD ligand[101]. T cells cultured in the 3D scaffolds were highly sensitive to the altered 3D stiffness; T cell proliferation, cytokines secretion (IL-2, IFN-γ, and TNF-α), and immune synapse with antigen presenting cells were activated in stiffer 3D matrix, demonstrating a trend similar to 2D conditions with regard to stiffness-dependency.

Fibrous structures are a key feature of native ECM; fibers offer anisotropy (directionality) along which cells are aligned and elongated. Therefore, fiber-structured 3D scaffolds have been widely used for the repair of tissues, including bone. Some earlier studies designed fibrous scaffolds with different porosities, pore sizes, and fiber sizes, and examined the effects on immune/inflammatory cells[102–104]. Macrophages cultured on electrospun polydioxanone fibers exhibited increased M2 polarization (thus decreased M1) with higher porosity and larger pore (or fiber) size[105], suggesting the importance of pore size and porosity of fibrous scaffolds in regulating macrophage phenotype, although biological mechanisms underlying the event was not detailed. One intriguing stud reported neutrophils were more activated (such as increase of NETosis) upon small-diameter (400 nm) fiber (vs. large-diameter one, 2.1 μm), whereby IgG, a robust NETosis activator, was adsorbed more on small fibers case[103]. It is thus reasoned that the different fiber size is related to the surface-associated molecular reactions that can alter the adhesion and mechanosignaling of neutrophils. On the other hand, the effect of fiber size on macrophages appeared to be somewhat dissimilar to the case in neutrophils; macrophage secretion of pro-inflammatory molecules (TNF-α, G-CSF, MIP-1R) was mitigated in smaller-sized PLA fiber (500~600 nm vs. 1500 nm)[106]. Although the exact mechanism on this was not demonstrated well, the smaller-sized fibers might activate integrin-mediated polarization or induce topography-driven cell confinement, which still needs further investigation. Another notable effect of fibers was found with alignment; when macrophages were cultured on aligned nanofibers (600~700 nm diameter), the pro-healing phenotype (M2 type) was enhanced when compared with that on random fiber[106]. The behaviors of immune/inflammatory cells involved in osteoimmunology under the 3D gel and fiber matrix culture conditions are summarized in **Table 2**.

Effects of combined matrix cues

Given the various matrix cues are coupled in real (*in vivo*) conditions, studies have examined the effects of combined matrix cues, such as stiffness coupled with adhesion ligand and nano/microtopography. First, matrix stiffness was shown to synergize with ligand density in the activation of inflammatory cells, such as neutrophils.

Neutrophil activity, such as NET formation and the release of pro-inflammatory cytokines, was revealed to increase with increasing stiffness of PDMS gel and the coating density of adhesive proteins, such as fibronectin and synthetic RGD peptide,[49] which is mediated by integrin-FAK signaling.

Stiffness also interplays with nanotopography in the activation of immune cells. For the stiffness-nanotopography coupling study, nano-textured PAA gels (800 nm width and 600 nm height to provide grooves and ridges) with different stiffness values (16 and 50 kPa) were designed which was coated with ICAM1[76]. T cells (CD4+) cultured on the gels sensed the nanotopography of grooves and ridges, showing both in-groove (uni-directional) and on-ridge (multi-directional) invasions. Of note, with increasing stiffness, the in-groove invasion became attenuated, and the phenomena were proven to be regulated by the dynamics of another mechanosensitive machinery microtubule. The T cell migration was further investigated within a 3D collagen matrix: a more ECM-mimicking environment[76]. Despite the difficulty in decoupling of matrix cues, such as stiffness and ligand density, the collagen is considered to provide a biomimetic 3D condition of a fibrous network. The T cell migration was found to be regulated by microtubule instability, i.e., depolymerizing microtubules activated Rho pathway-dependent cortical contractility and T cell migration. These results suggest that the matrix architecture and mechanics (stiffness) are key to driving T cell motility across a range of complex matrix cues that may be encountered *in vivo*.

The effect of stiffness coupled with topographical cue was further examined using a micro-pillar array system. Micro-pillars made of PDMS (height of 6 or 3 μm with diameter of 1 μm in straight features) gave two different spring constants (0.8 and 6.2 nN/μm for 6 and 3 μm height, respectively; considered as rigidity of pillars)[77]. CD4+ T cells were shown to recognize the microstructure of pillars, infiltrating with time. Moreover, cells could sense the pillar mechanics, i.e., secreting more inflammatory cytokine IFN-γ on higher spring constant pillars. Of note, the phenomena were found to be regulated by the microtubules. Overall, the microtopography coupled with stiffness made T cells respond in more a extensive and mechanically complex interaction with the underlying matrix. Further in-depth studies remain to be carried out, with regard to which matrix parameter (either stiffness or topography) would be dominant in T cell activation.

As witnessed in the above-discussed defined *in vitro* studies, the matrix cues play combinatory or synergistic roles in control over immune/inflammatory cell behavior, such as phenotype change, migration, and immune-activation. Given that cells *in vivo* are influenced under complex ECM cues, more *in vitro* studies are needed with combined matrix cues that facilitates more accurate interpretation of *in vivo* phenomena, such as immune/inflammatory cell-mediated bone repair processes.

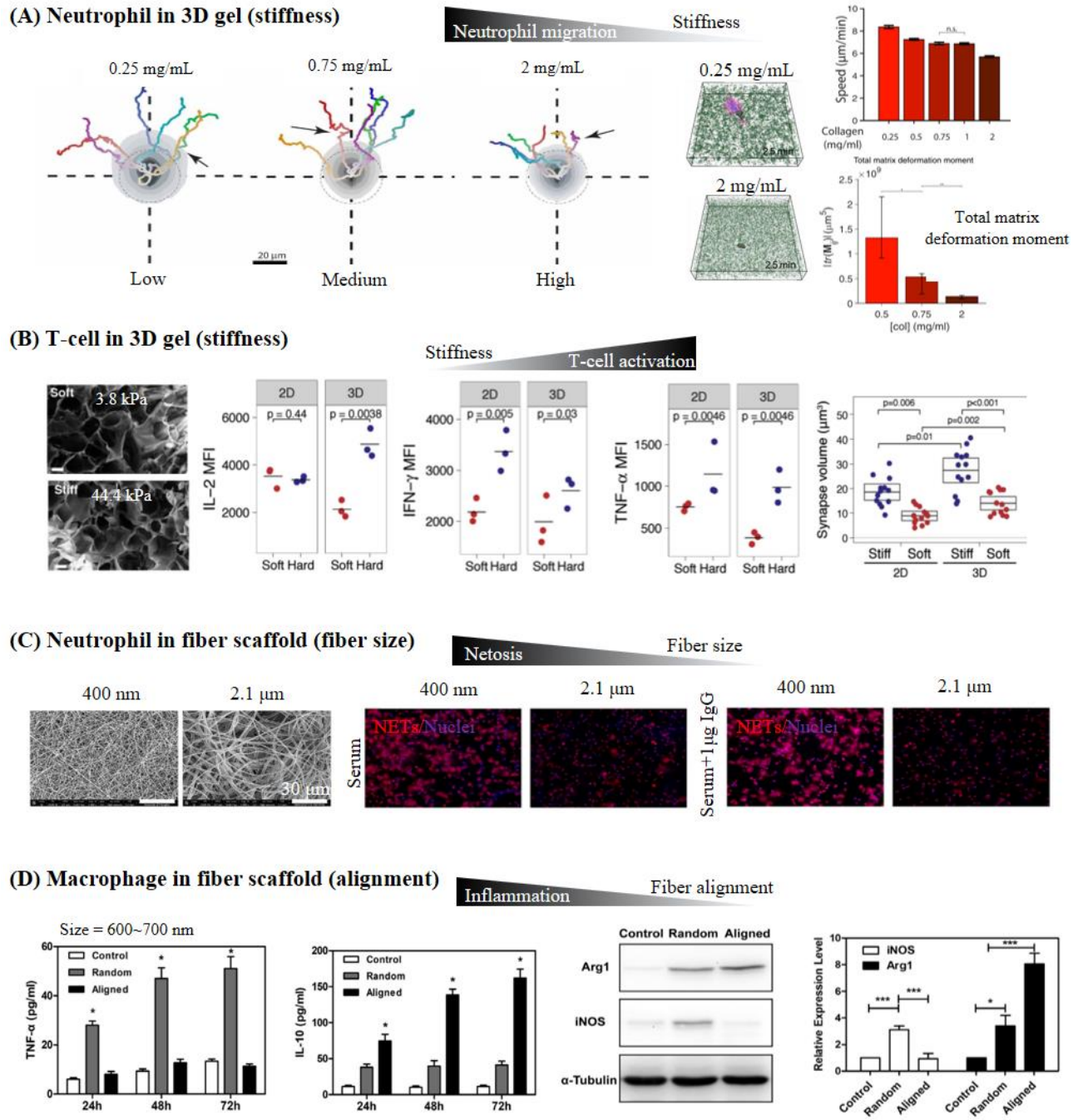

Fig. 7. Influence of three-dimensionality and anisotropy of gels and scaffolds on immune/inflammatory cells. (A) Neutrophils in 3D stiffness-varied collagen gels. When 3D-cultured in collagen gel with different stiffness levels (using 0.25~2mg/mL collagen), the cell migration was enhanced in lower stiffness gel. (B) T-cells in 3D stiffness-varied alginate gels. Two different stiffness levels (3.8 and 44.4 kPa) were enabled in 2D and 3D matrices of anti-CD3 and anti-CD28 coated microparticles, which act as antigen presenting cells and T cell activation. Secretion of cytokines (IL-2, IFN-r, TNF-a) increased in higher stiffness matrices along with enhanced synapse volume. (C) Neutrophils on fiber scaffolds with different fiber sizes. Cells were less activated on small diameter fiber (400 nm) than large diameter fiber (2.1 μm) under serum or 1 μg IgG. (D) Macrophages on fiber scaffolds with directional cue. Aligned fiber decreased inflammatory status and cytokines release in macrophages than random-directional fiber regardless of diameter (500~1500 nm). Adapted from Ref. [98] (François et al. in Science Advances, 2021) in (A), Ref. [101] (Majedi et al. in Biomaterials, 2020) in (B), Ref. [103] (Fetz et al. in Acta Biomaterialia, 2021) in (C), and Ref. [107] (Jia et al. in Acta Biomaterialia, 2019) in (D).

Table 2. Summarizing the effects of matrix cues on the *in vitro* behaviors of various types of cells involved in osteoimmunology (except bone cells).

Matrix cue	Range/value	Material used	Cell type	Summary	Reference
stiffness	5 to 100 kPa	fibronectin-coated PAA gel	neutrophil	Greater forces on stiffer substrates	Oakes *et al.*[15]
stiffness	1 to 128 kPa	collagen 1, fibrinogen-coated PAA gel	neutrophil	LPS-induced NETosis increase with substrate elasticity	Erpenbeck *et al.*[72]
stiffness	0.2~32 kPa	PDMS gel coated with various ligands	neutrophil	Increased NET formation, pro-inflammatory cytokines on higher stiffness	Abaricia *et al.*[73]
stiffness	~100 Pa, ~GPa	fibrin hydrogel, glass	macrophage	Lower YAP nuclear localization, decreased inflammatory response on soft gel	Meli *et al.*[16]
stiffness	1,20,40,280kPa	fibronectin-coated PAA gel	macrophage	Piezo1 channel and macrophage function activation on stiffer gel	Atcha *et al.*[74]
stiffness	11, 88, 323 kPa	collagen-coated PAA gel	macrophage	Phenotype and migratory mode determined by stiffness	Sridharan *et al.*[84]
stiffness	2.55, 34.88, 63.53 kPa	PAA gel	macrophage	M1 phenotype on soft gel, M2 phenotype on stiff gel	Chen *et al.*[85]
stiffness	50, 500 kPa, 2 MPa	PDMS, Nusil beads	T cell	Response differently to beads with varied stiffness	O'Connor *et al.*[78]
stiffness	0.5, 6.4, 100 kPa	ICAM-1 coated PAA gel	T cell	Sensing stiffness of gel and response accordingly	Saitakis *et al.*[79]
stiffness	7.1, 9.4, 50.6 kPa	anti- CD3, CD28 coated paa hydrogel	T cell	Secretion of IL-2 and spreading affected by stiffness and ligand density	Chin *et al.*[80]
stiffness	5, 110 kPa	anti- CD3, CD28 coated paa hydrogel	T cell	Proliferation and IL-2 secretion showing biphasic response with stiffness	Yuan *et al.*[18]
stiffness	4,40 kPa	alginate-RGD hydrogel	T cell	YAP induced by stiffness, decreasing proliferation	Meng *et al.*[83]

microtopography (roughness)	0.61, 3.22 μm roughness	titanium disk	neutrophil	More activation on roughened Ti	Abaricia *et al.*[87]
microtopography (pattern)	50,20 μm width	micropatterned substrate coated with fibronectin	macrophage	Cell elongation (shape) & M2 polarization on patterned surface	McWhorter *et al.*[88]
microtopography (pattern)	N/A	circular fibronectin-coated PDMS	macrophage	Spatial confinement negating pro-inflammatory gene expression	Jain *et al.*[89]
nanotopography (pattern)	90, 500, 1000, 5000 nm (diameter)	honeycomb-TiO2 structure	macrophage	Reduced scale of honeycomb-like structure activating M2 phenotype	Zhu *et al*.[90]
3D environment	0.25, 0.5, 0.75, 1, 2 mg/mL collagen	collagen 1-coated migration chamber	neutrophil	Low collagen concentration leading to decreased matrix deformation and linear trajectory of cell migration	François *et al.*[98]
3D environment (gel stiffness)	4-40 kPa	alginate-based 3D scaffold	T cell	Mechano-sensing 3D environment and actively altering behaviors	Majedi *et al.*[101]
3D environment (fiber)	60, 100, 140 mg/mL polymer concen.	electrospun polydioxanone fiber	macrophage	Correlation of fiber/pore size and M2 phenotype	Garg *et al.*[105]
3D environment (fiber)	50, 120 mg/mL polymer concen.	electrospun polydioxanone fiber	neutrophil	NET release regulated by IgG adsorption, engagement of FcγRIIIb, and signaling through TAK1	Fetz *et al.*[103]
3D environment (fiber)	1.53, 1.60, 0.61, 0.55 um size	electrospun PLLA fiber	macrophage	Proinflammatory molecules depending on fiber size	Saino *et al.*[106]

Importance of dynamic matrix cues (viscoelasticity and remodeling): Lessons from the behavior of other cells

Matrix interacts with cells and changes its physico-mechano-chemical properties over time. Natural ECM is viscoelastic, rather than elastic, thus its initial stiffness is not static but changes dynamically over time, allowing cells to experience dynamically-altering physico-mechanical environment (as depicted in **Fig. 8Ai**). Moreover, ECM degrades hydrolytically/enzymatically and deposits over time, thus providing dynamically-changing mechano-chemical environments to cells (as depicted in **Fig. 8Bi**). These dynamic environments of ECM have recently been highlighted as key determinants of the diverse behavior of cells[53,54,62,63]. Due to the lack of understadnign of dynamic matrix cues related with cells, most work are still undertaken primarily with more familiar cell types, such as fibroblasts, MSCs and cancer cells, which however, may inspire us to interpret the behaviors of immune/inflammatory cells in osteoimmunology under such dynamically-changing matrix conditions.

Due to matrix viscoelasticity, cell forces exerted on the matrix relax over time, a phenomenon called 'stress relaxation', which has recently been found to affect a diverse range of behaviors of cells[53,54,62]. For these studies, matrices should be fine-tuned to have a range of stress relaxation, independent of initial stiffness. Alginate-based gels have been intensively used for this purpose. Altering molecular weight of alginate or incorporation of PEG spacer into alginate network could alter the stress relaxation while equalizing initial stiffness; in this way, for example, $t_{1/2}$ (a time to reach 50% of stress relative to initial stress) was achieved to vary 70-to-3300s (from fast-to-slow stress-relaxing), at fixed initial stiffness and ligand density[62]. MSCs cultured within these 3D gels with varied stress-relaxation exhibited significantly different behaviors; cells in faster stress-relaxing gels had enhanced cell spreading, proliferation, and osteogenic differentiation (**Fig. 8Aii**), and furthermore, their behavior was less dependent on the initial stiffness level, suggesting stress relaxation might override the initial stiffness effect. Of note, such cellular responses were found to be mediated by mechanical clustering of adhesion ligands, actomyosin contractility, and subsequent ECM remodeling[62]; when considering that the time scales of these cellular responses are approximately seconds-to-minutes, the MSCs, placed within 3D gels with stress-relaxation of the corresponding time scales, should be more mechanically-competent and -active.

Such activated cellular behavior within stress-relaxing 3D gels was also seen in other systems, such as in chondrocyte growth and matrix synthesis, and cancer cell mitosis and migration[86,108,109]. These studies implicate that stress relaxation, as a key viscoelastic property of matrices, rather than its initial static stiffness, is decisive in favoring diverse cellular behavior, by means of offering matrix physico-mechanical conditions permissive to cell-exerting forces. The forces investigated in those studies, i.e., protrusive forces during mitosis, expansion forces during growth and matrix synthesis, and pushing/squeezing forces during migration, are not limited to specific types of cells, but are rather general to a broad spectrum of cells, including immune/inflammatory cells. Hence, the behavior of cells involved in osteoimmunology under such fine-tuned viscoelastic conditions need further to be investigated to fully understand the impact of matrix cues.

ECM remodels through dynamic interactions with cells, i.e., cells degrade matrix networks while synthesizing new composition. In this way, the matrix alters its chemo-mechanical properties over time. Recent studies have underscored the importance of matrix degradation in cellular behavior, such as MSC proliferation and differentiation, and neural progenitor cell stemness maintenance[63,110,111]. For the study of cell-degrading 3D matrices, proteolytically-cleavable crosslinker (MMP-degradable oligopeptide) was incorporated into covalently-crosslinked hyaluronic acid (HA) gels[63]. Of note, the cell-mediated degradation allowed MSCs to exhibit higher cell spreading, traction force, and ECM deformation, leading to enhanced osteogenesis[63] (**Fig. 8Bii**), which highlights the importance of matrix mechano-permissiveness to MSCs, incurred by cell-mediated degradation, in their lineage specification, especially osteogenesis. Other work showed that the higher MSC osteogenesis in void-forming 3D matrices also shares a similar concept that a degradable matrix (due to void-formation therein) is effective in driving MSC spreading and osteogenesis[112]

Matrix not only degrades but deposits also, i.e., remodels. Although the effects of matrix degradation on cell behavior have been well studied, those of deposited matrices has recently been appreciated for its significance[113,114]. When MSCs were cultured within HA gels they were shown to secrete new adhesive proteins

(e.g., fibronectin) quickly within a day which increased over culture time[113]. Hence, such a cell-secreted matrix alters the chemical composition and local mechanics, masking and overriding the initial matrix properties, which ultimately modulates cellular mechanotransduction. Indeed, MSCs could sense the newly-produced ECM, utilizing it for their subsequent mechano-signaling processes, such as spreading and YAP nuclear-translocation, which is ultimately helpful for osteogenic differentiation (**Fig. 8Biii**). Hence, the cell-deposited matrix is considered to provide cues that can complement the signals supplied by the material itself.

As discussed above, the dynamic matrix cues, including viscoelasticity (stress relaxation, dynamic stiffness) and remodeling (degradation, deposition), are key in regulating the diverse behavior of cells, such as initial adhesion, spreading, migration, and differentiation, through modulating mechanotransduction processes. Despite such behaviors being seen mainly with MSCs, the implications in immune/inflammatory cells are massive, and as yet unexplored, and is an important area for further investigation. Mounting evidence with other types of cells also point to the the necessity for research in this area, which however, should be context-dependent. For instance, the stress-relaxation range is highly variable ($t_{1/2}$: seconds to minutes to hours, and sometimes even higher), depending on tissue type and the condition which cells confront; for immune cells, protrusion and migration during extravasation of blood vessels and tissue migration are needed which takes seconds-to-minutes (contrasted to hours-to-days in expanding cells such as aggregated stem cells). Another note that is specific to bone tissue is the mineralization process over time, which is considered a dynamic stiffening of matrix during bone regeneration, which may need to be considered as a matrix cue in regulating cellular behavior in osteoimmunological events. In fact, several studies developed in situ stiffening matrices to interpret the pathological phenomena (e.g., fibrosis) and the underlying mechanobiological mechanisms [refs].

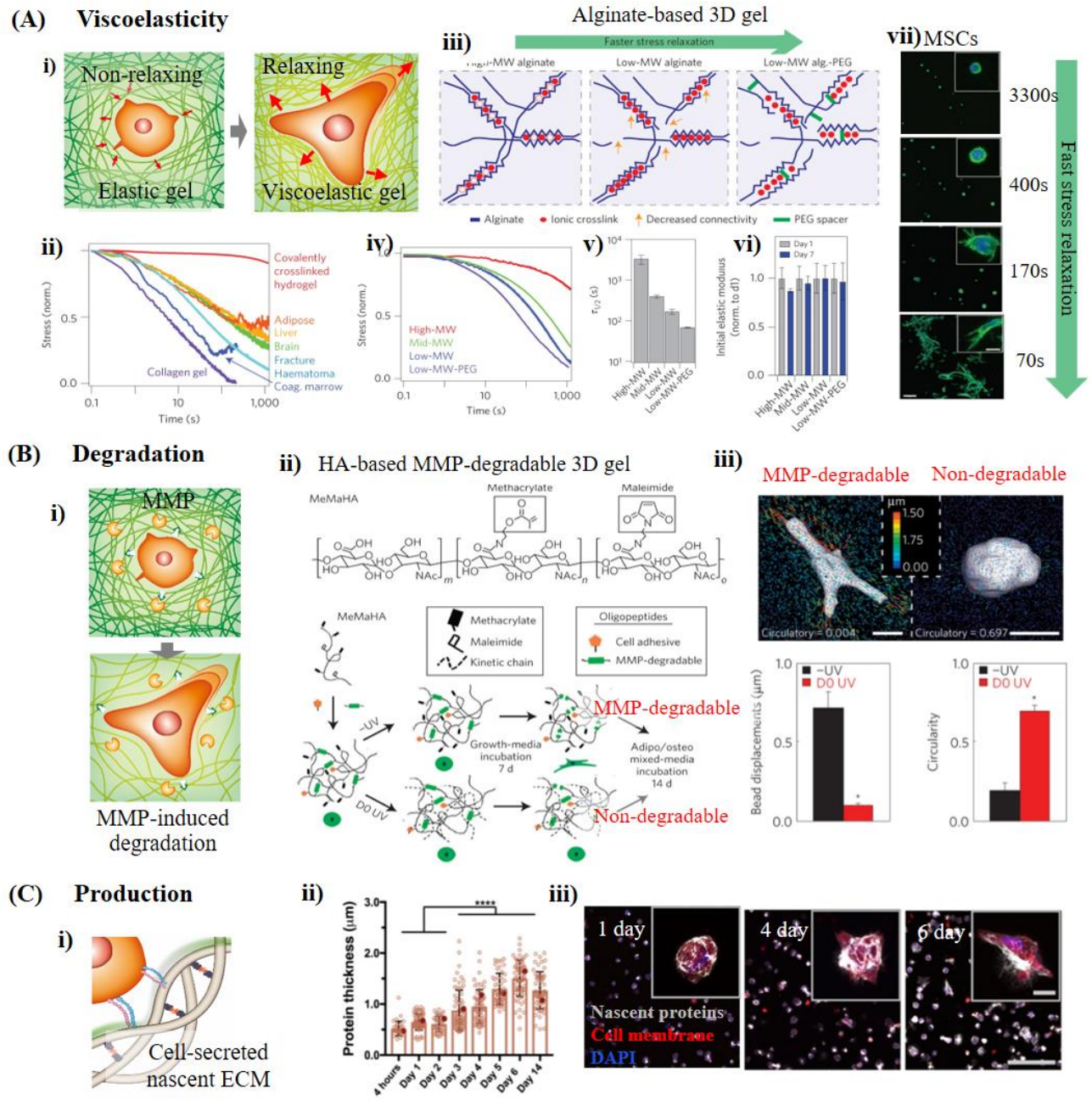

Fig. 8. Impact of dynamic matrix cues in cell behaviors (A) Matrix viscoelasticity. (i) Illustration showing that viscoelastic gel is stress-relaxing with time in response to cell-exerting force (red arrows), permitting cells to protrude, expand, and migrate. (ii) Viscoelastic native tissues show stress-relaxation behaviors over time, but elastic covalently crosslinked gel cannot. (iii) Exemplar study of designing alginate-based 3D gels with different stress-relaxation rates by changing molecular weight and PEG addition. (iv) Stress relaxes over time, showing an order of magnitude difference in $t^{1/2}$ (v) while the initial modulus is preserved (vi). (vii) MSCs cultured in the fast stress relaxing gels show higher cytoskeletal processes. **(B)** Cell-mediated matrix degradation. (i) Illustration showing that cell-secreted MMP degrades matrix network over time, altering the physico-mechanical properties. (ii) Exemplar study of designed hyaluronic acid (HA)-based MMP-degradable 3D gels. (iii) MSCs cultured in the MMP-degradable gel elongated better with higher bead displacement and less circularity than in the non-degradable gel with comparable stiffness value. **(C)** Cell-mediated matrix production. (i) Illustration showing that cells produce proteins over time (green color), altering the biochemistry of initial matrix. (ii) MSCs cultured in the 3D gels produced nascent proteins with increasing thickness over time, and (iii) the images of proteins in cells. Adapted from ref[62]. O. Chaudhuri et al. in Nat Mater 2016 for (A), ref[63]. S. Khetan et al. in Nat Mater 2013 for (B), ref[113] by Noebel et al. in Nat Mater 2019 for (C).

Matrix impacts on cellular crosstalks *in vitro* and *in vivo*: Immune/inflammatory cells with bone cells

Matrix-primed MSCs playing in regulation of immune/inflammation

As discussed above, dynamically-changing matrix properties, such as viscoelasticity and remodeling, have profound effects on regulating mechano-signaling of MSCs and their diverse behaviors, such as proliferation, migration, and osteogenic differentiation, which is obviously the case for other matrix cues, such as static stiffness, nano/microtopography, and ligand type/density. Because MSCs modulate the local immune/inflammatory milieu, the matrix-properties primed MSC responses are considered to determine osteoimmunological bone healing events.

Some recent work highlighted the matrix roles in priming MSCs behaviors, which subsequently modulate immune/inflammation events. When MSCs were cultured in alginate hydrogels over a range of elastic moduli, they were primed to express immunomodulatory markers, based on transcriptome-wide (RNA seq) analysis[115]. Indeed, MSCs mobilize from the bone marrow to vessel wall in response to inflammation and injury, and are prepared to egress into the vasculature, sensing the altered fluid and matrix mechanics. When shear-stressed, MSCs were activated to signal immune cells and modulate macrophage polarization, implying force as a critical cue to MSCs residing at the vascular interface which influence immunomodulatory and paracrine activity[116]. In addition, the surface topography regulates paracrine interactions that MSCs establish with macrophages[117]. MSCs grown in a 3D spatial arrangement with microporous topographical cues, decreased the production of inflammatory signals in macrophages with a concurrent increase in anti-inflammatory proteins (vs. dense 2D surface), underscoring the importance of matrix topographical cues in the soluble factor-guided communication between MSCs and macrophages. Recent work by Wong et al. added to the evidence that MSCs primed by 3D matrix mechanics could activate immune cells to ameliorate inflammation[118] (as shown in **Fig. 9A**). A soft matrix could maximize the ability of MSCs to produce paracrine factors that have been implicated in monocyte production and chemotaxis upon inflammatory stimulation. Actin polymerization and lipid rafts were found to be the key mechanosensitive mechanisms that regulate the mechano-activation of MSCs, implying the significance of matrix-induced signaling in moderating inflammatory activation of MSCs. This study lays a foundation for understanding how physical signals from ECM in the bone marrow microenvironment instructing MSCs to direct immune cell functions, and further for strategizing how to control inflammation and drive bone regeneration.

While most studies considered paracrine molecules as the key signaling factors that the MSCs primed by matrix cues can establish for crosstalk with immune/inflammatory cells, their biophysical interplay should also occur, because cell-secreted ECM molecules can mediate the intercellular biophysical communications, which, despite needing clarification with future studies, is likely to do, given the accumulating evidence, such as matrix-stiffness-mediated biophysical interactions between endothelial and hepatic satellite cells[119]. Also, the other stromal cells resident in osteo-niche, such as osteoblasts and endothelial cells, are also influenced by matrix cues[120], possibly working in the osteoimmuno-communications, which remains an unexplored area for further research. Given the reciprocal interactions between immune/inflammatory cells and stromal cells within the context of matrix cues, we discuss, in the following sections, more on the effects of immune/inflammatory cells modulated by matrix cues on MSCs and osteoblasts.

Matrix-regulated immune/inflammatory cells and the crosstalk with bone forming cells

As discussed in the previous sections, matrix cues are decisive for regulating immune/inflammatory cell responses, such as neutrophil extracellular trap formation, macrophage polarization, and T cell activation, which is mediated by the mechanotransduction process. Given the crosstalk between immune/inflammatory cells and stromal cells, such mechanically-altered immune/inflammatory signals should modulate bone healing

events by stimulation of cell proliferation or osteogenic differentiation.

Of note to consider is that the biological effects by the same matrix cue are often contradictory depending on cell type; for example, upon matrix stiffening, MSCs undergo osteogenic differentiation whereas macrophages polarize to be pro-inflammatory which can restrict the MSCs differentiation activity. One recent study provided experimental evidence for this. Using stiffness-varied (1.5, ~20, and ~60 kPa) 3D cell-encapsulating gels, made of transglutaminase-cross-linked gelatin, the macrophage polarization and MSC osteogenesis were analyzed (**Fig. 9B**)[121]. The macrophages cultured in soft gels were shifted towards a more M2-like phenotype (vs. stiff gel), suggesting mechano-priming of macrophage phenotype by the gel stiffness. In case of MSCs, as expected, a high-stiffness gel enhanced osteogenic differentiation. Of note, however, when the MSCs were co-cultured with the mechano-primed macrophages, high-stiffness gels reduced the osteogenic differentiation of MSCs, revealing an opposite effect of matrix stiffness on MSCs osteogenesis between mono- and co-culture system. The results signify the matrix role in macrophages and their indirect effects on MSCs which can override the direct role of matrices in MSCs. It should thus be borne in mind that matrix-mediated osteogenesis of MSCs should be interpreted in the context of crosstalk with immune/inflammatory cells.

The matrix nanotopography was also demonstrated to play a decisive role in osteogenic modulation by macrophages (**Fig. 9C**). Different scales of honeycomb-like topographies (90~5000 nm) made of TiO_2 were engineered on a Ti substrate[90]. Among others, 90-nm-scale surface activated macrophages to express higher anti-inflammatory signals (CD206, IL-4, IL-10, BMP-2), leading to M2 polarization. In this event, filopodia formation and RhoA pathway were found to be the key activated mechanotransduction signalings. Of note, was the fact that the MSCs were highly responsive to the macrophage-conditioned media, i.e., higher osteogenic gene expression and biomineralization were achieved with 90-nm-cultured macrophages, demonstrating the significant role of matrix-topographical-modulated macrophages in MSCs osteogenic functions.

Despite the significant implications of crosstalk between immune/inflammatory cells and bone forming cells (e.g., MSCs), studies in the context of matrix cues are still in its infancy, and have just begun with macrophages and previously known matrix cues (stiffness and nanotopography); therefore, studies on other cells less widely investigated (e.g., neutrophils, T cells), under dynamic matrix conditions (e.g., viscoelastic or remodeling matrix) or with combined cues, are envisaged to follow in the future.

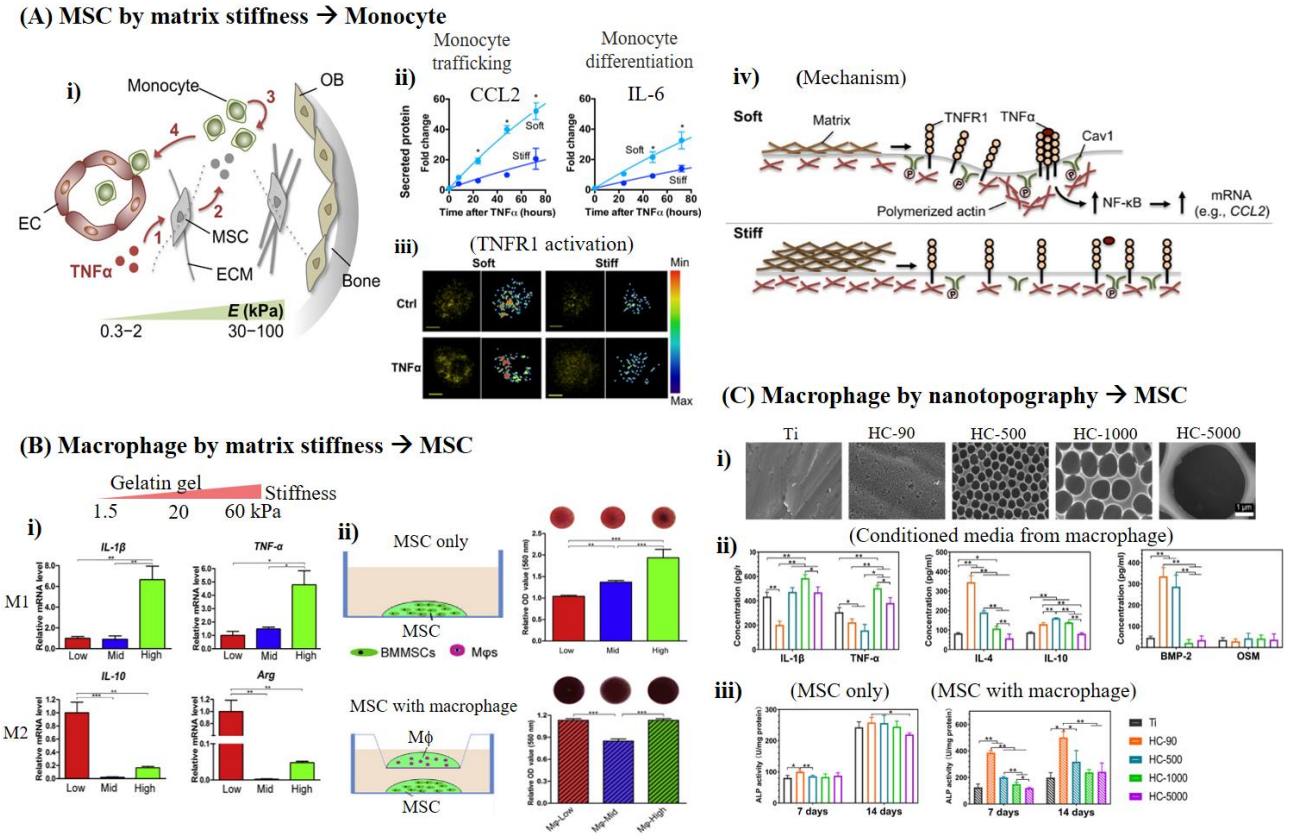

Fig. 9. **Matrix-cued mechanobiological-crosstalks between MSCs and immune cells**. **(A)** 3D Matrix-stiffness-induced MSCs paracrine signals modulate monocyte-mediated inflammatory responses. i) Scheme showing that MSCs experience stiffness dynamics from soft bone marrow (0.3~2 kPa) to stiff bone surface (30~100 kPa), and regulate monocytes upon TNF-α activation. Ii) Under TNF-α-induced inflammatory condition *in vitro*, soft matrix (~2 kPa) consisting of alginate-RGD causes more secretion of monocyte regulatory (trafficking and differentiation) cytokines (i.e. CCL2 and IL-6) than stiff matrix (~35 kPa), iii) which is facilitated by the activated TNFR1 (membrane embedded receptor for capturing TNF-α) clustering. vi) Mechanistic view of the activated TNFR1 in MSC within soft matrix condition, which is mediated by the polymerized actin and membrane rafts. **(B)** Matrix-stiffness-mediated macrophages responses influence MSCs osteogenesis. (i) Macrophages cultured upon different stiffness levels (low 1.5, mid 20, and high 60 kPa) of transglutaminase cross-linked gelatin gels show altered polarization, i.e., pro-inflammatory (IL-1β and TNF- α expression) on high, and anti-inflammatory (IL-10 and Arg expression) on low stiffness gel. (ii) Although MSCs show higher osteogenic differentiation on high stiffness matrix, the matrix-conditioned macrophages significantly alter the osteogenic behaviors. **(C)** Nanotopographical-sensing of macrophages regulates osteogenesis of MSCs. i) Honeycomb-like nano-topographies (diameters varied; 90 nm (HC90) ~ 5000 nm (HC5000)), reveal ii) nanotopography-dependent cytokines or bone-forming growth factors secretion from macrophages. iii) The conditioned media from macrophages on different nanotopographies influence the osteogenesis (ALP activity) of MSCs, i.e., higher osteogenesis by less-inflammatory geometry, HC90. Adapted from Ref. [118] (Wong et al. in Sci Adv, 2020) for (A), Ref. [121] (He et al. in Acta Biomaterialia, 2018) for (B), Ref. [90] (Zhu et al. in Sci Adv 2021) for (C).

Consideration of matrix effects related with osteoclasts

Compared with the well-known effects of matrix cues on bone forming cells, such as osteoblasts and osteogenic MSCs, those on osteoclasts are much less studied, but they are indeed sensitive to matrix cues, such as ligand composition, stiffness, and nano/microtopology. Due to their unique function of bone resorption at the mineralizing bone surface, osteoclasts can recognize the hardness of mineralizing collagenous matrix as well as the nanoscale topographical change related with mineralizing calcium phosphate

nanocrystals[66,122,123].

Natural bone surface exhibits a highly variable topography, ranging from smooth (Ra < 50 nm) to rough (Ra ~1 μm) surface landscape. Thus, some studies have investigated the topographical (specifically, nano/micro-roughness) effect on osteoclastic activity. When cultured on calcium phosphates with different roughness values, the osteoclastic functions, such as cell fusion and surface resorption, were greater on smoother surfaces (Ra=1 μm vs. 2 μm[66]; Ra=0.13 μm vs. 1.3 μm [123]). However, when the nano-surface was ultra-fine (12 nm, enabled with calcite crystals), the osteoclastic activity was reduced, exhibiting relatively small and unstable actin rings with fewer sealing zones compared with that on submicrometer-roughened (530 nm) surface. It is possible that the ultra-fine roughness (12 nm) might be too small for cell receptors to adopt the matrix topographical cue, i.e., difficult for integrins to cluster and thus to reinforce the intracellular mechano-signalings. Indeed, approximately 30 nm was proven as a threshold for cellular topographical mechanosensing, and a reinforcement of cellular mechano-response (e.g., focal adhesion, spreading, etc.) was enabled through integrin clusters (with a growing size of ~a hundred nm)[124].

The role of matrix stiffness in activating osteoclasto-genesis has also been implicated because osteoclasts showed highly activated YAP/TAZ when cultured on a stiff 2D matrix[125]; YAP/TAZ is a key mechanosensor that can further stimulate cellular mechano-responses. This phenomenon is also understandable in part by the fact that osteoclasts are active on hard mineralized surfaces. On the other hand, another recent study reported an opposite result, where the authors used preosteoblast-derived ECM after decellularization and controlled the stiffness at a relatively smaller range (0.24, 2.24, and 3.21 kPa) by using the lysyl oxidase-mediated crosslinker genipin[65]. Increasing stiffness was shown to decrease the gene expression and maturation of osteoclasts. Such a discrepancy was reasoned to be due to the tailored stiffness range being quite small (far less than mineralized hard matrix which corresponds at least to ~hundreds of kPa), and/or the matrix degradation related with altered crosslinking density might be possible, but needs further study to clarify.

Taking these findings, and despite the limited studies, the osteoclasts, which are programmed to resorb mineralized bone matrix, are considered to be able to sense the underlying substrate biophysical cues (topography, stiffness), and then to adopt their bone resorbing activity. The bone resorbing cells work in proximity to bone forming cells, reciprocally signaling paracrine factors. Thus, their interactions related with matrix cues are a topic of importance, in order to fully understand the fracture healing around bone fixation devices and the regenerating mechanisms at bone substitutes. Moreover, given that osteoclasts share the origin (hematopoietic lineage) in common with immune/inflammatory cells, their interactions at the interface of matrix cues would warrant future investigation.

In vivo observations with mechanobiological implications

While the findings were mainly with *in vitro* studies, some *in vivo* rsearch has evidenced the effect of matrix cues on immune/inflammatory responses, and their impact on bone repair process. One recent study elucidated the stiffness effect of a gel matrix on inflammatory events after implantation in an *in vivo* subcutaneous tissue. Soft materials (fibrin and PEGDA (1kPa) hydrogels) could reduce the expression of inflammatory markers as well as YAP in surrounding macrophages when compared with relatively stiff counterparts (Tegaderm and PEGDA (140 kPa), respectively)[16], supporting again the *in vitro* findings related to matrix stiffness effects on inflammatory cells (**Fig. 10A**).

With regard to matrix topographies, some earlier studies have reported the *in vivo* immune/inflammatory phenomena using porous or nanofibrous scaffolds. For instance, aligned nanofibers reduced the secretion of pro-inflammatory molecules more than random nanofibers[106]. Among other *in vivo* studies, the findings with micro-porous scaffolds (tens of micrometers) are notable. In fact, as previously discussed, the *in vitro* cultured macrophages could mechanically sense the underlying micro-topography, downsizing their pro-inflammatory activation, and this was reasoned to be due to the cellular mechanical confinement[89]. An *in vivo* study by the Ratner group has proven that scaffolds with specific pore sizes (20-30 μm) were less inflammatory in a tissue (myocardium), which was eventually helpful for vascularization; despite lacking mechanistic investigations, this study might be interpreted in a similar context with the above *in vitro* finding, *i.e.,* the mechanical confinement

role of microscale topographies. However, the *in vivo* environment is much more complex than *in vitro*, mainly due to the existence of a plethora of plasma proteins and different sorts of recruited cells. With regard to this, one recent finding explained the reduced macrophages activation in the *in vivo* tissue around microporous scaffolds was associated with the altered profile of proteins sequestered to the scaffolds, which, together with the combinatory role in endothelial cells and MSCs, resulted in significantly enhanced bone regeneration[11].

The *in vivo* impact of nanoscale topography on macrophage behavior and their implications in bone formation have also been demonstrated. Nano-to-micro-scale topographies of TiO_2 (90 nm~5 μm) were tailored on Ti, and the 90-nm nanotopographical surface showed expression of reduced inflammatory markers with upregulated anti-inflammatory signals, and led to enhanced matrix-to-bone osteointegration than other surfaces (dense or 5 μm) (**Fig. 10B**)[90]. Because inflammatory responses related with macrophages occur early at the bone defect (within 1-2 weeks), such a phenotypic alteration of macrophages might modulate the microenvironment favorable for a later stage of bone formation, where the role of other bone forming cells might be dominant.

As discussed above, the dynamic matrix cues, such as viscoelasticity, are of special importance in dictating cellular fate, which being of more relevance to *in vivo* conditions. For instance, fast stress-relaxing viscoelastic gels allow MSCs to be more contractile and mechanically active, which can even override the initial static stiffness cue in terms of osteogenic differentiation. While the impact of matrix viscoelasticity has been highlighted *in vitro* with many different cell cultures (e.g., MSCs, chondrocytes, fibroblasts, and cancer cells), the *in vivo* evidence has relatively been less explored. One of the studies by the Mooney group demonstrated the *in vivo* bone formation altered by stress-relaxation rate, where Ca–cross-linked alginate gels were engineered with two distinct stress relaxations (fast with $t_{1/2}$ ~50 s vs. slow ~800 s) but with a similar stiffness (~20 kPa). The bone formation was found to be greater in the fast-relaxing gel (**Fig. 10C**)[53]; although the underlying mechanism was not fully described in detail, it is plausible that, based on *in vitro* supporting experiments, MSCs that interact with the fast-relaxing gel would recruit more ligand clusters and exhibit higher myosin contractility, thus accelerating mechano-signalings for osteogenesis.

Not only the viscoelasticity, but matrix remodeling is also a key dynamic nature of ECM, and with regard to this, further studies may be needed. Moreover, the influence of dynamic matrix cues on immune/inflammatory cells, such as neutrophils, macrophages, or T cells, needs further investigation, and their interplay with bone forming cells in the context of matrix dynamic properties should help the understanding of bone inflammation and healing events related with implanted biomaterials, and help to bring further improvements.

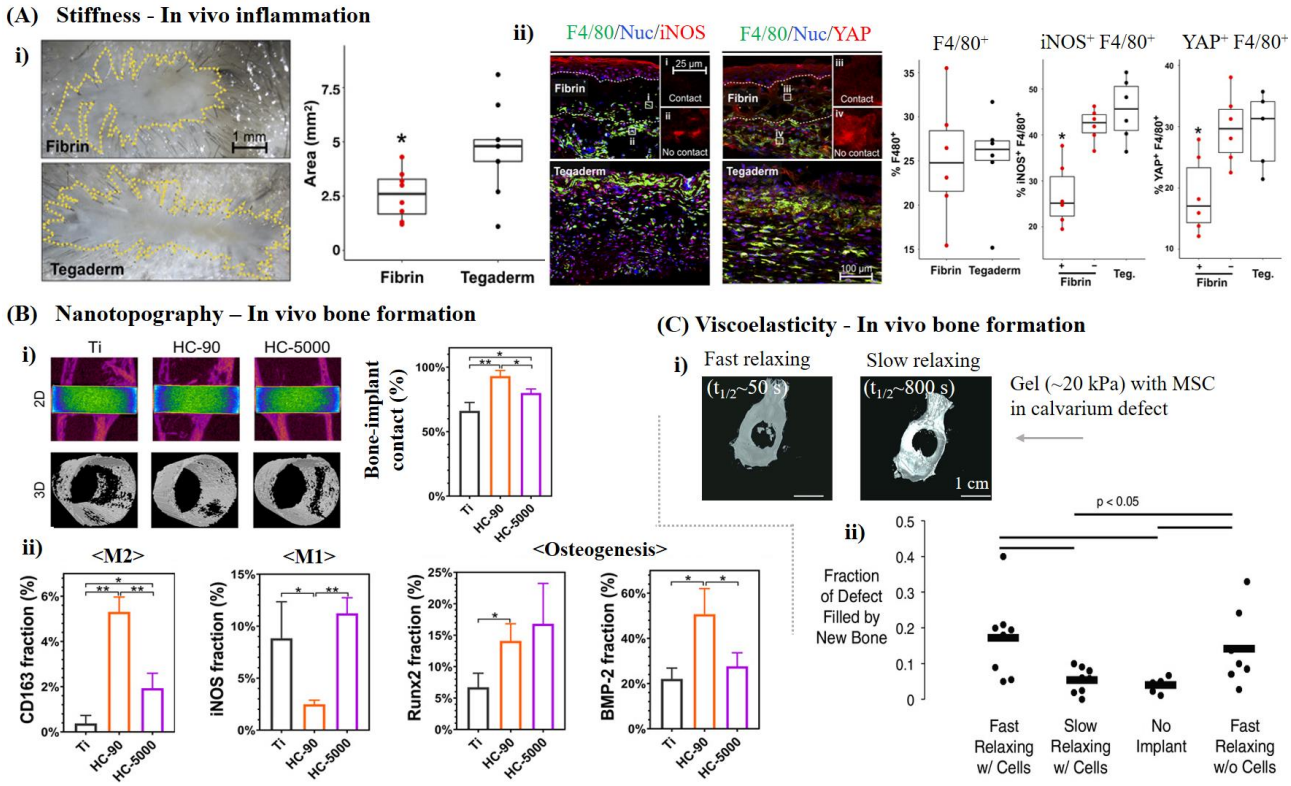

Fig. 10. ***In vivo*** **evidence of matrix-related mechanobiological regulation of inflammation and bone regeneration**. **(A)** Matrix-stiffness regulates inflammation in vivo. (i) Stiff Tegaderm shows higher scar formation area than soft fibrin gel. (ii) Immunohistochemical staining of tissue samples showing higher expression of iNOS (M1 macrophage) and YAP in stiff Tegaderm than in soft fibrin gel. + or – in fibrin group in the graphs shows contact or non-contact region, respectively. **(B)** Nano-topographical effects on in vivo bone formation, related with inflammatory responses. (i) Micro-CT images and the quantification of bone-implant contact reveal higher bone formation in nanotopographical surface, especially in HC-90. (ii) Analysis of tissue samples shows nanotopographical implant has higher M2 (CD163) and lower M2 (iNOS) phenotypic expression, together with higher osteogenic signals (Runx2, BMP-2). **(C)** Stress-relaxation of 3D gels governing *in vivo* bone regeneration. (i) MSCs-encapsulated gel with fast stress-relaxation ($t_{1/2}$ ~50 s) shows higher bone formation than that with slow stress-relaxation (~800 s). Both gels have similar initial stiffness (~20 kPa). (ii) New bone formation quantified shows fast-relaxing gels either with or without MSCs are more effective in bone regeneration. Adapted from Ref. [16] (Meli et al. in Sci Adv, 2020) for (A), Ref. [90] (Zhu et al. in Sci Adv, 2021) for (B), and Ref. [53] (Darnell et al. in Adv Health Mater, 2017) for (C).

Conclusions and future directions

The bone regeneration process is intimately mediated through reciprocal interactions of immune/inflammatory cells with bone forming cells. As discussed, cellular responses in the immune-skeletal axis are dictated by a diverse set of matrix properties, including 2D stiffness, nano/microtopography, ligand type and density, and 3-dimensionality and dynamic mechanics (e.g., viscoelasticity and degradation). Cellular mechano-sensing of the matrix cues and related mechanotransductory signals eventually shape and shift various cell behavior, including adhesion, migration, phenotypic change, and secretome profiling. Therefore, understanding such matrix-related mechanobiological phenomena in osteoimmunology guides us to develop materials, i.e., offers design principles of coordinating both immune and skeletal cells toward successful regeneration while minimizing detrimental inflammatory pathways that otherwise lead to bone impairment.

As witnessed, matrix properties (e.g., stiffness, nano/microtopography, and ligand type and density) have

recently been identified to govern the responses of immune/inflammatory cells, such as T cells, neutrophils, and macrophages of different origins. When considering bone and its surrounding microenvironment, the physico-mechanical properties vary dynamically depending on the anatomical site. For example, matrix stiffness scales over a wide range in the journey of immune/inflammatory cells, from fluid-like soft (initially in blood and bone marrow) to stiff (upon their entry to stromal and mineralized tissue) matrix. Also, immune/inflammatory cells face different levels of stiffness of artificial biomaterials (from soft hydrogels to stiff bioceramics or metallic implants). As such, immune/inflammatory cells taking part in bone repair can sense the stiffness dynamics over the process of attachment, extravasation, and migration, whereby they transduce the extracellular physical sensation to intracellular biochemical signalings. Not only matrix stiffness, but nano/microtopographies featured with different tissue topographies and cell curvatures are also potent regulators of the immune/inflammatory cell behavior. In addition, 3-dimensionality and the fibrous structure of the matrix – the key feature of native 3D ECM – dictate immune/inflammatory cell behavior. Above all, given the various matrix cues are coupled in real (*in vivo*) conditions, findings with combined matrix cues, such as stiffness coupled with adhesion ligand and nano/microtopography are notable; for instances, matrix stiffness was shown to synergize with ligand density in neutrophil activation, and was also found to interplay with nanotopography in T cell activation.

Recent work highlights the dynamic matrix cues, such as matrix viscoelastic properties (e.g., stress relaxation and creep) and remodeling (degradation and deposition), both of which are driven by the cellular interactive process with the matrix. Yet, most work in this area have been with MSCs, fibroblasts, or cancer cells, and based on these studies, the dynamic matrix cues were found to even override the static matrix effect; for instance, MSCs in fast stress-relaxing gels can undergo osteogenesis even under conditions with initially-high stiffness, which otherwise, restricts cellular extension and osteogenic process, hinting at a design strategy for bone regenerative 3D gel matrices. Cellular behavior in a degradable matrix (e.g., degradable by cell-secreted MMPs) can also be interpreted in a similar context. Furthermore, cell-secreted matrix alters the composition and local biomechanics, masking and overriding the initial matrix properties, which ultimately modulates cellular mechanotransduction. However, studies on the effects of dynamic-matrix-cued osteogenesis on immune/inflammatory cells or vice versa are largely limited, necessitating future in-depth investigations. Also, the fact that bone tissue undergoes mineralization over time, a sort of dynamic matrix stiffening process occurring uniquely in bone repair, underlines future work is needed in this area. These studies will substantiate our understanding of the matrix-induced bone regeneration mechanisms and guide us as to how to design artificial matrix for bone by regulated osteoimmunology.

One note here is that the process by which cells recognize the different matrix cues is through the mechanosensitive machineries, such as integrins, FA molecules, actomyosin, LINC molecules, lamins, and ion channels, which are key research elements in mechanobiology field. Recent works in mechanobiology have grown rapidly with some focused events; for instances, matrix-induced forces transmission to nucleus, altering chromatin accessibility, and profound key roles of stretch-activated ion channel Piezo1/2 in various pathophysiological conditions. Therefore, the studies on matrix-induced osteoimmunological phenomena need to be updated with research progress in mechanobiology. This will enlarge our views on mechanobiological mechanisms underlying the osteoimmunology events, and find possible therapeutic targets (e.g., mechanosensitive machinery or its repressor/enhancer) – a new therapeutic approach of how to modulate cellular mechanosensitivity for matrix cues.

While a wealth of studies were carried out in *in vitro* conditions using engineered matrix parameters with immune/inflammatory cellular events, such as phenotypic change, migration, and immune-activation, *in vivo* investigations are largely lacking. Thus far, only a fraction of *in vivo* work has been documented, which contributes to understanding the significance of matrix properties (e.g., pore size, fiber diameter, nanotopography) in inflammatory responses, and the resultant tissue healing or bone formation. As such, more in-depth *in vivo* studies would be of paramount importance, particularly focusing on *in vivo* immune/stromal cellular interactions in response to engineered matrix cues, such as combined cues of implants or dynamic cues of hydrogels, e.g., elucidation of the type of immune/inflammatory cells recruited and the degree of activation, such as NETosis, macrophage polarization, or T cell function, around different stress-relaxing gels with similar stiffness, and the resultant tissue healing, osteogenesis, and bone formation. This will deepen our understanding of why some of the bone scaffolds and implants have been found to be more bone-regenerative and osteo-integrative, which will advance the development of bone biomaterials.

As discussed, compelling evidence underlie that native ECM and engineered biomaterial cues are key to determining the interactions between immune/inflammatory cells and bone forming/resorbing cells, i.e., regulating osteoimmunology and bone regeneration. The agendas put forward in this communication are essential in advancing matrix-related osteoimmunology and bone regeneration, but are rarely explored yet, thus warranting future in depth studies. Advancing this will help us understand the matrix-enabled bone regeneration process, identify the mechanobiological mechanisms and therapeutic targets underlying the events, and design materials for bone regeneration by immune/inflammation modulation.

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Author contributions

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Declaration of interests

The authors declare no competing interests.

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