Location, location, location: understanding how the local tissue microenvironment drives inflammation in arthritis

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Abstract

Current treatments for rheumatoid arthritis (RA) do not work well for a large proportion of patients, they do not work at all in some people, nor can they cure or prevent this disease. One major obstacle to developing better drugs is lack of a complete understanding of how inflammatory joint disease arises and progresses. Here, we discuss emerging evidence as to how the tissue microenvironment impacts RA pathogenesis. Each tissue is made up of cells surrounded and supported by a unique extracellular matrix. These complex molecular networks define tissue architecture and provide environmental signals that programme site-specific cell behaviour. In the synovium, a major site of disease activity in RA, both positional

and disease stage-specific cellular diversity exists. Improved resolution of the architecture of the synovium, from gross anatomy to the single cell level, in parallel with evidence demonstrating how the synovial extracellular matrix is vital for synovial homeostasis, and how dysregulated signals from the matrix drive chronic inflammation and tissue destruction in the RA joint, have opened up new ways to think about RA pathogenesis, and offer novel therapeutic approaches for people with hard to treat disease, or as a means of disease prevention.

Introduction

Tissue specialization is essential for life. However, the fundamental principles that drive tissue-specific cell behaviour are not fully understood. For example, why are fibroblasts in the gut so different to those in the skin, and why do macrophages resident in the brain behave differently to those in the liver? Technologies that can interrogate tissues at the single cell level are being used to generate an encyclopedic inventory of the different cell populations comprising each tissue of the body, revealing extraordinary levels of cellular complexity and phenotypic plasticity. Mapping the anatomic location, and the interaction networks, of newly discovered cell subsets will be the next essential step towards understanding tissue structure and function. Moreover, cells do not exist in a vacuum. The tissue microenvironment is a key determinant of cell behaviour, enabling cells to perform distinct roles dictated by their anatomical location, as well as specifically by their location within tissues. But what defines the microenvironment? Cells in tissues are surrounded and supported by an extracellular matrix. In each tissue the matrix is made up of a combination of more than 1000 different secreted molecules that is unique to that

tissue, assembled into a complex 3D network, providing external cues that govern cell behaviour. Understanding how tissues function in health and disease therefore requires knowing both the identity of resident cell populations and how complex external microenvironments cohesively define cell phenotype in situ.

In this review we focus on the synovium, and examine how changes in both the cellular and extracellular compartments of this tissue play a causal role in driving chronic inflammation during rheumatoid arthritis (RA). We will review how recent single-cell transcriptional analysis has revealed extraordinary microanatomical complexity within the RA synovium, identifying at least 18 distinct cell phenotypes, amongst which diverse subpopulations exhibit striking positional and functional segregation. We discuss how these studies provide compelling new insights into the cellular basis of inflammatory joint disease. We also highlight the evidence that extracellular networks create anatomically distinct sub-synovial niches within which environmental cues dictate site-specific behaviour, that is behaviour that is unique to the position of any cell within a tissue. We detail how these networks directly contribute to chronic inflammation in the inflamed joint, and we examine why this information changes the way we think about how inflammatory joint disease arises and progresses, offering new methods of patient stratification, as well as novel classes of therapeutic drugs. Finally, we highlight the key questions and challenges that remain.

What exactly is the tissue microenvironment?

All tissues consist of cells surrounded by an intricate extracellular matrix. This 3D network of secreted molecules provides structural support for cells and dictates their spatial organization within tissues. However, the matrix is not simply an inert scaffold, it also a key determinant of cell phenotype, providing environmental cues that enable cells to move relative to each other as well as perform distinct roles determined by their anatomic location[1,2]. Extracellular matrices are made from a selection of more than 1000 molecules collectively called the matrisome. Genes in the matrisome code for all of the proteins that can be secreted by cells, encompassing extracellular matrix molecules, matrix-associated proteins, soluble growth factors, chemokines and cytokines, and enzymes including proteases and kinases[3] (http://matrisomeproject.mit.edu/).

Expression of site-specific combinations of matrisome molecules, and their assembly into networks around cells, creates unique tissue microenvironments, as well as local niches within tissues. Integrated mechanical and biochemical cues from each type of matrix provide essential context for cell behavior, wherein distinct combinations of extracellular molecules cohesively define cell differentiation and specialization. For example, joints are specialized multi-tissue organs that provide the structures by which bones move relative to each other, and by which muscles mediate coordinated locomotion. The components of a classical human synovial joint include tissues such as the synovium, tendons, muscle, ligaments, bursae, menisci, articular cartilage and subchondral bone. Each constituent tissue of the joint is made up of a unique combination of matrisomal molecules that confer the distinctive physical properties that together are essential for effective joint function (**Box 1**).

The extracellular matrix is as dynamic as it is complex, changing throughout development and ageing, as well as during inflammation and disease. However, for most human tissues, including the joint, we lack a detailed understanding of the molecular and topological organization of the extracellular networks surrounding cells. It is also not clear how tissue architecture changes during inflammation, nor the functional implications of these changes. Here, we review emerging data that highlight the importance of understanding the complex interplay between cells and their matrix microenvironment in defining cell behaviour within the synovium, and in controlling joint inflammation.

Complex tissue architecture within the synovium

The synovium is an intricate tissue, made up of a number of cell types including tissue resident macrophages, fibroblasts, nerve and endothelial cells. Even at the gross histological level, subcellular compartmentalization within the synovium is evident forming two distinct zones; the intima lining layer and the subintima (**Box 1**). In a healthy joint the intima is only 1-3 cells thick, and is composed of tissue resident macrophages and fibroblasts supported by a porous basement-like membrane. This zone of the synovium controls cellular and molecular ingress and egress between the synovium and the joint cavity, playing a key role in maintaining joint integrity and the composition of synovial fluid, ensuring effective joint lubrication and nutrient exchange. The subintima, comprising fibroblasts distributed throughout a looser collagenous extracellular matrix, and containing blood and lymphatic vessels, and

nerves serves to vascularise and enervate the synovium, and provide transport routes for cells, nutrients and lymph into and out of synovial tissue[4].

The synovium becomes markedly expanded in RA, with the intimal layer increasing up to as much as 10-20 cells in thickness. Infiltrating immune cells join resident macrophages and proliferating fibroblasts to cause synovial hyperplasia. This quantitative change in the cellular ecosystem is accompanied by qualitative changes in cell phenotype; expansion and activation of lymphocytic, myeloid and fibroblast subpopulations that promote inflammation and tissue destruction, alongside suppression of cell subsets that mediate the resolution of inflammation, occurs, driving the immune status of the joint towards chronic inflammation[5,6].

Changes in the organization of the synovial architecture are also evident in RA. There is not just vast and random cellular influx and expansion; a specific selection of cells only enter the joint, organized by the chemokine repertoire of the synovium. Moreover the tissue is markedly reorganized, creating new compartmentalized niches within which pathogenic cell behaviour is confined[5,6]. For example, ectopic (or tertiary) lymphoid structures develop in the synovium during RA in around 40% of patients, with around 10-25% of samples exhibiting germinal center-like structures[7]. These aggregates of lymphocytes resemble secondary lymphoid organs, albeit with varying degrees of organization, characterized by a T cell-rich zone enclosing a central B cell-rich zone, served by a network of high endothelial venules that enhances naïve T and B cell recruitment to the synovium (reviewed in [8]). Biopsy studies have shown the existence of gradients of CXCL13 and CCL19/CCL21 which

support cellular segregation, and where B cells differentiate in situ into plasma cells, supporting autoantibody production[8]. Lymphoid-rich synovitis, defined by a distinct transcriptomic profile, and by high serum CXCL13, represents a histologically distinct subset of patients with high disease activity, who are difficult to treat[9]. These data exemplify how disease pathotypes or endotypes can be categorized based on synovial cell ecosystems.

The pannus is also a well-described architectural feature of the inflamed synovium. Although used historically, the term pannus is likely to be replaced with 'activated aggressive RA synovium'. This region of hypertrophic synovium, often called the aggressive front, is composed of macrophages and fibroblasts that release tissue degrading enzymes responsible for invasion of cartilage and bone[6] (**Figure 1a**). Most interestingly is the fact that RA synovial fibroblasts attach to the cartilage matrix and invade it progressively and destructively, a close relationship that has been observed in studies of the MLR/lpr mouse model[10], as well as models using engraftment of human synovial tissue or isolated synovial fibroblasts together with human cartilage in SCID mice[11,12]. These areas of invasive pannus formation have been well studied at the molecular level, revealing that this tissue niche is hypoxic[13], and displays discreet patterns of gene expression. This encompasses upregaulation of genes such as MMPs[14,15], TLRs[16], p53[17,18] and SUMO/Sentrin[19], and down regulation of the tumor suppressor gene PTEN[20], which combine to create a destructive milieu in which aggressive pannus-resident cells are protected from apoptosis. Moreover, changes in epigenetic marks have been suggested to contribute to the aggressive phenotype of synovial fibroblasts at the site of invasion

into cartilage[21]. Expression of tissue degrading enzymes and apoptosis-inhibiting factors in RA synovial fibroblasts found at the sites of cartilage destruction is associated with gene hypomethylation; and this altered epigenetic landscape might explain why therapeutically targeting the progression of RA joint destruction is extremely difficult[22]. Some studies have also reported how the tissue microenvironment itself changes within the pannus, and the consequences of altered extracellular protein expression on localized tissue invasion. For example, galectin-3, a secreted beta-galactoside-binding protein that is elevated early in RA pathogenesis, localizes almost exclusively to the pannus in the inflamed synovium (**Figure 1b**)[23,24]. Galectin-3 directly activates synovial fibroblasts, stimulating secretion of inflammatory cytokines, such as interleukin-6 (IL-6), and chemokines, such as IL-8, CCL2, CCL3, and CCL5, as well as MMP3, via activation of MAPK and phosphatidylinositol 3-kinase (PI 3-kinase) signalling pathways[25]. Moreover, galectin-3 expression by RA synovial fibroblasts is required for IL6 synthesis downstream of TLR2[26], a pattern recognition receptor that also localizes to the pannus in inflamed synovia (**Figure 1c**)[16]. Together these data imply that local interplay between galectin-3 and TLR2 serves to activate pannus-resident synovial fibroblasts, in a cytokine-independent manner, and recruit immune cell infiltration to reinforce inflammation specifically at this key pathogenic site.

Thus it becomes apparent how localized changes in the tissue occurring in RA direct site-specific aspects of pathology, and might explain the fact that targeting cytokines in RA is not enough to cure this disease. However, a systematic cellular atlas that describes the spatio-temporal organization of synovial cells is missing; little is known

about how many different cell subsets make up this tissue, nor their organization into functional networks.

Single cell resolution of the RA synovium

A step change in our ability to perform a cellular census of the cell types present in synovial joints has occurred because of advances in minimally invasive ultrasound-guided biopsy techniques, coupled with tissue digestion and single cell (sc) RNA sequencing [27-29]. Using these precision molecular analytics, multiparameter imaging and state of the art bioinformatics, recent work from tissue in the inflamed joint has revealed further insight into the complexity of the synovium, showing the RA synovium to be comprised of at least 18 distinct types of types of T cells, B cells, macrophages and fibroblasts[29] and allowing us to compile for the first time a synovial map of the leucocyte and stromal cells in the synovium in diseases such as OA and RA[29,30](**Figure 2**).

These studies have revealed unprecedented insight into anatomical and functional specialization of synovial cells. It has long been known that not only T cell number, but also the balance amongst T cell polarization, is a key determinant of immune status, for example lower ratios of Tregs compared to Th17 subsets contribute to impaired immune restraint and chronicity of inflammation[31]. Now, in the human RA joint, the existence of a pathogenic T cell population (termed TPh) that express high levels of PD1 but not CXCR5, has been identified to be highly expanded in seropositive RA patients and not seronegative[32]. These data indicate complexity in the rheumatoid T cell compartment that have not been previously appreciated.

It is also now clear that synovial fibroblasts exhibit striking positional and phenotypic segregation, with inflammatory Thy1 positive populations predominating in the sublining layer and destructive populations in the intima or lining layer, together with a further, distinct, subpopulation populating the perivascular space. Moreover, inflammatory populations of synovial fibroblasts have been shown to expand in the synovial sublining layer in RA compared to OA, contributing to immune dysregulation, whilst destructive populations in the lining layer are responsible for cartilage and bone destruction during disease[30] (**Table 1, top panel**). This degree of cellular resolution and functional delegation starts to unravel disease progression at a new level**.**

New details are also emerging around macrophage populations in the RA joint. Evidence suggests that tissue resident macrophages in the intima serve a barrier function that maintains immune privilege in the joint. This becomes compromised in RA, allowing unrestricted infiltration of monocyte-derived cells, whilst preventing inflammation in OA. In contrast, subintimal macrophages comprise heterogeneous monocyte- and tissue-derived populations, amongst which pro-inflammatory phenotypes dominate in RA[33] (**Table 1, bottom panel**). An independent study also highlighted RA synovial macrophage heterogeneity, in this instance with a focus on comparative analysis of disease remission and disease flare. Four distinct subpopulations were identified, comprising nine discrete phenotypic states, amongst which two subpopulations (MerTK+TREM2hi and MerTK+LYVE1+) were enriched in people whose RA was in remission compared to those with active disease, and

whose contraction was associated with increased risk of disease flare. These subsets can induce synovial repair responses via production of inflammation-resolving lipid mediators[34]. Finally, the existence of HBEFG(+) macrophages and fibroblasts in the rheumatoid synovium that induce fibroblast invasiveness has provided insight into functional, pathogenic cellular interaction networks across subpopulations from different lineages[35].

Together these studies demonstrate how our understanding of the architecture of the joint has progressed from gross anatomy, through subsynovial structures, including pannus tissue and tertiary lymphoid structures, to the single cell level, and how this has enabled the emergence of a more complete cell atlas of the joint. These data have also shown how changes in the balance of synovial cellular ecosystems underpin chronic inflammation during the onset and progression of RA compared to OA. Some of the underlying drives of these changes are beginning to emerge, for example, the expansion of Thy1 positive fibroblasts in the RA sublining is NOTCH3 dependent[36], compared to the lining layer, where Thy1 negative fibroblasts, along with lining layer MerTK positive macrophages, contract in active disease. Moreover, the increases in the ratio of MertK positive to negative macrophages in the RA synovium in patients in disease remission suggests that lining layer macrophages regulate remission in RA[34].

These data may aid in therapeutic strategies that target pathogenic cell populations in RA. For example, functional subclasses of fibroblasts have proven difficult to define, characterize and study in health and disease. Consequently, there are no

approved drugs that specifically target fibroblasts in human diseases. The recent identification of "pathogenic" fibroblast subpopulations[30] offers an attractive new, non-immunosuppressive therapeutic target. However, fibroblasts are a functionally heterogeneous group of cells that support discrete biological functions within the joint tissue. This has led to a therapeutic dilemma: which fibroblast subsets should be targeted and suppressed and which should be retained and augmented? A clear understanding of the biology and clinical significance of fibroblast heterogeneity is therefore essential to provide a coherent rationale for their therapeutic targeting in treatment of diseases such as RA. The selective targeting of pathogenic fibroblast subsets using anti-fibroblast monoclonal antibodies, analogous to B cell depletion using CD20 (rituximab), would complement other targeted therapies commonly used against leucocytes and their cell products[37,38]. Improved resolution of RA synovial macrophage subsets also now offers the potential for additional arsenal in modulating pathogenic myeloid cell behaviour, with MerTK+ subsets, or anti-inflammatory mediators released by these cells during disease remission, offering tractable targets for boosting synovial repair processes[34].

However, despite a clearer picture of the cellular networks inhabiting the RA synovium, it still remains uncertain what initiates and maintains pathogenic behaviour in different cell subsets in RA.

Immunological geography

It is now clear that synovial cell networks compartmentalize in distinct microdomains within the healthy joint, and that distinct, sub-synovial, niches arise in the RA

synovium compared to OA during disease progression. It is also clear that synovial cells do not exist in a vacuum, and an understanding the microenvironmental cues that shape their phenotype will provide key insight into joint tissue homeostasis and disease. The extracellular matrix can impact cell behavior via a diverse range of mechanisms[39], all of which contribute to defining synovial tissue biology, discussed below and summarized in **Table 2 and Figure 3**.

Physical properties and mechanical cues

The extracellular matrix defines the physical properties of tissues. For example, synovial fluid is the richest source of hyaluronic acid (HA), a glycosaminoglycan (GAG) comprising polymeric disaccharide repeats, which protects cartilage from frictional damage[40]. Coating of articular surfaces with lubricin, or proteoglycan 4, a mucinous glycoprotein also found in synovial fluid, is the major means of effective joint lubrication[41]. Matrix molecules also bind to other matrix molecules to form complex, multicomponent structural networks. For example the thin membrane of the synovial lining layer comprises types III, IV, V and VI collagen and laminin, which supports intimal cells and acts as a molecular sieve, controlling bidirectional solute transfer between the synovium and synovial fluid[4,42]. This specific architecture is key to allowing controlled, bidirectional flow of cells and molecules between the synovium and the joint cavity, maintaining tissue structure and integrity, controlling synovial fluid content and volume, clearing up debris and maintaining immunological homeostasis[43].

In addition to structural functionalization, the mechanical properties of the matrix also provide key environmental cues to tissue resident cells. In this way, not only the molecular content of the matrix dictates cell behaviour, but also the physical structure of the matrix itself defines the mechanical cues derived from the tissue[44]. For example, interstitial cell migration within the fibrous synovial microenvironment is regulated both by tissue microstructure, such as matrix alignment and porosity, and tissue micromechanics, such as tensile, compressive and shear moduli, which cells use directly to sense biophysical cues via integrin receptors[45]. Emerging data also shows how changes in tissue mechanics controls immune cell plasticity and polarization. For example, spatial confinement restricts late events in the activation of pro-inflammatory macrophages[46], which may have implications in how immune responses are modulated as tissue stiffness changes with synovial hyperplasia and fibrosis. In a manner analogous to matrix stiffness within the tumor microenvironment emerging as a key determinant of cancer progression and treatment response[47,48], so too the influence of the mechanical properties of the synovium, derived from the matrix content and higher order organization, on disease progression in RA should be considered.

Tissue architecture and spatial positioning

The extracellular matrix controls the spatial positioning of cells within tissues. For example, both lubricin and HA exert anti-adhesive properties which prevents cell adhesion at smooth articulated surfaces within joints that would be impeded by cell occupancy[4]. Conversely, deposition of the pro-adhesive matrix molecule fibronectin within the synovial lining layer membrane helps to maintain cellular interaction

networks by anchoring synovial fibroblasts to their surrounding matrix[49]. Ectopic expression of fibronectin in the RA joint enables aberrant cell adhesion, for example, high levels of fibronectin in the pannus enhance synovial fibroblast adhesion to cartilage, stabilizing invadopodia, actin-rich protrusions of the plasma membrane that are associated with tissue degradation, by promoting coherent points of anchorage that facilitate cartilage invasion[50]. Expression of fibronectin at the basal lamina and at the endothelial surface in inflamed synovium has also been proposed to serve as a permissive migration track for infiltrating lymphocytes, enabling T cells to cross the endothelial basement membrane in RA[51,52]. The matrix also plays a key role in restricting cell migration, with the synovial membrane serving a barrier function to maintain immune privilege in the synovium, which is disrupted in RA[33].

Patterning of soluble factors

Soluble factors such as cytokines, chemokines and growth factors, by virtue of their being secreted by cells, are part of the matrisome (**Box 1**). The role of several of these inflammatory mediators in RA is well documented, and forms the basis for a number of key current biological therapies used to treat people with RA[53]. However, within tissues these molecules often require interaction with other matrisomal components to signal, and their presentation, concentration and bio-availability throughout the synovium provides key context for their function. Indeed, core matrisomal molecules have been shown to control the localization of soluble factors in tissues, and are key determinants of their activity. Chemokine immobilization by GAGs, in particular heparan sulfate proteoglycans (HSPGs), at the luminal endothelial surface of blood vessels establishes chemokine gradients for migrating leukocytes[54],

as well as protecting these soluble factors from degradation[55], and facilitating oligomerization required for optimal activity[56]. For example, in the RA synovium elevated expression of the HSPG syndecan-3 tethers CXCL8 in the endothelial lumen, and this interaction has been shown to promote leukocyte trafficking into the inflamed tissue in vivo during antigen-induced arthritis[57,58]. The matrix is an essential reservoir for other soluble factors including cytokines, bone morphogenetic proteins (BMPs), Wnts and growth factors, where binding is often promiscuous, but is specific. For example, fibronectin, vitronectin, tenascin-C, osteopontin, type I collagen and fibrinogen each bind to several soluble factors from amongst the vascular endothelial growth factor (VEGF), platelet derived growth factor (PDGF), fibroblast growth factor (FGF), transforming growth factor (TGF), insulin-like growth factor (IGF) and BMP families. However, each matrix molecule has a distinct set of soluble binding partners. Moreover, these molecules bind with different affinities across each family of growth factors; e.g. tenascin-C binds to VEGF-B but not VEGF-A, vitronectin binds to FGF-18, whilst tenascin-C does not, and neither bind to FGF-1 or -6 [59]. These interactions not only control tissue levels and locations of soluble factors, but are also essential for their function by serving as co-receptors. Proteoglycans in particular are well documented accessory molecules[60], with syndecans playing key roles in cartilage breakdown and synovial inflammation[61]. For example, optimal activity of FGF2, a growth factor up-regulated in RA, where it contributes to driving fibroblast activation during disease progression[62], requires the formation of a ternary complex between the HS chains of syndecan-4 and the FGF receptor, as well as signaling via cytoplasmic domain of syndecan-4 to strengthen the duration and intensity of downstream signaling upon ligand binding[63]. As such,

the role of many soluble factors may not be fully understood without examining how they interact with other extracellular tissue components. Moreover, simply targeting the activity of individual soluble factors in RA may not represent the most effective, or tissue-specific means of modulating their activity.

Direct signalling to cells

Matrix molecules provide key biochemical signals directly to cells. By virtue of their ability to interact with a large repertoire of cell surface receptors, including integrins, they can influence cellular behaviour ranging from proliferation to survival to cell death, and differentiation. Small, soluble effector molecules tend to evoke relatively simple signaling pathways, for example TNF at 17kDa activates just two receptors, TNFR1 and TNFR2[64]. In contrast, matrix molecules are much larger, multimodular molecules, with far more complex interaction partners. For example thrombospondin-1 is a 450kDa secreted glycoprotein with seven modular domains, that is elevated in RA serum and synovium[65,66], and which has at least 83 different ligands, including other matrix molecules and soluble factors, as well as a plethora of cell surface receptors[67]. Direct cues from the tissue microenvironment play a key in maintaining tissue homeostasis. Endogenous danger signals are immunologically silent in healthy tissues, but which trigger inflammatory responses upon cellular stress or tissue damage. These can include alarmins, intracellular molecules that are released to the extracellular milieu during cell activation or death[68], as well as extracellular matrix molecules whose expression is upregulated or modulated upon tissue injury, or which undergo post-translation modification[69]. These damage associated molecular patterns (DAMPs) are sensed by pattern receptors such as TLRs

and integrins, triggering innate immunity and shaping adaptive responses designed to restore homeostasis and activate tissue repair. In the joints of people who do not have RA, these signals are essential in order for cells to detect and respond to injury and insult. However, dysregulation of these pathways is emerging as a major cause of chronic inflammation and tissue destruction in RA. For example, tenascin-C is an extracellular matrix molecule that is not expressed in most healthy tissues including the joint, but is transiently upregulated following tissue injury where it activates TLR4-mediated inflammation. Typically downregulated and cleared from tissues following repair, tenascin-C accumulates at high levels in the synovium of people with RA. Expression of this pro-inflammatory matrix molecule is required for the persistence of joint inflammation and tissue destruction in several different models of arthritis[70-72].

These studies collectively exemplify how the extracellular matrix surrounding and supporting synovial cells plays a key role in dictating site-specific behavior within the synovium. Emerging data also indicate dysregulated signals from the matrix drive chronic inflammation in the joint during the pathogenesis of RA, and that targeting these signals may provide an effective means of restoring immune control.

The extracellular matrix in the pathogenesis of RA

Whole exome sequencing has identified new genetic variants associated with RA susceptibility, amongst which genes in extracellular matrix-receptor pathways were most highly enriched (COL4A4, COL6A5, COL11A1, COL11A2, HSPG2, ITGB5, LAMC1, THBS1, RASGRF1, FLNB, MYL5)[73]. Microarray analysis comparing healthy and RA

synovium also revealed differentially expressed genes involved in cell adhesion and organization of the extracellular matrix (PTPRC, SDC1, CD8A, CD2, HLA-DPA1, ITGA4, HLA-DMB, CD6, HLA-DOB, PDCD1LG2, COL3A1, SDC1, COL1A2, INTGB2)[74]. Whilst the impact of sequence variation, or up-regulation, of these genes in people with RA is not known, these data implicate changes in the matrix and microenvironment in disease pathogenesis.

Altered tissue turnover has long been a pathological hallmark of RA[5,6,75,76], and serum levels of matrix metabolites are commonly used biomarkers for joint remodeling and bone degradation[77,78]. For example, the C-telopeptide fragment of type I collagen (CTX-I) generated by osteoclast-derived cathepsin K reflects bone resorption[79], whilst osteocalcin produced by mature osteoblasts, and the N-terminal type I procollagen propeptide (PINP) released during collagen fibril synthesis, reflect bone formation[80]. Cartilage degradation is assayed by examining serum levels of cartilage oligomeric matrix protein (COMP)[81], the C-terminal telopeptide of type II collagen (CTX-II)[82], and C2M, a fragment of type II collagen[83]. Synovial remodelling is reflected by high circulating C1M, C3M and C4M, fragments of type I, type III and type IV collagen generated by MMP cleavage[84-87], or proteases implicated in tissue destruction, such as total MMP-3 or the activated form of MMP-3[88,89]. A reduction in serum matrix metabolites accompanies positive response to therapies including tocilizumab, etanercept, methotrexate, adalimumab, and tofacitinib (for example; [86,90-93]). Analysis of these biomarkers at baseline can also predict people who will respond well to tocilizumab[90], as well as predicting lack of efficacy of Syk inhibition via fostamatinib on structural end points[94]. These serological markers therefore

serve as reliable surrogates of tissue destruction in RA, and may prove useful in stratifying patient treatment response. Emerging data also show that matrix metabolites are not simply inert collateral damage released from joint tissue as disease progresses, but active players in RA pathogenesis.

Expression of the tissue-degrading enzyme MT1-MMP is elevated in the RA joint, at sites of pannus invasion into cartilage[15]. Collagen-induced upregulation of MT1-MMP via DDR2 activation on synovial fibroblasts is more pronounced in variants missing non-helical telopeptides compared with intact collagen fibrils, and is enhanced in response to damaged cartilage[95], suggesting a positive feedback loop in which collagen degradation reinforces further tissue destruction. Fragments of hyaluronic acid (HA) are also detected in RA synovial fluid[96]. The size of HA fragments dictates the function of this glycan, for example low molecular weight (MW), but not high MW, fragments activate TLR2-mediated inflammation in macrophages[97]. Fragments of osteopontin are also elevated in synovial fluid from people with RA[98]. Thrombin cleavage of this matrix molecule creates a C-terminal fragment that induces CD44-dependent macrophage chemotaxis, and an N-terminal fragment that promotes β3 integrin-mediated macrophage spreading and activation[99,100]. These data suggest that elevated levels of matrix metabolites contribute to both tissue remodeling and inflammation in RA.

The pro-inflammatory activity of osteopontin fragments is further regulated by phosphorylation; whilst the chemotactic activity of the C-terminal fragment is independent of modification, macrophage activation leading to cytokine and MMP

release by the N-terminal fragment requires phosphorylation[99,100]. Higher levels of phosphorylated osteopontin, and phosphorylated osteopontin fragments, were observed in synovial fluid from people with RA compared to OA patients, whilst total osteopontin levels did not discriminate RA from OA[101], suggesting that both proteolytic processing and post-translational modification of the matrix contributes to disease activity. Indeed, autoantibodies recognizing citrullinated proteins (ACPA), the post-translational conversion of arginine to citrulline catalyzed by peptidyl arginine deiminases, are gold-standard diagnostic markers for RA[102]. ACPA recognize a number of modified matrix molecules (reviewed in [103,104]), including citrullinated epitopes in type II collagen[105], well-established pathogenic drivers of joint disease in vivo[106,107]; citrullinated fibrinogen[108], levels of which predict higher DAS 28 scores[109]; citrullinated tenascin-C[110], which may delineate different disease aetiologies[111]; citrullinated aggrecan, which correlate with higher frequencies of cit-aggrecan-specific T cells in people with RA[112], and citrullinated fibronectin[113]. Intra-articular injection of citrullinated collagen and fibrinogen enhances their arthritogenic potential compared to unmodified protein[114-116]. Moreover, citrullination of fibrin(ogen) and fibronectin *in vitro* enhances their pro-inflammatory capabilities[117-119], whilst citrullination of collagen and fibronectin alters their integrin binding repertoire and capacity to support synovial cell adhesion[113,118,120]. Citrullinated fibronectin also effectively promotes cell survival, in contrast to induction of apoptosis by the native molecule [49,117], whilst the modified form exhibits increased affinity for VEGF but is less effective at binding to, and inhibiting, the aggrecanase ADAMTS4[121,122]. As such matrix modification can not only break tolerance, i.e. create novel antigen epitopes that lead to the generation of T and B cell responses

against endogenous molecules, it can also generate pathological protein variants that may exacerbate inflammation in the RA joint.

RA diagnosis: the truth is in the tissue

One question arising from the study of circulating matrix metabolites, or antibodies recognizing modified matrix, is how well these markers reflect tissue pathology in the joint. Examining collagen, fibrinogen and fibronectin ex vivo in synovial biopsies by immunohistochemistry has been used to assess the degree of fibrosis in the RA synovium[123]. This approach, whilst more invasive than serological analysis, takes into account that synovial pathology is compartmentalized, allowing examination of disease pathogenesis in the context of synovial anatomy. These details are likely to be important. For example, microfibrillar-associated protein 4 (MFAP4), a matrix molecule that associates with elastin and collagen, is implicated in stromal hyperplasia and fibrosis in liver and lung disease[124]. MFAP4 is found at similarly high levels in the serum and synovial fluid from people with RA and OA, compared to low levels in healthy controls. In the tissue, it is detected in synovial sub-lining arteriole vessel walls and in adventitial tissue at sites of immune cell infiltration. However, it is absent from the internal elastic membrane of vessels in RA synovia, whilst present at high levels at this site in OA synovia[125]. The consequences of differential distribution of MFAP4 in OA and RA synovia are not yet clear, but these data highlight that alterations in local tissue architecture are not always reflected in 'bulk' serum or tissue analysis.

Whilst circulating biomarkers therefore can be correlative with tissue pathology, they are not always causal, and it is clear that changes in the serum do not mirror the totality of changes in the synovium. Work examining the distribution of tenascin-C exemplifies how important mechanistic detail can be lost without the context of tissue anatomy. Levels of this pro-inflammatory matrix molecule are elevated in RA serum and synovial fluid[126,127], correlating with bone erosion during disease, and predicting poor improvement in pain in response to anti-TNF treatment[127]. In the RA synovium, tenascin-C is found predominantly in the sublining layer, where it is restricted to two specific niches; a dense matrix surrounding CD34 negative fibroblast populations, and close to CD34+ perivascular fibroblasts located underneath blood vessels at sites of lymphocyte infiltration[128]. This highlights specific cellular targets for tenascin-C in the RA joint, which may have remained obscured without anatomical analysis, and directs further mechanistic investigation, for example what role tenascin-C might play in promoting prolonged activation of inflammatory signaling in fibroblasts[71,129] or in modulating pericyte adhesion, migration[130] or differentiation[131] during RA.

Considering the advances in our knowledge of the cellular and molecular basis of synovial inflammation, it is clear that analysis of cell subset interaction networks in the tissue (for example inflammatory versus destructive fibroblasts, TPh cell or HBEFG(+) macrophage burden), together with the microenvironmental cues that instruct their behavior, is likely the most accurate way to assess the underlying events driving RA, enabling more precise disease classification, leading to process driven patient stratification and better targeted therapeutic intervention. However,

whilst advances in synovial biopsy methodology have enabled safer and more practicable tissue acquisition, sometimes involving two or more repeat samples[132], by design interrogation of tissue micro-niches may be subject to sampling heterogeneity, and approaches designed to image the synovium *in vivo* may provide a useful complement to tissue harvest. Positron emission tomography (PET) using targeted radiotracers to visualize specific matrix components including collagen[133] or fibronectin[134] is developing as a viable method to image tissue fibrosis in vivo (reviewed in [135,136]). PET imaging of GPVI-Fc, a fusion protein comprising the soluble human IgG1 Fc domain and the extracellular domain of platelet glycoprotein VI, a trans-membrane platelet glycoprotein that binds with high affinity to matrix molecules including collagen, fibronectin and fibrinogen is also emerging as a means to visualize changes in the synovium in vivo. This chimeric molecule has been used to image nascent exposure of extracellular matrix during tissue damage, and synthesis of new fibrous tissue in GPI-serum induced experimental arthritis[137]. These approaches constitute the first steps towards detailed molecular analysis of the synovial matrix in real time in vivo.

Exploiting the tissue microenvironment for improved disease treatment

Understanding the cells and the synovial microenvironment at unparalleled resolution not only illuminates our understanding of the tissue biology of the joint, and provides insight into disease status and disease mechanisms, it is also paving the way for new therapeutic strategies. Targeting the extracellular matrix is being used to develop a wide variety of new treatments[138], and these have been applied to RA in a number of different ways (**Table 3**).

Advances in drug delivery. Exploiting the tissue specificity of matrix molecule expression has led to new approaches in drug delivery. Linking established anti-inflammatory agents to antibodies that recognize matrix molecules, which are not found in healthy tissue but which are upregulated at disease sites, creates a new class of immunomodulatory agent that can home to areas of disease, and deliver localized, site-specific treatment. This approach has been comprehensively reviewed in [139], and is most recently exemplified by F8-IL10. F8-IL10, or DEKAVIL, is a cytokine-antibody fusion protein, comprising a single-chain antibody variable domain (Fv) fragment of antibody F8 and the anti-inflammatory cytokine IL10. F8 recognizes the extra domain A (EDA) of fibronectin, a foetally restricted splice variant of this matrix molecule, which is re-expressed in adults at sites of inflammation and in cancer. F8-IL10 exhibits targeted delivery of IL10 to the inflamed synovium in murine models of arthritis, and to both clinically and sub-clinically inflamed joints in people with RA[140]. Whilst PET-CT imaging revealed unexpected localization of F8-IL10 to the liver and spleen in people with RA, no safety issues were reported in Phase 1b clinical trials[141]. This approach may effectively overcome the lack of efficacy of systemically administered IL10. Indeed, this immunocytokine inhibited the progression of established arthritis in the collagen-induced mouse model when tested alone and in combination with methotrexate[142] and early signs of therapeutic benefit in over half of people treated at Phase 1b[141]. F8-IL10, and other immunocytokines designed to deliver anti-inflammatory agents directly to inflamed sites represent a novel class of therapeutic

598 agents that effectively target antigens at the site of inflammation, followed by local
599 activity of the cytokine[139].
600
601 ***Engineered matrix binding.*** Engineering matrix-binding capabilities to anti-TNF
602 antibodies also shows promise in improving the efficacy of targeting TNF following
603 intra-articular injection. Whilst systemic TNF blockade can induce generalized
604 immunosuppression, intra-articular administration of anti-TNF antibodies is limited
605 by rapid drug clearance from inflamed joints. Chemical conjugation of the heparin
606 binding domain of placenta-growth factor-1 (PIGF-2), which binds with high affinity
607 to many different matrix molecules, to murine monoclonal anti-TNF antibodies
608 increased antibody retention times in the joint and significantly improved clinical
609 scores in collagen antibody induced arthritis (CAIA) compared to unconjugated
610 antibody[143]. Similarly, conjugating anti-TNF antibodies to the collagen binding
611 domain of decorin improves antibody accumulation in inflamed paws during CAIA
612 and suppressing disease progression more effectively that unmodified antibody[144].
613 This approach might make feasible intra-articular drug administration for
614 monoarthritis, and help limit off target effects of systemic immune suppression. TNF
615 blockade has also been re-engineered using MMP-cleavable inhibitory peptides.
616 Construction of a chimeric TNF receptor linking the trimerization domain of
617 adiponectin (Acrp30) to the N-terminus of the extracellular domain of TNFR2 via an
618 MMP2/9 substrate sequence creates a cap which blocks TNF access to TNFR, which
619 is released by MMP cleavage. *In vitro* this successfully allows controlled binding of
620 TNFR2 to TNF. If this can be recapitulated *in vivo*, allowing elevated MMP activation
621 at sites of inflammation to enable TNF binding to soluble chimeric receptors,

precluding activation of cellular TNFR, this could provide a powerful means of conferring inflamed tissue selective TNF blockade[145].

Preventing matrix degradation. An altogether different strategy in treating RA has been to directly target the activity of matrix degradation in order to prevent excessive joint tissue destruction (reviewed in [146,147]). Whilst early approaches using broad-spectrum small molecule MMP inhibitors were fraught with unacceptable side effects, more recent attempts with specific protease inhibitors appear more promising. A recent phase 1b trial of MMP9 specific monoclonal antibodies showed this approach to be safe and well tolerated[148], and pre-clinical data show how combining TNF and MT1-MMP blockade confers long-term protection from inflammation and tissue damage in mice with collagen induced arthritis[149]. These data highlight how inhibiting both inflammatory and tissue destructive processes can exert synergistic effects in established disease. However, targeting these mediators hits targets comparatively late events in RA pathogenesis, and new data have begun to reveal the possibility of intervening earlier in disease, before mis-regulated cytokine networks and tissue destruction are evident.

Manipulating soluble factor binding to the matrix. One elegant way to intervene at the point of leukocyte invasion into the inflamed synovium may be to use decoy chemokines. Engineered to have a higher affinity for GAG interaction sites, but to be incapable of competent signaling via chemokine receptors, these agents can effectively displace wild type chemokines from essential matrix binding sites, acting as powerful dominant negative chemokine inhibitors. For example, CXCL8 variants

with enhanced HSPG binding, and ablated CXCR1 or CXCR2 binding, reduced peri-articular neutrophil infiltration and inhibited leucocyte adhesion on the venule at the site of joint inflammation, resulting in inhibited leucocyte transmigration into the knee cavity during mBSA-induced experimental arthritis[150]. Similarly, short-chain basic peptides representing the GAG-binding region of chemokines such as CXCL8 bind to HSPG with high affinity, reduced leukocyte migration through the endothelial cell layer in vitro, compete with intact CXCL8 for binding around the endothelium in human RA tissue, and reduce inflammation and neutrophil infiltration during antigen-induced arthritis *in vivo*[151]. Alternatively, administration of the soluble extracellular domain of syndecan-3 has been used to mop up unwanted chemokines in the joint. Soluble syndecan-3 inhibited CCL7-activated leukocyte migration in vitro, and ameliorated histological disease severity, concomitantly reducing the number of blood vessels staining positive for CCL7 in the inflamed synovium, during antigen- and collagen-induced models of RA[152].

Targeting chronic pro-inflammatory signals from the matrix. Matrix molecules, however, are more than just postcode proteins with which to deliver existing drugs, placeholders for chemokines, or substrates for proteolytic degradation; they also play a key role in driving disease. By creating distinct niches within the RA joint they deliver aberrant pro-inflammatory signal to resident cell networks. Targeting these networks can be useful in early disease modulation. For example, thrombin-cleaved osteopontin binding to fibronectin at the cell surface of synovial fibroblasts aids B cell adhesion and stimulates the production of inflammatory cytokines[153]. A scFV antibody recognizing osteopontin, which blocks its interaction with fibronectin,

effectively reduced synovial fibroblast migration and adhesion to B cells in vitro, and improved clinical score, synovial hyperplasia, cartilage damage, cytokine levels when given early during collagen-antibody induced arthritis[154]. These data show how targeting key matrix interactions during disease onset can be useful in preventing the formation of immune permissive environments. Moreover, it is increasingly apparent that changes in the synovial microenvironment take place long before any overt clinical symptoms. For example, serum levels of both tenascin-C and ficolin-1, both secreted endogenous TLR4 agonists[72], are elevated in people with early synovitis who go on to develop RA compared to people with synovitis that spontaneously resolves[155,156]. Moreover, baseline levels of ficolin-1 predict disease remission[155]. Furthermore, therapeutic monoclonal antibodies that inhibit TLR4 activation by the fibrinogen–like globe of tenascin-C prevent chronic inflammation and halt disease progression when given early during collagen-induced arthritis[128]. These data suggest that identifying and targeting key events that precede disease development might pave the way for better outcomes by early intervention, and even raise the possibility of disease prevention in pre-symptomatic individuals. This new matrix modifying drug class acts by blocking signals from the inflamed synovium, therefore also offering the advantage of selective blockade of tissue and disease specific cues, rather than global immune suppression, suppressing the true drivers of disease, but leaving intact our ability to respond to infection.

Challenges and perspectives

Whilst these therapeutic approaches appear promising, with some already in early clinical trials[140], and others opening up potential windows for very early disease

intervention or even prevention[157], many questions remain. At the most fundamental level, we do not yet have a full picture of which combination of the >1000 strong matrisomal gene subset are expressed in the synovium, nor how the resultant proteins and proteoglycans are organized at the subsynovial level. Advances in proteomic analysis of extracellular matrix (for example [158,159]) are providing much greater depth in interrogation of matrix constituents of tissues. However, proteomic deconstruction is challenging for the synovium because large amounts of tissue are rarely available, particularly from healthy joints or early RA.

RNA sequencing of single cells from RA joints has provided striking resolution of gene expression at the subpopulation level. However, this approach alone does not capture the full complexity of the tissue microenvironment, which necessitates understanding not only gene expression, but also post-transcriptional processing, and protein post-translational modification, all key factors in dictating matrix assembly and function. Furthermore, high-resolution cellular analysis at a single snapshot in time makes it difficult to discern whether cell populations identified in this way represent distinct cell types (and lineages), or the same cell types at distinct points on a spectrum of phenotypic polarization.

Another challenge lies in understanding precisely how target cells respond to the integrated biochemical and mechanical signals provided by multicomponent, 3D tissue microenvironments. Many approaches to assessing cell phenotype require the isolation of cells from tissues, in order to assess, for example, their transcriptional status. However, the process of cell isolation has a profound effect

on cell phenotype itself, accounting for as much as 40% of the transcriptome[160,161]. This makes it difficult to differentiate cell behaviour instructed in situ or that caused by the stress of cell purification. Technologies such as NICHE-seq[162] or spatial transcriptomics[163] can now provide information about localized gene expression programs, whilst matrix assisted laser desorption/ionization mass spectrometry imaging (MALDI MSI) can visualise the spatial distribution of molecules, such glycans, peptides or proteins, by their molecular masses[164]. Used in parallel with multiplex imaging and improved capabilities in optical sectioning provided by light sheet microscopy, which enables good resolution imaging of intact tissues and organs[165], these methods can now be applied to better resolve the content of the matrix of the joint, and its organization at the single cell level in situ, and with this a potentially rich source of tractable new targets with which to diagnose and treat inflammatory joint disease.

When thinking about cellular response to the tissue microenvironment, it is worth considering how external cues contribute both to programming cell identity, as well as to orchestrating transient cellular activation states required to respond to dynamically fluctuating tissue conditions. It has been shown that in tissue-resident macrophages from different organs, the tissue environment is crucial in the creation and maintenance of organ-specific macrophage functions[166], although the full extent of how integrated external signals programme this positional memory remains to be completely unravelled. Most likely tissue-derived signals also shape fibroblasts from different organs and differences in the epigenetic landscape, gene expression and response to stimulus were found by comparing cultured synovial and dermal

fibroblasts, suggesting a stable imprinting of organ-specific gene expression even when dissociated from tissue architecture[167-169]. On the other hand, in synovial[170], dermal[171] and intestinal fibroblasts[172] expression of HOX genes, which govern positional cellular identities during embryonic development, differs between different anatomical regions, which shows that also the anatomical site shapes cellular gene expression illustrated by the various differences found between hip, knee and ankle joints[170,173-177]. Mechanical stimulation of joint cells is a well-established driver of cell identity during embryonic development[178] as well as postnatally and also influences the composition of the extracellular matrix[179,180]. Together these data implicate that at different anatomical sites, differences in embryonic development as well as environmental cues induce changes in the content and structure of the synovial microenvironment and define cell behaviour at a transcriptomic and epigenetic level, which could at least partly explain the specific pattern of joint involvement seen in many joint diseases (**Figure 4**).

Conclusions

Interrogation of synovial cell populations using single cell transcriptomics, and mapping the location of cell subsets identified by this approach within tissues, is revealing detailed anatomical complexity in the synovium. Our understanding of the cellular basis of synovial health and disease has been accelerated by examination of how specialized cell networks function within discreet synovial neighbourhoods. In parallel, analysis of the role of microenvironment in defining synovial tissue structure and function is starting to reveal how extracellular cues are essential in organizing cell networks, and directing niche-specific cell behavior. These data also

change our thinking about how inflammatory joint disease arises and progresses, supporting more holistic consideration of synovial cell ecosystems, wherein communication between multiple different cell types and their surrounding matrix within discreet but interconnected neighbourhoods in the synovium, is essential for tissue homeostasis. Perturbations in any aspect of these symbiotic ecosystems are deleterious to synovial homeostasis, and can be pathogenic. We are already starting to see how this new perspective has the potential to change clinical practice. This is evident both in terms of disease diagnosis and classification, for example in efforts to use local changes in synovial tissue to better assess patient disease status, as well as in offering new treatment options. These may either improve the efficacy or specificity of drugs currently used to treat people with RA, or offer completely novel approaches to ameliorating disease.

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Competing interests

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Key points

- All tissues are made up of cells surrounded by an extracellular matrix; this intricate, 3D molecular network is a both a key determinant of tissue architecture and cell behaviour.
- The synovium is a complex anatomical tissue comprising many different cell (sub)populations, located in distinct subsynovial niches, where each are specialized to perform unique roles in synovial homeostasis.
- In RA, infiltrating immune cells join tissue-resident cells; a quantum change accompanied by qualitative changes in cell phenotype that promote inflammation and tissue destruction, and suppress the resolution of inflammation.
- The extracellular matrix plays a key role in dictating the organization of synovial cell ecosystems and in programming synovial cell specialization.
- Changes in the synovial microenvironment start to occur early in the development of RA, and these aberrant extracellular cues shape pathogenic cell behaviour during the onset and progression of disease.
- Analysing localized changes in the synovial microenvironment can improve disease classification and patient stratification, whilst targeting the extracellular matrix holds promise for the development of new strategies to treat and prevent RA.

Figure legends

Box 1 | Tissue specific extracellular matrix.

Tissues are made up of cells and extracellular matrix. The matrix consists of a 3D network of secreted molecules, coded for by genes that are collectively called the matrisome. Matrisomal genes can be classified as: ***1) core matrisomal genes***, including: *collagens*, *glycoproteins* (such as fibronectin, laminins, tenascins, thrombospondins), and *proteoglycans*, and ***2) matrisome-associated genes*** including *matrix-affiliated molecules* (such as mucins, lectins, syndecans, and galectins), *matrix regulators* (for example, crosslinking enzymes such as lysyl oxidases and transglutaminases, modifying enzymes such as kinases and sulfatases, proteases such as matrix metalloproteases (MMPs) and cathepsins, and protease inhibitors such as TIMPs and cystatins) and *soluble factors* (such as growth factors, Wnts, cytokines and chemokines). More than 1000 matrisomal genes exist. Each tissue is formed by the assembly of a unique selection of these molecules into a complex extracellular network. These matrices confer different physical properties to tissues, and dictate both cellular organization and cellular behaviour within tissues.

In the human synovial joint, subchondral bone consists of a layer of compact cortical bone and underlying cancellous bone. A hard, calcified, type I collagen-rich matrix enables bones to provide anatomical support (**a**). The articular surface of bone in synovial joints consists of a smooth layer of hyaline articular cartilage, which provides compressive resistance in the joint. A matrix rich in type II collagen and proteoglycans confers the shock absorbing capabilities of cartilage (**b**). Tendons are the key functional anatomic bridges between muscle and bone. They focus the force of muscle into localized areas on the bone, the enthesis, and by splitting to form a number of insertions distribute the force of muscle contraction to different bones. A matrix comprising tightly packed parallel bundles of type I collagen fibrils confer tensile strength to tendons (**c**). The synovium is a thin mesenchymal membrane that encapsulates the joint space and provides boundary layer lubrication to ensure frictionless movement. A healthy synovium is composed of two distinct layers; an

intimal layer that is 20-40 micron thick, and a fibrous-areolar subintima that can be up to 5mm in thickness. The intima is composed of tissue resident macrophages and fibroblasts, supported by a discontinuous membrane made of types III, IV, V and VI collagen and laminin, which controls joint lubrication and nutrient exchange via the synovial fluid. The subintima contains blood and lymphatic vessels, as well as nerves and fibroblasts, in a looser collagenous extracellular matrix (**d**). Understanding tissue biology therefore requires understanding patterns of matrisomal gene expression, and how the resultant proteins are organized and modified to create distinct microenvironments.

Fig. 1 | The pannus is a key architectural feature of the inflamed synovium.
The region in the inflamed joint where hypertrophic synovium invades into adjacent cartilage and bone is called the pannus, where synovial cells and chondrocytes are closely juxtaposed. The left hand panel shows the overall architecture of the inflamed synovium, and the red boxed area in the right hand panel focsues in on the specific zone of synovial-cartilage interaction (**a**). In this relatively small anatomical zone, exquisitely site-specific patterns of gene expression are observed. Examples of pannus restricted biology include galectin-3 (**b**) and TLR2 (**c**) expression, both of which are upregulated specifically at these sites of invasion into underlying bone, and mediate localized synovial fibrolast activation and MMP synthesis, as well as localized chemokine synthesis that recuits infiltrating immune cells to the area.

Fig. 2| Distinct fibroblast populations in the RA synovium inhabit distinct tissue niches.
Single cell transcriptional analysis reveals 5 different fibroblast populations in the inflamed mouse synovium (labelled F1-F5 here), three of which are conserved in human tissue.

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Fig. 3 | Tissue microarchitecture in the healthy and RA joint.

Within sub-synovial niches, distinct combinations of matrix molecules define local tissue structure and function. The matrix confers physical properties to tissues, for example, at the articular surface proteoglycans and GAGs ensure frictionless joint articulation, a property diminished in RA as these molecules become degraded, creating pro-inflammatory matrix fragments (a). The synovial membrane forms a porous meshwork, comprising points of anchorage which organize lining layer cells into a cohesive network, together creating a barrier restricting cell movement, whose integrity is lost in RA (b). The matrix provides mechanical cues that directly control cell phenotype, these become altered during synovial hyperplasia and fibrosis, where changes in the orgnaization of the fibrous interstitial matrix dictate stromal cell movement, whilst matrix stiffness impacts macrophage phenotype (c). As well as controlling the spatial positioning of cells by providing points of adhesion and migration barriers, the matrix also creates tracks which are permissive for cell migration, for example in and around the endothelial basement membrane. In RA, elevated expression of proteoglycans also pattern gradients of soluble factors around blood vessels, and serve as chemokine co-receptors, orchestrating enhanced cell infiltration via the perivascular niche (d). The matrix is a rich source of biochemical signals that are directly sensed by cell surface receptors to dictate cell behaviour, these signals may derive from complex multicomponent networks of extracellular molcules or fragments of matrix molecules generated during tissue remodelling. Both are exemplified in the pannus where ectopic matrix deposition provides a cell substrate permissive for immune cell activation and fibroblast spreading and invasion, whilst damaged matrix sustains signalling loops that perpetuate tissue destruction (f).

Fig. 4| Shaping of joint specific cellular phentoypes.

Positional memory in joint stroma cells can be modified at all stages of life. During embryonic development joint-specific pathways and stimulatory signals such as fetal movements work in concert with joint-specific HOX gene expression to shape the different joint regions[170]. In early childhood, the transition to walking upright is associated with substantial adaptation of motor and biomechanical processes that shape gene expression in the tissues involved. Later in life, unphysiological load, trauma or other environmental factors such as infection and inflammation, e.g. rheumatoid arthritis can lead to joint-specific changes.

Table 1 | Conserved cell populations in the RA joint.

Cell subset	Marker (human)	Marker (mouse)	Activation marker/effectors
Fibroblasts			
Lining layer	CD90- CD55+ PGR4+ F4	CD90- PGR4+ F5	RANKL:OPG ratio, CCL9, CLIC5, MMP1, MMP2, MMP3, MMP9, MMP13, HAS1, HTRA4, DNASE1L3
Immunomodulatory sublining layer	CD90+ CD34- HLA-DRA^{hi} F2 CD90+ CD34- DKK+ F3	CD90+ CD34- F1	IL6, IL33, IL34, IFI30, Lif, CXCL9, CXCL12, CXCL13, CCL2, CCL19, CCL21
Perivascular sublining layer	CD90+ CD34+ F1	CD90+ CD34+ F3	
Macrophages			
Lining layer		CX3CR1+ CFSR1-	TREM2, VSIG4, AXL, MFGE8, JAM1, ZO-1, CLDN5, FAT4, VANGL2
Interstitial	NURP1+ CD11c- CD38- M2	CX3CR1- CFSR1+ MHCII+ AQP1+	MERTK, CTSK, HTRA1, GPNMB, ITGB5
	C1QA+ CD11c+ CD38+ M3	CX3CR1- CFSR1+ RELMA+	MRC1, CD163, MARCO
Monocyte-derived infiltrating	SPP1+ IFN-activated CD11c+ CCR2+ CD38+ M4 IL1b+ CD11c+ CCR2+ CD38+ M1	CCR2+ Ly6c2- ARG1+ CCR2+ Ly6c2- IL1b+	ARG1, IFI6, IFI44L, LY6E, SPP1 NR4A2, HBEGF, PLAUR, RGS2, IL1b, HTF3, CXCl2, EREG

Single cell transcriptional analysis of the human RA synovium has identified at least 18 different cell types, including fibroblast and macrophage subsets that are conserved in the inflamed murine synovium. Each cell subpopulation exhibits strikingly different localization within the joint and distinct functional specialization. Data summarised from references [27-30,33,35].

1402 **Table 2 | How the tissue microenvironment can impact joint cell behaviour**

1403

Matrix	Effect and location	Reference
Physical properties and mechanical cues		
Hyaluronic acid	High levels in synovial fluid prevent friction	40
Lubricin	Distributed on the articular surface to lubricate the joint	41
Lining layer basement membrane	Maintains synovial integrity and immune privilege, by regulating and restricting, molecular and cellular exchange, that is lost in RA	4 43 33
Sub-intimal interstitial matrix	Controls matrix alignment and porosity, as well as tissue micromechanics, to regulate stromal cell adhesion and movement	45
	Dictates tissue stiffness which impacts macrophage polarization and activation	46
Spatial positioning		
Hyaluronic acid and lubricin	High levels in the synovial fluid prevent cell adhesion at the cartilage surface to facilitate unimpeded joint articulation	4
Fibronectin	Within the lining layer basement membrane promotes cell adhesion to create cohesive barrier function	49
	Ectopic expression in the RA pannus stabilizes cell invading machinery	50
	Up-regulation in the endothelial basement membrane in RA provides permissive tracks that support T cell infiltration	51,52
Soluble factor patterning and activity		
GAGs	High levels at the endothelial basement membrane in RA create chemokine gradients that enhance cell infiltration	54 55 56-58
HSPGs	Expression at the cell surface serves as a co-receptor for chemokines and growth factors, potentiating signalling	60 61 62 63
Direct signalling to cells		
Tenascin-C	Upregulation in the RA synovial sublining layer activates TLR4-mediated inflammation	70-72
Hyaluronic acid fragments	In RA synovial fluid, low molecular weight fragments activate TLR2-mediated inflammatory signalling	97
Osteopontin fragments	In RA synovial fluid, C-terminal fragments induce macrophage chemotaxis, and phosphorylated N-terminal fragments enhance macrophage spreading and activation	98 99,100
Damaged collagen	In the pannus, degradation of cartilage collagen increases localized MT1-MMP expression by synovial fibroblasts	95

Table 3 | Matrix targeting strategies in development for the treatment of RA

Approach	Mode of action	Development	Reference
Drug delivery			
Immunocytokine	Cytokine-antibody fusion protein DEKAVIL (F8-IL10): scFV of antibody F8 mediates delivery to inflamed joints via recognition of the EDA domain of fibronectin, where IL-10 exerts a localized anti-inflammatory effect.	Phase Ib	141
Chimeric antibodies	Anti-TNF antibodies fused to the heparin binding domain of PIGF-2, or to the collagen binding domain of decorin, are preferentially retained in the inflamed joint	Pre-clinical	143 144
Drug activity			
Chimeric cytokine receptors	Soluble TNFR fused to MMP cleavable adiponectin-derived cap creates controllable TNFR-TNF binding, activated at sites of high protease activity	In vitro	145
Inhibition of pathological processes			
Tissue destruction	Therapeutic monoclonal antibodies blocking the tissue degrading activity of specific proteases.	Phase 1b (MMP9) Pre-clinical (MT1-MMP)	148 149
Leukocyte infiltration	Decoy chemokines: signalling incompetent variants of CXCL8 with high HS affinity, or peptides comprising CXCL8 heparin binding domain, displace endogenous chemokine from tissue GAGs Decoy GAGs: soluble syndecan-3 competes for CXCL8 binding to endogenous syndecan at the endothelial lumen.	Pre-clinical Pre-clinical	150 151 152
Synovial inflammation	Therapeutic monoclonal antibodies that block osteopontin-fibronectin interactions, or that prevent activation of TLR4 by the fibrinogen like globe domain of tenascin-C	Pre-clinical	128,154