

1 **Heterogeneity of Inflammation-associated Synovial Fibroblasts in Rheumatoid Arthritis**
2 **and Its Drivers.**

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24 **Abstract**

25 Inflammation of non-barrier immunologically quiescent tissues is associated with a massive influx
26 of blood-borne innate and adaptive immune cells. Cues from the latter are likely to alter and
27 expand the spectrum of states observed in cells that are constitutively resident. However, local
28 communications between immigrant and resident cell types in human inflammatory disease
29 remain poorly understood. Here, we explored heterogeneity of synovial fibroblasts (FLS) in
30 inflamed joints of rheumatoid arthritis (RA) patients using paired single cell RNA and ATAC
31 sequencing (scRNA/ATAC-seq), multiplexed imaging, and spatial transcriptomics along with *in*
32 *vitro* modeling of cell extrinsic factor signaling. These analyses suggest that local exposures to
33 myeloid and T cell derived cytokines, $TNF\alpha$, $IFN\gamma$, $IL-1\beta$, or lack thereof, drive six distinct FLS
34 states some of which closely resemble fibroblast states in other disease-affected tissues including
35 skin and colon. Our results highlight a role for concurrent, spatially distributed cytokine signaling
36 within the inflamed synovium.

37 Rheumatoid arthritis (RA), a systemic autoimmune disease with predominantly articular
38 manifestations, is characterized by hyperplasia of both the synovial lining, which interfaces the
39 synovial sublining and the synovial fluid-filled joint space, as well as the synovial sublining, which
40 exhibits increased vascularization and an influx of leukocytes. Both the lining and sublining
41 fibroblast-like synoviocytes (FLS) undergo proliferation and activation, assuming a state, in which
42 they stimulate angiogenesis, produce pro-inflammatory cytokines and chemokines, and invade
43 adjacent articular cartilage and bone¹. Expression of MHC class II molecules by activated FLS is
44 associated with synovial inflammation^{2,3} and their expression by lining FLS correlates with active
45 disease⁴. HLA-DR⁺ FLS expression of soluble mediators, including the proinflammatory cytokines
46 IL-6 and IL-15, and chemokines CCL2, CXCL9, and CXCL12 along with adhesion molecules such
47 as ICAM1 and VCAM1 suggests that these features of FLS might be imparted by their interactions
48 with leukocytes². In support of this possibility, prior *in vitro* studies have shown that HLA-DR⁺ FLS
49 are capable of presenting antigens to CD4⁺ T cells⁵⁻⁷. Furthermore, production of the
50 aforementioned proinflammatory chemokines by FLS likely acts as a feedforward mechanism to
51 further facilitate recruitment of diverse immune cell types expressing the corresponding receptors.
52 Indeed, a recent study of the overall cellular makeup of synovial tissue from RA patients using
53 single cell RNA sequencing (scRNA-seq) analysis identified a diverse mix of migratory and
54 resident cell types of hematopoietic and non-hematopoietic origin including different CD4 and
55 CD8 T cell subsets, myeloid cells, and FLS². These observations suggest that states of FLS
56 activation and differentiation are likely modulated by diverse innate and adaptive immune cell
57 types found in inflamed joints of RA patients and that this modulation factors prominently in the
58 disease pathogenesis.

59

60 Thus, we sought to undertake an in-depth investigation of the spectrum of FLS states induced in
61 the inflamed RA synovium and potential drivers underlying the observed FLS heterogeneity
62 through paired scRNA and assay for transposase-accessible chromatin with sequencing
63 (scRNA/ATAC-seq) and *in vitro* modeling of FLS transcriptional responses to key immune cell-
64 derived proinflammatory cytokines. We then mapped the spatial distribution of FLS heterogeneity
65 and transcriptional responses by employing spatial transcriptomic (ST) analyses as well as
66 multiplex imaging. Our studies suggest that spatially constrained FLS responses to three major
67 leukocyte-derived cytokines, TNF α , IFN γ , and IL-1 β , or lack thereof, drive the formation of six
68 distinct FLS states associated with synovial inflammation in RA.

69

70 **Results**

71 **Inflammation is associated with heightened FLS heterogeneity**

72 To test the possibility that severe joint inflammation in RA patients leads to a marked expansion
73 of FLS heterogeneity, we sought to characterize their transcriptomes and chromatin accessibility
74 at a single cell resolution. Fluorescence-activated cell sorting (FACS)-sorted FLS (CD45⁺CD31⁻
75 PDPN⁺) were isolated from two RA patients with highly inflamed synovium, who had similar
76 disease characteristics as well as histologic findings, and subjected to paired scRNA/ATAC-seq
77 using the 10x Multiome platform (patients 1 and 2 in Table S1, Extended Data Fig. 1a). After
78 extensive filtering, we obtained 15,736 FLS that clustered into 14 clusters based on the scRNA-
79 seq datasets (Fig. 1a, Extended Data Fig. 1b-e, Table S2). Similar results were obtained with and
80 without Mutual Nearest Neighbors (MNN) batch correction (Extended Data Fig. 1f,g). Using
81 established characteristic markers², some of which are shown in Fig. 1b, we identified lining,
82 sublining and pan-synovial clusters. The latter clusters express genes characteristic of both
83 sublining and lining FLS. Consistent with the high degree of synovial inflammation, MHC class II
84 expression was widespread as HLA-DR expressing cells were found in all clusters except for
85 cluster 13 (Fig. 1c). Our observation of a high fraction of lining FLS expressing HLA-DR was at
86 odds with previous reports that HLA-DR⁺ FLS represent a subset within the sublining FLS
87 population². Therefore, we independently assessed HLA-DR expression using multicolor
88 immunofluorescence (IF) and confirmed the pan-synovial expression of HLA-DR on FLS (Fig. 1d).

89 In addition to capturing all FLS subsets previously identified in the RA synovium^{2,4} (Fig. 1e),
90 our focused analysis of FLS in highly inflamed tissue enabled identification of novel subsets of
91 both sublining (clusters 0, 10, 11) as well as lining (clusters 4, 6, 9) FLS (Fig. 1e). The large
92 number of lining FLS spanning multiple clusters included those sharing transcriptional signatures
93 characteristic of FLS derived from patients with both active and remission RA⁴. These results
94 suggest that a highly inflamed RA joint harbors a greatly expanded range of FLS states possibly
95 representing a broad spectrum of the disease.

96 While each cluster had specific features, such as the expression of *NOTCH3* in cluster 8 that
97 marks perivascular FLS⁸, gene set enrichment analysis (GSEA) of each cluster highlighted shared
98 functionality between groups of clusters (Fig. 1f and Table S3). In fact, the assessment of cluster-
99 by-cluster correlation, allowed us to define six FLS states each with distinct inferred functionality
100 (Fig. 1g). The identified resting lining FLS state shared a transcriptional profile with lining FLS
101 from RA patients in remission⁴. The cytokine-activated lining FLS state, which expressed elevated
102 levels of HLA-DR, displayed an IFN γ response gene signature and additional inflammatory
103 response gene signatures. Pan-synovial HLA-DR^{high} FLS were characterized by the highest
104 expression of HLA-DR, and also displayed an IFN γ response signature as well as

105 lysosome/endosome-related genes (*CD63*, *CTSD*, *NPC2*, *LAPTM4A*, *CTSK*, *CD68*, *CTSL*,
106 *CTSA*, *HEXA*, *CTSF*, *GUSB*, *LAMP1*) suggesting their potential role as antigen-presenting cells
107 in the inflamed synovium. A transcriptional profile of these pan-synovial HLA-DR^{high} FLS closely
108 resembled the one previously reported for lining FLS from patients with active RA⁴. The sublining
109 FLS were enriched for cytokine signaling pathway genes, most notably TNF α , but also IFN γ and
110 IL-6. Of note, both the sublining and cytokine-activated sublining FLS states show evidence of
111 STAT5 signaling possibly driven by IL-15. Finally, we observed that the transcriptomes of a subset
112 of sublining FLS exhibited features expected for progenitor cells including the highest level of
113 CD34 expression, and enrichment for expressed genes related to extracellular matrix (ECM)
114 homeostasis and epithelial mesenchymal transition (EMT) (*MFAP5*, *FBN1*, *VCAN*, *TGFBR3*,
115 *FBLN2*, *PRRX1*, *DCN*, *LAMA2*, *SFRP4*, *EDIL3*, *FBLN1*, *LGALS1*, *LOXL1*, *ADAM12*, *LOX*, *CD44*,
116 *IGFBP4*, *LRP1*). Notably, the most differentially expressed gene for the progenitor state was PI16,
117 which was recently described as a marker for one of two populations of universal mouse (“cross-
118 tissue”) fibroblasts that can give rise to specialized fibroblast populations during development and
119 upon perturbation⁹. Furthermore, the gene expression features of the progenitor FLS state we
120 characterized showed extensive similarity to the PI16⁺ cluster and enrichment for the “universal
121 fibroblast gene signature” identified in a human perturbed-state fibroblast atlas (Extended Data
122 Fig. 1h). Since tissue progenitor or “stem-like” cells can be frequently found as aggregates within
123 distinct specialized anatomical niches commonly associated with vasculature, we explored spatial
124 distribution of these CD34^{high}THY1⁺PDPN⁺ FLS using IF. However, we found them widely
125 dispersed as solitary cells throughout the inflamed synovium without conspicuous association
126 with the vascular endothelium (Extended Data Fig. 1i). This finding suggests a possibility that in
127 highly inflamed RA synovium FLS regenerative capacity is preserved in a non-compartmentalized
128 manner.

129

130 **Comparison with non-synovial fibroblasts shows shared functionality across tissues**

131 Previous cell population-based studies suggested distinct diversity of transcriptional features of
132 human fibroblasts in different anatomical locations and heritable imprinting of their “topography”¹⁰.
133 However, our observation of conserved “universal fibroblast” features of progenitor PI16⁺ FLS
134 suggested that there might be an overlap between disease-induced states of anatomically distinct
135 tissue fibroblasts affected by different pathologies. Thus, we next sought to explore whether other
136 FLS states we identified were tissue or disease-specific, namely, unique to the synovium or RA-
137 associated inflammation, or alternatively, were shared with fibroblast populations observed in
138 other diseases and tissues (Fig. 2, Extended Data Fig. 2). For this comparative analysis, we took

139 advantage of several recent scRNA-seq datasets of colonic^{11,12} and dermal fibroblasts^{13,14}, in
140 which at least 5 fibroblast clusters can be delineated.

141 As expected from the aforementioned presence of the “universal fibroblast signature”, the
142 progenitor FLS state shared transcriptional similarity with fibroblasts from both the colon and skin.
143 In the colon, this transcriptional signature was observed in two fibroblast populations implicated
144 in creating an intestinal stem cell niche: ECM producing fibroblasts and myofibroblasts. In the
145 skin, this transcriptional signature was observed in fibroblasts from unwounded skin responsible
146 for ECM homeostasis and in a fibroblast population that undergoes contraction in scleroderma as
147 compared to healthy skin.

148 Unexpectedly, we observed a remarkable transcriptional similarity between inflammatory
149 cancer associated fibroblasts in colorectal cancer and the HLA-DR⁺ cytokine-activated lining FLS.
150 The latter FLS state was also similar to transcriptional states of fibroblasts in diabetic foot ulcers
151 that were able to successfully heal.

152 On the other hand, transcriptional features of fibroblasts in the diseased skin or colon bore
153 only limited similarity to both the resting lining and cytokine-activated sublining FLS states we
154 observed. The resting lining FLS were characterized by high expression of genes involved in the
155 production of synovial fluid and ECM (*XYLT1*, *FN1*, *ITGB8*, *PRG4*) and axonal guidance
156 (*SEMA5A*, *ANK3*, *NTN4*, *SLIT2*), features which likely reflect their specialized functions within the
157 synovium. The cytokine-activated sublining FLS state also appeared distinct to the RA synovium,
158 however, it likely emerged due to the high degree of inflammation in the RA synovial samples we
159 analyzed. Accordingly, corresponding fibroblast states in other tissues are expected to be found
160 only under similarly highly inflamed conditions and may not have been enriched in the scRNA-
161 seq datasets used for the cross-comparison. Thus, inflammation-induced perturbations in the
162 overall composition of the FLS population and spectrum of FLS states were shared with
163 fibroblasts found in other tissues in a range of pathologies.

164

165 **FLS states exhibit distinct transcriptional regulation with evidence of differential cytokine** 166 **stimulation**

167 To gain insight into transcription factors and upstream signaling pathways which promoted FLS
168 heterogeneity in RA, we analyzed paired scATAC-seq datasets. Unsupervised clustering resulted
169 in seven clusters with lining and sublining FLS subtypes as well as an independent cluster of
170 progenitor-like FLS (Fig. 3a,b, Extended Data Fig. 3a-c). Distinct FLS states identified by scRNA-
171 seq analyses occupied divergent areas of the scATAC-seq UMAP (Fig. 3c) partially overlapping
172 with the identified scATAC-seq clusters (Extended Data Fig. 3d). To infer differential transcription

173 factor activity in identified FLS states, we performed chromatin accessibility variation analysis
174 using chromVAR. For this analysis, we used the paired scRNA-seq data as a filter to assess only
175 motifs for the transcription factor families whose members were expressed by >20% of cells in
176 the corresponding state. We observed marked differences in enrichment of distinct transcription
177 factor binding motifs within open chromatin sites with differential motif accessibility between states
178 (Fig. 3d,e). The cytokine-activated lining and HLA-DR^{high} pan-synovial FLS states were enriched
179 for activity of AP-1 transcription factor family members (JUN, JUNB, JUND, FOS, FOSL2), whose
180 increased contribution to gene regulation downstream of fibroblast growth factor (FGF) and
181 immune receptor signaling, such as IL-1, has been suggested to play a role in tissue-destructive
182 properties of FLS in RA^{15,16}. Interestingly, open chromatin sites characteristic of the cytokine-
183 activated sublining FLS state were enriched for STAT and IRF family motifs implicating a distinct
184 set of inflammatory pathways such as IFN signaling in establishing this state. Contrary to the two
185 major flavors of activated, inflammation-associated FLS, the resting lining FLS state was
186 distinguished by the accessibility of homeobox transcription factor family member motifs, which
187 besides serving as major regulators of development and organization, control fibroblast
188 quiescence (e.g., PRRX1)^{17,18}. In support of a role for homeobox transcription factors in
189 modulating FLS activation, the resting lining FLS state exhibited increased expression of
190 homeobox family member *CUX1*, which has been shown to bind to NF-κB and alter its activity
191 through either downregulation¹⁹ or upregulation²⁰ of specific NF-κB-regulated cytokines and
192 chemokines. Finally, the PI16⁺ progenitor FLS were distinguished by accessible *cis*-regulatory
193 elements enriched for KLF, SOX and TEAD family motifs, which play a role in maintaining
194 quiescent undifferentiated states in stem cells and early progenitors. In this regard, KLF4 has
195 been implicated in the induction of a pluripotent state in fibroblasts consistent with the likely role
196 of this FLS subset as progenitors²¹. These results suggest that distinct states of FLS in the
197 inflamed RA joint were dependent upon their local stimulation by immune cell derived factors,
198 foremost proinflammatory cytokines, or avoidance of these inflammatory exposures.

199

200 **Cytokine signaling drives transcriptional heterogeneity**

201 To test the above possibility and to elucidate influences of inflammatory factors on transcriptomes
202 of FLS states we sought to deconvolute their complex transcriptomes by establishing cell-type
203 specific cytokine-induced programs. For identification of immune cell derived cytokine
204 transcriptional responses and their contributions to distinct features of FLS state-specific
205 transcriptomes, we employed *in vitro* stimulation of cultured FLS by candidate pro-inflammatory
206 cytokines and other factors. For these experiments, FLS were isolated from four RA synovial

207 tissue samples and cultured for three passages prior to pooling FLS from all donors and
208 performing the stimulations in triplicate. FLS were stimulated with three major cytokines implicated
209 in RA – $\text{TNF}\alpha$, $\text{IFN}\gamma$ and $\text{IL-1}\beta$, either individually or in combination – and the resulting gene
210 expression changes were assessed using RNA-seq (Fig. 4a, Table S4). Additionally, to directly
211 compare cytokine stimulation effects on a per patient basis, we isolated FLS from the same RA
212 synovial tissue samples subjected to scATAC/RNA-seq (Figs. 1 and 3) stimulated them with
213 $\text{TNF}\alpha$, $\text{IFN}\gamma$ and $\text{IL-1}\beta$ or $\text{TNF}\alpha$ and $\text{IFN}\gamma$ and performed scATAC/RNA-seq. We found that *in vitro*
214 stimulation of FLS with both $\text{TNF}\alpha$ and $\text{IFN}\gamma$ induced expression of genes including *CCL2* and
215 *IL6*, which were highly expressed by *ex vivo* isolated cytokine-activated sublining FLS. On the
216 other hand, genes whose expression was markedly suppressed in response to these cytokines
217 (e.g., *VCAN*, *CCDC80*, *CD248* in Fig. 4a) were highly upregulated by the $\text{CD34}^{\text{high}}\text{THY1}^+\text{PI16}^+$
218 FLS suggesting that the progenitor FLS state is shielded from exposure to inflammatory mediators
219 and that these cytokines may lead to the loss of this state. In the same vein, *ANK3*, which is
220 upregulated in the resting lining FLS state, was also downregulated in response to *in vitro*
221 stimulation by $\text{TNF}\alpha$ and $\text{IFN}\gamma$ suggesting that besides the progenitor FLS in the sublining, resting
222 lining FLS also appear to be spared from the full effects of inflammatory cytokines. Finally, genes
223 induced in FLS subjected to *in vitro* stimulation by the combination of $\text{TNF}\alpha$, $\text{IFN}\gamma$ and $\text{IL-1}\beta$, which
224 included *MMP3* and *CXCL1*, were most differentially expressed in *ex vivo* isolated cytokine-
225 activated lining FLS.

226 Notch signaling induced by ligands expressed by the vascular endothelium have been
227 suggested to factor prominently in the differentiation of perivascular and sublining FLS in the RA
228 synovium⁸. This raised the question as to whether Notch signaling can potentially modulate
229 transcriptional responses of FLS to pro-inflammatory cytokines within the sublining. To explore
230 this possibility, we investigated changes in gene expression induced in FLS upon stimulation with
231 $\text{TNF}\alpha$, $\text{IFN}\gamma$ and $\text{IL-1}\beta$ in the presence or absence of plate-bound Notch ligand Delta like-4 (DLL4).
232 This analysis revealed a global dampening of transcriptional responses to all three cytokines (Fig.
233 4b, Table S5): for both up- and down-regulated genes, the observed changes were blunted across
234 the board. DLL4 similarly attenuated transcriptional responses to dual and triple combinations of
235 cytokines ($\text{TNF}\alpha + \text{IFN}\gamma$ and $\text{TNF}\alpha + \text{IFN}\gamma + \text{IL-1}\beta$, respectively) (data not shown). This finding
236 was unexpected considering that previous studies suggested that Notch signaling augments
237 macrophage responses to TLR ligands and increases production of pro-inflammatory cytokines²².
238 However, there may be multiple specific regulatory mechanisms in play as previous reports also
239 show that $\text{IFN}\gamma$ can inhibit Notch signaling in macrophages²³. These results raise an intriguing

240 question as to whether coincident engagement of inflammatory and developmental signaling
241 pathways may result in different functional outcomes depending on a given cell type.

242 Mapping of cytokine response gene signatures established by the above *in vitro* analyses
243 onto our scRNA-seq datasets revealed the most pronounced expression of these gene signatures
244 in the cytokine activated lining FLS state (Fig. 4c). In contrast, the dominant cytokine response
245 signatures in the cytokine-activated sublining FLS state included either genes modulated by a
246 dual combination of TNF α and IFN γ or by IFN γ alone. Notably, the progenitor state was devoid of
247 cytokine response gene signatures.

248 Previous scRNA-seq analysis suggests that CD8⁺ T cells represent the major source of IFN γ
249 in the RA synovium². In agreement with previous reports, our imaging showed predominant
250 localization of CD8⁺ T cells within lymphoid aggregates in the sublining region. Therefore, we
251 sought to investigate whether local FLS interferon responses correlated with CD8⁺ T cell
252 localization *in situ* by analyzing phosphorylated STAT1 (pSTAT1), the key downstream target of
253 interferon signaling, using IF (Fig. 4d). Interestingly, we observed nuclear pSTAT1 in PDPN⁺ FLS
254 within both lymphocyte aggregates in the sublining region as well as the T cell poor lining region.
255 Of note, some of the PDPN⁺pSTAT1⁺ FLS also express HLA-DR consistent with a well-recognized
256 role of IFN γ in driving MHC class II expression, while pSTAT1 observed in T cell poor regions
257 may reflect local type I IFN signaling.

258 To further validate the effect of cytokine stimulation on regulation of gene expression, we
259 performed scATAC-seq of cultured FLS stimulated with combinations of cytokines to cross-
260 reference the observed transcription factor motif activity at modulated chromatin accessibility sites
261 to that of distinct FLS states revealed by scATAC-seq analyses of *ex vivo* isolated cells (Fig. 4e).
262 We found that stimulation of FLS with a combination of TNF α and IFN γ resulted in enrichment of
263 IRF and STAT family transcription factor motifs at differentially accessible chromatin sites in
264 comparison to unstimulated FLS, closely matching those in the cytokine-activated sublining state
265 of FLS *ex vivo*. In contrast, triple combination of TNF α , IFN γ and IL-1 β resulted in differential
266 chromatin remodeling at sites enriched for AP-1 transcription factor family motifs. The latter
267 observation was consistent with a previous report of remodeling of chromatin regions containing
268 NF-kB and AP-1 binding motifs in response to IL-1 β ²⁴. Impressively, the differentially accessible
269 sites enriched for AP-1 family motifs were nearly identical to those observed in the *ex vivo* isolated
270 cytokine-activated lining FLS state. The comparison of cytokine-stimulated samples to the
271 unstimulated control explains the seemingly unexpected decrease in accessibility of *cis*-
272 regulatory elements containing STAT motifs in the cytokine-activated lining FLS (Fig. 3d) despite
273 the presence of a robust IFN γ response gene expression signature (Fig. 4c) and STAT1

274 phosphorylation (Fig. 3d) (Extended Data Fig. 4). Thus, this was most likely due to a relative
275 decrease in STAT accessibility of a subset of these elements caused by combined IL-1 β , IFN γ
276 and TNF α exposure while the corresponding transcript levels were not markedly impacted.
277 Together, these analyses of transcriptomes and epigenomes strongly support a role of
278 combinatorial stimulation by TNF α and IFN γ in facilitating the establishment of the cytokine-
279 activated sublining FLS state whereas the cytokine-activated lining FLS state was likely driven by
280 a triple combination of TNF α , IFN γ and IL-1 β .

281

282 **Cytokine signaling is spatially constrained and correlated with cellular localization**

283 The observations above suggest that distinct states of FLS in the inflamed synovium are
284 established in a spatial manner as the result of locally produced inflammatory cytokines and other
285 mediators by distinct types of immune cells invading the RA synovium. To define the spatial
286 distribution of identified FLS states within the RA synovium, we performed spatial transcriptomic
287 (ST) analyses using the 10x Visium platform combined with multiplex IF imaging of adjacent tissue
288 sections for two additional inflamed synovial tissue samples isolated from RA patients (patients 3
289 and 4 in Table S1). The hematoxylin and eosin (H&E) staining of the sections subjected to ST
290 analysis showed prominent lymphocyte aggregates as well as copious synovial lining (Fig. 5a).
291 IF imaging of the sections adjacent to those used for ST showed scattered PDPN⁺ FLS and CD68⁺
292 macrophages as well as lymphocyte aggregates in the sublining region, and multiple regions of
293 lining populated by FLS and macrophages (Fig. 5b). The ST datasets were integrated with our
294 scRNA-seq analyses to map the transcriptional signatures from the six FLS states on the ST
295 datasets. This showed that the resting and cytokine-activated lining FLS appeared to intermix
296 without forming well-defined regions. In contrast, the three identified sublining FLS subsets were
297 differentially localized (Fig. 5c). We next applied the *in vitro* FLS cytokine response gene
298 signatures to spatial gene expression maps derived from ST analysis. We found that the IL-1 β
299 response signature mapped predominantly to the synovial lining, while the other cytokine
300 response signatures were more scattered (Fig. 5d). Since multiple cells contribute to each RNA
301 capture spot on a Visium slide, we confirmed that the IL-1 β response gene signature observed in
302 ST analysis was contributed by FLS rather than other cell types by creating a modified IL-1 β
303 response gene signature that only contained genes uniquely expressed by FLS based on recent
304 scRNA-seq analysis of RA synovium². Integrated ST and IF imaging analysis of adjacent sections
305 showed that the cytokine-activated lining FLS state featuring the IL-1 β response gene signature
306 was near areas densely populated by CD68⁺ macrophages. Mapping cell type specific

307 transcriptional signatures from a recent scRNA-seq analysis of the RA synovium² onto the spatial
308 gene expression datasets confirmed the colocalization of monocyte/macrophages with the IL-1 β
309 response gene signature (Fig. 5e). Similar analyses of an independent RA sample confirmed
310 these results (Extended Data Fig. 5a-e). This association was further validated by an unbiased
311 correlation of the FLS states, cytokine response gene signatures, and cell type specific gene
312 signatures within individual spots that showed a correlation between monocytes/macrophages,
313 lining FLS and the IL-1 β response gene signature (Extended Data Fig. 5f). These results suggest
314 that cytokine signaling shapes multiple spatially distinct microenvironments within the inflamed
315 RA synovium with IL-1 β from either resident macrophages or infiltrating monocytes defining the
316 synovial lining FLS.

317

318 **Discussion**

319 Phenotypic and functional heterogeneity of parenchymal cells within a given tissue depends on
320 both constant cell-intrinsic differentiation programs and cell extrinsic cues afforded by stable and
321 transient interactions with tissue resident and infiltrating cells. The latter are represented for the
322 most part by diverse types of immune cells, which, when activated, produce cytokines and other
323 mediators that can act on the parenchymal cells changing the range of their physiological or
324 homeostatic states. In RA, the synovium, which is in health a non-barrier immunologically
325 quiescent tissue, experiences a massive influx of both innate and adaptive immune cells. In this
326 setting, FLS experience differentiation signals, such as the endothelium-derived Notch signaling
327 gradient that shapes FLS in the synovial sublining region⁸, and coincident exposures to multiple
328 cytokine and other inflammatory mediators. Using paired scRNA/ATAC-seq and ST analyses
329 assisted by *in vitro* generated cytokine response signatures, we demonstrated that leukocyte-
330 derived cytokines play a key role in the formation of discrete, spatially defined, but likely dynamic
331 FLS states with distinct inferred functionality. We also observed, contrary to a reported
332 potentiation of inflammatory cytokine responses in macrophages²², Notch activation in FLS
333 attenuated cytokine responses. This finding may explain the relatively dampened cytokine
334 response gene signatures observed in the sublining FLS state, which was distinguished by the
335 highest *NOTCH3* expression, as compared to the cytokine-activated sublining FLS state.

336 Our analysis of rich paired datasets of single cell transcriptomes and epigenomes allowed us
337 to detect previously unappreciated heterogeneity of FLS in highly inflamed RA synovium,
338 including an inflamed sublining state driven by IFN γ and TNF α and two lining states defined by
339 differential cytokine responses. The observation that the cytokine-activated lining FLS
340 transcriptome was enriched for genes downstream of IFN γ , TNF α and IL-1 β was consistent with

341 the expression of the latter two cytokines by synovial macrophages², and the coincident
342 positioning of macrophages with the local IL-1 β signature in the synovial lining in our ST analysis.
343 Potential sources of IFN γ and STAT1 activation within the synovial lining remained less clear.
344 While CD8⁺ T cells have been described as the dominant producers of IFN γ within the synovium,
345 it is possible that other cells such as NK cells³ or myeloid cells²⁵ may contribute. Finally, it is
346 probable that the prominent pSTAT1 signal observed in the synovial lining FLS (Fig. 4c), which
347 has also been reported previously²⁶, is the result of the action of alternative drivers of STAT1
348 activation including type I IFN²⁶, whose expression can be driven by IL-1 β ²⁷.

349 The prominent IL-1 β response signature in the cytokine-activated lining FLS state is notable
350 given its possible functional and therapeutic implications. First, IL-1 β is the primary inducer of
351 MMPs, which have been implicated in FLS invasiveness²⁸. This invites the possibility of a
352 functional dichotomy between sublining and lining FLS in RA parallel to that observed in an
353 experimental arthritis model in mice, where the lining FLS are uniquely responsible for destruction
354 of cartilage and bone²⁹. Blocking IL-1 may antagonize the capacity of FLS to assume this MMP
355 expressing lining state and, thus it is possible that for a subset of patients the addition of IL-1
356 inhibition during flares could prove effective for preventing joint destruction. Second, the cytokine-
357 activated lining FLS state may drive migration of neutrophils into the synovial fluid, where there is
358 a surfeit of neutrophils during RA flares. In this regard, the inflammatory cancer-associated
359 fibroblasts in colorectal adenocarcinomas, which we found to share extensive transcriptional
360 similarity with the cytokine-activated lining FLS state, express neutrophil chemoattractants
361 including *CXCL1* and *CXCL8* and their location was spatially correlated with the accumulation of
362 neutrophils¹¹. In RA, IL-1 β produced by macrophages in the lining region likely drives the high
363 expression of *CXCL1* observed in the cytokine-activated lining FLS. Finally, the prominence of
364 the cytokine-activated lining FLS state observed in highly inflamed RA synovium may have
365 prognostic implications. A recent study of pathotypes in inflammatory bowel disease showed an
366 association of an IL-1 β -activated fibroblast signature with a lack of response to multiple
367 therapies³⁰.

368 Besides the cytokine-driven FLS states, we defined a CD34^{high}THY1⁺PDPN⁺ progenitor-like
369 FLS population that was devoid of cytokine response signatures and shared extensive
370 transcriptional similarity with fibroblast populations found in both the colon and skin. The
371 abundance of this progenitor-like population appears to vary between synovial tissues of
372 individual patients and may depend on disease activity or treatment. Future studies will help
373 determine if the paucity of cytokine response gene signatures in these FLS owes to their

374 sequestration from cytokine-producing immune cells, or cell-intrinsic attenuation of cytokine
375 signaling.

376 In conclusion, we established a spatial atlas of heterogeneity of synovial fibroblast states in
377 RA defined by their distinct transcriptional signatures and patterns of chromatin accessibility
378 driven by differential local exposure to immune cell-derived pro-inflammatory cytokines. The
379 resulting datasets will serve as a rich resource for future investigation of RA pathogenesis through
380 integration of unique and shared characteristics of inflamed synovial fibroblasts described herein
381 with other disease-associated signals, such as complement activation³¹, and potential antigen
382 presentation via HLA-DR.

383

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397

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403

404 **Author contributions:**

405 MHS conceived the project, collected clinical data, designed experiments, performed
406 experiments, prepared figures, wrote the manuscript.

407 VRG analyzed sequencing data, prepared figures and edited the manuscript.

408 MS analyzed sequencing data.

409 AK performed experiments.

410 EFD scored histology slides.

411 SMG collected clinical data.

412 TMN designed experiments, supervised research experiments, analyzed sequencing data and
413 prepared figures.

414 CSL oversaw the computational analysis and edited the manuscript.

415 LTD conceived the project, acquired funding, designed experiments, supervised research
416 experiments, oversaw data analysis and edited the manuscript.

417 AYR conceived the project, acquired funding, designed experiments, supervised research
418 experiments, oversaw data analysis and wrote the manuscript.

419

420 **Competing interests:** AYR is an SAB member, has equity in Sonoma Biotherapeutics and
421 Vedanta Biosciences, and is a co-inventor or has IP licensed to Takeda that is unrelated to the
422 content of the present study. The remaining authors declare no competing interests.

423

424 **Code availability:** The customized code used in the present study is publicly available at:
425 https://github.com/viannegao/RA_Fibroblast_Analysis

426

427 **Data availability:** All sequencing data generated in this paper were deposited in the Gene
428 Expression Omnibus (GEO) under accession numbers: (accession number to be generated)

429

430 All materials and data transferred between HSS and MSKCC were covered under material
431 transfer agreements.

432

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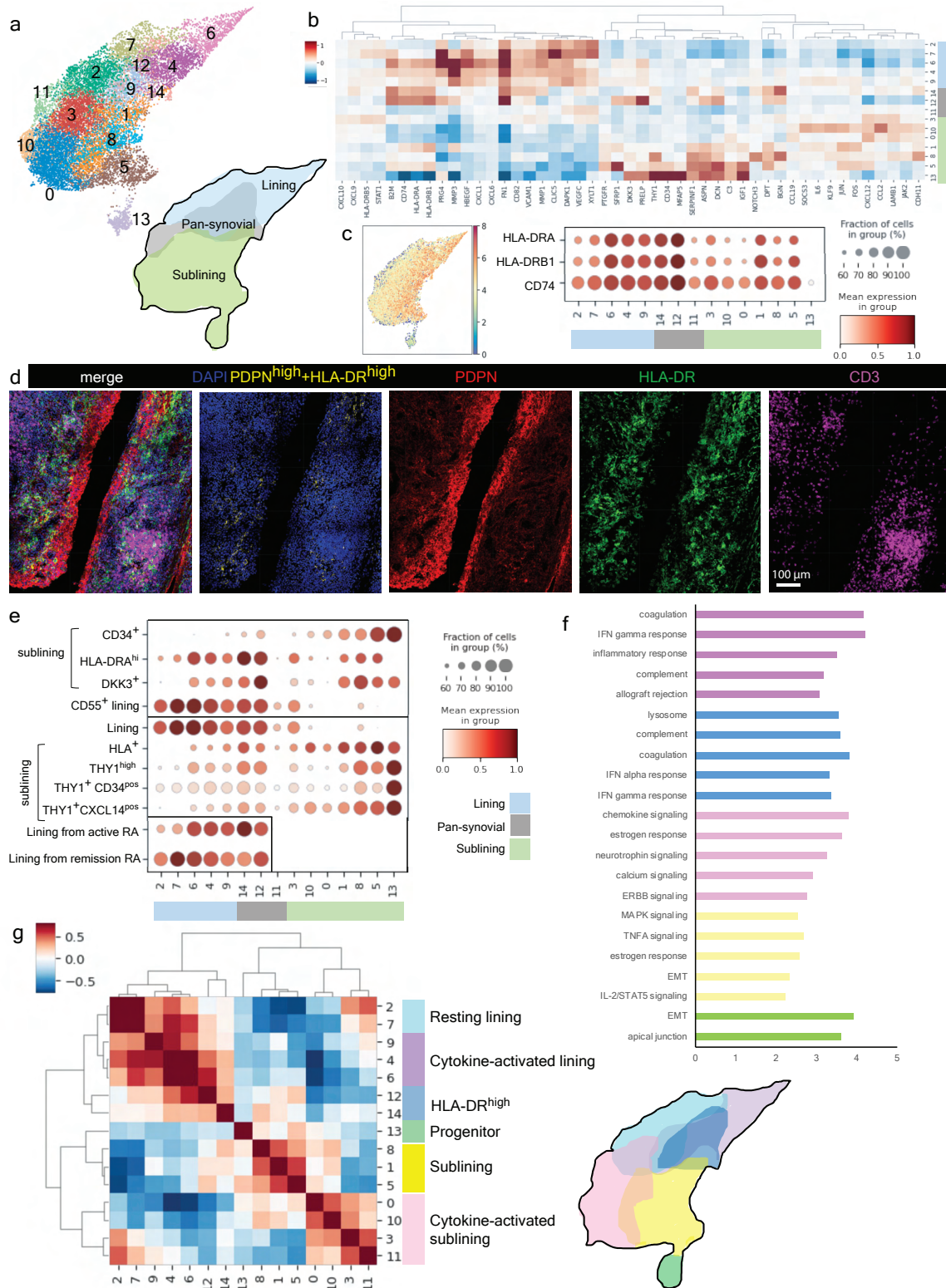
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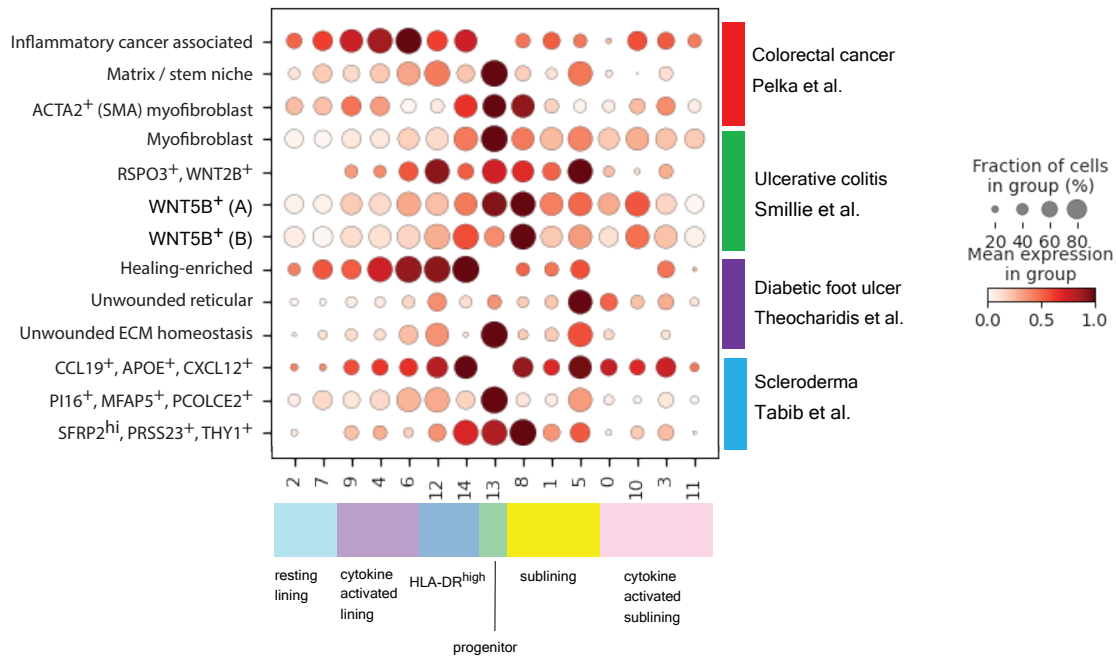
516

517 **Figures:**



518

519 **Figure 1. Heterogeneity of HLA-DR⁺ FLS in the inflamed RA synovium.** **a**, UMAP of 14 FLS
520 clusters identified by scRNA-seq analysis with annotations of synovial localization. **b**, Heatmap of
521 selected DEGs for each cluster colored by synovial localization. **c**, UMAP colored by logged,
522 library size-normalized expression of HLA-DRA and dot plot showing relative expression of HLA-
523 DRA, HLA-DRB1 and CD74. **d**, Representative confocal microscopy of PDPN (red), HLA-DR
524 (green), CD3 (magenta) and nuclear marker (blue) from RA synovial tissue (N = 5 tissues). Pixels
525 with the highest intensity (top 3%) for both PDPN and HLA-DR are colored in yellow. **e**, Dotplot
526 showing the relative per cluster expression of previously published cluster-derived gene
527 signatures: Zhang et al² above and Alivernini et al⁴ below horizontal line. Box (bottom left) shows
528 lining FLS signatures from patients in remission or with active disease from Alivernini et al. **f**,
529 GSEA showing top 5 pathways from KEGG with FDR <0.1 for each of the states defined in G.
530 The resting lining state did not have any statistically enriched pathways. **g**, Cluster by cluster
531 correlation of the mean expression of highly variable genes in clusters from Fig. 1a with defined
532 FLS states colored on UMAP.
533



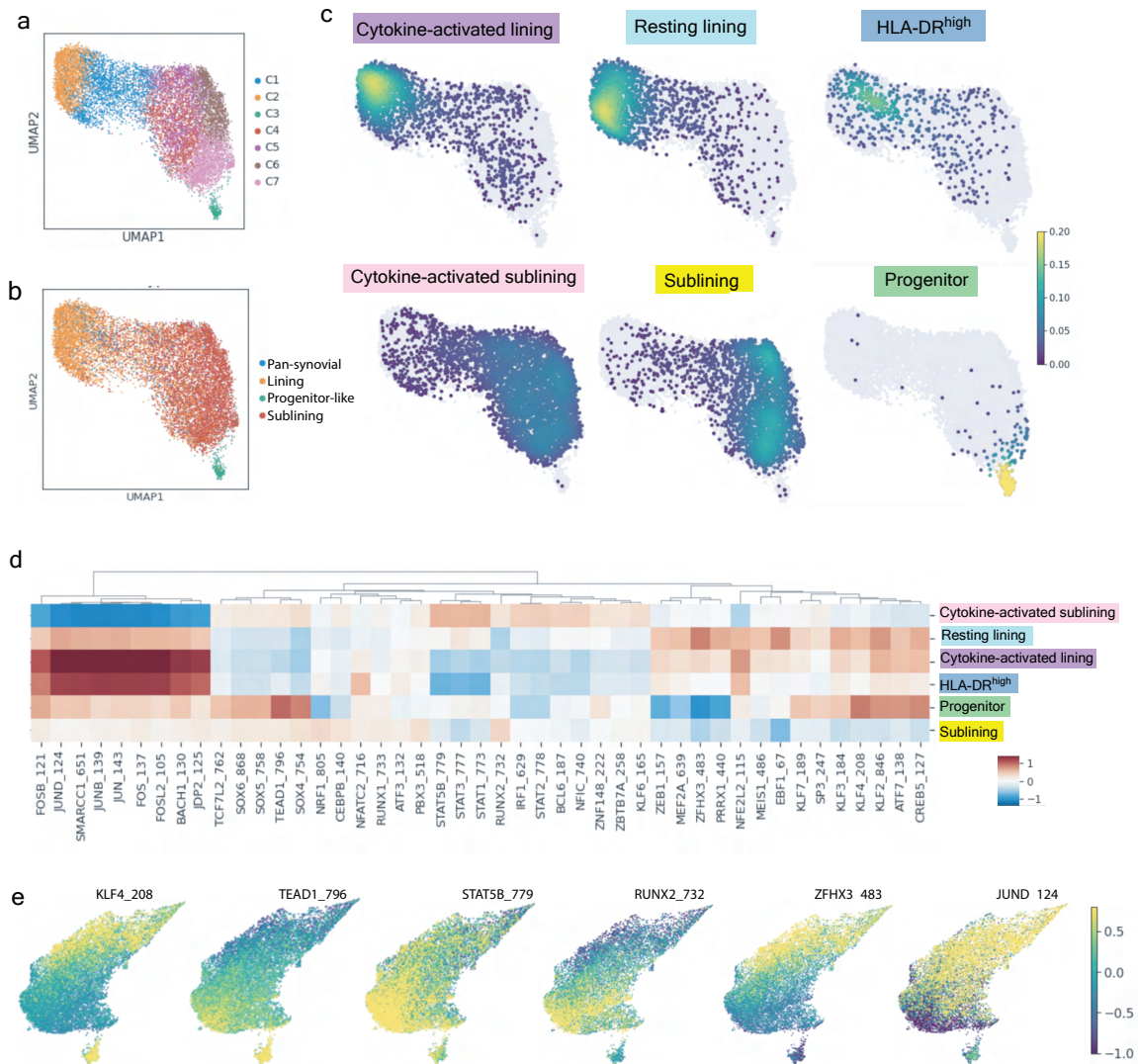
534

535 **Figure 2. Shared functional gene expression programs in FLS and non-synovial fibroblasts**

536 **across tissues and diseases.** Dot plot showing relative expression of selected gene signatures

537 from published tissue fibroblast populations¹¹⁻¹⁴ in FLS clusters shown in Fig. 1a colored

538 according to FLS states defined in Fig. 1g.



539

540 **Figure 3. Chromatin accessibility analysis of FLS states reveals their distinct**

541 **transcriptional regulation. a**, UMAP of 7 FLS clusters identified by tile-based scATAC-seq

542 analysis. **b**, Annotations of synovial localization as well as the progenitor state on the scATAC-

543 seq UMAP. **c**, Projection of six FLS states onto scATAC-seq UMAP. **d**, Heat map with top 10

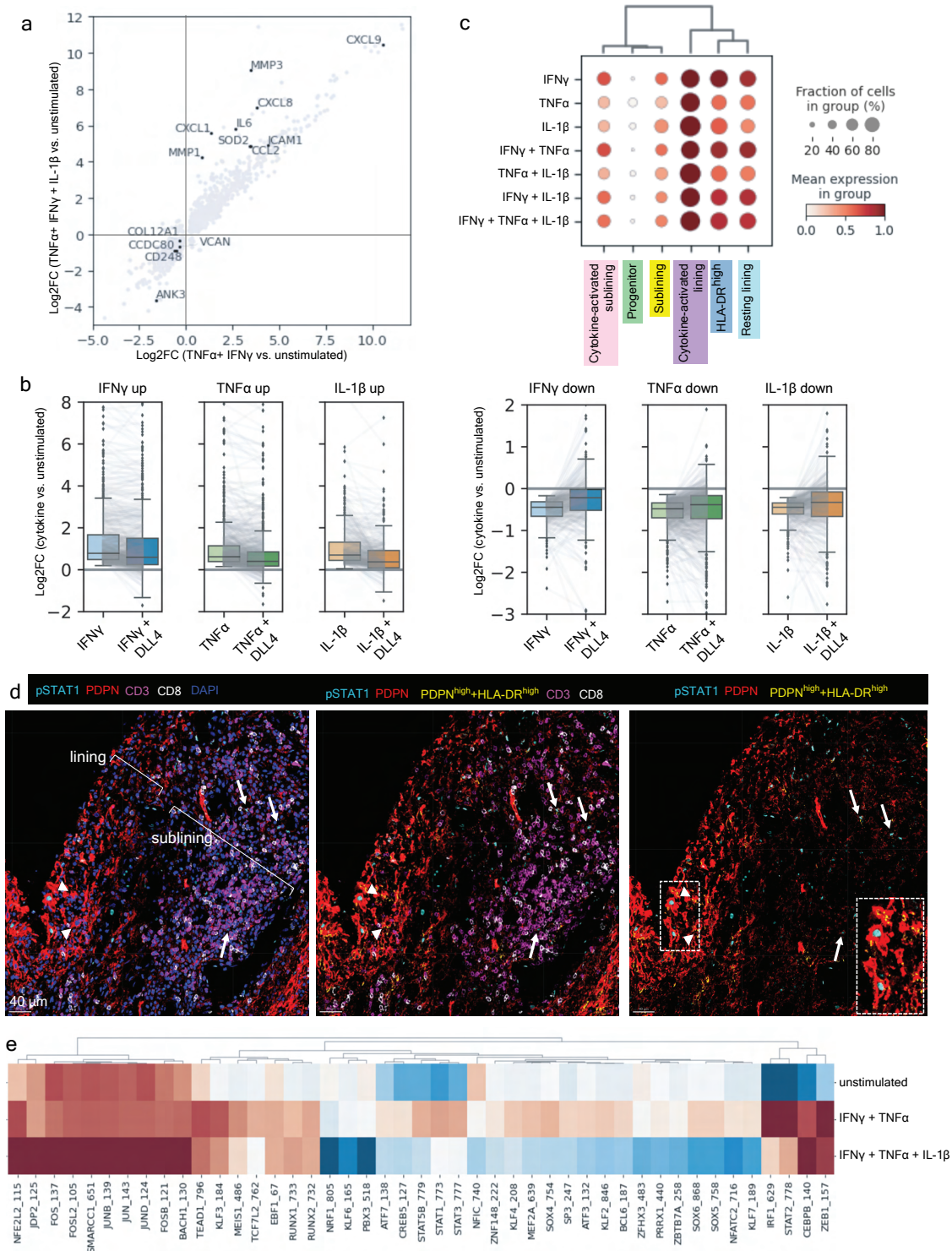
544 differentially accessible transcription factor motifs identified by ChromVAR for each FLS state

545 defined in Fig. 1g. Motifs filtered to include only those for which the corresponding transcription

546 factor was expressed by >20% of cells in the corresponding state. **e**, ChromVAR z-score projected

547 onto scRNA-seq UMAP for a selection of top differentially accessible transcription factor motifs

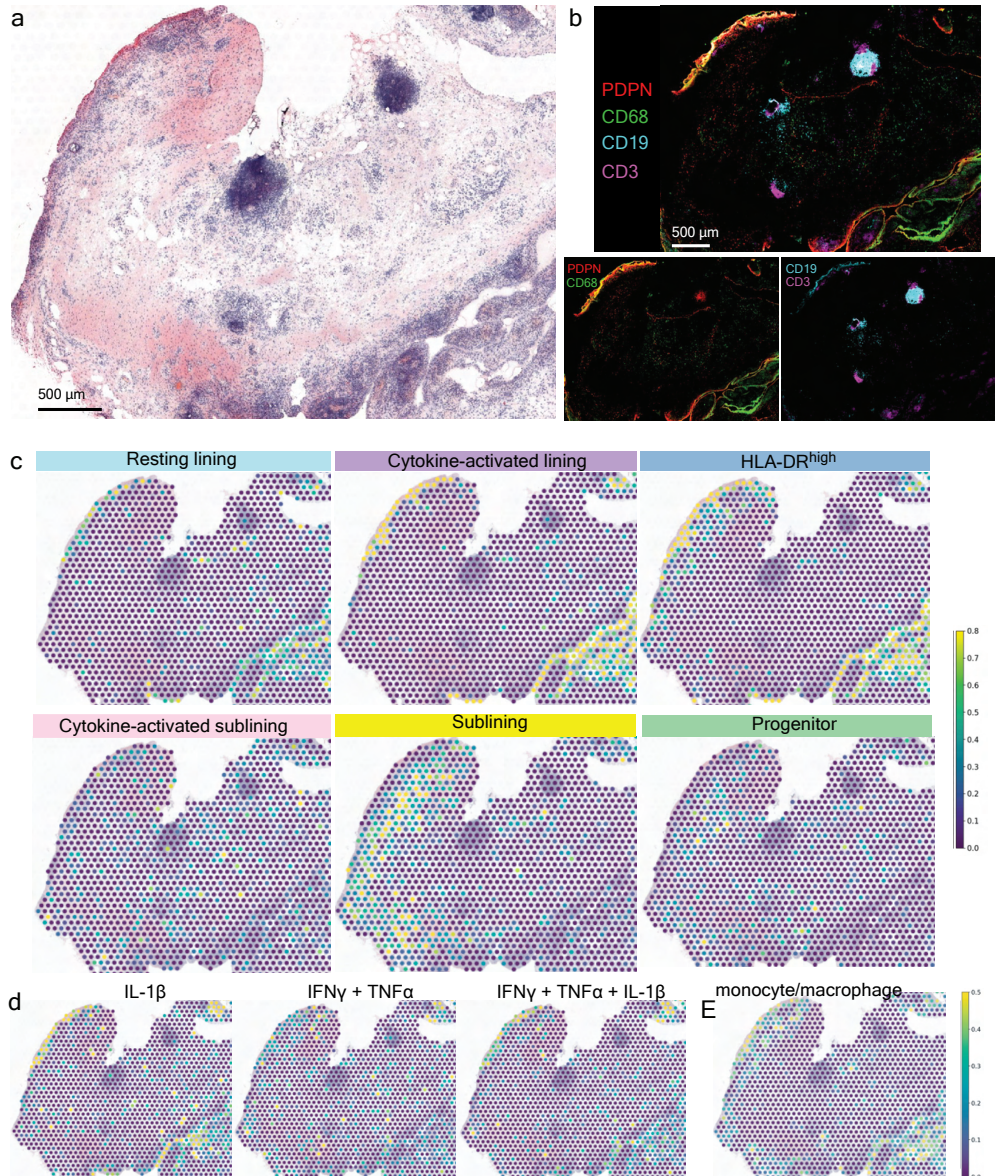
548 derived from each FLS state.



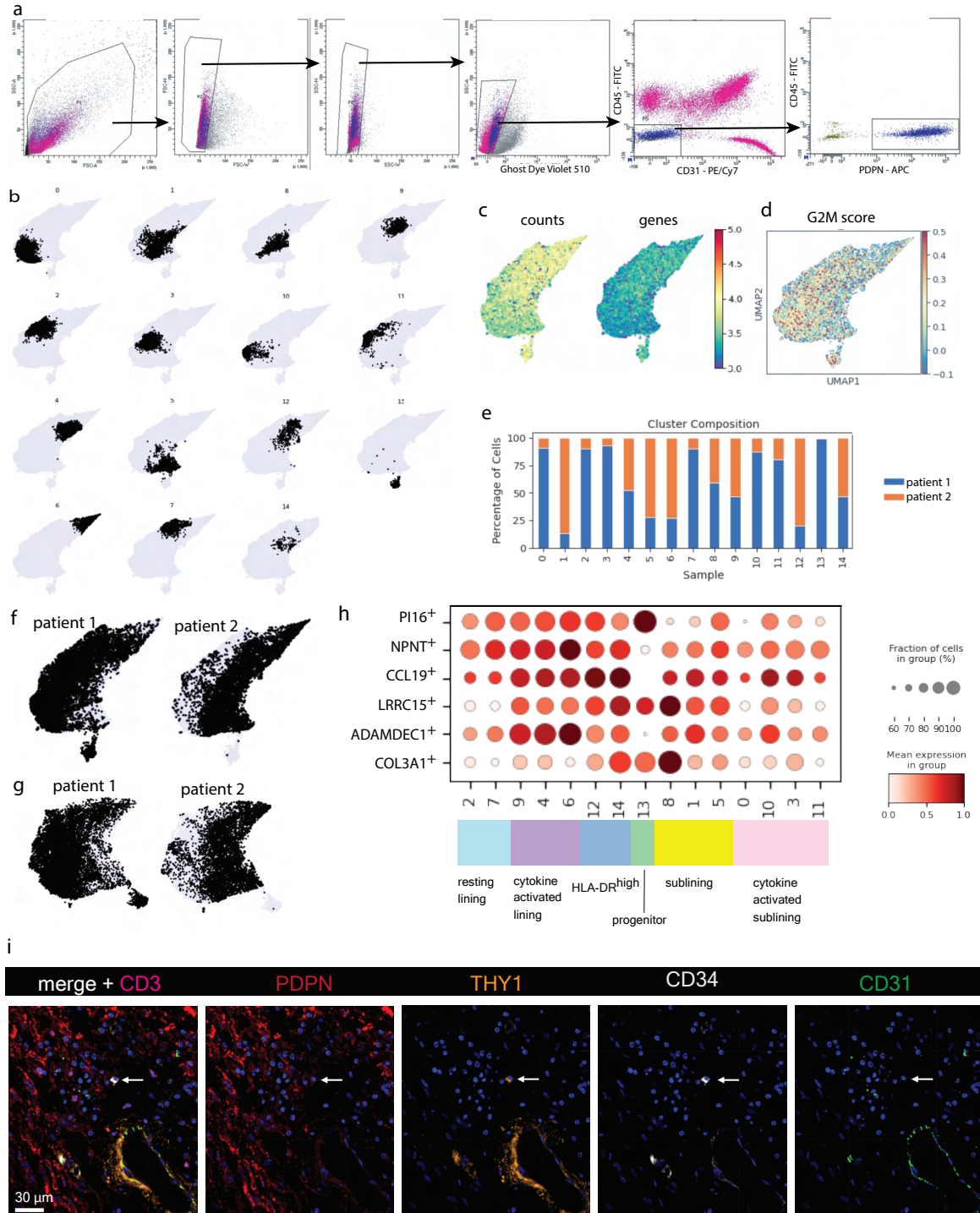
549

550 **Figure 4. Cytokine signaling drives transcriptional FLS heterogeneity.** **a**, Changes in gene
 551 expression (log fold change) after combinatorial stimulation of cultured FLS by cytokines
 552 indicated. **b**, Cultured FLS were treated with the individual cytokines indicated *in vitro* and

553 subjected to 3' RNA sequencing to identify genes that were up- (left) or down- (right) regulated.
554 Box plots compare the distribution of log₂ fold changes in the expression of these genes (in
555 stimulated versus control) in FLS treated with each cytokine alone or in combination with DLL4.
556 Gray lines connect individual genes across conditions. **c**, Dot plot showing relative expression of
557 the identified cytokine response signatures in each of the FLS states defined in Fig. 1g. **d**,
558 Representative confocal images of phosphorylated STAT1 staining (cyan), PDPN (red), CD3
559 (magenta), CD8 (white) and nuclear marker (blue) (N = 4 tissues). Pixels with the highest intensity
560 (top 3%) for both PDPN and HLA-DR are colored in yellow. White arrows indicate FLS with
561 nuclear pSTAT1 staining in the sublining and white arrowheads indicate FLS with nuclear pSTAT1
562 staining in the lining. **e**, ChromVAR z-score of motifs from Fig. 3d in cultured FLS that were
563 unstimulated, simulated with TNF α + IFN γ , or stimulated with TNF α , IFN γ and IL-1 β .
564



565
566 **Figure 5. Cytokine signaling is spatially constrained and correlated with cellular**
567 **localization.** **a**, H&E staining of a representative tissue section used for ST (patient 4 in Table
568 S1). **b**, IF image from a serial tissue section directly adjacent to that used for ST analysis. The
569 down staining is for the following markers: PDPN (red), CD68 (green), CD19 (cyan), and CD3
570 (magenta). (N = 2 tissues for ST with 2 adjacent sections each) **c**, Relative expression of FLS
571 states defined in Fig. 1g in each RNA capture area on the ST slide. **d**, Relative expression of FLS
572 cytokine response signatures in each RNA capture area on the ST slide. **e**, Relative expression
573 of synovial macrophage specific gene signature from Zhang et al² in each RNA capture area on
574 the ST slide.



575

576 **Extended Data Figure 1: scRNA-seq analysis of FLS isolated from inflamed RA synovium.**

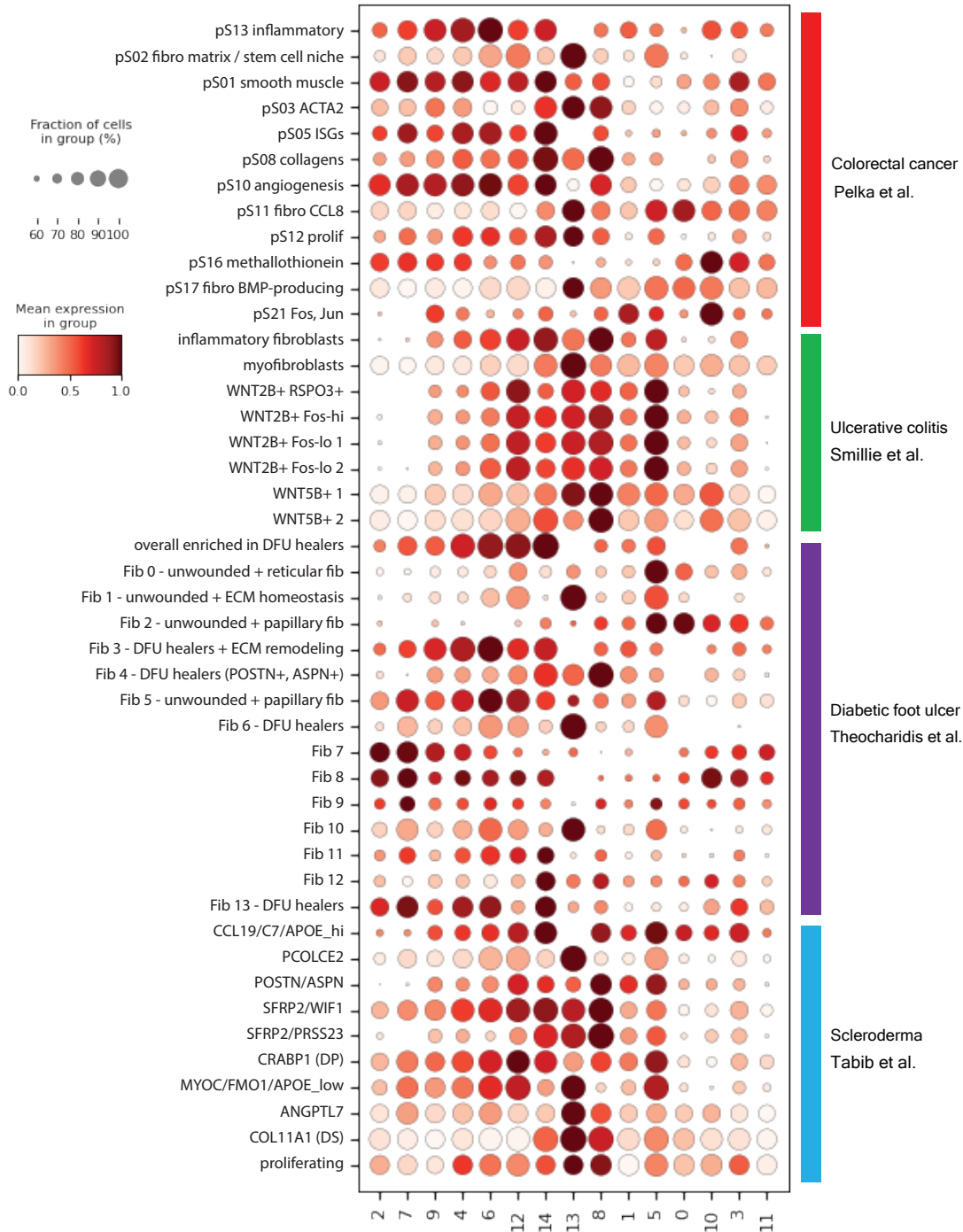
577 **a**, Gating strategy for FACS sorting of FLS. **b**, Individual FLS clusters shown on UMAP. **c**,

578 Numbers of UMI counts and unique genes expressed in each cell shown on UMAP. **d**, Cell cycle

579 G2M score per cell shown on UMAP. **e**, Cluster composition by patient. **f**, Distribution of cells from

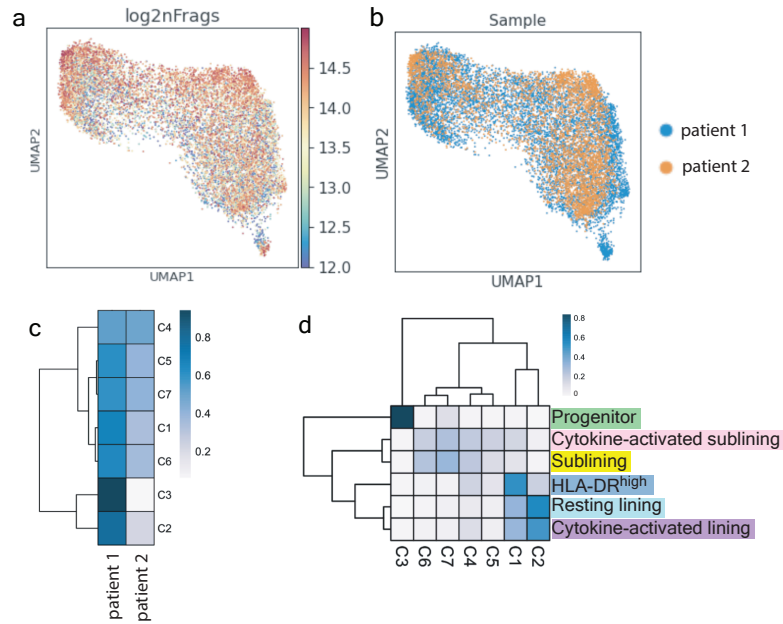
580 each patient without batch correction. **g**, Distribution of cells from each patient with MNN batch

581 correction. **h**, Dot plot with relative expression of fibroblast signatures from all clusters defined by
582 the human perturbed fibroblast atlas from Buechler et al⁹. **i**, Representative confocal image of
583 PDPN (red), THY1 (orange), CD34 (white), CD31 (green), CD3 (magenta) and nuclear marker
584 (blue) from RA synovial tissue (N = 4 tissues). White arrow indicates individual CD34⁺, THY1⁺,
585 PDPN⁺ cell not within the vasculature.
586



587

588 **Extended Data Figure 2: Expression of non-synovial fibroblast gene signatures in FLS.** Dot
 589 plot showing relative expression of all gene signatures from published tissue fibroblast
 590 populations¹¹⁻¹⁴ in FLS clusters shown in Fig. 1a colored according to FLS states defined in Fig.
 591 1g.



592

593 **Extended Data Figure 3: Paired scATAC-seq analysis of isolated FLS. a,** Logged number of

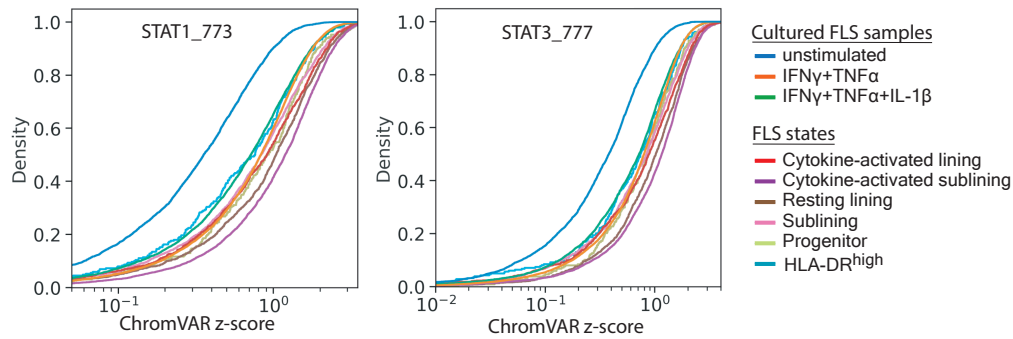
594 fragments detected in each cell shown on UMAP. **b,** Distribution of cells from each patient. **c,**

595 scATAC-seq cluster composition by patient. **d,** scATAC-seq cluster composition by FLS states.

596

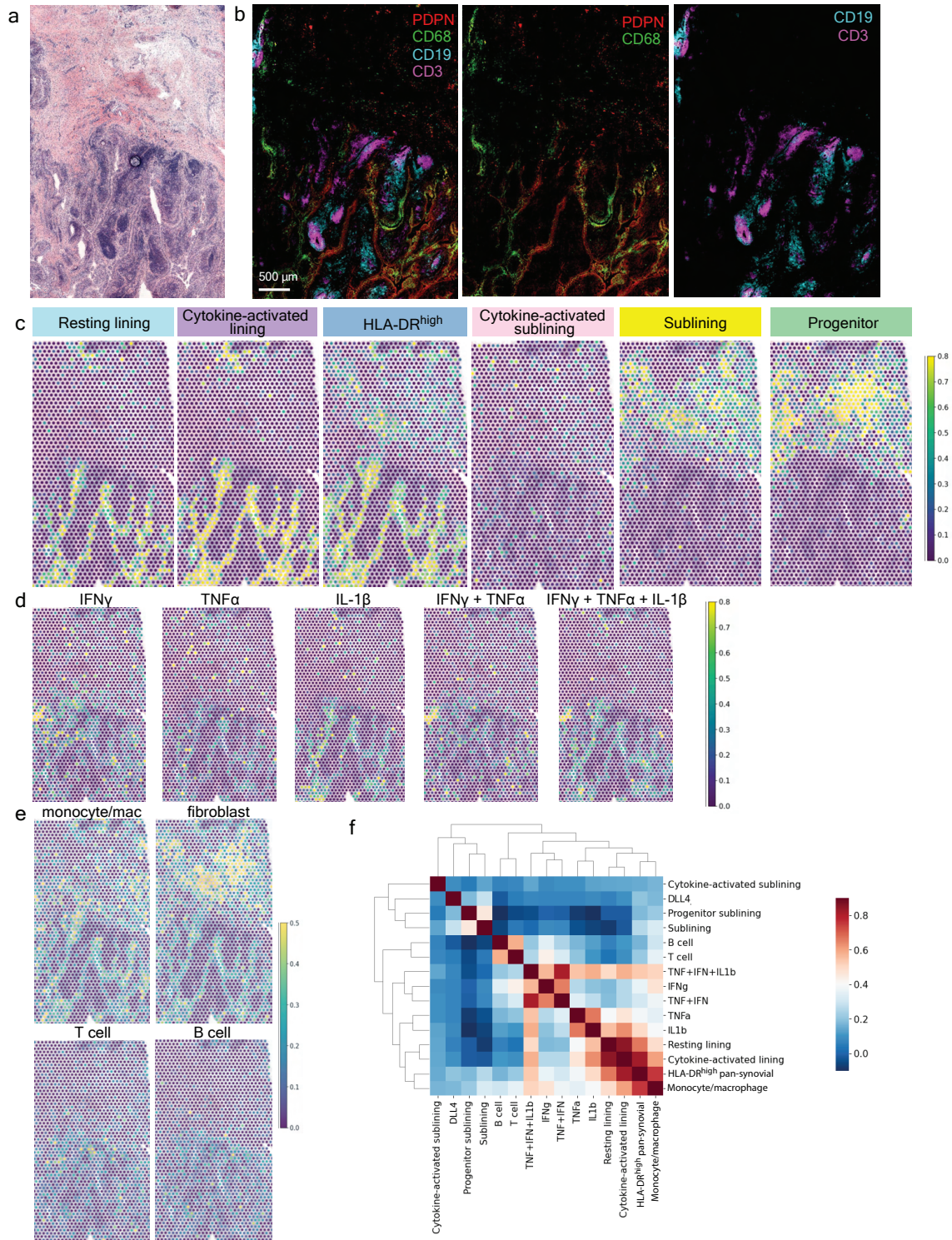
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598



599

600 **Extended Data Figure 4: Dynamics of chromatin accessibility of STAT motif containing cis-**
601 **regulatory elements across *in vivo* FLS states and *in vitro* cytokine stimulated or**
602 **unstimulated FLS.** Empirical cumulative distribution function (ECDF) 600 plots of ChromVAR z-
603 scores for STAT motifs from scATAC-seq analysis of the indicated FLS samples or states are
604 shown.



605

606 **Extended Data Figure 5: Spatial transcriptomic analysis of inflamed RA synovial tissue. a,**

607 H&E staining of a tissue section used for ST (patient 3 in Table S1). **b,** IF image from a serial

608 tissue section directly adjacent to that used for ST stained for: PDPN (red), CD68 (green), CD19

609 (cyan), and CD3 (magenta). **c**, Relative expression of gene signatures of FLS states defined in
610 Fig. 1g in each RNA capture area on the ST slide. **d**, Relative expression of FLS cytokine
611 response gene signatures in each RNA capture area on the ST slide. **e**, Relative expression of
612 synovial cell type specific gene signatures from Zhang et al² in each RNA capture area on the ST
613 slide. **f**, Correlation between FLS state gene signatures derived from scRNA-seq data (from c), *in*
614 *vitro* cytokine response gene signatures (from d), and cell type specific gene signatures (from e)
615 within individual RNA capture spots.

616

617

618 **Supplementary Information**

619

620 **Table S1:** Patient characteristics for synovial tissue samples used in this study. Samples from
621 patients 1 and 2 were used for the paired scRNA and scATAC sequencing. Samples from patients
622 3 and 4 were used for spatial transcriptomics.

623 **Table S2:** Differentially expressed genes for clusters defined in Fig. 1a and states defined in Fig.
624 1g.

625 **Table S3:** GSEA results for each state defined in Fig. 1g.

626 **Table S4:** Differentially expressed genes for RA FLS stimulated with cytokines in vitro.

627 **Table S5:** Genes differentially expressed with the addition of Notch ligand DLL4 to cytokine
628 stimulation.

629 **Methods**

630

631 **Human synovial tissue:** Synovial tissue was obtained from patients consented into the HSS
632 FLARE study of RA patients undergoing arthroplasty or synovectomy (IRB no 2014-233). On day
633 of surgery, samples were cryopreserved in as small fragments in CryoStor CS10 (Stem Cell
634 Technologies #07959). Synovial tissue quality and grading of synovitis³² were evaluated by
635 histologic analysis (H&E).

636

637 **Sample preparation for single cell sequencing:** Synovial tissue samples were disaggregated
638 into a single-cell suspension as described previously². Briefly, fragments were minced and
639 enzymatically digested (Liberase TL (Sigma-Aldrich) 100 µg/mL and DNaseI (New England
640 Biolabs) 100 µg/mL in RPMI) for 30 min at 37°C. Disaggregated cells were assessed for quality
641 and viability (Nexcelom Cellometer Auto 2000) and then stained with antibodies to CD45 (2D1),
642 CD31 (WM59), PDPN (NZ-1.3) and Ghost Dye Violet 510 (Tonbo) for fluorescence activated cell
643 sorting (BD FACSAria III Cell Sorter). Synovial fibroblasts (CD45⁻, CD31⁻, PDPN⁺ were collected
644 directly into FACS buffer. Individual nuclei were prepared using the 10x Genomics protocol
645 CG000365- Rev A. Nuclei were submitted for sequencing via Chromium Single Cell Multiome
646 ATAC + Gene Expression (10x Genomics) by the Integrated Genomics Operation core facility at
647 the Sloan Kettering Institute.

648 For cultured cytokine stimulated FLS, synovial tissues were dissociated into single cells as above,
649 cultured in MEM alpha (ThermoFisher Scientific Gibco 12561056) with 10% Fetal bovine serum
650 (R&D systems S11550) as well as 1% penicillin/streptomycin (ThermoFisher Scientific 15070063)
651 and 1% L-glutamine (ThermoFisher Scientific 25030081). Cells were passaged using TrypLE
652 Express Enzyme (ThermoFisher Scientific Gibco 12605010) until a FLS monoculture was present
653 (>3 passages). At passage 4 with TNFα (20 ng/mL) + IFNγ (5 ng/mL) or TNFα (20 ng/mL) + IFNγ
654 (5 ng/mL) + IL-1β (1 ng/mL) for 24 hours prior to harvesting and isolating nuclei as above.

655 Cytokine sources: recombinant human TNFα from PeproTech (#300-01A), recombinant human
656 IFNγ from Roche (#11040596001), recombinant human IL-1β from PeproTech (#200-01B).

657

658 **Quantification and Statistical Analysis**

659

660 **Pre-processing of single cell multiome ATAC + gene expression data:** RNA and ATAC
661 libraries for each patient were aligned using cellranger-arc software (v1.0.0, 10x Genomics)
662 against 10x genomics reference refdata-cellranger-arc-GRCh38-2020-A using default

663 parameters. The output files `fragments.tsv.gz` and `filtered_feature_matrix.h5` were utilized for
664 downstream processing and quality control analysis. We then perform the following additional cell
665 filtering steps: 1) cells with a high fraction of mitochondrial molecules were filtered ($> 20\%$); 2)
666 clusters resembling contaminating immune cell populations were removed and 3) clusters with
667 low library complexity were filtered (cells that express very few unique genes). Putative doublets
668 were removed using the `DoubletDetection` package (<https://doi.org/10.5281/zenodo.2658729>).
669 Cells or nuclei that passed these QC cutoffs were used to generate sparse count matrices and
670 filtered `fragments.tsv.gz` files for downstream analysis.

671

672 **Single-cell RNA-seq data analysis**

673

674 *Preprocessing, dimensionality reduction, clustering:* Combining the two patient samples yielded
675 a filtered count matrix of 15736 cells by 36391 genes, with a median of 4156 molecules per cell.
676 The count matrix was then normalized by library size and scaled to 100,000 per cell for analysis
677 of the combined dataset. Highly-variable genes were identified using the `Scanpy`
678 `highly_variable_genes` function with `batch_key='sample'`. Principal component analysis (PCA)
679 was performed on the normalized expression of highly-variable genes with the top 30 principal
680 components (PCs) retained. We first performed clustering on the combined dataset using
681 `Phenograph` with $k = 30$ to identify 15 clusters. To aid subtype annotation, we merged these
682 clusters into meta-clusters based on the correlation in cluster mean expression of highly-variable
683 genes. We then annotated these meta-clusters based on enriched gene pathways identified by
684 GSEA and previously-published datasets (Alivernini et al⁴ and Zhang et al²). To evaluate the
685 amount of batch effect between the two patients, we performed mutual nearest neighbor
686 correction using `Scanpy`'s `mnn_correct` function. Specifically, we limited the analysis to only the
687 highly-variable genes and used `svd_dim = 50`.

688

689 *Visualization of single-cell RNA-seq:* To visualize single cells of the two patients, we used UMAP
690 projections (McInnes et al., 2018) to generate lower dimensional representations using `knn = 30`
691 and `min_dist = 0.2`.

692

693 *Differential expression in scRNA-seq:* We performed differential expression for the following
694 comparisons: 1) samples from each cytokine-stimulation conditions vs samples from non-
695 stimulated, cultured samples (Tables S4), 2) each fibroblast state vs rest (Table S2 tab 2), and 3)
696 each unsupervised cluster vs rest (Table S2 tab 1). All differential expression was performed

697 using MAST (version 1.8.2)³³, which provides a flexible framework for fitting a hierarchical
698 generalized linear model to the expression data. We used a regression model that adjusts for
699 cellular detection rate (cngeneson, or number of genes detected per sample):

$$700 \quad Y_{i,j} \sim \text{condition} + \text{cngeneson}$$

701 where condition represents the condition of interest and $Y_{i,j}$ is the expression level of gene i in
702 cells in cluster j , transformed by natural logarithm with a pseudocount of 1.

703 To homogenize cell sampling per condition, we downsampled such that the cell complexity (i.e.
704 the number of genes per cell) was evenly matched across groups. We partitioned cells from each
705 group into 10 equally sized bins based on cell complexity and subsampled from each bin to match
706 cell complexity distribution across samples. We downsampled to at most m cells per group, where
707 m is the median number of cells per group. We considered genes to be significantly differentially
708 expressed for Bonferroni-adjusted p -value < 0.05 .

709

710 *Identifying enriched gene pathways in single-cell RNA-seq data:* Enriched gene pathways were
711 identified using pre-ranked GSEA, as implemented by the R package fGSEA³⁴ using 10,000
712 permutations. Gene ranks were calculated using $-\log(p\text{-value}) \times \log \text{fold change}$ based on MAST³³
713 differential expression. To assess enriched pathways in clusters, we used HALLMARK and KEGG
714 subset of Canonical Pathways in MSigDB v 7.1³⁵. We considered pathways with Benjamini-
715 Hochberg adjusted p -values < 0.1 to be significant.

716

717 *Scoring Gene Signature Expressions:* To score the single-cell expression of gene signatures, we
718 first transformed the library size-normalized, log-transformed data by z-score and calculated the
719 average expression of each curated gene set per cell type subtracted from the average
720 expression of a reference set of genes using the score_genes function in scanpy. The subsequent
721 cell type scores were transformed again by z-score. For comparisons to published datasets, we
722 used the top 30 genes after sorting by adjusted p value (padj) for top differentially expressed
723 genes for each unsupervised cluster/cell type.

724

725 *Correlating Spatial Gene Signature Expression:* To correlate the spatial localization of gene
726 signatures of interest, we computed the pearson correlation coefficient of their expressions across
727 individual spots for all samples combined. The signatures are generated as described above.

728

729 **Single-cell ATAC-seq data analysis**

730

731 *Preprocessing, dimensionality reduction, clustering:* We preprocessed the filtered fragments.tsv
732 file using the ArchR package³⁶ v1.0.1. Specifically, we binarized sparse accessibility matrices
733 binned at 500bp tiles across the genome. We then perform iterative latent semantic indexing (LSI)
734 on the tile matrix to generate 30 components. For downstream analysis, we filter 3 components
735 strongly correlated ($\text{cor} > 0.5$) with the number of fragments detected per cell, as well as one
736 component that is strongly correlated with batch. For visualization, we used the addUMAP
737 function in ArchR with the following parameters: nNeighbors = 150, minDist = 0.05, metric =
738 cosine. Clustering was done using the addClusters function in ArchR with the following
739 parameters: method = "Seurat", knnAssign = 50.

740

741 *Peak-calling and TF motif accessibility scoring:* Filtered fragments for cells in each sample were
742 aggregated and used as input to the MACS2 peak caller³⁷; parameters -f BED, -g 2.7e9, --no-
743 model, --shift -75, --extsize 150, -q 0.05). Peaks are filtered using an IDR cutoff of 0.05. We
744 subsequently added motif annotations using "addMotifAnnotations" with the CisBP motif database
745 and computed chromVAR deviations for each single cell with "addDeviationsMatrix".

746

747 *Identifying enriched motifs per cluster:* To identify differentially accessible motifs for each group
748 of interest, we used the rank_genes_groups function in scanpy with method='wilcoxon' and
749 corr_method = 'benjamini-hochberg' on the chromVAR zscore matrix. Motifs were filtered to
750 include only those for which the corresponding transcription factor was expressed by >20% of
751 cells in the corresponding FLS state. The top 10 motifs after ranking by 'score' were then selected
752 for plotting in the heatmap in Fig. 3d.

753

754 **In vitro FLS culture and stimulation for bulk RNA sequencing:** Synovial tissues from 4 donors
755 (RF⁺ and/or CCP⁺) were disaggregated and cultured as above. At passage 4 or 5, cells from the
756 4 donors were pooled and were plated into 12 well plates at 70,000 cells/well. Cells were allowed
757 to adhere and were then stimulated with TNF α (0.1 ng/mL), IFN γ (0.05 ng/mL), or IL-1 β (0.01
758 ng/mL) alone or in combination for 24 hours in triplicate. For DLL4 treatment, cell culture plates
759 were coated with recombinant DLL4-FC (R&D systems) overnight at 4°C at 0.5 $\mu\text{g/mL}$ prior to the
760 addition of FLS and cytokines. Concentrations for all stimuli were determined based on an initial
761 titration experiment with 4 concentrations per stimulus (10x dilutions starting with 100 ng/mL
762 TNF α , 50 ng/mL IFN γ , 10 ng/mL IL-1 β and 5 $\mu\text{g/mL}$ DLL4) and bulk 3' RNA sequencing to
763 determine the number of differentially expressed genes relative to untreated cells. After
764 stimulation, we lysed cells, isolated RNA (Zymo Research R1052), prepared libraries (Lexogen

765 QuantSeq 3' mRNA-Seq Library Prep Kit (FWD) for Illumina 015.96) and the Integrated Genomics
766 Operation at the Sloan Kettering Institute sequenced samples (bulk RNA sequencing).

767

768 **Stimulated FLS bulk RNA sequencing data analysis:** Reads from 3' RNA sequencing of
769 fibroblasts treated with cytokines were processed using version 2.5.3 of the snakePipes mRNA-
770 seq pipeline³⁸ using the flags "--reads '_R1_001' '_R2_001' --mode 'alignment' --trim --
771 trimmerOptions '-a A{10}N{90}'". In brief, this pipeline trims reads using Cutadapt, aligns them
772 using STAR to the genome (release 34 of GRCh38 with Gencode annotations), and then
773 aggregates gene-level counts using featureCounts. Differentially expressed genes for each
774 condition were then defined relative to control cells using DESeq2. Genes that were up- or
775 downregulated at $p < 0.05$ following correction for multiple hypothesis testing for each single
776 cytokine treatment were used to define expression signatures for each cytokine. The distributions
777 shown in Fig. 4b are of the (shrunk) \log_2 fold change estimates of these genes relative to control
778 cells estimated by DESeq2 in cells treated with the indicated cytokines or combinations of
779 cytokines.

780

781 **Multicolor immunofluorescence:** Synovial tissue was fixed in 1:4 dilution
782 Fixation/Permeabilization solution (BD Biosciences Cytofix/Cytoperm Cat No. 554714) in PBS pH
783 7.4 for 16-20 hours at 4°C. Tissue was washed 3x with PBS then placed in 30% sucrose in 0.1M
784 sodium phosphate buffer pH 7.4 until the tissue fell to the bottom of the tube (~6 hrs) at which
785 point tissue was embedded in optimal cutting temperature compound (OCT), frozen on dry ice
786 and stored at -80°C until sectioning (10 μ m thick). For staining, tissues were rehydrated on slides,
787 permeabilized with 0.1% triton in PBS for 10 min and blocked with 5% normal goat serum
788 (ThermoFisher Scientific 31873) in PBS for 45 min prior to staining with primary antibodies (5 hrs
789 RT or 21 hrs at 4°C) followed by secondary antibodies (2 hours RT). Appropriate isotype controls
790 were used on a separate section. After antibody stains, slides were washed, stained for nuclei
791 (DAPI – ThermoFisher Scientific 62248 – 1:2000 for 5 min RT) and mounted with Fluoromount G
792 (ThermoFisher 00-4958-02). Images were acquired with a Leica SP8 confocal microscope (40x
793 oil immersion). Image analysis (merging of channels) was performed with Imaris cell imaging
794 software.

795

796 **Spatial Transcriptomics:** Fresh synovium was immediately embedded in OCT and frozen using
797 isopentane cooled by liquid nitrogen. We used Visium Spatial Gene Expression platform (10x
798 Genomics) in conjunction with the Integrated Genomics Operation and Molecular Cytology core

799 facilities at the Sloan Kettering Institute. For this, tissue was sectioned (10 μ m sections, 2 tissue
800 sections in 2 replicates each per slide, capture area 6.5 x 6.5 mm), stained with H&E, and
801 permeabilized. This was followed by cDNA library construction with spatial barcoding and
802 sequencing.

803

804 **Spatial transcriptomics data analysis**

805

806 *Preprocessing, dimensionality reduction:* Spatial sequencing data from 2 patients (2 samples
807 each) were aligned using the Space Ranger (v1.2.2, 10x genomics) pipeline to the 10x genomics
808 reference genome refdata-gex-GRCh38-2020-A using default parameters to derive a feature
809 spot-barcode gene expression matrix. Combining the 4 samples yielded a filtered count matrix of
810 12257 spots by 19809 genes, with a median of 1754 molecules per spot. The count matrix was
811 then normalized by library size and scaled to the median of total counts of all cells before
812 normalization for analysis of the combined dataset. We then natural-log transformed the reads
813 with a pseudocount of 1. Seurat-v3.2 package was then used to select top variable genes for
814 spatial RNA-seq clustering. Highly-variable genes were identified using the Scanpy
815 `highly_variable_genes` function with `batch_key='sample'` and `n_top_genes = 2000`. PCA was
816 performed on the normalized expression of highly-variable genes with the top 50 principal
817 components (PCs) retained.

818

819 *Scoring Gene Signature Expressions:* To score the single-cell expression of gene signatures, we
820 further transformed the data by z-score and calculated the average expression of each curated
821 gene set per cell type subtracted from the average expression of a reference set of genes using
822 the `score_genes` function in scanpy. The subsequent cell type scores were transformed again by
823 z-score. Gene signature expressions were visualized using the `scanpy.pl.spatial` function.

824

825 **Antibodies used:**

826 Antibody (vendor; catalog number; clone; lot; dilution – final concentration)

827

828 For sorting FLS:

829 Anti-CD45-FITC (eBiosciences; 11-9459-42, 2D1; lot 4271593; 1:100)

830 Anti-PDPN-APC (Invitrogen; 17-9381-42; NZ-1.3; lot 1988690; 1:100)

831 Anti-CD31- PE/Cy7 (Biolegend; 303118; WM59; lot B276836; 1:100)

832 Ghost Dye Violet 510 (Tonbo; 13-0870-T100; no clone; lot D0870040521133; 1:1000)

833

834 For immunofluorescence:

835 Primary:

836 Anti-PDPN (Invitrogen; 14-9381-82; NZ-1.3; lot 2400405; 1:100 – final 5 µg/mL)

837 Anti-HLA.DR-AF488 (Biolegend; 307620; L243; lot B271228; 1:100 – 2 µg/mL)

838 Anti-CD3-BV480 (BD biosciences; 566105; UCHT1; lot 0079903; 1:100)

839 Anti-CD8-AF647 (Biolegend; 344725; SK1; lot B270006; 1:50 – final 1 µg/mL)

840 Anti-pSTAT1-PE (Biolegend; 686403; A15158B; lot B327686; 1:50 – final 0.12 µg/mL)

841 Anti-CD68-AF488 (Biolegend; 333812; Y1/82A; lot B278908; 1:10 – final 2.4 µg/mL)

842 Anti-CD19-PE (Biolegend; 302208; HIB19; lot B273506; 1:20 – final 2.5 µg/mL)

843 Anti-CD90-AF700 (R&D systems; FAB2067N; Thy1A1; lot 1569061; 1:50 – final 4 µg/mL)

844 Anti-CD34-AF647 (Biolegend; 343507; 581; lot B312791; 1:100 – final 2 µg/mL)

845

846 Secondary:

847 Anti-rat-AF594 (Biolegend; 405422; polyclonal; lot B302011; 1:1000)