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### REVIEW

# Pathogenic stromal cells as therapeutic targets in joint inflammation

(NRR-17-203V<u>4</u>)

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Telephone: 01865 227374 The authors declare no competing financial interests

Key words: stroma, inflammation, musculoskeletal, fibroblast, joint, mesenchymal

#### 1 Abstract

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3 Knowledge of how the joint functions as an integrated unit in health and disease requires an understanding of the stromal cells populating the joint mesenchyme, 4 5 including fibroblasts, tissue resident macrophages and endothelial cells. Physiological and pathological mechanisms in these mesenchymal cells that define 6 the joint have begun to cast new light on why joint inflammation persists. In this 7 review, we highlight how the shared embryological origins of fibroblasts and 8 9 endothelial cells may shape the behaviour of these cell types in diseased adult tissues. We review the molecular mechanisms by which cells of mesenchymal origin 10 sustain inflammation in the synovial membrane and tendons, highlighting the 11 importance of recently discovered fibroblast subtypes and their associated cross talk 12 with endothelial cells, tissue resident macrophages and leukocytes. Finally, we 13 14 discuss how this knowledge shapes the future therapeutic landscape, emphasising the requirement for new strategies to address the pathogenic stroma and associated 15 cross talk of leukocytes with cells of mesenchymal origin. 16

### 17

#### 18 Key points

- 19
- Joint inflammation and tissue damage are mediated by stromal cells-derived
   from of embryonic-mesodermal origin
- Stromal activation and <u>inflammation</u> "memory" of previous inflammatory
   <u>insults</u> are shared <u>disease</u> mechanisms exhibited by fibroblasts, <u>tissue</u>
   <u>resident macrophages and -and endothelial cells</u>
- Recent advances characterising the phenotype and function of cells of
   mesenchymal origin highlight <u>thee</u> distinct fibroblast subtypes mediatinge joint
   inflammation and tissue damage
- Mesenchymal stromal cell niches and their interactions with leukocytes are implicated in the persistence of joint inflammation
- To be effective, strategies to treat residual joint disease should target
   pathogenic stroma and associated immune cell cross talk
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#### 34 Introduction

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Chronic inflammatory diseases affecting joint soft tissues include arthritis 36 37 (synovium and cartilage), enthesopathy and tendinopathy. Collectively, these diseases comprise a significant global economic burden<sup>1</sup>. Each are characterized 38 39 by inflammation of mesenchymal tissues that form the synovium, tendons, ligaments and joint capsule and in some cases structural damage to bone and 40 cartilage. Inflammation of these tissues is broadly characterized by leuckocyte 41 infiltration, fibroblast accumulation and neovascularization supporting cell 42 43 expansion. In this article, we first review the pathophysiological basis of 44 inflammation and tissue damage with respect to the embryological origins of joint mesenchymal tissues. We next discuss the stromal cell types populating joint 45 46 mesenchymal tissues, including fibroblasts, tissue resident macrophages (TRM) 47 and endothelial cells (vascular and lymphatic), highlighting their contribution and roles in chronic synovial inflammation and tissue damage. Finally, we discuss 48 49 potential future therapeutic strategies to target inflammation across joint mesenchymal tissues that address the pathogenic stroma and associated immune 50 51 cell cross talk.

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## 1.0 Embryological origins of the tissues that mediate inflammation and damage across the whole joint organ

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56 Inflammation and tissue damage are pivotal pathological processes affecting tissues structures across the whole joint organ. To further understand the mechanisms and 57 inter-relationships underpinning these fundamental disease processes, it is important 58 59 to consider the origins of joint tissues, given that an organ is best defined by its 60 embryological origin as well as function. This section discusses how the embryological and anatomical origins of the tissues that comprise the joint might 61 shape inflammation and tissue damage, highlighting how this knowledge informs 62 understanding of 'disease patterns' across the joint. 63

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The anatomical basis of inflammation and tissue damage relative to their embryological origin is summarized in Figure 1. <u>Although parts of the axial skeleton</u>

derive from neural crest, Embryonic mesoderm is the precursor for mesenchymal 67 68 tissues comprising the axial and appendicular skeleton, synovium, cartilage, 69 tendons, ligaments, joint capsule and their associated lymphatics and vasculature. These joint soft tissues are predominantly composed of cells of mesenchymal origin, 70 71 including fibroblasts, vascular and lymphatic endothelial cells and TRM. The shared embryological origins of stromal fibroblasts and endothelial cells may shape the 72 behaviour of these cell types in diseased adult tissues. Notably, mesoderm derived 73 74 fibroblast and endothelial cell populations both undergo sustained phenotypic 75 changes after exposure to inflammatory stimuli, exhibiting stromal activation and a form of tissue 'memory'<sup>2,3</sup>. However, the distinct molecular markers expressed by 76 these cell types vary, as we later discuss. TRM also exhibit complex activation 77 states and "memory"<sup>4</sup>. The origins and renewal of TRM have been extensively 78 reviewed and will not be repeated here <sup>5-8</sup>. The majority of TRM are established 79 during embryonic development and persist into adulthood, rather than replacement 80 from circulating adult monocytes <sup>7,9-14</sup>. During early gestation, macrophages are first 81 observed and expand in the extraembryonic yolk sac during primitive hematopoiesis. 82 Yolk sac derived hematopoietic stem cells (HSCs) emerge to form bone marrow 83 precursor cells, which subsequently gives rise to all immune cell lineages <sup>7,15</sup> (Figure 84 1). Importantly, yolk sac derived TRM are phenotypically distinct from HSC derived 85 progeny <sup>10</sup>. The subspecialized adult tissue niches which TRM occupy dictate 86 heterogeneity in the phenotype and functions of these cells in health and disease <sup>16</sup>. 87 We next review how these mesenchymal cell populations are implicated in mediating 88 89 inflammation and tissue damage in joint disease.

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### 2.0 Cells of mesenchymal origin in the healthy and diseased joint

In this section, we focus exclusively on cells of mesenchymal origin including fibroblasts, endothelial cells and TRM rather than on haematopoietically derived cells whose role in these processes (in particularly inflammation and damage) has been well documented<sup>17-19</sup>. We discuss the roles of these cells in normal joint physiology and their impact on inflammation and damage in joint disease. We highlight the recently identified mechanisms implicated in sustaining synovial inflammation,

discussing the molecular features and pathological phenotypes of fibroblast 99 100 subtypes, endothelial cells and TRM.

#### 101 2.1 Fibroblasts and the healthy joint

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103 The term 'stroma' was originally derived from the Greek word describing "a platform 104 on which to lie" and is used to describe the supporting substance of tissue. Its 105 principle role is to maintain the microenvironment required by the parenchyma; the 106 important functional elements of each body system. The stroma comprises 107 connective tissue, nerves, vessels and the extracellular matrices (ECM) and fluids which these cells produce<sup>20</sup>. Joint soft tissues including synovium, capsule, tendon 108 109 and enthesis are predominantly composed of mesenchymal stromal cellscomprised of cellular and acellular ECM. Fibroblasts are the most abundant cell type populating 110 these joint connective issues<sup>21</sup> and synthesise the highly organized collagen rich 111 scaffold necessary for joint structure and movement. 112

113

114 Fibroblasts are defined by their spindle shaped morphology, the absence of specific 115 lineage markers of leukocytes, endothelium and epithelium and their ability to adhere to tissue culture plastic in vitro<sup>22</sup>. They are believed to arise from 3 distinct cellular 116 origins; primary mesenchyme, local epithelial-mesenchymal transition (EMT) or bone 117 marrow derived precursors (circulating fibrocytes)<sup>23,24</sup>. It is widely accepted that the 118 119 majority of fibroblasts originate from primary mesenchymal cells and that fibroblasts can proliferate to generate new progeny<sup>25,26</sup>. In physiological conditions, fibroblasts 120 provide mechanical strength to tissues by producing ECM components (type I, III 121 122 and V collagen and fibronectin) as well as factors that regulate ECM turnover, 123 including metalloproteinases (MMPs) and proteins involved in the formation of basement membranes (type IV collagen and laminin)<sup>27,28</sup>. Fibroblasts synthesise an 124 array of paracrine factors<sup>29</sup> and exhibit mechanosensitive properties<sup>30</sup> to effect 125 126 functional adaptation in normal joint physiology. The intimate relationship between 127 fibroblasts and mesenchymal stromal cells (MSC) and the clinical use of MSC to 128 repair damaged tissues has driven a renewed interest in fibroblasts as new therapeutic targets<sup>21</sup>. 129

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#### 2.2 Mechanisms sustaining joint inflammation based on pathogenic stroma 131 132

#### 133 **2.2.1** Fibroblasts and the diseased joint

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135 Traditionally, the diversity of stromal cells and in particular fibroblasts and their roles 136 beyond those of space filling and ECM homeostasis have been underexplored in 137 inflammation. Mesenchymal tissues in the joint including the synovium, capsule, enthesis and tendons undergo phenotypic changes as a consequence of 138 inflammation<sup>31-33</sup>. These include molecular and structural changes to the ECM, 139 impacting upon the functional quality of the healed tissue<sup>34</sup>. Whilst it remains 140 challenging to discern which is the initiating pathogeneic cell type, it is clear that 141 stromal cells populating these tissues provide a niche conducive to sustaining 142 chronic inflammation<sup>2,35,36</sup>. Recent work shows that fibroblasts vary phenotypically 143 and functionally at different anatomical sites and contribute significantly to the 144 identity of individual tissues, providing a so-called 'stromal postcode'<sup>26</sup>. Furthermore, 145 it is known that, rather than acting as a bystander, fibroblasts are capable of actively 146 participating and indeed orchestrating inflammation and immunity<sup>36-38</sup>. We next 147 review how fibroblasts sustain inflammation, highlighting the mechanisms 148 149 underpinning their activation, "memory" and phenotypic diversity, with particular focus on the synovial microenvironment. 150

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#### 152 Fibroblast activation and memory

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154 Fibroblast activation is a recognized feature of diseases affecting the joint, whereby fibroblasts adopt a pro-inflammatory phenotype. This pathological feature has been 155 identified in cancer <sup>39</sup>, rheumatoid synovium<sup>32,33</sup> and tendon disease<sup>31</sup>. Fibroblast 156 157 activation and memory therefore span boths innate and adaptive immune responses, suggesting this is a highly conserved disease mechanism common to tissues of 158 mesenchymal origin. There is now a growing list of cell surface molecules and 159 160 secreted products which collectively provide a fibroblast activation marker "cassette". These include CD90 (Thy1), CD44, decay accelerating factor (CD55), VCAM-1 161 (CD106), uridine diphosphoglucose dehydrogenase, and prolyl-4-hydroxylase, 162 Podoplanin (PDPN/gp38), endosialin (CD248) and Fibroblast Activation Protein 163 (FAP) <sup>31,36,37,40-42</sup>. Fibroblast activation markers therefore represent important 164 phenotypic alterations implicated in effecting the switch from resolving to persistent 165 inflammation <sup>42</sup>. 166

168 Epigenetic changes are implicated in fibroblast activation and memory. New insights into the epigenetics of inflammatory rheumatic diseases have been recently 169 reviewed in detail elsewhere <sup>43</sup>. Prolonged exposure of RA synovial fibroblasts to 170 TNF $\alpha$  reduce histone H4 levels and promote H4 acetylation <sup>44</sup>. This study showed 171 that TNF $\alpha$  removed the chromatin barrier from the CXCL10 promoter, permitting 172 abundant binding of NF-kB family transcription factors and recruitment of 173 transcriptional machinery<sup>44</sup>. DNA methylation is another important epigenetic 174 modification identified in RA synovial fibroblasts occurring during the early stage of 175 disease <sup>45</sup>. Further studies are required to identify the mechanisms underpinning 176 DNA methylation and there appears to be important prognostic potential for 177 differentially methylated genes as disease biomarkers <sup>45</sup>. The activated and 178 aggressive phenotype of RA synovial fibroblasts is associated with global DNA 179 hypomethylation<sup>46</sup>. Gaur *et al.* investigated if microRNAs moderate the methylation 180 status of RA synovial fibroblasts, showing L-methionine increased DNA methylation 181 compared to betaine <sup>47</sup>. Collectively these studies advance our understanding of how 182 epigenetic changes are implicated in fibroblast activation and memory, informing 183 future strategies to selectively target pathogenic fibroblasts. 184 Recent work shows that tissue resident fibroblasts help define the pattern of joints 185

186 involved, not only in arthritis but in other diseases with a prominent stromal
 187 component<sup>39</sup>.

Importantly, this concept of epigenetically-driven anatomical diversity of synovial 188 fibroblasts provides an attractive mechanism to explain the clinical observations that 189 190 different types of arthritis affect distinct types of joints. For example, OA and PSA often involve the distal interphalangeal joints, whereas RA is frequently symmetrical 191 and more commonly affects the MCP joints. In contrast, AS mainly targets spinal 192 ligaments and entheseal tissue<sup>49</sup>. Such studies have prompted improved 193 characterization of the phenotypes of fibroblast subsets and their different proposed 194 roles. In RA, synovial fibroblasts undergo distinct changes in function, including loss 195 196 of immunosuppressive response in early disease, followed by later acquisition of an immuno-stimulatory phenotype<sup>41</sup>-197

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Fibroblasts from different joint tissues maintain their phenotype, positional memory 199 200 and topographic differentiation despite culture ex vivo. Fibroblasts isolated from RA 201 synovium or diseased tendon exhibit stromal 'memory', whereby these cells show an enhanced subsequent capacity to respond to an additional inflammatory stimulus 202 <sup>2,31,44</sup>. Therefore, sustained expression of activation markers by fibroblasts in the joint 203 reflects their 'primed' status after exposure to an inflammatory stimulus. In addition to 204 205 fibroblast activation, this concept of stromal memory also spans innate and adaptive 206 immunity, suggestive of a highly conserved disease mechanism across tissues of mesenchymal origin. The processes underpinning innate memory have been 207 extensively reported for leukocytes 48,49 and are gaining acceptance in tissue 208 209 resident cells of mesenchymal origin. Engagement of TLR4 and downstream activation of the NFkB pathway is a prominent pathological feature of fibroblasts 210 populating inflamed joint tissues <sup>2,31,44</sup>. These studies suggest that fibroblast memory 211 is associated with altered NF $\kappa$ B responsiveness to an inflammatory stimulus <sup>50</sup>. 212 213 Given the longevity of fibroblasts as tissue resident cells and the relatively low rates of tissue-cell turnover in the joint <sup>51</sup>, the effects of stromal memory in tissues such as 214 215 synovium and tendon are likely to be long lived. In contrast, dermal fibroblasts show 216 higher rates of turnover and do not exhibit stromal memory, suggesting this disease mechanismprocess of stromal memory may vary according to anatomical location 217 2,52,53. Rheumatic diseases follow a characteristic anatomical pattern of joint and 218 219 organ involvement. Mechanisms regulating the predilection of specific joints for 220 developing particular forms of arthritis (for example osteoarthritis (OA) compared to rheumatoid arthritis (RA)) have been reviewed in detail <sup>54</sup>. These include site-specific 221 local cell types driving disease, systemic triggers affecting local cell types and site-222 223 specific exogenous factors activating cells locally. Therefore the mechanisms underpinning activation of stromal cells depends on the local anatomical tissue 224 niche<sup>54</sup>. 225

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#### 227 Fibroblast diversity

Recent work shows that tissue resident fibroblasts help define the pattern of joints
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250 The synovium is composed of lining and sub-lining layers of fibroblasts which vary in 251 terms of phenotype and function-according to their anatomical sub-location. Single 252 cell RNA sequencing and immunohistochemistry have revealed that RA synovial 253 fibroblasts can be broadly characterized into 3 subsets, highlighted in Figure 2. Synovial lining fibroblasts are CD34 CD90 CD55<sup>+</sup> and Cadherin 11<sup>+</sup>. This lining 254 subset synthesizes MMP-1 and MMP-3 which mediate tissue damage in the 255 256 inflamed joint <sup>62</sup>. Fibroblasts populating the synovial sublining are predominantly 257 comprised of 2 populations. CD34<sup>+</sup>CD90<sup>-</sup> fibroblasts release CXCL12, CCL2 and IL-258 6 and mediate-drive fibroblast accumulation cell proliferation and invasion. A second 259 population of CD34<sup>-</sup>CD90<sup>+</sup> fibroblasts with a pro-inflammatory phenotype highly express markers of stromal fibroblast activation <sup>62,63</sup>. These 'pathogenic' fibroblast 260 261 subsets between them degrade articular cartilage, mediate stromal memory, sense 262 tissue damage via TLR4 activation and have altered responsiveness to signalling pathways converging on NF $\kappa$ B responsiveness<sup>26,33,50,62</sup> (Figure 2). Having 263 highlighted the complexity of discrete synovial fibroblast subtypes, we next discuss 264

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the phenotypes and functions of other mesenchymal cell types including endothelial
cells and TRM and their respective roles in joint health and disease.

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#### 2.2.2 The endolymphatic niche in the healthy and diseased joint

270 Other mesenchymal stromal tissues including the vasculature and lymphatics 271 contribute to sustaining inflammation across the joint organ. Neo-angiogenesis is a 272 prominent feature of disease of mesenchymal joint tissues and impacts upon changes in tissue architecture and pain perception<sup>64</sup>. In health, vascular endothelial 273 274 cells regulate blood flow, vessel wall permeability and leukocyte extravasation into tissues, regulating the inflammatory process <sup>65-68</sup>. In lymph nodes and tertiary 275 276 lymphoid tissues, high endothelial vessels (HEVs) provide specialized 277 microenvironments for efficient entry of lymphocytes into tissues in an L-selectin dependent process<sup>69</sup>. The phenotypes of endothelial cells change as inflammation 278 transitions from acute to chronic and also between activation of innate and adaptive 279 280 immune systems <sup>67</sup>. Endothelial cell phenotypes are poorly characterized in tendon 281 and entheseal tissues. However, in RA synovium, these cells have been described as activated, angiogenic, apoptotic and leaky, a process found in many tumour 282 283 microenvironments <sup>70</sup>. During prolonged exposure to inflammatory stimuli endothelial cells become activated, exhibit memory and express adhesion molecules including 284 ICAM, VCAM-1 and CD31 (PECAM-1) <sup>3,71-73</sup> (Figure 2). These activated endothelial 285 cells subsequently also present chemokines and initiate leukocyte migration from 286 blood to local tissues<sup>70</sup>. Endothelial activation is a cause and consequence of 287 endothelial dysfunction<sup>74,75</sup>, culminating in increased microvascular permeability, 288 289 extravasation of plasma and joint oedema. Release of angiogenic factors including VEGF triggers angiogenesis, provide necessary nutrients and oxygen to meet the 290 291 metabolic demands of the inflamed tissue. Importantly, neo-angiogenesis further promotes the retention and survival of immune cells at inflamed sites, thereby 292 sustaining chronic inflammation <sup>38</sup>. These angiogenic processes occur during normal 293 inflammatory immune responses (i.e vaccination)<sup>76</sup>, however whether angiogenesis 294 that occurs in joint disease is a cause or effect of pathology remains unclear. 295

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297 Stromal lymphatic vessels form a one-way conduit for tissue fluid and leukocytes in 298 health and disease <sup>77</sup>. During adaptive immune responses, antigen presenting cells

travel to lymph nodes via lymphatic vessels, which highly express PDPN, implicated 299 in-stromal fibroblast activation<sup>78</sup>. The permeability of lymphatic vessels is a tightly 300 regulated dynamic process that alters during health and disease <sup>79</sup>. Lymphatic 301 vessel growth (lymphangiogenesis) is a primary response during acute inflammation. 302 which becomes dysregulated in chronically inflamed adult tissues<sup>80</sup>. In experimental 303 murine models of inflammatory arthritis, lymphatic vessels and nodes draining the 304 diseased joint undergo an initial expansion phase to expedite lymphatic clearance. 305 306 This expansion phase is followed by a collapsed phase, characterized by structural damage to lymphatic vessels and reduced lymphatic clearance <sup>79,81</sup>. Studies 307 demonstrate alteration in lymphatic vessel function and lymph node volume also 308 occur in patients with RA flare <sup>82</sup>. Therapies targeting aberrant lymphatic function 309 have shown promise in preclinical models of inflammatory arthritis and may prove 310 efficacious in RA 79. 311

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#### 2.2.3 Tissue Resident Macrophages in the healthy and diseased joint

TRM mediate a diverse range of biological actions. They are appropriately positioned and transcriptionally primed to respond to local environmental challenges, maintaining tissue homeostasis. TRM direct immune surveillance, induce inflammation and promote subsequent resolution, reviewed in detail elsewhere <sup>34,83</sup>. Given the biological complexity of these roles, TRM are highly heterogeneous and exhibit diverse phenotypic and functionally distinct subtypes within a single tissue type <sup>5,84</sup>.

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323 In inflamed synovium, TRM mediate immune surveillance through expression of a variety of pattern recognition receptors DAMPs, notably Toll-like receptors (TLR) 324 TLR2 and TLR4 and facilitate the recruitment of infiltrating leukocytes, incuding 325 monocyte derived macrophages <sup>85-87</sup>. TRM induce joint inflammation through release 326 of TNFα, IL-1β IL-6, GM-CSF and PGE<sub>2</sub>, driving fibroblast accumulation proliferation, 327 angiogenesis, leukocyte recruitment and tissue damage via protease secretion 328 (Figure 2). The essential role of non-classical Ly6C-monocytes has been reported in 329 murine arthritis models <sup>88</sup>. This study highlights the phenotypic heterogeneity of 330 synovial TRM, demonstrating how macrophage activation status regulates disease 331

progression and resolution. In support of this, human RA synovial macrophages
exhibit distinct transcriptional profiles associated with disease activity and therapy <sup>89</sup>.
However, distinction between TRM and infiltrating macrophages is currently
hampered by a lack of specific markers that distinguish between these populations in
diseased human tissues.

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The pro-inflammatory milieu in the inflammed synovium triggers an active process of 338 339 lipid mediator class switching and the subsequent release of families of specialized 340 proresolving mediators (SPM). These include lipoxins, resolvins, protectins and 341 maresins, that are generated via transcellular biosynthesis and are concerned with mediating resolution of inflammation<sup>90-94</sup>. These bioactive lipid mediators initiate 342 programmes which halt neutrophil infiltration, potentiate monocyte recruitment, 343 344 moderate vascular permeability and promote phagocytosis and drainage of apoptotic cells<sup>95</sup>. The mechanisms mediating resolution in inflammatory arthritis have been 345 reviewed in detail and are not covered here are reviewed in detail elsewhere <sup>96</sup>. TRM 346 are key regulators of repair and fibrosis across all tissue types <sup>34</sup> and are also 347 implicated in mediating resolution of inflammation. Distinct populations of resolution 348 phase macrophages have been identified in systemic murine inflammation models 349 that express Alox15, Timd4 and Tgfb2, which terminate leukocyte recruitment and 350 promote clearance <sup>97</sup>. However, the precise phenotypes of TRM mediatingeffecting 351 resolution in human joint disease requires further investigation. 352

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### 356 2.2.4 Cross talk between cells of mesenchymal origin

Having highlighted the molecular features and phenotypes of mesenchymal cells and their roles in mediating joint pathology, we next discuss how cross talk between these cell populations sustains inflammation. Damage sensing mechanisms, cytokine and chemokine gradients are pivotal pathological processes involving cross talk between fibroblast, endothelial cell, TRM and leukocyte populations that sustain inflammation in the diseased joint <sup>26,98,99</sup>.

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RA synovial fibroblasts act as sentinel cells that can "sense" tissue damage. This 365 366 occurs via the binding of damage associated molecular patterns (DAMPs) including HMGB1, heat shock and S100 proteins<sup>100,101</sup>. Tenascin-C a matrix protein induced 367 upon tissue damage also activates TLR4 mediated sterile inflammation <sup>102</sup>. Binding 368 of these ligands to TLR4 induces a high alert state, favouring the development of 369 chronic inflammation <sup>50,103</sup>. Engagement of TLR4 activates Myd88 signalling 370 pathways, inducing pro-inflammatory cytokine release via NF $\kappa$ B activation <sup>48</sup>. 371 372 Consequently, activated synovial fibroblasts are primed to release a broad range of pro-inflammatory mediators. These localised cytokine and chemokine gradients 373 promote the migration, retention and survival of leukocytes and TRM,<sup>42,104</sup> creating a 374 complex functional syncytium conducive to sustaining inflammation, highlighted in 375 376 Figure 3. The processes mediating leukocyte trafficking between stromal compartments in RA are recently reviewed in detail elsewhere <sup>105</sup>. 377

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#### 379 Fibroblast – immune cell cross talk

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381 RA synovial fibroblasts promote leukocyte retention via release of cytokines and 382 chemokines and via contact with other cells of mesenchymal origin. Pro-383 inflammatory cytokines released by retained monocytes, T cells and TRM including IFNy, TNF $\alpha$  and IL-1 $\beta$  induce activated synovial fibroblasts to release high levels of 384 PGE<sub>2</sub>, GM-CSF, IL-6. These cytokines exert differing effects on leukocyte activation. 385 PGE<sub>2</sub> moderates chemokine production and promotes Th2, Th17 and Treg 386 responses <sup>106</sup>. IL-6 drives CD4+ T cells towards Th17 activation <sup>107</sup>, whereas GM-387 CSF promotes neutrophil survival and monocyte differentiation in the inflamed 388 synovium <sup>26,108</sup>. Nguyen et al. demonstrated that IL-6 and other inflammatory 389 390 cytokines and chemokines are regulated by a positive feedback loop that selectively operates in fibroblasts involving leukemia inhibitory factor (LIF), LIF receptor and 391 STAT4 <sup>109</sup>. TGFβ, also found at high levels in RA synovium induces persistent 392 expression of CXCR4 on synovial T cells, leading to their active CXCL12 mediated 393 retention, providing an additional mechanism for immune cell retention <sup>110</sup>. RA 394 395 synovial fibroblasts also release a repertoire of chemokines, generating a gradient consisting of CCL2, CCL4, CCL5, CCL8, CXCL8, CXCL12 and IFNB<sup>26,111,112</sup>. This 396 397 chemokine gradient actively promotes the recruitment, retention and survival of

monocytes and CD4+ T cells at the inflamed synovial site (Figure 3). CXCL12,
 VCAM-1 (CD106) and IL-6 therefore constitute part of a 'stromal address code',
 critical for leukocyte survival and differentiation <sup>26</sup>.

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402 Endothelial cell cross talk

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Resident stromal cells populating inflamed synovium modulate the ability of 404 405 endothelial cells to recruit leukocytes via release of soluble mediators or direct cell-406 cell contact. - Stromal Fibroblasts isolated from healthy patients are known to 407 regulate the cytokine-sensitivity of vascular endothelium, while fibroblasts associated with chronic inflammation adopt a pro-inflammatory phenotype <sup>29,113</sup>. Cytokine and 408 409 chemokine gradients mediate and sustain cross talk between endothelial cell, synovial fibroblast and TRM populations. IL-6, TGF $\beta$ 1 and VEGF released from TRM 410 provide the necessary cues to promote an angiogenic environment required to 411 412 sustain endothelial cell activation and dysfunction (Figure 3). This is supported by antibody neutralisation of IL-6, which diminished the ability of endothelial cells to bind 413 lymphocytes in co-cultures with RA fibroblasts <sup>29</sup>. 414

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The RA synovial fibroblast milieu further sustains an angiogenic environment through 416 chemokine gradients comprising CXCL1-5 and CXCL8 <sup>26</sup> (Figure 3). RA fibroblasts 417 regulate expression of endothelial cell adhesion molecules, potentiate leukocyte 418 extravasation <sup>58</sup> and induce unstimulated HUVEC to bind flowing lymphocytes via a 419 CXCR4-CXCL12 dependent manner <sup>29</sup>. Consequently, the interactions between cells 420 of mesenchymal origin create and sustain an inflammatory milieu, whereby synovial 421 422 inflammation persists and potentially becomes independent of its inciting cause. We 423 next consider how persistent inflammation culminates in tissue damage across soft tissues that comprise the joint. 424

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#### 426 **2.3 Mesenchymal cells and their role in joint damage**

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In health, early damage repair mechanisms maintain the integrity of joint soft tissues.
In joint disease, sustained inflammation, tissue remodeling and fibrosis ensue,
resulting in irreversible tissue damage. We next discuss how cells of mesenchymal

431 origin mediate fetal scarless healing and highlight the mechanisms by which they432 induce damage across adult joint tissues.

In contrast to normal adult tissues, early human and murine fetal wounds and 433 wounds in Nude (FoxN1 deficient) mice heal without scar formation<sup>114</sup>. Fetal wounds 434 435 show diminished numbers of immune cells and lower levels of cytokines compared to adult tissues <sup>115-118</sup>. Differences between embryonic and adult tissue healing are 436 also attributed to the milieu of pro-fibrotic growth factors released by TRM, including 437 438 those of the TGF $\beta$  family. TGF $\beta$ 1 levels are reduced and this growth factor shows accelerated clearance in embryonic compared to adult tissue repair <sup>119-121</sup>. 439 440 Collectively these studies indicate a role for immune cell derived cytokines including TNF $\alpha$  and TGF $\beta$  in tissue scarring and healing<sup>122</sup>. Other studies highlight differences 441 between fetal and adult fibroblasts and localized production of MMP-9 and MMP-13 442 in the scarring process <sup>114</sup>. Fetal fibroblasts show enhanced synthetic function, 443 increased rate of turnover of collagen, hyaluronic acid, ECM components and 444 445 increased migration velocity compared to adult fibroblasts, suggesting rapid healing may also play a role in scarless tissue repair<sup>123-125</sup>. 446

In adult tissues, fibroblasts and TRM directly contribute to joint destruction, bony 447 erosions and remodeling through expression of enzymes such as MMPs<sup>126</sup>. MMP-2, 448 MMP-9 and MMP-13 have been specifically implicated in the pathogeneis of RA and 449 OA<sup>127</sup>. MMP-9 is also upregulated by CXCL12 (SDF-1) a key chemokine secreted by 450 synovial fibroblasts<sup>128</sup>. FAP is highly expressed within RA synovium and co-localises 451 with MMP-13, where it appears to play a role in tissue degradation<sup>129</sup>. Cathepsins, a 452 major group of proteases involved in joint remodeling are also upregulated in the 453 diseased joint<sup>130</sup>. Additionally fibroblasts can indirectly contribute through cross talk 454 with TRM and lymphocytes, further amplifying processes driving tissue damage 455 (Figure 3), whilst also presenting antigen to tissue infiltrating lymphoctyes<sup>131</sup>. 456

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Pathological conditions in which cells of mesenchymal origin play a role include chronic inflammation (e.g. RA, chronic skin wound healing), tissue fibrosis (e.g. COPD) and cancer (e.g. breast cancer). Interestingly, while these diseases differ dramatically in aetiology and genetic predispositions, they converge in terms of phenotype and function of the stromal component. Fibroblasts expand in the RA 463 synovial tissue and in the tumor parenchyma, while fibrosis is characterized by 464 profound changes in myofibroblast phenotype and function across different organs such as the lungs and kidneys <sup>132</sup>. Whether these fibroblast properties are intrinsic 465 phenotypic changes acquired as a consequence of exposure to chronic 466 467 inflammation, or are derived from the conditioning of the pathogenic infiltrating cells is still under investigation and seems to differ in the different conditions<sup>37</sup>. Lafevre et 468 al reported epigenetically programmed aggressive cells may "spread" arthritis from 469 inflamed to uninflamed joints in the early stages of disease, <sup>133</sup>. PDPN expressing 470 lining synovial fibroblasts are migratory and mediate release of cartilage destructive 471 472 MMPs <sup>33,62</sup>. Collectively, these data raise the possibility of distinct mesenchymal cell subsets implicated in mediating the effects of tissue damage in the diseased joint. 473 474 We next discuss how the possibility of selectively targeting pathogenic stromal subpopulations mediating inflammation and tissue damage informs the development 475 476 of future strategies to successfully treat joint disease.

- 478 3.0 Shaping the future landscape: therapeutic targeting of
  479 mesenchymal cells
- 480

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481 Cells of mesenchymal origin including fibroblasts, TRM and endothelial cells 482 constitute the major cell types populating joint soft tissues. We have discussed the 483 roles and mechanisms by which these cells mediate joint inflammation, highlighting 484 their ability to act as immune sensing sentinel cells, their capacity for activation, 485 positional memory and their altered phenotypes comprising multiple cellular subpopulations. Multidirectional cross talk between stromal cell populations further fuels 486 487 the development of persistent inflammation. Given these important roles and 488 associated biological complexities, it is likely that residual disease activity in patients 489 treated with immune therapies may be attributable to stromal mediated inflammatory responses, which are refractory to current therapies that target immune cell 490 populations <sup>134</sup>. New therapeutic approaches are therefore required to 'break the 491 492 cycle and reset the system', particularly in scenarios where inflammation becomes 493 independent of the inciting stimulus. Given the limited capacity of joint tissues to 494 regenerate once damaged, there are significant challenges associated with curbing 495 tissue damage, which might be accomplished through moderating persistent 496 inflammation as a driver of fibrosis. We next discuss the requirement for future 497 strategies to address the pathobiology concerned with the stromal 498 microenvironment, targeting cells of mesenchymal origin. We review the drug classes in current clinical use, those in early phase clinical trials and strategies with 499 500 pre-clinical potential to target stromal mediated joint disease. The cellular and 501 molecular targets and the mechanism of action through which these drug classes 502 function are summarized in Table 1.

503

#### 504 Existing licensed therapies

505

506 Nonsteroidal anti-inflammatory drugs (NSAIDs) and corticosteroids provide 507 symptomtic relief for a broad array of conditions targeting inflammation and pain. Their clinical use in the management of a multitude of diseases affecting the joint is 508 well established <sup>135-138</sup>. These therapies target fibroblasts, TRM and endothelial cells 509 via differing biological modes of action. Inhibition of COX activity by NSAIDs 510 511 dampens release of prostaglandins, leukotrienes and thromboxane A<sub>2</sub>. 512 Corticosteroids act via the glucocorticoid receptor to inhibit cPLA<sub>2</sub>, regulate expression of NFkB / MAPK target genes and dampen release of inflammation 513 514 initiating eicosanoids. Whilst NSAIDs and corticosteroids continue to provide 515 background anti-inflammatory therapy for many rheumatic diseases, they are both associated with well documented adverse systemic effects. Importantly, COX-2 516 selective NSAIDs also dampen protective endogenous resolution responses <sup>139,140</sup>, 517 518 which may paradoxically impede the capacity of inflamed joint tissues to heal.

519

Monoclonal antibodies enable precise molecular targeting of cytokines mediating 520 521 joint inflammation. The biological modes of action and efficacy of therapeutic inhibitors of IL-1, IL-6, TNFa and IL-17 in current clinical use are well reported and 522 523 listed in Table 1. One disadvantage associated with selective cytokine inhibition is 524 the failure of this approach to fully target stromal mediated inflammatory responses and address the complex multidirectional cross talk between mesenchymal cell 525 populations. Similarly targeting chemokine gradients is an attractive strategy to 526 moderate leukocyte retention <sup>141</sup>. However chemokine antagonists including 527 AMD3100 targeting CXCR4 are associated with adverse systemic effects <sup>142</sup> and the 528

plethora of chemokines mediating stromal inflammatory responses presents a furthertherapeutic challenge.

531

#### 532 Therapies in early phase clinical trials

533

534 GM-CSF, predominantly produced by activated T cells, monocytes and macrophages is also released by tissue resident cells of mesenchymal origin <sup>143</sup>. 535 Humanised IgG1 monoclonal antibodies to GM-CSF prevent interaction of this 536 537 cytokine with its receptor, reducing downstream signalling pathways converging on NFκB. GM-CSF has shown potential as a therapeutic target in autoimmune and 538 inflammatory disorders, including RA. Early phase clinical trials demonstrated 539 540 disease activity scores reduced in mayrilimubab treated patients with moderate RA. Therapies targeting GM-CSF or its receptor have shown encouraging results in more 541 recent pre-clinical studies and are reviewed in detail elsewhere <sup>143</sup>. Recent phase IIb 542 studies have demonstrated that long term mavrilimumab treatment maintained 543 clinical responses and was well tolerated in RA patients with inadequate response to 544 DMARD's<sup>144</sup>. Further investigation is required to determine the efficacy of GM-CSF 545 targeted therapies to modulate stromal mediated inflammatory responses in the joint. 546 Kinase inhibitors targeting JAK and SYK signalling pathways have been investigated 547 for their therapeutic utility to reduce cytokine release through JAK STAT <sup>145,146</sup> or 548 MAPK / PKC <sup>147,148</sup> blockade respectively (Table 1). Baricitinib, an oral reversible 549 550 inhibitor of JAK1 and JAK2 has shown therapeutic value in RA patients. This 551 treatment was associated with significant clinical improvements in patients with an inadequate response to methotrexate compared with placebo and adalimumab 552 treated groups <sup>149</sup>. Protein kinase inhibitors target a broad range of cells types with 553 554 reported off target effects, highlighting the importance of understanding the pharmacology of these drugs beyond the kinome <sup>150</sup>. 555

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#### 557 Potential future strategies to target pathogenic stroma

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559 Developments in cancer medicine targeting cancer associated fibroblasts populating 560 tumour stroma have informed potential future strategies to target pathogenic stroma 561 in rheumatic disease <sup>151,152</sup>. Targeting pathogenic stroma presents a considerable therapeutic challenge due to the biological complexity underpinning activation, memory and phenotypic diversity exhibited by these mesenchymal cell populations. Potential future strategies to treat residual rheumatic disease might include targeting activated fibroblast subtypes, use of epigenetic modifiers or resolution agonists to target stromal mediated inflammation. Pre-clinical evidence supporting these approaches are discussed below.

568

569 Selective targeting of distinct fibroblast subtypes mediating joint inflammation and 570 tissue damage is a potential therapeutic strategy to target pathogenic stroma. Cadherin-11 is known to regulate synovial fibroblast inflammation, synergizing with 571 IL-1 $\beta$  and TNF $\alpha$  to regulate IL-6 release <sup>153</sup>. This study showed that cad-11 deficient 572 mice or anti-cad-11 mAb therapies reduced inflammation in arthritic mice, 573 suggesting that cadherin expression regulates the inflammatory capacity of synovial 574 fibroblasts. Cyclin dependent kinases regulate cell proliferation and survival via 575 576 specific inhibitors (CDKi) and are potential therapies to target fibroblast accumulationproliferation in RA synovium (Table 1). CDK pathways become 577 578 dysregulated in cancer, leading to the development of anti-cancer drugs including the CDKi Roscovitine <sup>154</sup>. In synovial fibroblasts, IL-6 and MMP-1 are known to be 579 regulated by CDKi p21<sup>155</sup>. Given that CD34<sup>+</sup>CD90<sup>-</sup> 'immunoregulatory' fibroblasts 580 are highly proliferative, invasive and produce IL-6<sup>62</sup>, CDKi therapies are a potential 581 strategy to target this fibrolast subset mediating joint disease. 582

583

584 We previously discussed how epigenetic changes are implicated in mediating 585 stromal fibroblast activation and memory. Epigenetic alterations in RA synovial fibroblasts are listed in Table 1, identifying DNA methylation, histone modification 586 and miRNA as potential processes to the rapeutically target <sup>43,45-47,156</sup>. Moderating the 587 epigenetic landscape is likely to have broad ranging effects on a variety of cell types, 588 589 with off target effects. Hence improved understanding of the pharmacology of these drugs beyond the epigenome is essential before we can appreciate their potential 590 591 utility to treat joint disease.

592

593 The roles of proresolving mediators in joint health and disease are increasingly 594 understood, identifying resolution agonists as potential therapies to moderate joint

inflammation and promote tissue repair 96. The biological modes of action of 595 596 proresolving mediators or 'immunoresolvents' are well established from in vitro and 597 in vivo studies and include limiting PMN infiltration, stimulating efferocytosis and activation of endogenous tissue protective mechanisms <sup>90-93,157,158</sup>. Whilst 598 immunoresolvents target leukocytes, their biological actions are not associated with 599 immunosuppression<sup>83,159</sup>. Importantly, proresolving mediators also target fibroblasts, 600 TRM and endothelial cells types <sup>160-162</sup> and therefore possess the capacity to 601 602 modulate stromal mediated inflammatory responses across joint tissues. Approaches 603 to potentiate resolution processes include dietary supplementation with proresolving precursors, blocking catabolism of proresolving mediators or local delivery of stable 604 analogues binding proresolving receptors <sup>96</sup>. The pro-resolving mediator RvD3 was 605 found to limit leukocyte infiltration and paw joint eicosanoid levels in murine 606 inflammatory arthritis <sup>163</sup>. The stable epimer 17R-RvD1 significantly attenuated 607 arthritis severity, cachexia, paw oedema, leukocyte infiltration and shortened the 608 remission interval, showing cartilage protective actions in murine models of acute 609 inflammatory arthritis<sup>164</sup>. In vitro studies also highlight the capacity of 15-epi-LXA<sub>4</sub> 610 611 and MaR1 stable epimers to regulate PDPN, STAT-1 and IL-6 in IL-1ß stimulated diseased human tendon stromal cells <sup>35,165</sup>. Collectively these studies suggest 612 resolution pharmacology may be an important future therapeutic tool to address 613 614 stromal pathobiology in the joint.

615

#### 616 **Conclusions**

617

Stromal cells of mesenchymal origin including fibroblasts, tissue resident 618 619 macrophages and endothelial cells are pivotal populations regulating health and 620 disease in musculoskeletal tissues. New insights are beginning to reveal the mechanisms underpinning the activation and dysfunction of mesenchymal stromal 621 622 cells and their contribution to sustaining chronic joint inflammation. The discovery that distinct synovial fibroblast subsets mediate joint inflammation and damage will 623 inform precision therapeutic targeting of pathogenic stromal cell populations. These 624 discoveries shape the future therapeutic landscape, presenting exciting new 625 approaches to address the pathogenic stromal microenvironment. Harnessing the 626 capacity to modulate cross talk between leukocyte and pathogenic stromal cell 627

| 628               | populations is a critical barrier to overcome in our quest to advance therapeutic   |
|-------------------|---|
| 629               | strategies for patients with refractory joint disease.  |
| 630               |   |
| 631               | Glossary of terms   |
| 632               |   |
| 633<br>634<br>635 | Mesoderm: Middle embryonic primary germ layer residing between ectoderm and endoderm  |
| 636<br>637        | Mesenchymal: Embryonic connective tissue derived from the mesoderm  |
| 638<br>639<br>640 | Mesenchymal tissue: Tissue of the musculoskeletal, circulatory and lymphatic systems  |
| 641<br>642        | Stromal cell: Non-haematopoietic, tissue resident cells.  |
| 643               | Stromal cell activation: Process whereby stromal cells including fibroblasts, tissue  |
| 644<br>645<br>646 | resident macrophages and endothelial cells adopt a pro-inflammatory phenotype and<br>express distinct molecular markers after exposure to an inflammatory stimulus. |
| 647               | Stromal cell memory: A change in the capacity of stromal cells to respond to  |

648 inflammatory stimuli

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## Table 1: Drugs to target the pathogenic stroma and associated immune cellcross talk in joint disease

| Drug                   | g Class                            | Target                              | Molecular                  | Mechanism of Action   | References  |                                       |
|------------------------|------------------------------------|-------------------------------------|----------------------------|---|-------------|---------------------------------------|
|                        |                                    | Mesenchymal                         | Target                     |   |             |                                       |
| NS                     | SAIDs                              | Fibroblast (F)                      |                            | Selective / non-selective   | 135,136     | -                                     |
|                        |                                    | Tissue Resident<br>Macrophage (TRM) | COX-1<br>COX-2             | inhibition of COX to reduce   |             |                                       |
| Ocatio                 |                                    | Endothelial Cell (EC)               |                            | leukotrienes, thromboxane   | 137.138     |                                       |
| Cortic                 | osteroids                          | F, TRM, EC                          | glucocorticoid<br>receptor | cPLA2 inhibition<br>regulate NFkB / MAPK target                                   | ,           |                                       |
|                        |                                    |                                     |                            | genes<br>reduce prostaglandins.   |             |                                       |
|                        |                                    |                                     |                            | leukotrienes, thromboxane   |             |                                       |
| Mono                   | clonal Ab                          |                                     |                            |   |             |                                       |
|                        | IL-1                               | TRM (F)                             | IL-1R                      | Reduce effects of inflammasome and caspase  | 166,167     |                                       |
|                        | IL-6                               | TRM, F                              | IL-6R                      | activation<br>Reduce STAT-3 signalling  | 168-172     |                                       |
| -                      | TNF                                | TRM (F)                             | TNFR 1/2                   | Reduce NFκB / MAPK<br>signalling  | 173-178     |                                       |
| GN                     | M-CSF                              | TRM, F, EC                          | GM-CSFR                    | Reduce JAK STAT, PI3K,<br>MAPK and NFκB signalling                                | 179,180     |                                       |
| <u>1</u>               | <u>L-17</u>                        | <u>TRM</u>                          | IL-17R family              | <u>Reduce TRAF6, MAPK, TAK1 &amp;</u><br><u>NFκB signalling</u>                   | 181-183     | <b>Comment [MOU2]:</b> New references |
| Kinase                 | Inhibitors                         |                                     |                            |   |             | for the treatment of PsA              |
| JAK i                  | inhibitors                         | F, TRM                              | JAK1 JAK2 JAK3<br>TYK2     | Blockade of cytokine signalling<br>via JAK STAT                                   | 145,146,149 |                                       |
| SYK                    | inhibitors                         | F, TRM                              | Fcy receptor               | Reduce IL-6 via MAPK / PKC  | 147,148     |                                       |
| Fibrobla               | st activation                      |                                     |                            |   |             |                                       |
| Cadhei                 | rin-11 mAb                         | F                                   | Cadherin-11                | Reduce MAPK, NFkB, IL-6   | 153         |                                       |
| Cyclin<br>kinase<br>(0 | dependent<br>e inhibitors<br>CDKi) | F                                   | CDK1,2,4,6                 | Inhibit cell proliferation & survival, induce apoptosis                           | 142,154,155 |                                       |
| Epigene                | tic Modifier                       |                                     | DNA methylation            | Hypomethylation LBH enhancer  | 45,47,156   | Comment [MOU3]: New references        |
|                        |                                    |                                     | modification               | <del>region</del>   | 44          | supporting therapies targeting DNA    |
| 1                      |                                    | F                                   |                            | Increase H4 acetylation<br>CXCL10 promoter  | 184         |                                       |
|                        |                                    |                                     | miRNA                      | Increase H4 acetylation IL-6  | 185<br>186  | Formatieu: Leit                       |
|                        |                                    |                                     |                            | Reduce miR-22<br>Reduce miR-20a<br>Reduce miR203                                  | 187         |                                       |
| Pro-r                  | resolving                          |                                     |                            |   |             |                                       |
| 17-I                   | R RvD1                             |                                     | ALX, DRV1                  | Chondroprotective   | 164         |                                       |
| Ann                    | nexin A1                           | F, TRM, EC                          | ALX                        | Chondroprotective, increased TGF $\beta$ , prevent apoptosis                      | 188         |                                       |
| F                      | RvD3                               |                                     | ALX                        | Reduce leukocyte infiltration,<br>prostaglandins, leukotrienes<br>and thromboxane | 163         |                                       |
| 15-e                   | epi-LXA <sub>4</sub>               |                                     | ALX                        | Reduced STAT-1, IL-6,   |             |                                       |

| Figures | s: |
|---------|----|

## Figure 1. Embryological origins of mesenchymal tissues in the whole joint organ.

Podoplanin

To further understand the mechanisms and inter-relationships underpinning inflammation and tissue damage across the joint, it is important to consider the embryonic origins of joint tissues, which may shape the behaviour of these cell types in diseased adult tissues. Embryonic Mesoderm is the precursor for mesenchymal tissues comprising the axial and appendicular skeleton, synovium, cartilage, tendons, ligaments, joint capsule and their associated lymphatics and vasculature. Adult joint soft tissues are predominantly composed of cells of mesenchymal origin, including fibroblasts, endothelial cells and tissue resident macrophages (TRM). The shared embryological origins of fibroblasts and endothelial cells shape the behavior of these cell types in diseased adult tissues in terms of their ability to exhibit activation and memory after exposure to inflammatory stimuli. Yolk sac derived TRM are phenotypicallygenetically distinct from HSC derived lineages. TRM occupy subspecialized niches which dictate their heterogeneity and phenotype in adult tissues.

### Figure 2. Molecular features of cells of mesenchymal origin in <u>Rheumatoid</u> pathological synovium.

Inset shows topographical location of cell types comprising RA synovium, consisting of lining and sublining layers. Synovial lining fibroblasts (blue) are CD34<sup>-</sup>CD90<sup>-</sup>, express PDPN, CD55 and release MMP-1 and MMP-13 implicated in tissue destruction. Fibroblast subsets concerned with proliferation, <u>accumulation and inflammationproliferation</u> and <u>inflammation occupy</u> the synovial sublining. Proliferative limmunoregulatory fibroblasts (green) promote fibroblast accumulation and invasion. These cells express CD34 and release chemokines and cytokines generating gradients that promote leukocyte retention. Pathogenic fibroblasts (red) are a CD34<sup>-</sup>CD90<sup>+</sup> subpopulation that highly express markers of stromal</sup> fibroblast activation and exhibit <u>inflammationstromal</u> memory. Pathogenic fibroblasts express TLR4 which mediates the damage sensing properties of these cells and downstream activation of the NFκB pathway via MAPK, JNK and JAK-STAT signalling pathways.

These phenotypic features sustain the pro-inflammatory pathogenic phenotype of this fibroblast subset. Fibroblasts in the synovial sublining are in close proximity to activated endothelial cells, expressing CD31, VCAM-1 and ICAM-1 and CD68<sup>+</sup> tissue resident macrophages (TRM) which release pro-inflammatory mediators and proteases.

## Figure 3: Mechanisms sustaining synovial inflammation, highlighting cross talk between cells of mesenchymal origin and leukocytes.

Cells of mesenchymal origin including fibroblast subsets, endothelial cells and tissue resident macrophages (TRM) are engaged in multidirectional cross talk, which sustains synovial inflammation. RA synovial fibroblasts promote leukocyte retention via release of cytokines and chemokine gradients and via contact with other cells of mesenchymal origin. Pro-inflammatory cytokines released by retained monocytes, T cells and TRM including IFN $\gamma$ , TNF $\alpha$  and IL-1 $\beta$  induce activated synovial fibroblasts to release high levels of PGE<sub>2</sub>, GM-CSF and IL-6. TGF $\beta$  released by TRM induces persistent expression of CXCR4 on synovial T cells, leading to their active CXCL12 mediated retention. RA synovial fibroblasts also release chemokines including CCL2, CCL4, CCL5, CCL8, CXCL8, CXCL12 and IFN $\beta$  that promotes the recruitment, retention and survival of monocytes and CD4+ T cells. IL-6, TGF $\beta$ 1 and VEGF released from TRM provide the necessary cues to promote an angiogenic environment required to sustain endothelial cell activation and dysfunction. The RA synovial fibroblast milieu further sustains an angiogenic environment through chemokine gradients comprising CXCL1-5 and CXCL8.

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