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DOI:
10.1038/s41584-020-00570-2

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Document Version
Peer reviewed version

Citation for published version (Harvard):
Buckley, C, Ospelt, C, Gay, S & Midwood, KS 2021, 'Location, location, location: how the tissue microenvironment affects inflammation in RA', *Nature Reviews Rheumatology*. https://doi.org/10.1038/s41584-020-00570-2

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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?

74 All tissues consist of cells surrounded by an intricate extracellular matrix. This 3D
75 network of secreted molecules provides structural support for cells and dictates
76 their spatial organization within tissues. However, the matrix is not simply an inert
77 scaffold, it also a key determinant of cell phenotype, providing environmental cues
78 that enable cells to move relative to each other as well as perform distinct roles
79 determined by their anatomic location[1,2]. Extracellular matrices are made from a
80 selection of more than 1000 molecules collectively called the matrisome. Genes in
81 the matrisome code for all of the proteins that can be secreted by cells,
82 encompassing extracellular matrix molecules, matrix-associated proteins, soluble
83 growth factors, chemokines and cytokines, and enzymes including proteases and
84 kinases[3] (http://matrisomeproject.mit.edu/).
85
86 Expression of site-specific combinations of matrisome molecules, and their assembly
87 into networks around cells, creates unique tissue microenvironments, as well as local
88 niches within tissues. Integrated mechanical and biochemical cues from each type of
89 matrix provide essential context for cell behavior, wherein distinct combinations of
90 extracellular molecules cohesively define cell differentiation and specialization. For
91 example, joints are specialized multi-tissue organs that provide the structures by
92 which bones move relative to each other, and by which muscles mediate
93 coordinated locomotion. The components of a classical human synovial joint include
94 tissues such as the synovium, tendons, muscle, ligaments, bursae, menisci, articular
95 cartilage and subchondral bone. Each constituent tissue of the joint is made up of a
96 unique combination of matrisomal molecules that confer the distinctive physical
97 properties that together are essential for effective joint function (**Box 1**).

98

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

108

109 Complex tissue architecture within the synovium

110 The synovium is an intricate tissue, made up of a number of cell types including
111 tissue resident macrophages, fibroblasts, nerve and endothelial cells. Even at the
112 gross histological level, subcellular compartmentalization within the synovium is
113 evident forming two distinct zones; the intima lining layer and the subintima (**Box 1**).
114 In a healthy joint the intima is only 1-3 cells thick, and is composed of tissue resident
115 macrophages and fibroblasts supported by a porous basement-like membrane. This
116 zone of the synovium controls cellular and molecular ingress and egress between the
117 synovium and the joint cavity, playing a key role in maintaining joint integrity and the
118 composition of synovial fluid, ensuring effective joint lubrication and nutrient
119 exchange. The subintima, comprising fibroblasts distributed throughout a looser
120 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

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

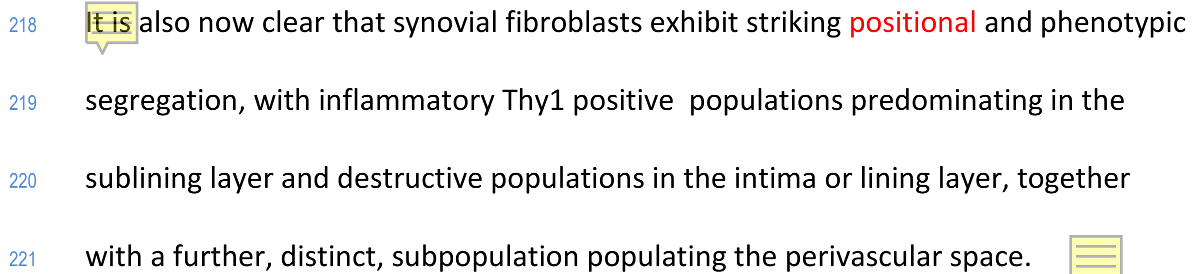
151

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

169 into cartilage[21]. Expression of tissue degrading enzymes and apoptosis-inhibiting
170 factors in RA synovial fibroblasts found at the sites of cartilage destruction is
171 associated with gene hypomethylation; and this altered epigenetic landscape might
172 explain why therapeutically targeting the progression of RA joint destruction is
173 extremely difficult[22]. Some studies have also reported how the tissue
174 microenvironment itself changes within the pannus, and the consequences of
175 altered extracellular protein expression on localized tissue invasion. For example,
176 galectin-3, a secreted beta-galactoside-binding protein that is elevated early in RA
177 pathogenesis, localizes almost exclusively to the pannus in the inflamed synovium
178 (**Figure 1b**)[23,24]. Galectin-3 directly activates synovial fibroblasts, stimulating
179 secretion of inflammatory cytokines, such as interleukin-6 (IL-6), and chemokines,
180 such as IL-8, CCL2, CCL3, and CCL5, as well as MMP3, via activation of MAPK and
181 phosphatidylinositol 3-kinase (PI 3-kinase) signalling pathways[25]. Moreover,
182 galectin-3 expression by RA synovial fibroblasts is required for IL6 synthesis
183 downstream of TLR2[26], a pattern recognition receptor that also localizes to the
184 pannus in inflamed synovia (**Figure 1c**)[16]. Together these data imply that local
185 interplay between galectin-3 and TLR2 serves to activate pannus-resident synovial
186 fibroblasts, in a cytokine-independent manner, and recruit immune cell infiltration to
187 reinforce inflammation specifically at this key pathogenic site.
188

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

193 about how many different cell subsets make up this tissue, nor their organization
194 into functional networks.
195
196 **Single cell resolution of the RA synovium**
197 A step change in our ability to perform a cellular census of the cell types present in
198 synovial joints has occurred because of advances in minimally invasive ultrasound-
199 guided biopsy techniques, coupled with tissue digestion and single cell (sc) RNA
200 sequencing [27-29]. Using these precision molecular analytics, multiparameter imaging
201 and state of the art bioinformatics, recent work from tissue in the inflamed joint has
202 revealed further insight into the complexity of the synovium, showing the RA
203 synovium to be comprised of at least 18 distinct types of types of T cells, B cells,
204 macrophages and fibroblasts[29] and allowing us to compile for the first time a
205 synovial map of the leucocyte and stromal cells in the synovium in diseases such as
206 OA and RA[29,30](**Figure 2**).
207
208 These studies have revealed unprecedented insight into anatomical and functional
209 specialization of synovial cells. It has long been known that not only T cell number,
210 but also the balance amongst T cell polarization, is a key determinant of immune
211 status, for example lower ratios of Tregs compared to Th17 subsets contribute to
212 impaired immune restraint and chronicity of inflammation[31]. Now, in the human RA
213 joint, the existence of a pathogenic T cell population (termed TPh) that express high
214 levels of PD1 but not CXCR5, has been identified to be highly expanded in
215 seropositive RA patients and not seronegative[32]. These data indicate complexity in
216 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

241 whose contraction was associated with increased risk of disease flare. These subsets
242 can induce synovial repair responses via production of inflammation-resolving lipid
243 mediators[34]. Finally, the existence of HBEFG(+) macrophages and fibroblasts in the
244 rheumatoid synovium that induce fibroblast invasiveness has provided insight into
245 functional, pathogenic cellular interaction networks across subpopulations from
246 different lineages[35].
247
248 Together these studies demonstrate how our understanding of the architecture of
249 the joint has progressed from gross anatomy, through subsynovial structures,
250 including pannus tissue and tertiary lymphoid structures, to the single cell level, and
251 how this has enabled the emergence of a more complete cell atlas of the joint.
252 These data have also shown how changes in the balance of synovial cellular
253 ecosystems underpin chronic inflammation during the onset and progression of RA
254 compared to OA. Some of the underlying drives of these changes are beginning to
255 emerge, for example, the expansion of Thy1 positive fibroblasts in the RA sublining is
256 NOTCH3 dependent[36], compared to the lining layer, where Thy1 negative fibroblasts,
257 along with lining layer MerTK positive macrophages, contract in active disease.
258 Moreover, the increases in the ratio of MertK positive to negative macrophages in
259 the RA synovium in patients in disease remission suggests that lining layer
260 macrophages regulate remission in RA[34].
261
262 These data may aid in therapeutic strategies that target pathogenic cell populations
263 in RA. For example, functional subclasses of fibroblasts have proven difficult to
264 define, characterize and study in health and disease. Consequently, there are no

265 approved drugs that specifically target fibroblasts in human diseases. The recent
266 identification of "pathogenic" fibroblast subpopulations[30] offers an attractive new,
267 non-immunosuppressive therapeutic target. However, fibroblasts are a functionally
268 heterogeneous group of cells that support discrete biological functions within the
269 joint tissue. This has led to a therapeutic dilemma: which fibroblast subsets should
270 be targeted and suppressed and which should be retained and augmented? A clear
271 understanding of the biology and clinical significance of fibroblast heterogeneity is
272 therefore essential to provide a coherent rationale for their therapeutic targeting in
273 treatment of diseases such as RA. The selective targeting of pathogenic fibroblast
274 subsets using anti-fibroblast monoclonal antibodies, analogous to B cell depletion
275 using CD20 (rituximab), would complement other targeted therapies commonly used
276 against leucocytes and their cell products[37,38]. Improved resolution of RA synovial
277 macrophage subsets also now offers the potential for additional arsenal in
278 modulating pathogenic myeloid cell behaviour, with MerTK+ subsets, or anti-
279 inflammatory mediators released by these cells during disease remission, offering
280 tractable targets for boosting synovial repair processes[34].
281
282 However, despite a clearer picture of the cellular networks inhabiting the RA
283 synovium, it still remains uncertain what initiates and maintains pathogenic
284 behaviour in different cell subsets in RA.
285

286 **Immunological geography**

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

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

295

296 ***Physical properties and mechanical cues***

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

311

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

336 networks by anchoring synovial fibroblasts to their surrounding matrix[49]. Ectopic
337 expression of fibronectin in the RA joint enables aberrant cell adhesion, for example,
338 high levels of fibronectin in the pannus enhance synovial fibroblast adhesion to
339 cartilage, stabilizing invadopodia, actin-rich protrusions of the plasma membrane
340 that are associated with tissue degradation, by promoting coherent points of
341 anchorage that facilitate cartilage invasion[50]. Expression of fibronectin at the basal
342 lamina and at the endothelial surface in inflamed synovium has also been proposed
343 to serve as a permissive migration track for infiltrating lymphocytes, enabling T cells
344 to cross the endothelial basement membrane in RA[51,52]. The matrix also plays a key
345 role in restricting cell migration, with the synovial membrane serving a barrier
346 function to maintain immune privilege in the synovium, which is disrupted in RA[33].
347

348 ***Patterning of soluble factors***

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

360 as well as protecting these soluble factors from degradation[55], and facilitating
361 oligomerization required for optimal activity[56]. For example, in the RA synovium
362 elevated expression of the HSPG syndecan-3 tethers CXCL8 in the endothelial lumen,
363 and this interaction has been shown to promote leukocyte trafficking into the
364 inflamed tissue in vivo during antigen-induced arthritis[57,58]. The matrix is an
365 essential reservoir for other soluble factors including cytokines, bone morphogenetic
366 proteins (BMPs), Wnts and growth factors, where binding is often promiscuous, but
367 is specific. For example, fibronectin, vitronectin, tenascin-C, osteopontin, type I
368 collagen and fibrinogen each bind to several soluble factors from amongst the
369 vascular endothelial growth factor (VEGF), platelet derived growth factor (PDGF),
370 fibroblast growth factor (FGF), transforming growth factor (TGF), insulin-like growth
371 factor (IGF) and BMP families. However, each matrix molecule has a distinct set of
372 soluble binding partners. Moreover, these molecules bind with different affinities
373 across each family of growth factors; e.g. tenascin-C binds to VEGF-B but not VEGF-A,
374 vitronectin binds to FGF-18, whilst tenascin-C does not, and neither bind to FGF-1 or
375 -6 [59]. These interactions not only control tissue levels and locations of soluble
376 factors, but are also essential for their function by serving as co-receptors.
377 Proteoglycans in particular are well documented accessory molecules[60], with
378 syndecans playing key roles in cartilage breakdown and synovial inflammation[61]. For
379 example, optimal activity of FGF2, a growth factor up-regulated in RA, where it
380 contributes to driving fibroblast activation during disease progression[62], requires the
381 formation of a ternary complex between the HS chains of syndecan-4 and the FGF
382 receptor, as well as signaling via cytoplasmic domain of syndecan-4 to strengthen
383 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

408 and integrins, triggering innate immunity and shaping adaptive responses designed
409 to restore homeostasis and activate tissue repair. In the joints of people who do not
410 have RA, these signals are essential in order for cells to detect and respond to injury
411 and insult. However, dysregulation of these pathways is emerging as a major cause
412 of chronic inflammation and tissue destruction in RA. For example, tenascin-C is an
413 extracellular matrix molecule that is not expressed in most healthy tissues including
414 the joint, but is transiently upregulated following tissue injury where it activates
415 TLR4-mediated inflammation. Typically downregulated and cleared from tissues
416 following repair, tenascin-C accumulates at high levels in the synovium of people
417 with RA. Expression of this pro-inflammatory matrix molecule is required for the
418 persistence of joint inflammation and tissue destruction in several different models
419 of arthritis[70-72].
420
421 These studies collectively exemplify how the extracellular matrix surrounding and
422 supporting synovial cells plays a key role in dictating site-specific behavior within the
423 synovium. Emerging data also indicate dysregulated signals from the matrix drive
424 chronic inflammation in the joint during the pathogenesis of RA, and that targeting
425 these signals may provide an effective means of restoring immune control.
426
427 **The extracellular matrix in the pathogenesis of RA**
428 Whole exome sequencing has identified new genetic variants associated with RA
429 susceptibility, amongst which genes in extracellular matrix-receptor pathways were
430 most highly enriched (COL4A4, COL6A5, COL11A1, COL11A2, HSPG2, ITGB5, LAMC1,
431 THBS1, RASGRF1, FLNB, MYL5)[73]. Microarray analysis comparing healthy and RA

432 synovium also revealed differentially expressed genes involved in cell adhesion and
433 organization of the extracellular matrix (PTPRC, SDC1, CD8A, CD2, HLA-DPA1, ITGA4,
434 HLA-DMB, CD6, HLA-DOB, PDCD1LG2, COL3A1, SDC1, COL1A2, INTGB2)[74]. Whilst the
435 impact of sequence variation, or up-regulation, of these genes in people with RA is
436 not known, these data implicate changes in the matrix and microenvironment in
437 disease pathogenesis.
438
439 Altered tissue turnover has long been a pathological hallmark of RA[5,6,75,76], and
440 serum levels of matrix metabolites are commonly used biomarkers for joint
441 remodeling and bone degradation[77,78]. For example, the C-telopeptide fragment of
442 type I collagen (CTX-I) generated by osteoclast-derived cathepsin K reflects bone
443 resorption[79], whilst osteocalcin produced by mature osteoblasts, and the N-terminal
444 type I procollagen propeptide (PINP) released during collagen fibril synthesis, reflect
445 bone formation[80]. Cartilage degradation is assayed by examining serum levels of
446 cartilage oligomeric matrix protein (COMP)[81], the C-terminal telopeptide of type II
447 collagen (CTX-II)[82], and C2M, a fragment of type II collagen[83]. Synovial remodelling is
448 reflected by high circulating C1M, C3M and C4M, fragments of type I, type III and
449 type IV collagen generated by MMP cleavage[84-87], or proteases implicated in tissue
450 destruction, such as total MMP-3 or the activated form of MMP-3[88,89]. A reduction
451 in serum matrix metabolites accompanies positive response to therapies including
452 tocilizumab, etanercept, methotrexate, adalimumab, and tofacitinib (for example;
453 [86,90-93]). Analysis of these biomarkers at baseline can also predict people who will
454 respond well to tocilizumab[90], as well as predicting lack of efficacy of Syk inhibition
455 via fostamatinib on structural end points[94]. These serological markers therefore

456 serve as reliable surrogates of tissue destruction in RA, and may prove useful in
457 stratifying patient treatment response. Emerging data also show that matrix
458 metabolites are not simply inert collateral damage released from joint tissue as
459 disease progresses, but active players in RA pathogenesis.
460
461 Expression of the tissue-degrading enzyme MT1-MMP is elevated in the RA joint, at
462 sites of pannus invasion into cartilage[15]. Collagen-induced upregulation of MT1-
463 MMP via DDR2 activation on synovial fibroblasts is more pronounced in variants
464 missing non-helical telopeptides compared with intact collagen fibrils, and is
465 enhanced in response to damaged cartilage[95], suggesting a positive feedback loop in
466 which collagen degradation reinforces further tissue destruction. Fragments of
467 hyaluronic acid (HA) are also detected in RA synovial fluid[96]. The size of HA
468 fragments dictates the function of this glycan, for example low molecular weight
469 (MW), but not high MW, fragments activate TLR2-mediated inflammation in
470 macrophages[97]. Fragments of osteopontin are also elevated in synovial fluid from
471 people with RA[98]. Thrombin cleavage of this matrix molecule creates a C-terminal
472 fragment that induces CD44-dependent macrophage chemotaxis, and an N-terminal
473 fragment that promotes β3 integrin-mediated macrophage spreading and
474 activation[99,100]. These data suggest that elevated levels of matrix metabolites
475 contribute to both tissue remodeling and inflammation in RA.
476
477 The pro-inflammatory activity of osteopontin fragments is further regulated by
478 phosphorylation; whilst the chemotactic activity of the C-terminal fragment is
479 independent of modification, macrophage activation leading to cytokine and MMP

480 release by the N-terminal fragment requires phosphorylation[99,100]. Higher levels of
481 phosphorylated osteopontin, and phosphorylated osteopontin fragments, were
482 observed in synovial fluid from people with RA compared to OA patients, whilst total
483 osteopontin levels did not discriminate RA from OA[101], suggesting that both
484 proteolytic processing and post-translational modification of the matrix contributes
485 to disease activity. Indeed, autoantibodies recognizing citrullinated proteins (ACPA),
486 the post-translational conversion of arginine to citrulline catalyzed by peptidyl
487 arginine deiminases, are gold-standard diagnostic markers for RA[102]. ACPA recognize
488 a number of modified matrix molecules (reviewed in [103,104]), including citrullinated
489 epitopes in type II collagen[105], well-established pathogenic drivers of joint disease in
490 vivo[106,107]; citrullinated fibrinogen[108], levels of which predict higher DAS 28 scores[109];
491 citrullinated tenascin-C[110], which may delineate different disease aetiologies[111];
492 citrullinated aggrecan, which correlate with higher frequencies of cit-aggrecan-
493 specific T cells in people with RA[112], and citrullinated fibronectin[113]. Intra-articular
494 injection of citrullinated collagen and fibrinogen enhances their arthritogenic
495 potential compared to unmodified protein[114-116]. Moreover, citrullination of
496 fibrin(ogen) and fibronectin *in vitro* enhances their pro-inflammatory capabilities[117-
497 119], whilst citrullination of collagen and fibronectin alters their integrin binding
498 repertoire and capacity to support synovial cell adhesion[113,118,120]. Citrullinated
499 fibronectin also effectively promotes cell survival, in contrast to induction of
500 apoptosis by the native molecule [49,117], whilst the modified form exhibits increased
501 affinity for VEGF but is less effective at binding to, and inhibiting, the aggrecanase
502 ADAMTS4[121,122]. As such matrix modification can not only break tolerance, i.e.
503 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.

527 Whilst circulating biomarkers therefore can be correlative with tissue pathology,
528 they are not always causal, and it is clear that changes in the serum do not mirror
529 the totality of changes in the synovium. Work examining the distribution of
530 tenascin-C exemplifies how important mechanistic detail can be lost without the
531 context of tissue anatomy. Levels of this pro-inflammatory matrix molecule are
532 elevated in RA serum and synovial fluid[126,127], correlating with bone erosion during
533 disease, and predicting poor improvement in pain in response to anti-TNF
534 treatment[127]. In the RA synovium, tenascin-C is found predominantly in the sublining
535 layer, where it is restricted to two specific niches; a dense matrix surrounding CD34
536 negative fibroblast populations, and close to CD34+ perivascular fibroblasts located
537 underneath blood vessels at sites of lymphocyte infiltration[128]. This highlights
538 specific cellular targets for tenascin-C in the RA joint, which may have remained
539 obscured without anatomical analysis, and directs further mechanistic investigation,
540 for example what role tenascin-C might play in promoting prolonged activation of
541 inflammatory signaling in fibroblasts[71,129] or in modulating pericyte adhesion,
542 migration[130] or differentiation[131] during RA.
543
544 Considering the advances in our knowledge of the cellular and molecular basis of
545 synovial inflammation, it is clear that analysis of cell subset interaction networks in
546 the tissue (for example inflammatory versus destructive fibroblasts, TPh cell or
547 HBEFG(+) macrophage burden), together with the microenvironmental cues that
548 instruct their behavior, is likely the most accurate way to assess the underlying
549 events driving RA, enabling more precise disease classification, leading to process
550 driven patient stratification and better targeted therapeutic intervention. However,

551 whilst advances in synovial biopsy methodology have enabled safer and more
552 practicable tissue acquisition, sometimes involving two or more repeat samples[132],
553 by design interrogation of tissue micro-niches may be subject to sampling
554 heterogeneity, and approaches designed to image the synovium *in vivo* may provide
555 a useful complement to tissue harvest. Positron emission tomography (PET) using
556 targeted radiotracers to visualize specific matrix components including collagen[133]
557 or fibronectin[134] is developing as a viable method to image tissue fibrosis in vivo
558 (reviewed in [135,136]). PET imaging of GPVI-Fc, a fusion protein comprising the soluble
559 human IgG1 Fc domain and the extracellular domain of platelet glycoprotein VI, a
560 trans-membrane platelet glycoprotein that binds with high affinity to matrix
561 molecules including collagen, fibronectin and fibrinogen is also emerging as a means
562 to visualize changes in the synovium in vivo. This chimeric molecule has been used
563 to image nascent exposure of extracellular matrix during tissue damage, and
564 synthesis of new fibrous tissue in GPI-serum induced experimental arthritis[137]. These
565 approaches constitute the first steps towards detailed molecular analysis of the
566 synovial matrix in real time in vivo.
567

568 **Exploiting the tissue microenvironment for improved disease treatment**

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

575

576 ***Advances in drug delivery.*** Exploiting the tissue specificity of matrix molecule
577 expression has led to new approaches in drug delivery. Linking established anti-
578 inflammatory agents to antibodies that recognize matrix molecules, which are not
579 found in healthy tissue but which are upregulated at disease sites, creates a new
580 class of immunomodulatory agent that can home to areas of disease, and deliver
581 localized, site-specific treatment. This approach has been comprehensively
582 reviewed in [139], and is most recently exemplified by F8-IL10. F8-IL10, or DEKAVIL, is a
583 cytokine-antibody fusion protein, comprising a single-chain antibody variable
584 domain (Fv) fragment of antibody F8 and the anti-inflammatory cytokine IL10. F8
585 recognizes the extra domain A (EDA) of fibronectin, a foetally restricted splice
586 variant of this matrix molecule, which is re-expressed in adults at sites of
587 inflammation and in cancer. F8-IL10 exhibits targeted delivery of IL10 to the
588 inflamed synovium in murine models of arthritis, and to both clinically and sub-
589 clinically inflamed joints in people with RA[140]. Whilst PET-CT imaging revealed
590 unexpected localization of F8-IL10 to the liver and spleen in people with RA, no
591 safety issues were reported in Phase 1b clinical trials[141]. This approach may
592 effectively overcome the lack of efficacy of systemically administered IL10. Indeed,
593 this immunocytokine inhibited the progression of established arthritis in the
594 collagen-induced mouse model when tested alone and in combination with
595 methotrexate[142] and early signs of therapeutic benefit in over half of people treated
596 at Phase 1b[141]. F8-IL10, and other immunocytokines designed to deliver anti-
597 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,

622 precluding activation of cellular TNFR, this could provide a powerful means of
623 conferring inflamed tissue selective TNF blockade[145].
624
625 ***Preventing matrix degradation.*** An altogether different strategy in treating RA has
626 been to directly target the activity of matrix degradation in order to prevent
627 excessive joint tissue destruction (reviewed in [146,147]). Whilst early approaches using
628 broad-spectrum small molecule MMP inhibitors were fraught with unacceptable side
629 effects, more recent attempts with specific protease inhibitors appear more
630 promising. A recent phase 1b trial of MMP9 specific monoclonal antibodies showed
631 this approach to be safe and well tolerated[148], and pre-clinical data show how
632 combining TNF and MT1-MMP blockade confers long-term protection from
633 inflammation and tissue damage in mice with collagen induced arthritis[149]. These
634 data highlight how inhibiting both inflammatory and tissue destructive processes can
635 exert synergistic effects in established disease. However, targeting these mediators
636 hits targets comparatively late events in RA pathogenesis, and new data have begun
637 to reveal the possibility of intervening earlier in disease, before mis-regulated
638 cytokine networks and tissue destruction are evident.
639
640 ***Manipulating soluble factor binding to the matrix.*** One elegant way to intervene at
641 the point of leukocyte invasion into the inflamed synovium may be to use decoy
642 chemokines. Engineered to have a higher affinity for GAG interaction sites, but to be
643 incapable of competent signaling via chemokine receptors, these agents can
644 effectively displace wild type chemokines from essential matrix binding sites, acting
645 as powerful dominant negative chemokine inhibitors. For example, CXCL8 variants

646 with enhanced HSPG binding, and ablated CXCR1 or CXCR2 binding, reduced peri-
647 articular neutrophil infiltration and inhibited leucocyte adhesion on the venule at the
648 site of joint inflammation, resulting in inhibited leucocyte transmigration into the
649 knee cavity during mBSA-induced experimental arthritis[150]. Similarly, short-chain
650 basic peptides representing the GAG-binding region of chemokines such as CXCL8
651 bind to HSPG with high affinity, reduced leukocyte migration through the endothelial
652 cell layer in vitro, compete with intact CXCL8 for binding around the endothelium in
653 human RA tissue, and reduce inflammation and neutrophil infiltration during
654 antigen-induced arthritis *in vivo*[151]. Alternatively, administration of the soluble
655 extracellular domain of syndecan-3 has been used to mop up unwanted chemokines
656 in the joint. Soluble syndecan-3 inhibited CCL7-activated leukocyte migration in
657 vitro, and ameliorated histological disease severity, concomitantly reducing the
658 number of blood vessels staining positive for CCL7 in the inflamed synovium, during
659 antigen- and collagen-induced models of RA[152].
660

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

670 effectively reduced synovial fibroblast migration and adhesion to B cells in vitro, and
671 improved clinical score, synovial hyperplasia, cartilage damage, cytokine levels when
672 given early during collagen-antibody induced arthritis[154]. These data show how
673 targeting key matrix interactions during disease onset can be useful in preventing
674 the formation of immune permissive environments. Moreover, it is increasingly
675 apparent that changes in the synovial microenvironment take place long before any
676 overt clinical symptoms. For example, serum levels of both tenascin-C and ficolin-1,
677 both secreted endogenous TLR4 agonists[72], are elevated in people with early
678 synovitis who go on to develop RA compared to people with synovitis that
679 spontaneously resolves[155,156]. Moreover, baseline levels of ficolin-1 predict disease
680 remission[155]. Furthermore, therapeutic monoclonal antibodies that inhibit TLR4
681 activation by the fibrinogen–like globe of tenascin-C prevent chronic inflammation
682 and halt disease progression when given early during collagen-induced arthritis[128].
683 These data suggest that identifying and targeting key events that precede disease
684 development might pave the way for better outcomes by early intervention, and
685 even raise the possibility of disease prevention in pre-symptomatic individuals. This
686 new matrix modifying drug class acts by blocking signals from the inflamed
687 synovium, therefore also offering the advantage of selective blockade of tissue and
688 disease specific cues, rather than global immune suppression, suppressing the true
689 drivers of disease, but leaving intact our ability to respond to infection.
690

691 ***Challenges and perspectives***

692 Whilst these therapeutic approaches appear promising, with some already in early
693 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

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

778

779 **Total word count:** 7733

780

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Acknowledgements

This report includes independent research supported by the National Institute for Health Research through the Birmingham Biomedical Research Center and Wellcome Trust Clinical Research Facility at University Hospitals Birmingham NHS Foundation Trust. The views expressed are those of the author(s) and not necessarily those of the NHS, the NIHR, our funding bodies or the Department of Health. Funding was also provided by the Versus Arthritis RACE Rheumatoid Arthritis Pathogenesis Centre of Excellence (grant 20298), a Versus Arthritis Programme grant to CDB (grant 19791) and a Versus Arthritis Senior Fellowship to KSM (grant 20003).

Competing interests

SG declares no competing interests. CO has received consultancy fees from Gilead Sciences Switzerland and funding from Novartis. CDB is a founder of MesTag Ltd and has received funding from MesTag. KSM is the founder and director of Nascient Ltd, and has received research funding from Nascient.

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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1355 **Fig. 3 | Tissue microarchitecture in the healthy and RA joint.**

1356 Within sub-synovial niches, distinct combinations of matrix molecules define local tissue
1357 structure and function. The matrix confers physical properties to tissues, for example, at the
1358 articular surface proteoglycans and GAGs ensure frictionless joint articulation, a property
1359 diminished in RA as these molecules become degraded, creating pro-inflammatory matrix
1360 fragments (a). The synovial membrane forms a porous meshwork, comprising points of
1361 anchorage which organize lining layer cells into a cohesive network, together creating a
1362 barrier restricting cell movement, whose integrity is lost in RA (b). The matrix provides
1363 mechanical cues that directly control cell phenotype, these become altered during synovial
1364 hyperplasia and fibrosis, where changes in the orgnaization of the fibrous interstitial matrix
1365 dictate stromal cell movement, whilst matrix stiffness impacts macrophage phenotype (c).
1366 As well as controlling the spatial positioning of cells by providing points of adhesion and
1367 migration barriers, the matrix also creates tracks which are permissive for cell migration, for
1368 example in and around the endothelial basement membrane. In RA, elevated expression of
1369 proteoglycans also pattern gradients of soluble factors around blood vessels, and serve as
1370 chemokine co-receptors, orchestrating enhanced cell infiltration via the perivascular niche
1371 (d). The matrix is a rich source of biochemical signals that are directly sensed by cell surface
1372 receptors to dictate cell behaviour, these signals may derive from complex multicomponent
1373 networks of extracellular molcules or fragments of matrix molecules generated during tissue
1374 remodelling. Both are exemplified in the pannus where ectopic matrix deposition provides a
1375 cell substrate permissive for immune cell activation and fibroblast spreading and invasion,
1376 whilst damaged matrix sustains signalling loops that perpetuate tissue destruction (f).

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1378 **Fig. 4| Shaping of joint specific cellular phentoypes.**

1379 Positional memory in joint stroma cells can be modified at all stages of life. During
1380 embryonic development joint-specific pathways and stimulatory signals such as fetal
1381 movements work in concert with joint-specific HOX gene expression to shape the different
1382 joint regions[170]. In early childhood, the transition to walking upright is associated with
1383 substantial adaptation of motor and biomechanical processes that shape gene expression in
1384 the tissues involved. Later in life, unphysiological load, trauma or other environmental
1385 factors such as infection and inflammation, e.g. rheumatoid arthritis can lead to joint-
1386 specific changes.
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1391 **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

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1393 Single cell transcriptional analysis of the human RA synovium has identified at least
1394 18 different cell types, including fibroblast and macrophage subsets that are
1395 conserved in the inflamed murine synovium. Each cell subpopulation exhibits
1396 strikingly different localization within the joint and distinct functional specialization.
1397 Data summarised from references [27-30,33,35].

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

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

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

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