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Follicular lymphoma triggers phenotypic and functional remodeling of the human lymphoid stromal cell landscape

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1 **SUMMARY**

2 Lymphoid stromal cells (LSCs) are essential organizers of immune responses. We
3 analyzed tonsillar tissue by combining flow cytometry, *in situ* imaging, RNA-
4 sequencing, and functional assays, thus defining three distinct human LSC subsets.
5 The integrin CD49a designated perivascular stromal cells exhibiting features of local
6 committed LSC precursors. CD49a also segregated cytokine/chemokine-producing
7 fibroblastic reticular cells (FRCs) supporting naive B cell and T cell survival. The
8 follicular dendritic cell (FDC) transcriptional profile reflected active responses to B cell
9 and non-B cell stimuli. We therefore examined the impact of B cell stimuli on LSCs in
10 follicular lymphoma (FL). FL B cells interacted primarily with CD49a⁺ FRCs.
11 Transcriptional analyses revealed LSC reprogramming *in situ* downstream of the
12 cytokines tumor necrosis factor (TNF) and transforming growth factor β (TGF- β),
13 including increased expression of the chemokines CCL19 and CCL21. Our findings
14 define human LSC populations in healthy tissue and reveal bidirectional crosstalk
15 between LSCs and malignant B cells that may present a targetable axis in lymphoma.
16

17 **INTRODUCTION**

18 Secondary lymphoid organs (SLOs) are immunological meeting places where antigen-
19 dependent adaptive immunity is controlled through finely regulated interactions
20 between immune cells, endothelial cells, and specialized fibroblastic cells of
21 mesenchymal origin called lymphoid stromal cells (LSCs) (Mueller and Germain,
22 2009). LSCs are essential coordinators of the functionally distinct microenvironmental
23 niches that support lymphocyte recruitment, survival, activation, and differentiation
24 (Krishnamurthy and Turley, 2020). Expression of gp38/Podoplanin (Pdpn) and lack of
25 the endothelial marker CD31 define mouse mature LSCs within the nonhematopoietic
26 compartment, as opposed to Pdpn⁻CD31⁻ LSCs (Malhotra et al., 2012). However,
27 Pdpn⁺ LSCs encompass different cell subsets. CD21⁺ follicular dendritic cells (FDCs),
28 populating the center of B cell follicles, regulate selection of antigen-specific B cells.
29 CD21⁻ fibroblastic reticular cells (FRCs), residing outside follicles, support antigen
30 delivery, naive lymphocyte and mature dendritic cell recruitment, and T cell survival
31 and activation (Perez-Shibayama et al., 2019). Similarly, the Pdpn⁻CD31⁻ LSC
32 population is heterogeneous, including myofibroblastic progenitors of the perivascular
33 space that are proposed to be adult LSC progenitors (Chai et al., 2013; Cheng et al.,

34 2019; Krautler et al., 2012). LSC diversity is only partially resolved (Rodda et al., 2018;
35 Takeuchi et al., 2018). In particular, specific FRC subsets support naive B cell survival
36 in the T cell zone (Cremasco et al., 2014), contribute to plasmablast exit from the
37 germinal center (GC) (Zhang et al., 2018), or support plasma cell survival in the
38 medulla (Huang et al., 2018), thus increasing the diversity of LSC subsets contributing
39 to B cell fate. In this rapidly evolving field, human LSCs remain poorly defined at both
40 transcriptomic and functional levels. Studies on human FRCs utilize long-term *in vitro*
41 expanded cells, introducing potential culture-related artifacts (Bar-Ephraim et al., 2016;
42 Knoblich et al., 2018; Severino et al., 2017). Moreover, the lack of data on native FDCs
43 and PDPN⁻CD31⁻ LSCs preclude any conclusion on human LSC heterogeneity, origin,
44 and pathogenic role.

45 Besides cell-intrinsic heterogeneity, LSCs possess dynamic plasticity in response to
46 extrinsic stimuli. Once an adaptive immune response is initiated, FRCs proliferate and
47 relax their actomyosin cytoskeleton, thus accommodating the rapid increase of
48 lymphocyte number (Astarita et al., 2015; C.-Y. Yang et al., 2014). Such physiological
49 responses could be hampered in many pathological settings, including chronic
50 infections, autoimmunity, and cancers (Fletcher et al., 2015). In particular, FRCs within
51 tumor-draining lymph nodes (LNs) can undergo structural remodeling and
52 transcriptional modifications reminiscent to that of cancer-associated fibroblasts
53 (CAFs). Such stroma reprogramming then impacts local immune-cell composition and
54 promotes an immunosuppressive tumor-supportive niche (Riedel et al., 2016).

55 In follicular lymphoma (FL), the most frequent indolent lymphoma, malignant B cells
56 are strongly dependent on a permissive lymphoid microenvironment including follicular
57 helper T cells (Tfh), and LSCs (Amé-Thomas and Tarte, 2014; Huet et al., 2018a;
58 Lamaison and Tarte, 2019). FL is thus one of the best paradigms of LSC-related
59 cancer. Whereas FL B cells exhibit the general hallmark of GC centrocytes, FL LNs
60 are characterized by a disappearance of CD21⁺ FDCs and an amplification of LSCs
61 overexpressing CXCL12 and the FRC marker transglutaminase (Pandey et al., 2017).
62 However, FL-infiltrating LSC heterogeneity and origin are largely unexplored. *In vitro*
63 expanded FL stromal cells exhibit specific phenotypic, transcriptomic, and functional
64 features associated with direct tumor B cell supportive functions. FL stromal cells also
65 display an increased capacity to recruit and activate other components of the tumor
66 cell niche (Grégoire et al., 2015; Guilloton et al., 2012). FL B cells likely participate in
67 the activation of LSC meshwork within malignant LNs as highlighted by their capacity

68 to trigger *in vitro* the commitment of mesenchymal precursors into FRC-like cells able
69 in turn to support their survival (Amé-Thomas et al., 2007). How native human FRCs
70 could promote FL tumor cell survival and how FL-infiltrating LSCs display tumor-
71 induced modifications *in situ* remain unknown.

72 Here, we characterized sorted human LSC subsets by a combination of phenotypic,
73 transcriptomic, and functional approaches. The integrin $\alpha 1$ subunit, CD49a, identified
74 a subset of clonogenic PDPN⁻ cells with a perivascular localization and endowed with
75 a LSC precursor phenotype. CD49a also highlighted immunologically competent FRCs
76 able to support naive T and B cell survival. We then revealed that FL B cells primarily
77 interact with human CD49a⁺ FRCs. Moreover, native FL-infiltrating LSC subsets
78 displayed specific alterations of their gene expression profiles (GEP), validated *in situ*
79 in a large series of FL patients, and consistent with an enhanced capacity to interact
80 with malignant B cells, that in turn contribute to their early reprogramming. Altogether,
81 these data identify FL-LSCs as interesting therapeutic targets.

82

83 **RESULTS**

84 *The integrin CD49a identifies discrete subsets of human LSCs*

85 To comprehensively study human LSCs, we used an enzymatic digestion protocol to
86 reproducibly identify viable stromal cell subsets from tonsils, including CD31⁺ PDPN⁻
87 blood endothelial cells (BEC), CD31⁺ PDPN⁺ lymphatic endothelial cells (LEC), and
88 CD31⁻ LSCs (Figure 1A). Human CD31⁻ LSCs were further divided into 3 main subsets
89 that expressed increasing amounts of the adhesion molecules VCAM1 and ICAM1:
90 PDPN⁻CD21⁻ cells (double negative (DN) LSCs), PDPN⁺CD21⁻ cells (PDPN LSCs),
91 and PDPN⁺CD21⁺ cells (CD21 LSCs). Histological analyses confirmed *in situ* the
92 PDPN^{hi}VCAM1^{hi}ICAM1^{hi} phenotype of follicular CD21⁺ LSCs. In comparison, VCAM1
93 and ICAM expression was reduced on extrafollicular PDPN^{int}CD21⁻ LSCs (Figure S1A-
94 C). Human PDPN LSCs specifically expressed transglutaminase (TGM2), a marker of
95 extrafollicular LSCs *in situ* (Thomazy et al., 2003), whereas DN LSCs were negative
96 for *PDPN*, *CR2* (encoding CD21), and *TGM2* (Figure S1B-D).
97 Evaluation of previously described LSC markers revealed that, whereas CD21 LSCs
98 were homogeneously ICAM1^{hi}VCAM1^{hi}PDGFR α ⁻PDGFR β ⁺CD146⁺, both PDPN LSCs
99 and DN LSCs displayed varied expression of these markers, suggesting additional
100 levels of heterogeneity (Figure 1B). To investigate the distinctive features of human

101 LSC subsets, we tested several classical markers of human mesenchymal
102 stem/stromal cells (MSCs) including CD73, CD271, and integrin α 1 subunit (CD49a)
103 (data not shown). Flow cytometry analyses identified CD49a as the most discriminant
104 marker of discrete subsets of both DN LSCs and PDPN LSCs, whereas follicular CD21
105 LSCs were CD49a⁻ (Figure 1C-D and S1E). First, CD49a identified a subset of DN
106 LSCs expressing high levels of CD146 and PDGFR β in the absence of PDGFR α . Initial
107 depletion of CD45⁺ cells revealed that PDPN⁻CD49a⁻ DN cells mostly corresponded
108 to residual hematopoietic cells and they were not further studied (Figure S1F). Second,
109 CD49a⁺ PDPN LSCs, unlike CD49a⁻ PDPN LSCs, were homogeneously PDGFR α ⁺
110 and PDGFR β ⁺, similarly to mouse FRCs (Malhotra et al., 2012). These data support
111 CD49a as a marker segregating phenotypically homogeneous human DN and FRC
112 subsets.

113

114 *Human LSCs can be divided into 3 subsets with GEP suggesting unique functionality*

115 To evaluate the relationship between LSC phenotype and function, we sorted human
116 LSC subsets and performed single-cell QPCR for a panel of 80 genes known to be
117 associated with stromal cell origin and function. Gene expression segregated CD49a⁺
118 DN LSCs, CD21 LSC, CD49a⁺PDPN LSC, and CD49a⁻PDPN LSCs onto a t-
119 distributed stochastic neighboring embedding (tSNE) plot (Figure 2A). At the single-
120 cell level, 72 genes were differentially expressed between the 4 cell populations (P
121 <.01, ANOVA) including 34 classical LSC markers (Figure 2B and S2A). CD49a⁺ DN
122 LSCs expressed genes associated with pericytic identity, like *ABCC9*, *NOTCH3*,
123 *MCAM*, *ITGA7*, *CSPG4*, together with *ACTA2*, encoding the α -SMA vascular smooth
124 muscle contractility marker, suggesting that they may correspond to the DN LSC
125 subset initially described in mice (Malhotra et al., 2012). The CD21 LSC cluster largely
126 expressed *CR1*, *CXCL13*, the Fc α / μ receptor *FCAMR*, playing an important role in
127 humoral immune responses (Shibuya and Honda, 2015), and the costimulatory
128 molecule *TNFRSF9*, regulating the GC B cell response (Middendorp et al., 2009), in
129 the absence of *COL4A1*, *COL1A1*, and *CCL19*. Concerning PDPN LSCs, expression
130 of CD49a identified a cluster enriched for FRC markers including *CCL19*, *CCL21*,
131 *CXCL12*, *COL1A1*, and *COL4A1*. Human CD49a⁺PDPN LSCs strongly expressed
132 *TNFSF13B*, encoding the B cell survival factor BAFF. CD49a⁻PDPN LSCs did not
133 express FDC or FRC-related cytokines and chemokines, were thus considered as non-

134 immunologically competent LSCs, and were not further characterized. Collectively,
135 these data support the identification of three phenotypically distinct subsets of human
136 functional LSCs: CD21⁺ LSCs corresponding to FDCs, CD49a⁺PDPN⁺ LSCs
137 corresponding to FRCs, and CD49a⁺DN LSCs hereafter called DN cells.

138 To better investigate the molecular makeup of these 3 human LSC populations, we
139 examined the GEP of sorted FDCs (n=4), FRCs (n=5), and DNs (n=4) by RNA-seq.
140 Principal Component Analysis (PCA) and Pearson correlation analysis adequately
141 segregated the 3 cell subsets (Figure 2C-D). We next explored FDCs, FRCs, and DNs
142 for their expression of cytokines, growth factors, adhesion molecules, extracellular
143 matrix (ECM) components, and immunologically relevant receptors. The differential
144 expression of key immune response regulators depicted the molecular landscape of
145 human LSC functional features (Figure S3). The comparison of our human data to the
146 reference mouse data set (Malhotra et al., 2012) using GSEA approach highlighted
147 the enrichment for mouse DN signature in human DN cells. Conversely, human FRC
148 and FDC profiles were both enriched for the signature of mouse Pdpn⁺ mature LSCs
149 (Figure S2B). We next assessed the 3536 differentially expressed genes between the
150 3 LSC subsets (Table S1). DN LSCs expressed some genes usually attributed to
151 mature FRCs (685 genes, including *CCL19*, *CCL21*, and *IL6*) or FDCs (49 genes,
152 including *MFGE8*), raising the hypothesis that human DN LSCs behave as local
153 precursors of FRCs and FDCs (Figure 2E).

154 To further characterize the molecular pathways that underline the relationship and
155 specificities between human DNs, FRCs, and FDCs, we applied an unsupervised
156 weighted gene co-expression network analysis (WGCNA) to generate transcriptional
157 modules of co-expressed genes and evaluate their relationship to LSC subsets (Figure
158 2F and S2C). Three modules were significantly associated with a specific LSC subset
159 with an eigengenes value, summarizing the gene expression profile of these modules,
160 above 0.3: DN module (MEblue, 1986 genes, P= .0028), FDC module (MEyellow, 3354
161 genes, P= .0028), and FRC module (MEpink, 1364 genes, P= .0016) (Figure 2F). We
162 analyzed using Ingenuity Pathway Analysis the genes overexpressed in each LSC-
163 specific module (Figure 2G). The DN module revealed enrichment for genes related to
164 stromal supportive niche functions and the FRC module for genes associated with
165 inflammation and fibrosis. The FDC module highlighted a strong enrichment for
166 metabolic pathways related to the mevalonate metabolism. Collectively, our findings

167 provide a comprehensive molecular characterization of human LSC populations
168 allowing further functional studies in normal and malignant settings.

169
170 *The DN LSC subset contains putative human lymphoid stromal precursors*

171 Given the molecular traits of DNs, we assessed their *in situ* localization and identified
172 cells co-expressing α -SMA, CD146, and CD49a in direct contact with the CD31⁺
173 endothelium of extrafollicular vessels (Figure 3A). Pericytes and vascular smooth
174 muscle cells, collectively referred to as perivascular cells, are heterogeneous cell
175 subsets playing critical roles in vascular development and homeostasis, and
176 expressing features of mesenchymal progenitors (Armulik et al., 2011). DNs shared
177 with human bone marrow MSCs (BM-MSCs) the expression of CD49a, CD146, and
178 PDGFR β , in the absence of PDGFR α (Gindraux et al., 2007; Li et al., 2014; Sacchetti
179 et al., 2007). We thus tested *in vitro* the proliferation and differentiation capacities of
180 DNs, looking for potential MSC-like properties. The clonogenic potential of sorted DNs,
181 reflected by the number of colony forming unit-fibroblastic (CFU-F) after 3 weeks of
182 culture, was strongly increased under low oxygen pressure, a common feature with
183 BM-MSCs (Bahsoun et al., 2018) (Figure 3B). Moreover, DNs were able to differentiate
184 into adipocytes, as evaluated by lipid droplet formation, and osteoblasts, as revealed
185 by mineralized matrix deposition, under appropriate culture conditions. In agreement,
186 DNs overexpressed the *NES* progenitor marker, as well as osteoblastic (*DLX5*, *DLX6*)
187 and pre-adipocytic (*CD36*) markers compared to FRCs and FDCs (Figure 3C).
188 Altogether, these data characterized human DNs as perivascular cells with MSC-like
189 precursor properties and expressing the FRC markers *CCL19* and *CCL21* as well as
190 the FDC-progenitor marker *MFGE8*.

191 To better characterize the putative phylogenetic relationship between DNs and mature
192 LSCs, we then compared the GEP of DNs, FRCs, and FDCs with that of purified BM-
193 MSCs (n=4), representing the standard human stromal cell precursors. The 4 cell
194 subsets could be distinguished in an unsupervised PCA (Figure 3D) and evaluation of
195 the Euclidean distance between them revealed that DNs were significantly closer to
196 mature LSCs than BM-MSCs (Figure 3E). Moreover, the PCA1 axis, that organized a
197 continuum from BM-MSCs, to DNs, FRCs, and ultimately FDCs (Figure 3F), reflected
198 a significant enrichment for genes induced in human stromal cells by tumor necrosis
199 factor alpha (TNF) and lymphotoxin α 1 β 2 (LT) (Dumontet et al., 2021), the two non-

200 redundant factors triggering LSC differentiation and maintenance (Figure 3G). In
201 agreement, the genes triggering the PCA1 axis were enriched for transcription factor
202 targets of the NF- κ B pathway, the main molecular driver of LSC commitment (Figure
203 3H). *CCL19*, *CCL21*, and *MFGE8* belonged to the 1671 genes overexpressed in DNs
204 compared to BM-MSCs (Table S2) whereas *CXCL13*, *TGM2*, and *PDPN* were not
205 expressed by the two cell subsets (Figure 3I) thus confirming that local mesenchymal
206 precursors harbor tissue-specific transcriptomic and functional features (Ménard et al.,
207 2019; Sacchetti et al., 2016). Since DNs also expressed *LTBR* (Figure 3C), we
208 stimulated them after a short-term *in vitro* expansion with a combination of TNF/LT.
209 Under TNF/LT activation, human DNs strikingly upregulated adhesion molecules
210 (*ICAM1*, *VCAM1*), FRC specific factors (*CCL19*, *CCL21*, *IL7*), and *TNFSF13B*, while
211 the pericytic markers *CSPG4* and *RGS5* were downregulated (Figure 3J). Altogether,
212 our data argue for human DNs being tissue-specific perivascular precursors of
213 lymphoid stromal cells.

214 215 *Human immunologically-competent FRCs support T cell and B cell survival*

216 In order to further characterize PDPN LSCs, we first applied Uniform Manifold
217 Approximation and Projection (UMAP) dimensionality reduction on publicly available
218 scRNA-seq data obtained from mouse LN LSCs (Rodda et al., 2018). These data were
219 overlaid with the gene expression specific signatures of human native DNs, FRCs, and
220 FDCs defined by our RNA-seq profiling (Figure 4A and Figure S4A). Our FRC-specific
221 signature (Figure 2E) highlighted the 7 murine FRC clusters and excluded PvC and
222 FDC clusters suggesting that we captured resting and activated FRC heterogeneity in
223 the absence of DN and FDC contamination.

224 FRCs construct a unique reticular conduit network delivering small antigens to the
225 cortex and organized as a collagen-rich core enveloped by a microfibrillar zone forming
226 a basement membrane ensheathed by FRCs. In agreement, human FRCs shared with
227 DNs a high expression of Collagen I, III, V, XII, XIV, XV, XVIII (Table S1 and Figure
228 S4B). Moreover, human FRCs specifically produced large amounts of small leucin-rich
229 proteoglycans, including decorin (*DCN*), fibromodulin (*FMOD*), osteomodulin (*OMD*),
230 lumican (*LUM*) and podocan-like protein 1 (*PODNL1*), known to regulate matrix and
231 elastic fiber assembly (Figure 4B). FRCs also overexpressed *LOXL1*, and *LOXL4*
232 members of the lysyl oxidase family involved in the crosslinking of collagen and elastin,
233 and *MFAP2*, *FBLN2*, *HMCN2*, and *EFEMP1/FBLN3*, four other ECM protein-bridging

234 factors, thus contributing to ECM stiffness and mechanical properties. Finally, matrix
235 metalloproteinases (MMP), which are crucial for the extensive turnover of the ECM
236 required to accommodate expansion and contraction of SLOs during immune
237 response, were highly expressed by FRCs (Table S1 and Figure S3). As a whole,
238 these data depicted the complete picture of conduit structure and ECM organization
239 and maintenance created by pure native immunologically-competent FRCs.
240 Human FRCs expressed high amounts of *THBD* and *JAM2*, two markers previously
241 described as highlighting chemokine-producing mouse FRCs (Frontera et al., 2011)
242 (Figure 4B). In agreement, FRCs produced high amounts of *CCL19*, *CCL21* (Figure
243 3C) and *CXCL13*, together with factors involved in T cell and B cell survival, namely
244 *IL7* and *BAFF*, and the alarmin *IL33* (Figure 4B). The expression of both *CCL21* and
245 *CXCL13* by Transglutaminase-expressing FRCs was confirmed by
246 immunohistofluorescence, highlighting a discrete population of *CXCL13*⁺ FRCs around
247 follicles (Figure 4C). We thus evaluated how human FRCs might promote the survival
248 of naive B and T cells. We used *CD49a*⁻PDPN LSCs as a control, since these cells
249 shared FRC basic phenotype (Figure S4C) but were devoid of *TNFRSF13* and *IL7*
250 expression (Figure 2). *In vitro* coculture experiments revealed a higher number of
251 viable tonsil-derived naive *CD4*⁺ T cells and *CD20*⁺ B cells after 7 days in the presence
252 of *CD49a*⁺PDPN LSCs compared to *CD49a*⁻PDPN LSCs (Figure 4D). Such T cell and
253 B cell pro-survival activity is related in mouse stromal cells to the production of IL-7
254 (Link et al., 2007) and BAFF (Cremasco et al., 2014), respectively. These data argue
255 for a key role of human *CD49a*⁺PDPN LSCs as functional FRCs involved in B and T
256 cell homeostasis.

257
258 *Human FDC profile suggests active response to B cell and non-B cell stimuli*

259 The GEP of a pure FDC population is yet to be established, due to the difficulty of
260 isolating these very rare cells that are firmly associated with GC B cells. In the FDC
261 signature, we first identified a strong upregulation of genes involved in the response to
262 external stimuli (Figure 5a). In particular, FDCs expressed *CSF2RA*, *CSF2RB*, and
263 *FGFR2*, revealing the capacity of FDCs to respond to GM-CSF and FGF. Moreover,
264 FDCs expressed receptors involved in the crosstalk with Tfh. In particular, *IL21R* and
265 *IL4R* are the receptors for the two main Tfh-derived cytokines (Crotty, 2019). Moreover,
266 the poliovirus receptor (*PVR*) is involved in the adhesion and activation of TIGIT⁺ Tfh
267 (Boles et al., 2009; Godefroy et al., 2015) and is able to transduce a cell-intrinsic

268 reverse signal promoting adhesion, migration, proliferation, and survival (O'Donnell et
269 al., 2019). Human FDCs also overexpressed *TLR4*, that triggers murine FDC
270 maturation and cytokine production (Garin et al., 2010; Suzuki et al., 2010). Finally,
271 *TNFRSF14*, a member of the TNF superfamily sharing with $LT\beta R$ the capacity to
272 interact with LIGHT and activate NF- κB pathway, was also strongly expressed by
273 FDCs. The overexpression of *FGFR2*, *IL21R*, *IL4R*, and *TLR4* on human FDCs was
274 confirmed at the single-cell level (Figure 5B).

275 As the most transcriptionally distinct LSC subset, FDCs expressed a specific pattern
276 of transcription factors, including *SOX8* and *SOX9*, previously reported in mouse FDCs
277 (Rodda et al., 2018), but also *FOXP4*, whose nuclear expression in CD21 FDCs was
278 confirmed *in situ* and *ex vivo* on FDC-enriched cells (Figure 5C and Figure S5). *FOXP4*
279 favors cell survival (Wu et al., 2019), suggesting specific mechanisms regulating the
280 long-term persistence of FDCs. Accordingly; the Bcl-2 family members were finely
281 regulated in FDCs with a significantly lower level of *BCL2* and higher level of *BCL2A1*,
282 a known NF- κB target gene (Figure 5C).

283 FDCs are known to support GC reaction and besides the well-defined FDC markers
284 related to antigen presentation, including *CR1*, *CR2*, *FCAMR*, *FCER2*, *ICAM2*, and
285 *CSTA* (Table S1), FDCs expressed high levels of *TNFRSF9*, *PTGIS*, *PTGES2*,
286 *WNT5A*, and *DLL1*, which are all involved in GC B cell survival, proliferation, and
287 differentiation (Jini Kim et al., 2013; Jungtae Kim et al., 2012; Middendorp et al., 2009;
288 Yoon et al., 2009). FDCs overexpressed several immunosuppressive molecules
289 including *CD200*, *IL4I1*, and *CD274* that could restrain T cell activation and
290 proliferation, and increase GC response stringency (Figure 5D). Finally, FDCs are
291 supposed to regulate the GC B cell response through the production of chemotactic
292 molecules. Human purified FDCs exhibited the highest level of *CXCL13* whose
293 migratory activity is enhanced by the FDC-specific soluble molecule FDC-SP (Al-Alwan
294 et al., 2007), and the lowest level of *CXCL12* (Figure 5E and 4B). Conversely, FRCs
295 overexpressed *CXCL12* as well as *CXCL14*, another CXCR4 ligand with synergistic
296 activity with *CXCL12* (Collins et al., 2017). In addition, analysis of human FDCs
297 confirmed the current model of regulation of follicular B cell guidance through gradients
298 of lipid mediators (Lu and Cyster, 2019). FDCs repressed *CH25H*, the rate limiting
299 enzyme in the synthesis of the EB12 ligand $7\alpha,25$ -dihydroxycholesterol ($7\alpha,25$ -HC),
300 known to guide the movement of recently activated B cells in interfollicular regions (Lu

301 and Cyster, 2019). Moreover, FDCs displayed low expression of *SPNS2*, involved in
302 the secretion of sphingosine-1 phosphate (S1P), and high expression of *GGT5*,
303 triggering the degradation of S-geranylgeranyl-L-glutathione (GGG) thus contributing
304 to a higher level of S1P and GGG in the outer follicle and a confinement of GC B cells
305 in the follicle center through the triggering of the inhibitory receptors S1PR2 and
306 P2RY8. Altogether, we provide a comprehensive picture of FDC GEP, shedding light
307 on the mechanisms of FDC-GC B cell crosstalk under steady-state conditions.

308
309 *Malignant FL B cells and FL-LSCs are engaged in a bidirectional crosstalk*

310 FL is the best paradigm of a malignancy dependent on a preserved lymphoid
311 microenvironment harboring a retained follicular pattern (Lamaison and Tarte, 2019).
312 Moreover, FRC-like cells obtained by *in vitro* differentiation of mesenchymal precursors
313 support FL B cell growth (Amé-Thomas et al., 2007). We thus decided to evaluate the
314 capacity of malignant FL B cells to specifically interact with LSC subsets by combining
315 transcriptomic data previously obtained from purified FL B cells (Desmots et al., 2019)
316 and our RNA-seq data from purified native human LSCs. This analysis was performed
317 using the CellPhoneDB repository making it possible to study biologically relevant
318 ligand-receptor interactions in order to infer cell-cell communication networks. When
319 we defined the interactome between FL B cells and LSC subsets, a higher number of
320 bidirectional interactions were predicted to occur between lymphoma cells and FRCs
321 compared to FDCs (Figure 6A). DNs exhibited lower capacity to interact with FL cells
322 than mature LSCs.

323 To further explore the lymphoma-supportive capacity of FRCs we evaluated their
324 functional impact on the survival of purified FL B cells (n=8) using CD49a⁻PDPN LSCs
325 as a control (Figure 6B). Whereas CD49a⁻PDPN LSCs have a limited impact on FL B
326 cell survival *in vitro*, FRCs strongly increased the number of viable FL B cells at day 7.
327 This activity was restricted to malignant B cells since FRCs were not able to support
328 survival of normal GC B cells. These data suggest that, even if FL B cells display a
329 GEP very close to that of normal centrocytes, they also exhibit specific capacities to
330 interact with surrounding stromal cells. FL-FRCs and FL-FDCs display poorly
331 characterized phenotypic changes that could impact their crosstalk with FL cells
332 (Pandey et al., 2017). After checking that CD49a expression on FL-LSCs mimicked
333 that of SLO LSCs (Figure S6A), we characterized FL-LSC subsets using RNA-seq of
334 isolated FL-DNs (n=4), FL-FRCs (n=4), and FL-FDCs (n=3). An unsupervised PCA

335 revealed that FL-LSCs were first segregated according to their differentiation stage
336 (Figure 6C). However, each FL-LSC subset also exhibited a distinct GEP compared to
337 its normal counterpart as highlighted by the second PCA axis, a result confirmed by a
338 Pearson correlation analysis (Figure 6D).

339 We next defined the FL-LSC signature by merging the genes differentially expressed
340 between the three LSC subsets obtained from healthy donors and FL patients (943
341 genes, Figure 6E and Table S3, adjusted P-value < .01, fold-change>2). Using a list
342 of genes upregulated in FL B cells compared to normal centrocytes (Desmots et al.,
343 2019) (FL-B signature, Figure 6E, Table S4), we then applied NicheNet computational
344 method to delineate the specific interactions underlying the crosstalk between FL-
345 LSCs and FL B cells (Figure 6F). NicheNet incorporates, beyond ligand-receptor
346 interactions, prior knowledge on intracellular signaling and resulting target gene
347 regulation. This strategy could thus prioritize how sender cell ligands affect target
348 genes in receiver cells. In addition, we used bootstrapping with randomized genes
349 (1000 iterations) to estimate the p-value of each NicheNet-predicted active ligand to
350 highlight the most significant crosstalk pathways (Figure 6G). We first defined FL-LSCs
351 as sender cells and FL B cells as receiver cells (Figure 6F). Several chemokine-related
352 pathways were found enriched at the interface between FL-LSCs and FL B cells (all
353 with a P-value < .0001). Moreover, FL-FRCs and FL-FDCs overexpressed *CCL19*,
354 *CCL21*, and *CXCL12* compared to FRCs and FDCs obtained from reactive SLOs and,
355 simultaneously, FL B cells expressed higher levels of *CCR7* and *CXCR4* than normal
356 CC (Figure S6B-C). Together with their reduced expression of the GC-confinement
357 receptors *S1PR2* and *P2RY8*, and their increased expression of the *S1PR1* egress
358 receptor (Figure S6D), these data suggested that FL B cells are prone to interact with
359 FL-LSCs both within and outside follicles. Apart from chemokines, the analysis of the
360 pathways specifically activated in FL B cells by FL-LSCs underlined a role for the TGF-
361 β /BMP/activin signaling (P<.0001). In particular, we identified a simultaneous
362 upregulation of *SMAD1*, *SMAD3*, the common signaling co-factor *SMAD4*, and the
363 activin receptors *ACVR1*, *ACVR1B*, and *ACVR2B* compared to normal centrocytes
364 (Figure S6E). To decipher how lymphoma cells could directly contribute to the
365 modification of FL-LSC phenotype, we next evaluated the impact of FL B cells defined
366 as sender cells on FL-LSCs considered as receiver cells (Figure 6f). Among the
367 mechanisms of B cell dependent LSC polarization, TNF emerged as a key driver factor
368 and we confirmed the upregulation of *TNF* and decreased expression of *LTA* in FL B

369 cells compared to CC (Pandey et al., 2017) whereas FL-FRCs overexpressed
370 *TNFRSF1A* and *TNFRSF1B*, able to bind TNF but not LT (Figure S6F). FL B cells were
371 also found to contribute to the activation of TGF β signaling in FL-LSCs and
372 interestingly, this hypothesis was reinforced by the identification of an upregulation of
373 *TGFB1* and *INHBA* in FL B cells, whereas FL-FRCs and FL-FDCs overexpressed
374 TGF β receptors (Figure S6F-G). Our bootstrap strategy further confirmed that TNF and
375 TGF β signaling pathways were the most discriminant FL B cell-derived drivers of FL-
376 LSC polarization ($P < .01$). Altogether we identified a combination of B cell-derived
377 factors, including TNF/LT and TGF- β /INHBA that could contribute to the specific
378 phenotype of FL-LSCs.

379 FL-DNs overexpressed several markers of mature LSCs including *PDGFRA*, *CCL19*,
380 *CCL21*, *TNFRSF13B*, *VCAM1*, and *SOX8* (Figure S6H). We thus compared the
381 Euclidean distance between DNs and mature LSCs in normal and malignant settings
382 and demonstrated that FL-DNs were significantly closer to both FL-FRCs and FL-FDCs
383 than DNs to FRCs and FDCs (Figure 6H). In FL tissues, we cannot detect TRANCE⁺
384 marginal reticular cells, one of the other proposed FDC precursors (Jarjour et al., 2014)
385 (Figure S7). These data suggest that infiltration by malignant FL B cells triggers early
386 LSC reprogramming towards cancer-supportive CAFs.

387

388 *TGF- β contributes to FL-LSC commitment in situ*

389 To further evaluate the role of TNF and TGF- β in FL-LSC polarization, we performed
390 *in vitro* stimulation of purified DNs for 3 days ($n=5$, Figure 7A). TNF/LT and TGF- β
391 synergized to induce FL-LSC markers, including the chemokines *CXCL12*, *CCL19*,
392 and *CCL2*, the adhesion molecules *ICAM2* and *VCAM1*, and *TNFRSF13B*. *CCL19*
393 expression was also increased in FRCs stimulated *in vitro* by TNF/LT + TGF- β (Figure
394 7B, $n=7$). We then explored the relevance of these observations *in situ* in FL patients.
395 For that purpose, we analyzed RNA-seq data obtained from fresh-frozen tumor
396 biopsies of a cohort of 148 untreated patients with high-tumor-burden FL (PRIMA trial,
397 NCT00140582) (Huet et al., 2018b). A significant correlation between *CCL19* and both
398 *TGFB1* and *LTB* levels was identified, suggesting again that TNF/LT and TGF- β could
399 contribute to the overexpression of *CCL19/CCL21* in FL (Figure 7C). We next analyzed
400 tumor microarrays obtained from 115 untreated patients with low-tumor-burden FL
401 (FLIRT trial, NCT02303119) for *CCL21* and *CD21* expression by multiplex

402 immunohistofluorescence. In this cohort of early FL, 37% of patients retained an intact
403 dense FDC network, whereas about 16% were completely devoid of FDC; the
404 remaining 47% harboring a disrupted FDC organization (Figure 7D). We highlighted
405 elevated CCL21 expression in FL compared to tonsils (n=18, Figure 7E). Moreover, a
406 careful analysis of CCL21 expression revealed, besides a very bright staining of FL-
407 FRCs, a specific expression of CCL21 by CD21⁺ FL-FDCs (Figure 7F-G), thus
408 confirming our transcriptomic data (Figure S6B). Collectively, these data support a role
409 for TNF/LT and TGF- β in the crosstalk between FL B cells and FL-LSCs and confirmed
410 the modification of FL-LSC functional properties *in situ*.

411

412 **DISCUSSION**

413 CD31⁻ LSCs are paramount to maintain SLO structural integrity and support adaptive
414 immune response (Krishnamurty and Turley, 2020). Our data provide a thorough
415 characterization of human LSC subsets, making it possible to analyze LSC alterations,
416 or ectopic induction of LSC-like cells, in pathological contexts. In particular, we
417 identified CD49a, an integrin expressed on human clonogenic pluripotent BM-MSCs
418 (Gindraux et al., 2007), as a marker of functional LSCs. CD49a expression highlighted
419 perivascular DN cells with molecular, phenotypic, and functional features of human
420 LSC precursors. CD49a also identified cytokine/chemokine-producing human FRCs
421 within inflamed SLOs. At least 5 FRC subsets are identified within mouse LNs as
422 highlighted by scRNA-seq analysis (Rodda et al., 2018). Analysis of *Cd49a/Itga1*
423 expression in the *gp38*⁺ fraction of these murine FRC subsets revealed a huge
424 variability with no significant difference in the chemokine/cytokine gene expression
425 pattern between *Cd49a*⁺ and *Cd49a*⁻ cell subsets (data not shown). *Cd49a/Itga1*
426 expression seems thus not able to distinguish immunologically-competent LSC
427 subsets in mouse.

428 We also provided a deep GEP of mature FDCs. FDCs expressed a specific pattern of
429 apoptosis regulators, in agreement with their unique capacity to retain and present
430 intact antigens to B cells for extended periods (Heesters et al., 2014). Moreover, human
431 FDCs expressed numerous receptors involved in the sensing of their surrounding
432 microenvironment and the control of GC response fitness. Besides FDCs, other murine
433 LSC subsets underpin B cell movement and survival, establishing a panel of B cell
434 interacting LSCs defined by their expression of CXCL13 (Cremasco et al., 2014; Pikor

435 et al., 2020). Similarly, both FDCs and perifollicular FRCs expressed CXCL13 and
436 TNFSF13B in human SLOs. Normal B cells and LSCs are engaged in a bidirectional
437 crosstalk. The production of TNF and LT by GC B cells is required for proper FDC
438 development and maintenance, and is closely dependent on FDC-derived CXCL13
439 (Ansel et al., 2000; Endres et al., 1999). Moreover, IL-4R-expressing B cells promote
440 FRC proliferation and activation, whereas B cells sustain prolonged LSC remodeling
441 after infection (Dubey et al., 2016; 2019; Gregory et al., 2017). FL B cells are
442 characterized by a TNF^{hi}LT^{lo} profile compared to normal CC, sustaining the
443 progressive disappearance of phenotypically mature FDCs (Chang et al., 2003;
444 Pandey et al., 2017; Pepe et al., 2018). Furthermore, FL B cells disseminate outside
445 follicles, thus contacting directly the various LSC subsets with putative impact on whole
446 stroma reprogramming. In agreement, our interactome analysis predicted a
447 preferential interaction of FL B cells with FRCs, unlike FDCs. Together with the
448 identification of ectopic FDC-like cells within FL B cell aggregates in invaded bone
449 marrow (Pandey et al., 2017), these data could support the hypothesis of a strong
450 dependency towards FDC network in the early stage of the disease or upon arrival of
451 FL B cells in a non-lymphoid microenvironment, whereas FDCs become dispensable
452 for later stages of FL progression, as recently proposed in chronic lymphocytic
453 leukemia (CLL) (Heinig et al., 2014). The lack of cells expressing classical markers of
454 marginal reticular cells within FL tissues raised the hypothesis of their differentiation
455 into FDCs in response to chronic activation.

456 Based on the purification of the three main LSC subsets, we were able to draw a picture
457 of the stromal landscape in FL, highlighting major qualitative changes in FDCs, FRCs,
458 and LSC precursors associated with their conversion to CAFs. Chemokines were
459 strongly deregulated and, besides the already-described CXCL12 induction, CCL19
460 and CCL21 were also found upregulated in FL-LSCs, unlike CXCL13. Moreover, we
461 confirmed in untreated early FL patients that CCL21 was upregulated in FL-LSCs *in*
462 *situ*, including in FL-FRCs and FL-FDCs. Conversely, in CLL, tumor B cells directly
463 trigger aberrant CXCL13 expression in non-FDC infiltrating stromal cells, through LTβ-
464 receptor activation and retinoic acid signaling (Farinello et al., 2018; Heinig et al.,
465 2014), demonstrating the specificity of tumor cell-stromal cell crosstalk. The relevance
466 of these observations is sustained by the demonstration that, in turn, FL B cells
467 exhibited a CXCR4^{hi}CCR7^{hi}S1PR1^{hi}S1PR2^{lo}P2RY8^{lo} phenotype compared to normal
468 CC, supporting their disseminated profile and preferential capacity to interact with

469 converted CAFs. The genes coding for the GC-confinement receptors *S1PR2* and
470 *P2RY8*, as well as their signaling molecule *GNA13*, are frequently mutated in
471 transformed FL and GC-like diffuse large B cell lymphomas (Muppidi et al., 2014). Our
472 demonstration that this pathway is also affected in early FL reinforces the role of G-
473 protein-coupled receptors in GC-derived lymphomas. Another interesting observation
474 was the bidirectional implication of the TGF β family. In solid cancers, TGF β signaling
475 activates CAFs, synergizing with NF- κ B triggering by inflammatory stimuli, and is
476 produced by CAFs to modulate tumor phenotype (Sahai et al., 2020). In FL, SMAD1
477 is upregulated and activated in malignant B cells (Munoz et al., 2004) and expression
478 of TGF- β by LN FL B cells is involved in the alteration of intratumoral T cell function
479 (Z.-Z. Yang et al., 2014; 2013). Moreover, FL tumor niche GEP is enriched for gene
480 networks related to TGF- β signaling (Pangault et al., 2020), whereas TGF- β could
481 contribute to the priming, by FL B cell-derived extracellular vesicles (EVs), of BM-
482 MSCs into tumor-supportive stromal niche (Dumontet et al., 2021). We identified here
483 a correlation between *CCL21* and *TGFB1* levels in the PRIMA cohort, suggesting that
484 TGF- β could also contribute to the stromal phenotype within FL LN. We cannot exclude
485 that other cell subsets of FL tumor microenvironment contribute to TGF- β production
486 and activation. In agreement, other components of FL cell niche participate in the
487 reprogramming of infiltrating stromal cells. In particular, FL-Tfh overexpress TNF/LT
488 but also IL-4 (Amé-Thomas et al., 2012), that triggers CXCL12 expression in FL-CAFs
489 (Pandey et al., 2017). An additional unsolved question is whether CAF phenotype is
490 instructed by oncogenic mutations within cancer cells. In particular, *TNFRSF14/HVEM*
491 mutations, found in 40% of FL patients, favor activation of pro-tumoral TNF^{hi}LT^{hi} Tfh,
492 able to sustain directly malignant B cell growth, and to activate FRCs and FDCs (Boice
493 et al., 2016). Conversely, *EZH2* mutations, occurring in 25-30% of FL patients, impair
494 requirement for Tfh help, while driving expansion of GC B cells dependent on an intact
495 FDC network (Béguelin et al., 2020). It is tempting to speculate that other recurrent
496 genetic alterations affecting B-T crosstalk, including mutations of *CREBBP* (Green et
497 al., 2015), *CTSS* (Bararia et al., 2020), or *RRAGC* (Ortega-Molina et al., 2019), would
498 also impact FL-CAF features. However, numerous non-mutually exclusive genetic
499 alterations coexist in FL, creating a dynamic mosaic of malignant B cell subclones, and
500 making it difficult to conclude on the individual impact of each of them on
501 microenvironment reprogramming. Despite this high level of potential heterogeneity,

502 we could identify some shared pathways in our limited series of patients, thus providing
503 some clues for understanding lymphomagenesis and design new therapeutic
504 strategies.

505 The targeting of pro-survival signals delivered by infiltrating stromal cells could
506 sensitize tumor cells to chemotherapy in CLL and mantle cell lymphomas (Park et al.,
507 2020). Moreover, standard immunochemotherapy regimens in FL are based on the
508 recruitment and activation of anti-tumor immune cells, a process profoundly affected
509 by stroma organization and activation (Salmon et al., 2012). Altogether, our study
510 suggests that therapeutic targeting of CAFs or CAF functions in FL could be a valuable
511 approach in this incurable malignancy.

512

513 **LIMITATIONS OF THE STUDY**

514 Despite elegant cell-fate mapping strategies, the identity, localization, and
515 differentiation potential of mouse LSC progenitors remain controversial, and we cannot
516 exclude that, besides DNs, other precursors exist within human SLOs or in the
517 surrounding adipose tissue. Human tonsils are widely used as a normal counterpart
518 for studying FL microenvironment, given their chronically inflamed profile, with similarly
519 high amounts of GC B cells and Tfh compared to FL LNs (Pangault et al., 2010) and
520 the presence of mature activated FDC and FRC networks. However, further studies
521 using different sources of inflamed and non-inflamed SLOs will be required to
522 characterize, through scRNA-seq and spatial transcriptomics, the whole spectrum of
523 human LSC heterogeneity, looking for the various B cell interacting reticular cell
524 subsets recently proposed in mice (Pikor et al., 2020). Finally, mechanistic insights into
525 the role of TNF and TGF- β pathways in FL-CAF polarization would require the use of
526 mouse models allowing cell tracing and cell targeting. However, existing FL mouse
527 models recreate single alterations in B cells and mimic only the early stage of the
528 disease. Detailed analysis of LSCs obtained from large numbers of patients accounting
529 for FL mutational landscape is highly challenging but would allow to definitively
530 correlate FL stroma functional features to FL B cell genetic background.

531

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552

553 **AUTHOR CONTRIBUTIONS**

554 FM: designed and performed experiments, analyzed data, and contributed writing;
555 DRou: analyzed data, and contributed writing; LV, RA, CP: generated transcriptomic
556 data from stromal cells and B cells; CL, MS: analyzed the TMA from the FLIRT cohort;
557 SH, GS: provided the RNA-seq data on the PRIMA cohort; CM, MG: provided technical
558 assistance; SB, PL: run the RNA sequencing; FMG: provided knowledge on CAF; VS,
559 BT, DRos, SL: analyzed data; GC: provided the TMA from the FLIRT trial; TF: provided
560 samples and discussed results; KT: designed and supervised research, analyzed data,
561 and wrote the paper.

562

563 **DECLARATION OF INTERESTS**

564 The authors declare no competing interests.

565

566 **INCLUSION AND DIVERSITY STATEMENT**

567 One or more of the authors of this paper self-identifies as an underrepresented ethnic
568 minority in science. One or more of the authors of this paper self-identifies as a member
569 of the LGBTQ+ community. One or more of the authors of this paper self-identifies as
570 living with a disability.

571

572

573 **FIGURES LEGENDS**

574

575 **Figure 1. Human LSC subset identification**

576 **A)** Gating strategy used for human tonsil LSCs. Three subsets were identified:
577 PDPN⁺CD21⁻ (PDPN LSC, Purple), PDPN⁺CD21⁺ (CD21 LSC, Green), and PDPN⁻
578 CD21⁻ (DN LSC, Red). Contour plot shows ICAM1 and VCAM1 expression on the 3
579 LSC populations.

580 **B)** Representative flow cytometry plots showing the expression of lymphoid stroma
581 markers on the 3 LSC subsets: isotype control (red), expression of the corresponding
582 marker (blue).

583 **C)** Human CD21⁻ LSCs gated on CD49a including CD49a⁻ PDPN LSC (Orange),
584 CD49a⁺ PDPN LSC (Blue), CD49a⁺ DN LSC (Red), and CD49a⁻ DN LSC (TN, Grey).
585 Lymphoid stroma marker expression was evaluated and representative flow cytometry
586 plots are shown: isotype control (red), expression of the corresponding marker (blue).

587 **D)** Immunofluorescence on tonsil sections for TGM2/Transglutaminase (Green)
588 CD49a (Red), and CD21 (Blue). Nuclei were counterstained with SytoxBlue (White).
589 Scale bar, 100µm. Boxes indicate areas magnified in right panels including GC (A) and
590 extrafollicular zone (B) where scale bars represent 50µm.

591 See also Figure S1.

592

593 **Figure 2. Transcriptomic characterization of human LSC subsets.**

594 **A)** t-SNE analysis of single cell Q-PCR data obtained from the various purified LSC
595 subsets (CD49a⁻ PDPN LSC, n=29; CD49a⁺ DN LSC, n=53; CD49a⁺ PDPN LSC,
596 n=79; CD21 LSC, n=47) computed on the 80-gene expression matrix.

597 **B)** Violin plot of LSC subset gene expression. Shown is the Log2-normalized
598 expression scaled for each gene.

599 **C-D)** GEP of human sorted LSCs including DNs (n=4), FRCs (n=5), and FDCs (n=4),
600 was obtained by RNA-seq. Unsupervised analysis was performed by Principal
601 Component Analysis (**C**), and by Pearson correlation matrix (**D**).

602 **E)** Differentially expressed genes represented through a Venn diagram.

603 **F-G)** GEP of human sorted LSCs were analyze by weight correlation network analysis
604 (WGCNA). The relationship between the module eigengenes and the traits (LSC
605 subsets) was evaluated based on a Mann-Whitney rank test and was represented as

606 a heat-map color-coded accordingly to the $-\text{LOG}_{10}(\text{FDR})$ (**F**). The genes upregulated
607 in the DN module (MEblue), FDC module (MEyellow), and FRC module (MEpink) were
608 analyzed using Ingenuity Pathway Analysis. Shown are the top twenty pathways
609 according to the p-value (**G**).

610 See also Figure S2, Figure S3, and Table S1.

611

612 **Figure 3. Characterization of human DNs.**

613 **A)** Localization of DNs was studied by immunohistofluorescence on tonsil sections.
614 *Left:* alpha-smooth Muscle Actin (α -SMA, Green), CD31 (Red), CD146 (Blue) and
615 Nucleus (White). White arrow indicates a perivascular cell co-expressing α SMA and
616 CD146 but not CD31. *Right:* α -SMA (Green), CD146 (Red), CD49a (Blue) and Nucleus
617 (White). White arrow indicates a triple positive cell co-expressing α -SMA, CD146, and
618 CD49a. Scale Bars, 5 μ m.

619 **B)** DNs were evaluated for their clonogenic and differentiation potential. CFU-F were
620 counted after 3 weeks of culture of DNs seeded in culture directly after sorting in 21%
621 O_2 versus 1% O_2 (left, n=3 independent experiments). Adipocytic and osteoblastic
622 differentiations were initiated after a short amplification of sorted DNs by seeding them
623 in specific culture media for 3 weeks before staining by Oil Red O, revealing lipid
624 droplets, and Alizarin Red, revealing calcium deposit.

625 **C)** Expression of DN-specific genes by the three LSC subsets as determined by RNA-
626 seq analysis.

627 **D)** GEP of human sorted LSCs were combined with that of sorted BM-MSCs (n=4)
628 obtained by RNA-seq. Unsupervised analysis was performed by Principal Component
629 Analysis.

630 **E)** Euclidean distance between DNs and mature LSCs and between BM-MSCs and
631 mature LSCs. ****P<. 0001

632 **F)** Scatter plot visualization of the PCA1 eigengene value of each stromal cell sample.
633 *P<.05, **P<.01, ****P<.0001.

634 **G)** GSEA enrichment plots of the TNF/LT signature (Dumontet et al., 2021) in the
635 genes ranked alongside the PCA1 axis.

636 **H)** Enrichment for transcription factor (TF) targets in the Top 5% genes positively
637 driving PCA1 as determined with the TRRUST V2 database.

638 **I)** Expression of LSC markers by DNs and BM-MSCs as determined by RNA-seq
639 analysis. * $P < .05$; **** $P < .0001$.

640 **J)** Effect of TNF/LT treatment on DNs (n= 5). DNs amplified for 10 days in culture were
641 stimulated or not (medium) by TNF/LT for 7 days before analysis of the expression of
642 markers related to lymphoid stroma or mesenchymal precursors by Q-PCR. The
643 arbitrary value of 100 was assigned to unstimulated DNs. ** $P < .01$

644 See also Table S2.

645

646 **Figure 4. Characterization of human FRCs**

647 **A)** Comparison between human and mouse LSC subsets. Mouse scRNA-seq data
648 (Rodda et al., 2018) were reanalyzed by UMAP and compared to our human RNA-seq
649 data. FDC-specific, FRC-specific, and DN-specific gene signatures were highlighted
650 on the UMAP.

651 **B)** Expression of FRC-specific genes by the three LSC subsets as determined by RNA-
652 seq analysis (all $P < .05$).

653 **C)** Tonsil section was stained for CCL21 (Green), CXCL13 (Red), and
654 Transglutaminase (Blue). Nuclei were counterstained with SytoxBlue (White). Scale
655 Bar, 100 μ m. Box indicates the T/B border area with higher magnification (scale bar,
656 20 μ m).

657 **D)** Purified naive CD4⁺ T cells (left) and naive B cells (right) were co-cultured for 7 days
658 in the presence or not (medium) of CD49a⁺ PDPN⁺ LSCs (FRCs) or CD49a⁻ PDPN⁺
659 LSCs before counting the DAPI⁻ viable lymphocytes by flow cytometry. Colors
660 represent paired independent experiments (n= 6). * $P < .05$.

661 See also Figure S4.

662

663 **Figure 5. Characterization of human FDCs**

664 **A)** Expression of FDC-specific genes related to response to external stimuli by the 3
665 LSC subsets as determined by RNA-seq analysis.

666 **B)** Relevant genes were validated by Q-PCR at the single-cell level in sorted FDCs.
667 Shown is the Log2-normalized expression scaled for each gene (all $P < .05$).

668 **C)** Expression of FDC-specific genes related to transcription factors or apoptosis
669 regulation by the three LSC subsets as determined by RNA-seq analysis (all $P < .05$).
670 Tonsil sections were stained for Transglutaminase (Green), FOXP4 (Red), and CD21L
671 (Blue). Nuclei were counterstained with SytoxBlue. Scale bar, 100 μ m.

672 **D)** Expression of FDC-specific genes related to immunomodulatory molecules by the
673 three LSC subsets as determined by RNA-seq analysis (all $P < .05$).

674 **E)** Expression of FDC-specific genes related to B cell migration by the three LSC
675 subsets as determined by RNA-seq analysis (all $P < .05$).

676 See also Figure S5.

677

678 **Figure 6. Interactions between FL B cells and LSCs.**

679 **A)** Heatmap showing the number of bidirectional interactions between FL B cells and
680 LSC subsets as evaluated using the CellPhoneDB repository to combine RNA-seq
681 data from human LSCs and from published microarray data from FL B cells (Desmots
682 et al., 2019).

683 **B)** FL B cells or GC B cells were co-cultured for 7 days or 2 days respectively in the
684 presence or not (medium) of CD49a⁺ PDPN⁺ LSCs (FRCs) or CD49a⁻ PDPN⁺ LSCs
685 before counting the number of DAPI⁻ viable lymphocytes by flow cytometry. Colors
686 represent independent experiments (n= 8). * $P < .05$, ** $P < .01$.

687 **C)** Principal component analysis obtained by RNA-seq analysis of purified DNs, FRCs,
688 and FDCs obtained from tonsils in FL samples.

689 **D)** Pearson correlation matrix of RNA-seq data obtained from tonsil-LSCs and FL-
690 LSCs.

691 **E)** Venn diagrams indicating the number genes differentially expressed in at least one
692 comparison of LSC subsets purified from tonsils *versus* FL samples as evaluated by
693 RNA-seq data, and the number of genes differentially expressed between purified FL
694 B cells *versus* centrocytes (CC) as evaluated by microarray data.

695 **F)** Circos plots showing predicted interactions between FL B cells and FL-LSCs. On
696 the left, FL-LSCs were defined as senders and FL B cells as receivers. On the right,
697 FL B cells were defined as senders and FL-LSCs as receivers.

698 **G)** Bootstrapping with randomized genes (1000 iterations) to estimate the p-value of
699 each NicheNet-predicted interaction, represented as a heatmap of -LOG₁₀(pvalue).

700 **H)** Euclidean distance between DNs and FRCs and between DNs and FRCs in normal
701 and FL settings. *** $P < 0.001$, **** $P < 0.0001$.

702 See also Figure S6, Figure S7, Table S3, and Table S4.

703

704

705

706 **Figure 7. FL-LSC commitment *in situ***
707 **A)** DNs amplified for 10 days in culture were stimulated or not (CTR) for 3 days by
708 TGF- β , TNF/LT, or a combination of TGF- β /TNF/LT before assessment of FL-LSC
709 commitment by QPCR. Data were represented as a mean of 5 individual experiments.
710 **B)** FRCs (n=7) amplified for 10 days in culture were stimulated or not (CTR) for 3 days
711 by TGF- β , TNF/LT, or a combination of TGF- β /TNF/LT before quantification of *CCL19*
712 expression by QPCR.
713 **C)** The level of *CCL21* was correlated with that of *TGFB1*, *LTB*, and *TNF* evaluated in
714 bulk RNA-seq from fresh-frozen tumor biopsies of 148 patients with high-tumor-burden
715 FL. Shown is linear regression and p-value.
716 **D)** FDC patterns were classified according to the expression of CD21 by
717 immunohistofluorescence. FL tissue sections were stained for CCL21 (Red) and CD21
718 (White). Nuclei were counterstained with DAPI (Blue). Scale Bar, 250 μ m. The FDC
719 network was classified a TMA with 115 low-tumor-burden FL samples depending on
720 the network integrity: dense, disrupted, and absent.
721 **E)** Quantification of CCL21 expression on FL LN and tonsil sections. *Left.* tissue
722 sections were stained for CCL21 (Red) and CD21 (Green). Nuclei were counterstained
723 with DAPI (Blue). Scale Bar, 250 μ m. *Right.* cumulative analysis of the CCL21
724 expression on a TMA with 115 low-tumor-burden FL samples compared to 18 tonsils.
725 Shown is mean +/- SD. *** $P < .001$
726 **F-G)** Analysis of the colocalization between CCL21 (Red) and CD21 (Green) staining
727 on a FL section (**F**) and a tonsil section (**G**). Scale Bar, 250 μ m. Boxes represent a
728 higher magnification (scale bar, 50 μ m). The white color indicates the colocalization
729 between CCL21 and CD21 to the related boxes.
730
731
732

733 **STAR METHODS**

734

735 **Resource Availability**

736 **Lead contact.** Further information and requests for resources and reagents should be
737 directed to and will be fulfilled by the Lead Contact, Karin Tarte (karin.tarte@univ-
738 rennes1.fr)

739 **Materials availability.** This study did not generate new unique reagents.

740 **Data and code availability.** The datasets generated during this study are available
741 under GEO accession number GSE148656.

742

743 **Experimental Models and Subject Details**

744 **Human subjects and samples.** Patients were recruited under institutional review
745 board approval following informed consent process according to the declaration of
746 Helsinki and the French National Cancer Institute ethic committee recommendations.
747 Tissue samples included tonsils collected from routine tonsillectomy, reactive LNs with
748 follicular hyperplasia, LNs from FL patients, and BM from patients undergoing cardiac
749 surgery. Human native BM-MSCs were sorted as previously described and
750 immediately used for RNA extraction (Ménard et al., 2019). For functional assays, FL
751 B cells were purified using the B Cell Isolation Kit II (Miltenyi Biotec), whereas tonsil
752 GC B cells were first enriched by the B Cell Isolation Kit II before purification by anti-
753 CD10 antibody (Immunotech) and anti-mouse IgG microbeads (Miltenyi Biotec). Tonsil
754 naive B cells were purified using the Naive B Cell Isolation Kit II, and tonsil naive CD4⁺
755 T cells using the Naïve CD4 T Cell Isolation Kit II (Miltenyi Biotec). All immune cell
756 purifications were performed on an autoMACS Pro separator (Miltenyi Biotec) and
757 purity of resulting populations was >98%. Tissue microarrays were obtained from a
758 series of 115 early FL patients included in the FLIRT clinical trial (NCT02303119)
759 sponsored by the Lymphoma Academic Research Organization (LYSA).

760

761 **Method Details**

762 **Preparation of tonsil stromal cell subsets.** Fresh tonsil tissue was cut in 3mm³
763 cubes that were incubated during 20 minutes at 37°C in dissociation buffer (DB)
764 containing α -MEM supplemented with 10U/mL DNase (Roche), 200U/mL Collagense
765 IV (Worthington), 1.6 U/mL Dispase (Worthington), and 5mM MgCl₂. Mechanical

766 dissociation was applied on a GentleMACS Dissociator (Miltenyi Biotec). A second
767 round of 40-minute dissociation at 37°C in fresh DB with a 350 rpm agitation was
768 performed. After enzymatic dissociation, the cell suspension was filtered on a 100µm
769 cell strainer (Fisherbrand), and washed with washing buffer (WB) containing PBS
770 supplemented with 1% Human serum albumin (HSA) and 0.6% of Sodium Citrate. Red
771 blood cells were lysed using EasyLyse solution (Dako) before two additional washes
772 with WB. For flow cytometry characterization, cells were resuspended in WB
773 containing DAPI (0.5 mg/mL) and directly stained before acquisition on a Beckman
774 Coulter Gallios and analysis using FlowJo (v10.4). The detailed list of antibodies used
775 for flow cytometry is provided in the Key Resources Table. The
776 CD45/CD11b/CD235a/EPCAM/CD31-negative fraction was defined as LSCs. For cell
777 sorting experiments, stromal cells were first enriched by depleting CD45⁺
778 hematopoietic cells using CD45 Dynabeads (Thermo Fischer). Cells were then stained
779 with a backgating cocktail (CD45/CD11b/CD235a/EPCAM/CD31), CD49a, PDPN, and
780 CD21 antibodies for 20 minutes at 4°C. Importantly, the anti-CD21 antibody used is
781 specific for the long isoform of CD21 (CD21L), which is expressed only by FDC unlike
782 B cells (Liu et al., 1997). After washing, cells were filtered on a 70µm cell strainer and
783 resuspended in WB containing DAPI (0.5 mg/mL). Cell sorting was performed on a
784 FACS Aria II (Becton Dickinson) using the 100µm nozzle. When indicated, FDCs were
785 enriched after enzymatic dissociation for immunofluorescence. Tonsil cell suspension
786 was centrifuged on a discontinuous Percoll gradient (Amersham) as previously
787 described (Amé-Thomas et al., 2007). Briefly, the FDC-enriched fraction was collected
788 from the interface of 15% and 25% layers, washed and resuspended in WB. Cell
789 suspension was then incubated on a poly-L-lysine (Merck) precoated slide during 30
790 minutes at 37°C, and fixed with 4% paraformaldehyde (PFA, Merck) for 15 minutes.

791
792 **Immunohistofluorescence.** Human samples were embedded in Optimal Cutting
793 Temperature Compound (OCT, Sakura) and frozen at -80°C. Cryostat sections (18µm
794 thick) were fixed with 4% PFA for 10min before incubation for 1 hour with a blocking
795 solution (PBS, 2% Bovine serum albumin, 4% donkey serum and 0.1% saponin).
796 Slides were then incubated in a humidified chamber overnight at 4°C with primary
797 antibodies. After washes in PBS/0.1% saponin, slides were incubated with
798 corresponding secondary antibodies for 1 hour at RT. Finally, tissue sections were
799 mounted with Mowiol (Merck) antifade reagent containing 20µM of SytoxBlue nucleic

800 acid stain (Thermo Fischer) and analyzed by confocal microscopy on a SP8 (Leica
801 Microsystems). ImageJ software (National Institutes of Health) was used for image
802 analysis. The list of primary and secondary antibodies used for
803 immunohistofluorescence is provided in the Key Resources Table.

804

805 **Real time quantitative PCR.** RNA was extracted by using NucleoSpin RNA plus xs
806 Kit (Macherey-Nagel) and gene expression level was assessed using the Fluidigm
807 BioMark HD system. Briefly, cDNAs were obtained using the Fluidigm reverse
808 transcription Master Mix and were then preamplified for 14 cycles in the presence Pre-
809 Amp Master Mix and pooled Taqman assay mix. Gene expression was then measured
810 with the Taqman Gene Expression Master Mix on a 96.96 Dynamic Array IFC.
811 Expression level was calculated with the CT calculation method and RPLPO and
812 RSP17 as housekeeping genes. The list of assay-on-demand used is provided in
813 Supplemental Table S5.

814

815 **Single Cell RT-QPCR.** Single-cell experiments were performed using the Fluidigm
816 C1 systems according to manufacturer instructions. Briefly, sorted stromal cells were
817 captured with C1 integrated fluidic circuits (IFC) medium-cell (10-17 μ m) chips
818 (Fluidigm). Cells were then lysed, and reverse transcription and pre-amplification
819 was done within the C1 system. Gene expression was then assessed by qPCR with
820 80 selected Taqman assays and 3 housekeeping genes using the TaqMan Gene
821 Expression Master Mix on a 96.96 Dynamic Array IFC and the Fluidigm BioMark HD
822 system. After removal of cell outliers using the R package fluidigmSC (Fluidigm
823 SINGuLAR Analysis Toolset, R package version 3.6.3), the gene expression profile
824 of remaining cells was determined. The Raw Ct values were converted into
825 expression level on a log2 scale. Significant genes were selected using an ANOVA
826 with built-in module of the SINGuLAR Analysis Toolset package. The list of assay-
827 on-demand used is provided in Supplemental Table S5.

828

829 **RNA-seq analysis.** Sorted tonsil FDCs (n=4), FRCs (n=5), DNs (n=4), sorted BM-
830 MSCs (n=4), and sorted FL-FDCs (n=3), FL-FRCs (n=4), and FL-DNs (n=4) were used
831 for RNA-seq analysis. RNA extractions were performed with the Single Cell RNA
832 Purification Kit (Norgen Biotek, Canada). Library for RNA-sequencing was then
833 prepared with the SMARTer Low Input RNA kit (Takara). After quality control, libraries

834 were sequenced with 2 x 100-bp paired-end reads on the Illumina Hiseq2500
835 sequencer generating more than 47 million reads per sample with > 80% of sequences
836 achieving > Q30 Phred quality scores. Raw data were checked for quality using
837 FastQC (v0.11.5) and aligned to the reference genome GRCh38 release 90 using
838 STAR (v2.5.2) (Dobin et al., 2013). Read summarization, gene and transcript counts
839 were performed using featureCounts (Liao et al., 2014) (version 1.5.0) from the
840 Subread R package.

841

842 **Colony-forming unit assays and in vitro differentiation.** Sorted DNs were
843 resuspended in α -MEM supplemented with 10% FCS (Hyclone), 100 IU/mL penicillin
844 and 100 μ g/mL streptomycin and seeded at 50 cells/cm² in 1% O₂ or 21% O₂. Fresh
845 medium was replaced every week. After 3 weeks, cells were stained with May-
846 Grunwald-Giemsa, and colonies with > 50 cells were counted. Each experiment was
847 performed in triplicate. To evaluate the differentiation potential of DNs, sorted cells
848 were first plated at 15000 cells/cm² in α -MEM supplemented with 10% FCS (Hyclone),
849 100IU/mL penicillin, 100 μ g/mL streptomycin and cultured in 1% O₂ during 10 days. For
850 adipogenic differentiation, cells were then cultured in StemMACS AdipoDiff Media
851 (Miltenyi) for 21 days before fixation and staining with Oil-red O (Sigma-Aldrich).
852 Osteoblast differentiation was achieved after 21 days of culture in StemMACS
853 OsteoDiff media (Miltenyi). Cells were then washed, fixed, and stained with Alizarin
854 red (Sigma-Aldrich). For the differentiation of DNs toward FRC, cells were stimulated
855 by TNF (10 ng/mL) and LT (100 ng/mL; R&D Systems) for 7 days. RNA was extracted
856 followed by RT-qPCR using assay-on-demand primers for indicated genes. For
857 differentiation of DNs and FRCs toward FL-LSC-like cells, DNs were stimulated by
858 TGF β (8 ng/ml; R&D Systems) alone or in combination with TNF and LT for 3 days.
859 RNA was extracted followed by RT-qPCR using assay-on-demand primers for
860 indicated genes.

861

862 **Cell survival assays.** CD49a⁺ PDPN LSCs and CD49a⁻ PDPN LSCs were sorted and
863 resuspended in α -MEM supplemented with 10% FCS (Hyclone), 100IU/mL penicillin,
864 and 100 μ g/mL streptomycin at 15000 cells/cm² in a 96-well plate until confluence. After
865 washing in PBS, naive B cells, naive CD4 T cells, GC B cells, and FL B cells were
866 added at 150,000 cells/well in RPMI supplemented with 2% FCS (Thermo Fischer),

867 100 IU/mL penicillin, and 100 µg/mL streptomycin. After 2 days of culture for GC B
868 cells or 7 days of culture for other immune cell types, the absolute number of
869 CD20⁺DAPI⁻ viable B cells or CD4⁺DAPI⁻ viable T cells was quantified using Flow-
870 Count beads (Beckman Coulter) by flow cytometry.

871

872 **Analysis of TMA from FL patients.** For multiplex fluorescence microscopy and
873 analysis, 18 FFPE human tonsils and TMA of 115 patients with a follicular lymphoma
874 from the FLIRT cohort (NCT02303119) were used. Four-micrometer-thick sections
875 obtained with a microtome (Histocore multicut Leica) were transferred onto plus-
876 charged slides (VWR international), followed by multiplex immunofluorescence
877 staining with a U DISCOVERY 5 plex immunofluorescence (Roche Diagnostics). Two
878 sequential rounds of staining were performed, each including heat deactivation step,
879 followed by incubation with primary antibody and corresponding HRP secondary
880 antibody. Staining for CCL21 (primary antibody from Biorad) and CD21 (primary
881 antibody from Biotechne) were visualized on the same section. HRP enzyme mediated
882 deposition of the tyramide coupled to respectively rhodamine and FAM corresponding
883 species fluorophores (kit Ventana Medical Systems). After two sequential reactions,
884 sections were counterstained with DAPI and coverslipped using Fluoromount
885 (CliniSciences). Visualization was performed with the Nanozoomer (Hamamatsu
886 Photonics) equipped with the multicolor fluorescence module. CCL21 detection and
887 quantification was performed using an automated analysis by machine learning with
888 the HALO software (Indicalabs). Four zones in each tonsil were quantified and the
889 average was used for the comparison with FL patients. The FDC network pattern was
890 classified by a certified pathologist as (1) absence; (2) disrupted: scattered or
891 peripheral presence of FDC; (3) dense: almost all tumor follicles were packed by FDC.
892 The colocalization between CD21 and CCL21 staining was evaluated using the plugin
893 Colocalization Threshold in FIJI software.

894

895 **Quantification and Statistical Analysis**

896 **RNA-seq analysis.** Data normalization and differential gene expression were
897 performed with the DESeq2 R package (v1.22.2). Significant genes were defined as
898 $p < 0.01$ after adjustment for multiple testing by Bonferroni correction and used in
899 downstream analysis. Euclidean distance was calculated using the R package stats

900 (v4.0.4) with the function `dist`. Pearson correlations were performed on the top 25%
901 most variable genes. Correlation matrix were visualized using the `heatmap()` function
902 of the `made4` R package (v1.58.0). Principal Component Analysis (PCA) was done
903 after scaling of the top 25% most variable genes, with the `prcomp()` function and
904 visualized using the `Factoextra` R package (v1.0.7). Visualization of differentially
905 expressed gene clusters was done using the `heatmap` function and Venn Diagrams.

906

907 **Weighted Gene Correlation network analysis (WGCNA).** We used the WGCNA R
908 package (v1.68)(Langfelder and Horvath, 2008) to identify modules of co-expressed
909 genes among the 13 tonsil LSC samples analyzed by RNA-seq. For this analysis, we
910 retained the top 75% most variable genes. Expression matrix was \log_2 transformed
911 and the network was constructed by Pearson correlation considering a soft threshold
912 power(β) of 8 identified with the function `Pick Soft Threshold`. Then, a topological
913 overlap metric (TOM) matrix was derived from the gene expression matrix and the
914 dissimilarity of TOM was calculated. Hierarchical clustering tree was created and
915 modules were generated by the dynamic tree cut method, with a minimum module size
916 of 100 and a `deepSplit` of 2. Each module was marked by a color. In order to reduce
917 the module numbers, modules with a similarity of more than 80% were merged with the
918 `MergedModule` function and only 13 modules were used for subsequent analysis. The
919 module eigengenes (ME) were then assigned to each cell types and visualized as a
920 box-plot. To determine if co-expression modules were associated with LSC subtypes,
921 we performed a Mann-Whitney test, and a heatmap visualization was then created and
922 colored according to the $-\text{LOG}_{10}(\text{padj})$, `padj` being the FDR-adjusted p-value (FDR:
923 False Discovery Rate). Finally, module gene lists were used for downstream
924 enrichment pathway using the Ingenuity Pathway Analysis software (QIAGEN
925 Bioinformatics).

926

927 **Comparison with mouse LN stromal cell GEP.** Raw microarray data corresponding
928 to FRC and DN mouse skin-draining LNs were downloaded from the NCBI GEO
929 database (GSE 15907)(Malhotra et al., 2012). Mouse gene symbols were mapped to
930 human gene symbols using the `BiomaRt` R package (v2.40.5). GSEA preRanked
931 analysis was conducted on the ranked gene lists generated for each pairwise
932 comparison of stromal cell phenotypes, the ranking being based on the moderated t-
933 test statistics (`limma` R package). Raw single cell RNA-seq data and clustering results

934 (*i.e* cell type annotation) from mouse lymph node stromal cells were downloaded from
935 the NCBI GEO database (GSE112903)(Rodda et al., 2018) and UMAP dimensionality
936 reduction was performed using the RunUMAP function of the Seurat package (v3.1.5),
937 based on 10 aligned correlation components (Stuart et al., 2019). Clusters of genes
938 up-regulated in DNs, FDCs, and FRCs computed from the RNA-seq data analysis were
939 then used as signatures for geneset scoring. Gene names were converted to their
940 mouse equivalent with a home-made R script and the homologene package. Signature
941 scores were computed with the AddModuleScore function of the Seurat R package.
942 This function calculates for each individual cell the average expression of each gene
943 signature, subtracted by the aggregated expression of control gene sets. All the
944 analysed genes were binned into 25 bins based on their averaged expression, and for
945 each gene of the gene signature, 100 control genes were randomly selected from the
946 same bin as the gene. For each gene signature, cell scores were visualized on UMAP
947 and violin plots using Seurat and Vioplot (v0.3.0) packages respectively. Student's t
948 tests were performed to calculate statistical significance between cell clusters with p-
949 value adjustment by the FDR correction of Benjamini-Hochberg.

950
951 ***Interactome analysis.*** For interactome analysis, CellPhoneDB (v2.0) (Efremova et
952 al., 2020) and Nichenet (v0.1.0) (Browaeys et al., 2020) scripts were used accordingly
953 to author's recommendations. We first created a data matrix containing the RNA-seq
954 data from the 13 tonsil LSC samples and the microarray data from 10 FL B cell
955 samples, keeping all genes that were not shared between the two expression datasets.
956 We then used the CellphoneDB python script with the following parameters: .8 for ratio
957 of cell in a cluster, a p-value of .05, and a number of iterations of 1000. The numbers
958 of significant interactions for which both a ligand and a receptor were present were
959 then summarized for each cell type and direction (*eg.* stromal cells toward FL-B cells
960 and FL-B cells toward stromal cells) and represented through a heatmap using the
961 pheatmap R package.

962 In another approach, we used the Nichenet R package, to identify top ligand-receptor
963 interactions between FL-LSCs and FL-B cells. RNA-seq from tonsil- and FL-LSCs were
964 normalized and each FL-LSC subset was compared to its normal counterpart using
965 Deseq2 R package, with significant genes defined as a fold change>2 and a p < 0.01
966 after adjustment for multiple testing by Bonferroni correction. Genes upregulated in at
967 least one FL-LSC subset compared to its normal counterpart were keep for subsequent

968 NicheNet analysis. For B cells, differentially expressed genes were obtained from
969 microarray data already published (purified centrocytes, GSE136248, n=5; purified FL
970 B cells, GSE85233, n=10) after limma analysis with a FDR of 5% and a fold
971 change >1.5. Upregulated genes in each cell type were then used to infer active ligand-
972 target links using NicheNet algorithm. An expression table was created by merging
973 gene expression in B cells and LSC in HD and FL contexts. Expressed genes were
974 then defined in sender and receiver cells based on an average log₂ expression above
975 7.5 in LSC samples and 4 in B cell samples. Genesets of interest (upregulated genes
976 in FL condition compared to the healthy condition) were used to predict how ligands
977 can impact target genes and ranked receptors accordingly to their activity. Then the
978 best upstream ligands were filtered by the genes that were expressed in sender cells.
979 In addition, we used a bootstrap test with 1000 random gene permutations (among the
980 receiver-expressed genes) to create a distribution of each ligand activity and estimate
981 a P-value for each NicheNet observed activity. Ligand-target interactions were
982 represented through Circos visualizations (R circlize package v0.4.9) with the best
983 upstream ligands.

984

985 ***Analysis of RNA-seq from FL patients.*** Normalized counts from RNA-seq data of a
986 cohort of 148 untreated patients with high-tumor-burden FL enrolled in the phase 3
987 randomized PRIMA trial (Huet et al., 2018b) were used to perform correlation analysis
988 with selected target genes. Correlation tests were performed with the rcorr function
989 from the Hmisc R (v1.0.0) package.

990

991 ***Statistical analysis***

992 Statistical analyses were performed with Prism software version 8.3.1 (GraphPad
993 Software) using the non-parametric Wilcoxon signed-rank test for paired samples, the
994 non-parametric Mann-Whitney test for independent samples, or one-way Anova
995 whenever appropriate.

996

997 **Additional Resources**

998 The PRIMA trial and the FLIRT trial are registered at ClinicalTrial.gov (NCT00140582
999 and NCT02303119; respectively).

1000

1001

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Figure 1. Human LSC subset identification

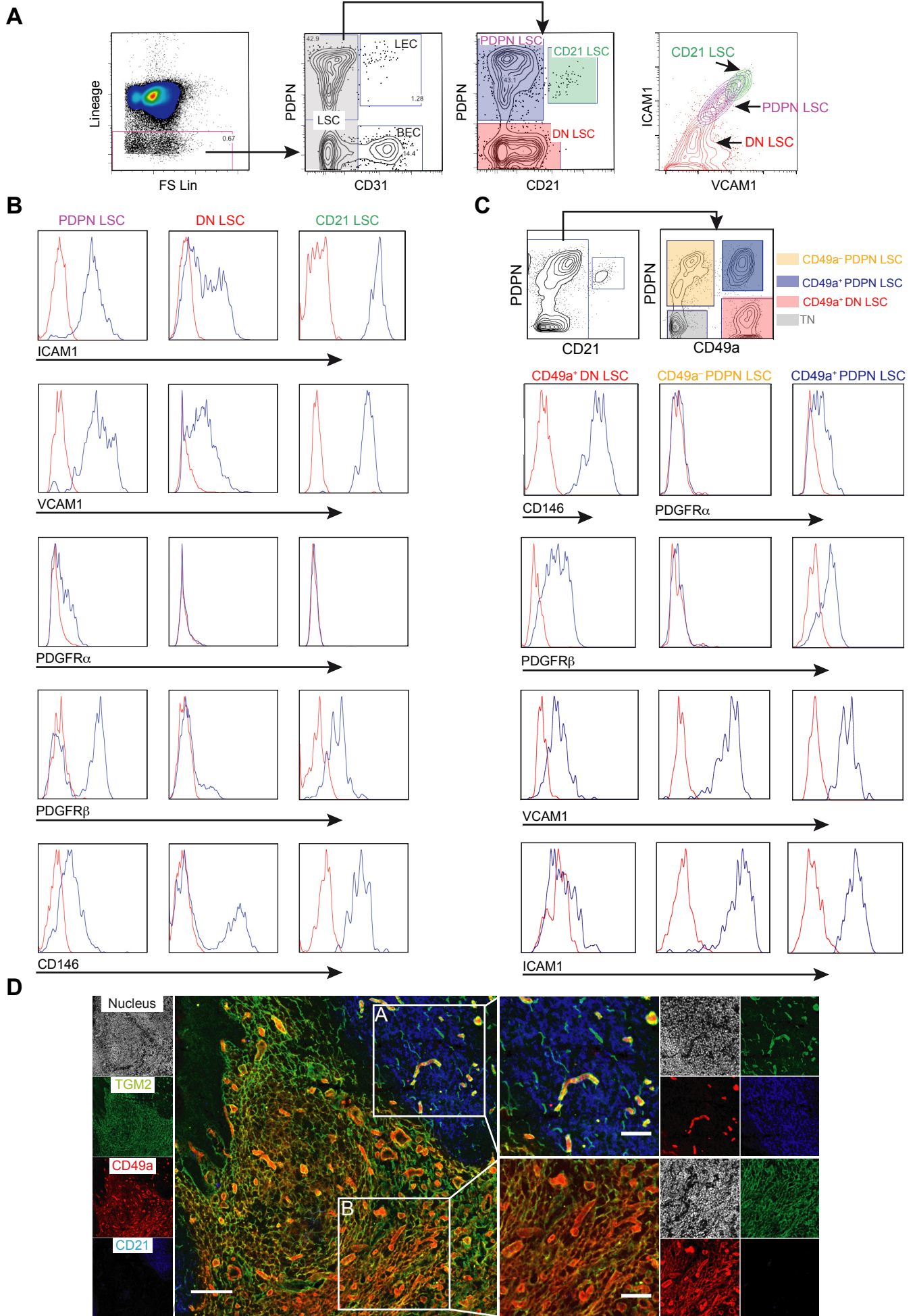


Figure 2. Transcriptomic characterization of human LSC subsets

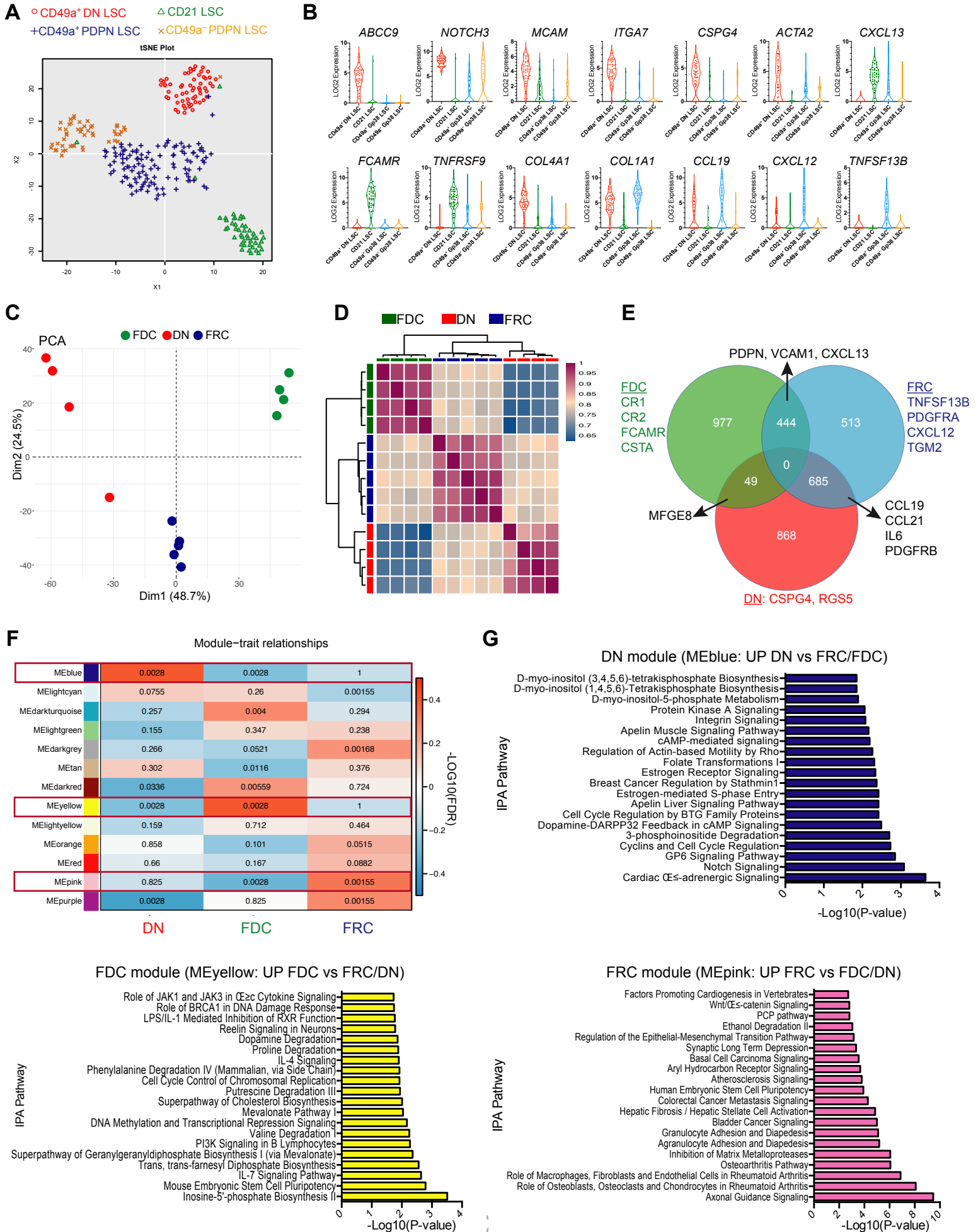


Figure 3. Characterization of human DNs

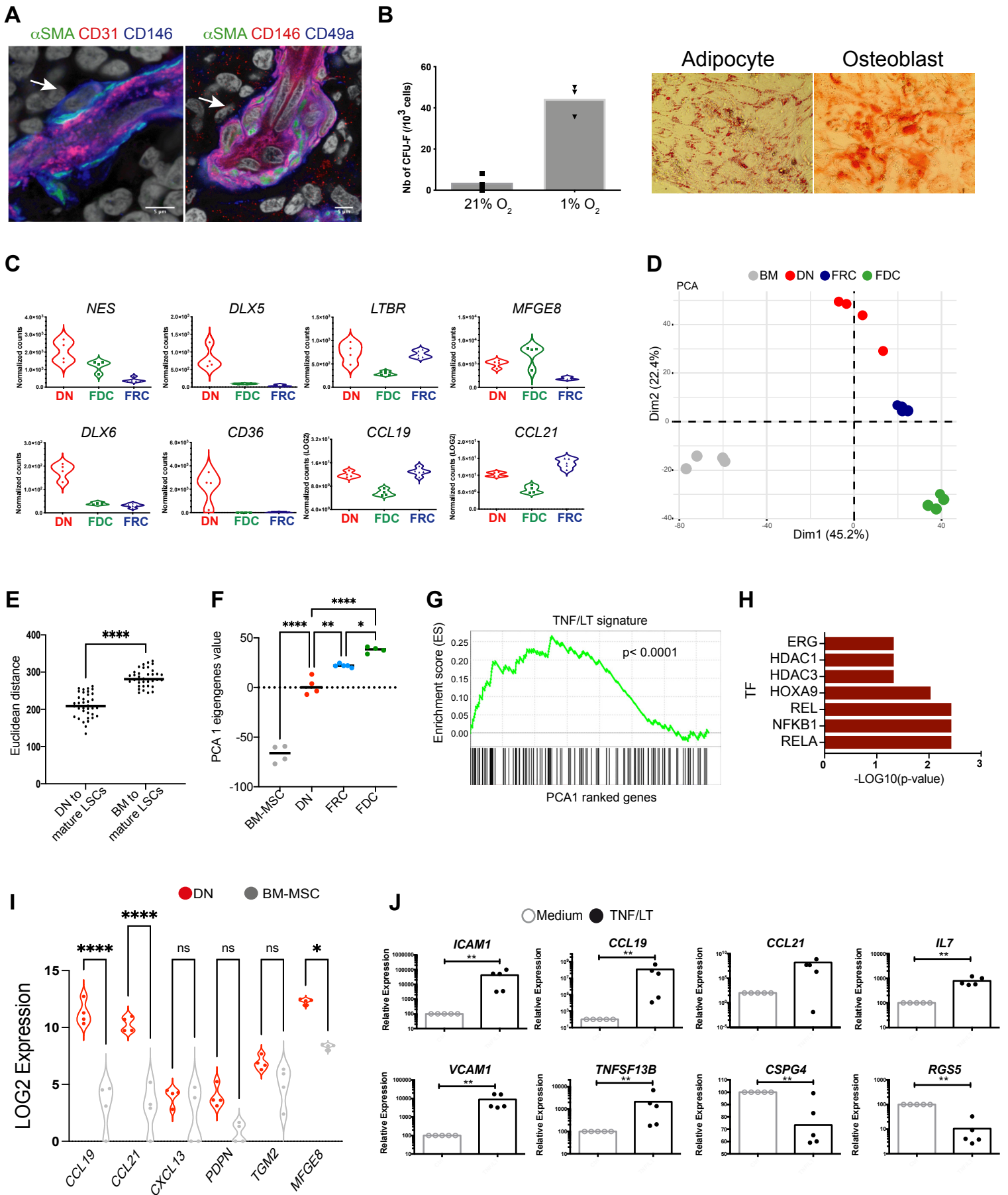
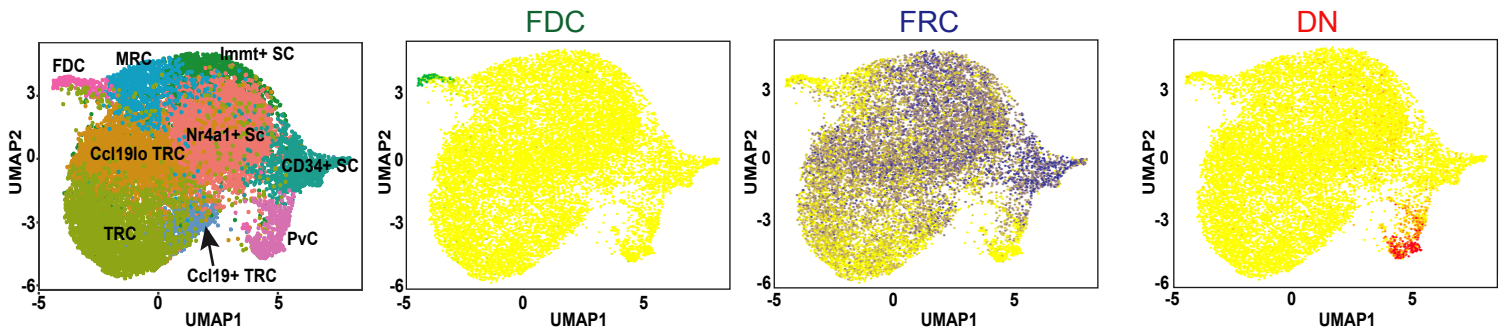
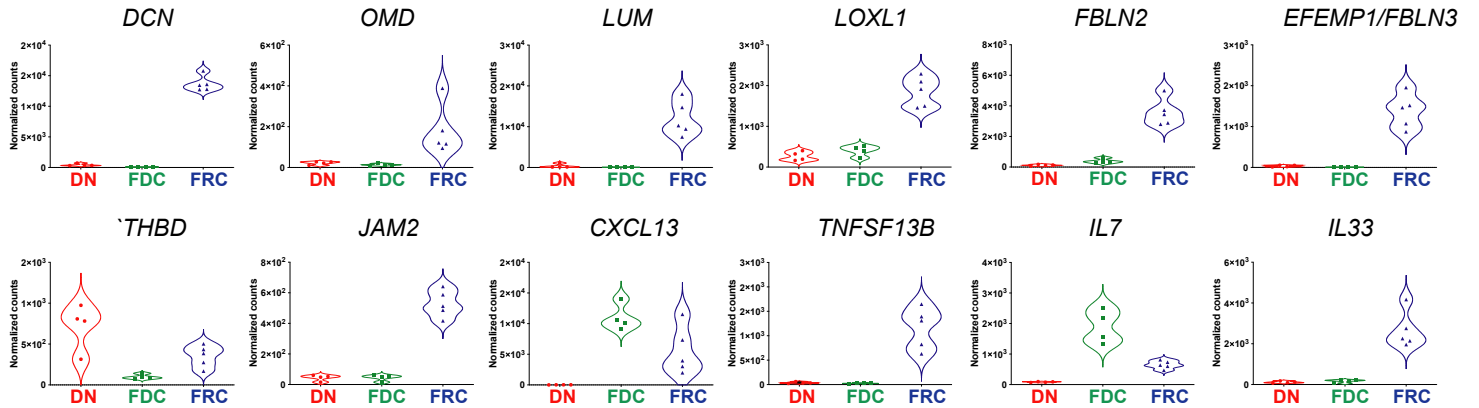


Figure 4. Characterization of human FRCs

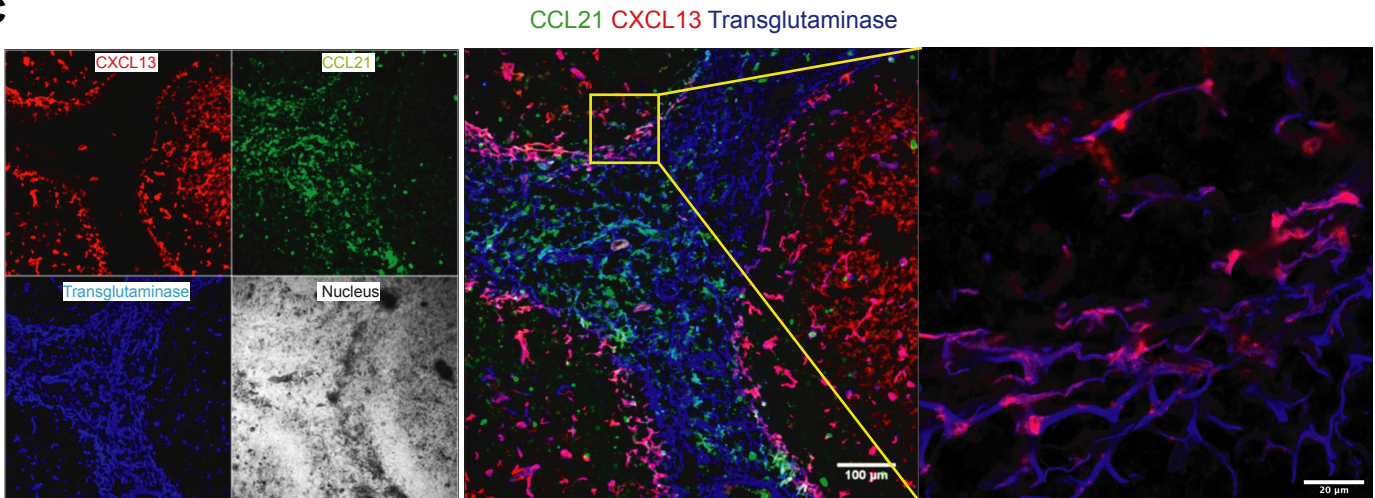
A



B



C



D

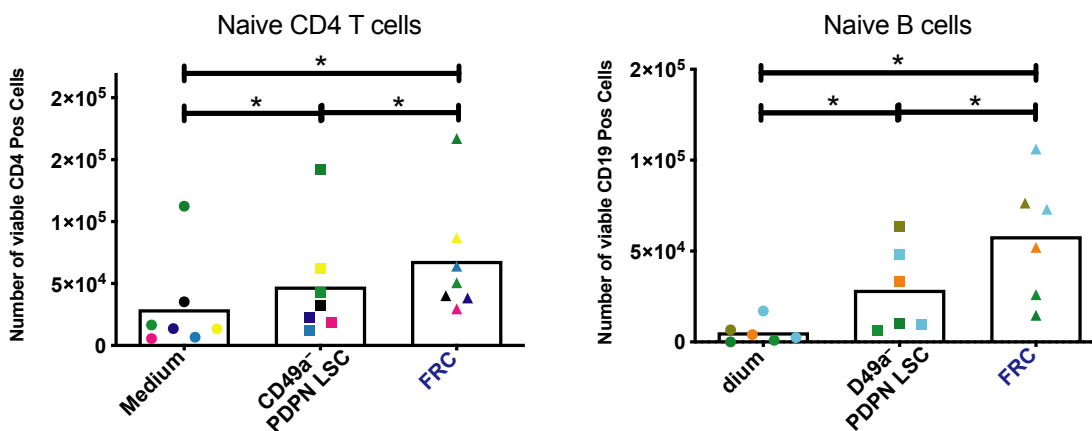


Figure 5. Characterization of human FDCs

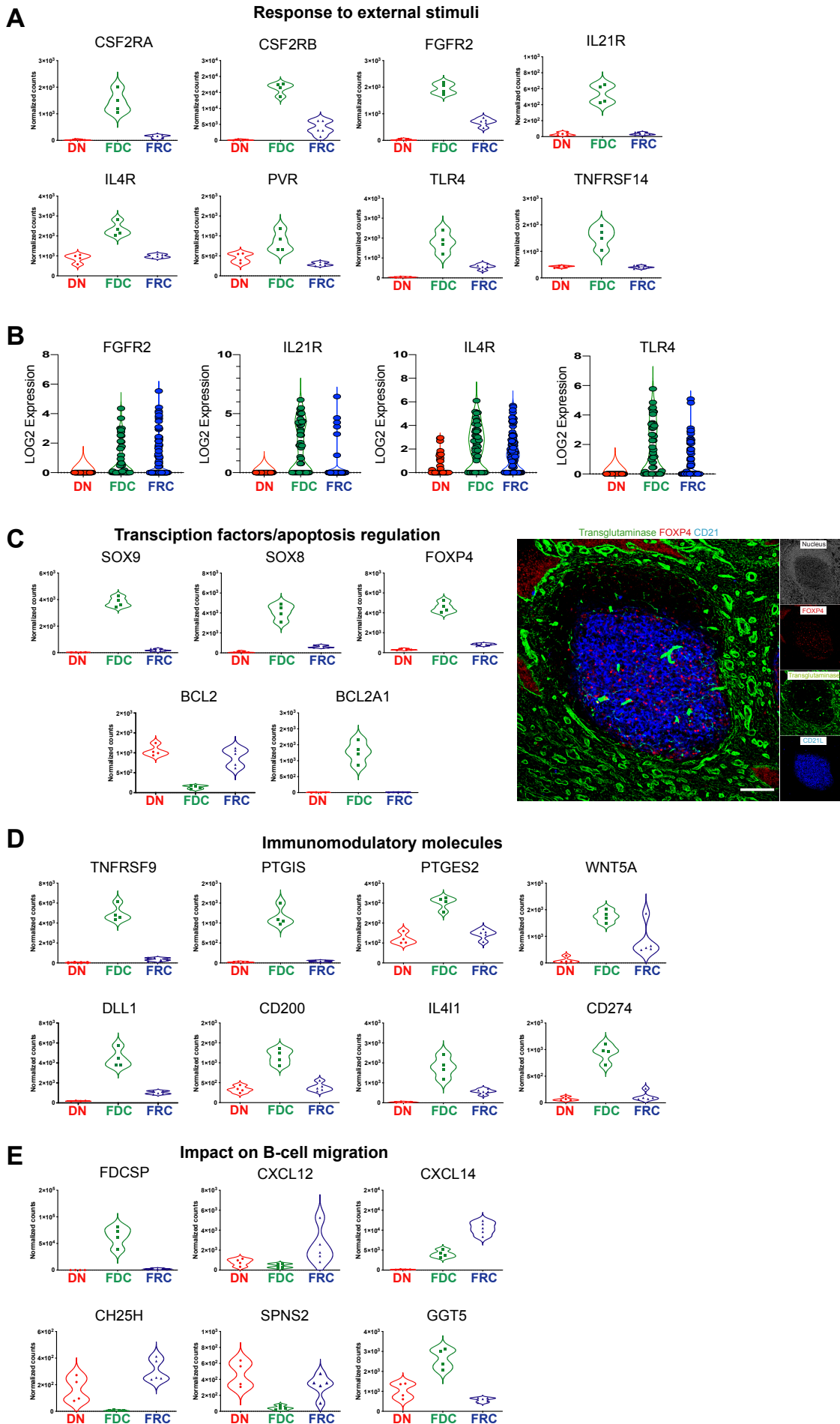


Figure 6. Interactions between FL B cells and LSCs

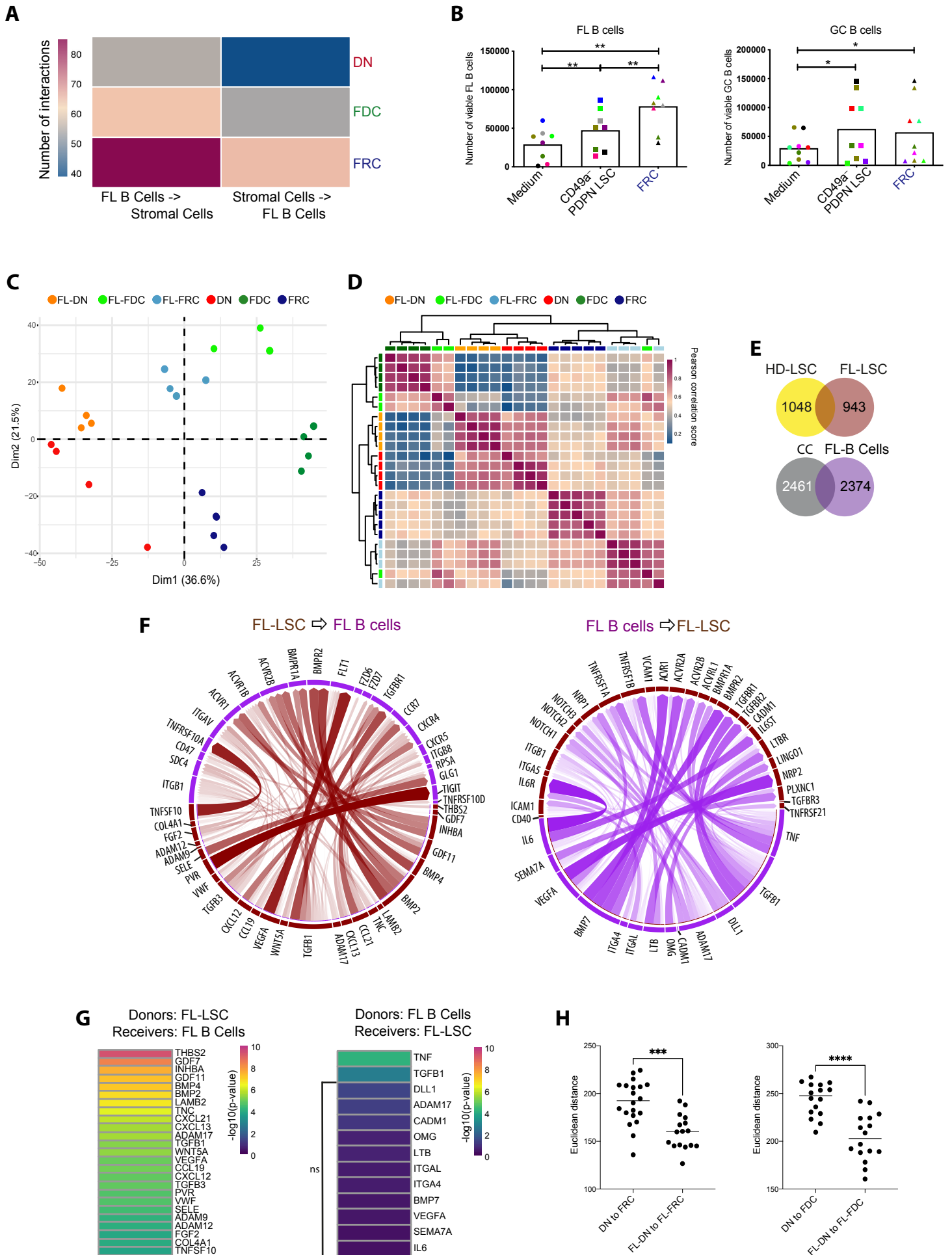
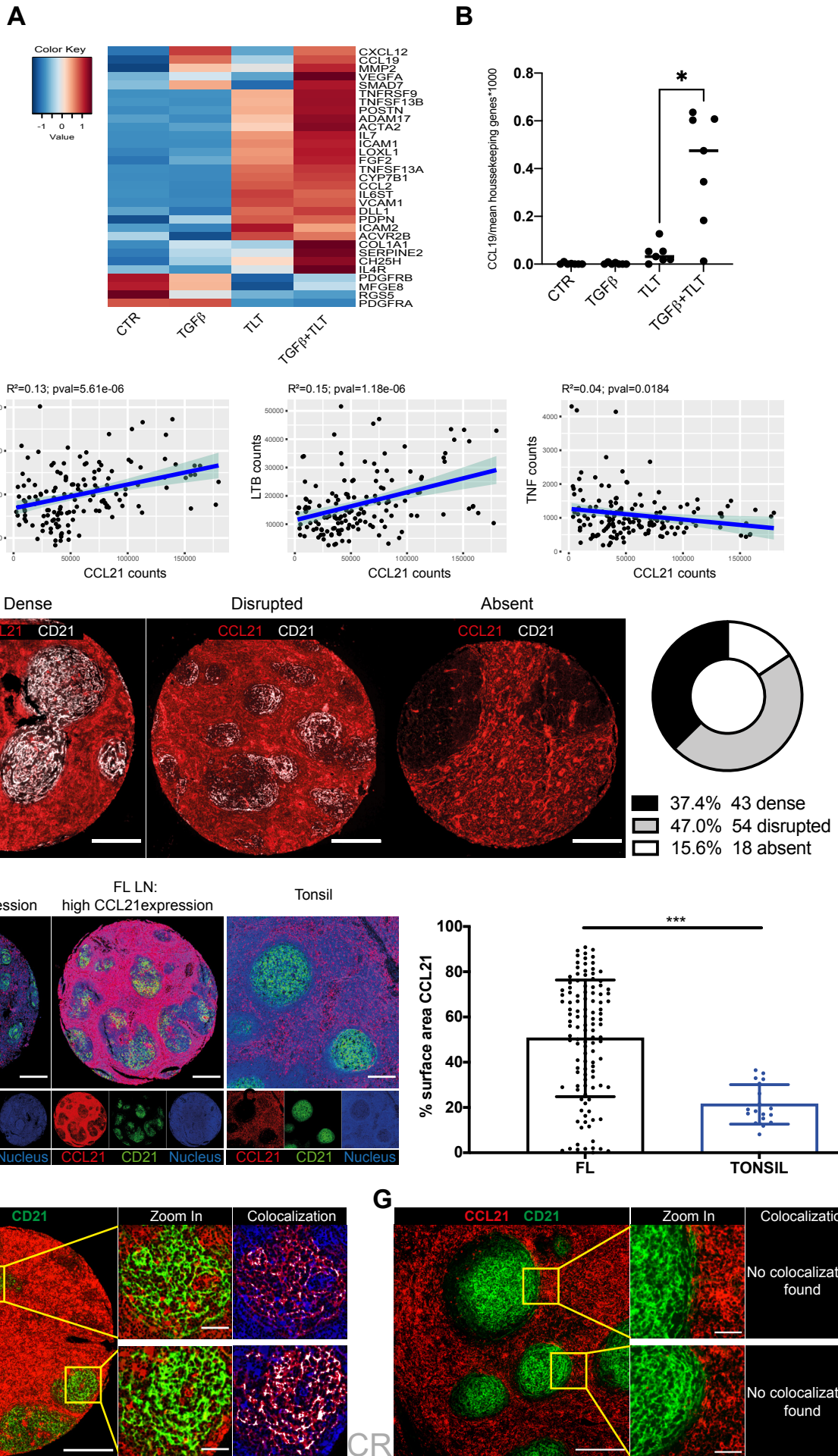


Figure 7. FL-LSC commitment in situ



Supplemental Figure 1. *In situ* and *ex vivo* characterization of human LSCs (related to Figure 1).

A) Architecture of a tonsil section obtained by immunofluorescence after staining for B cells (CD79a, Green), PDPN (Red), and CD21 (Blue). Nuclei were counterstained with SytoxBlue (White). Scale bars, 100 μ m.

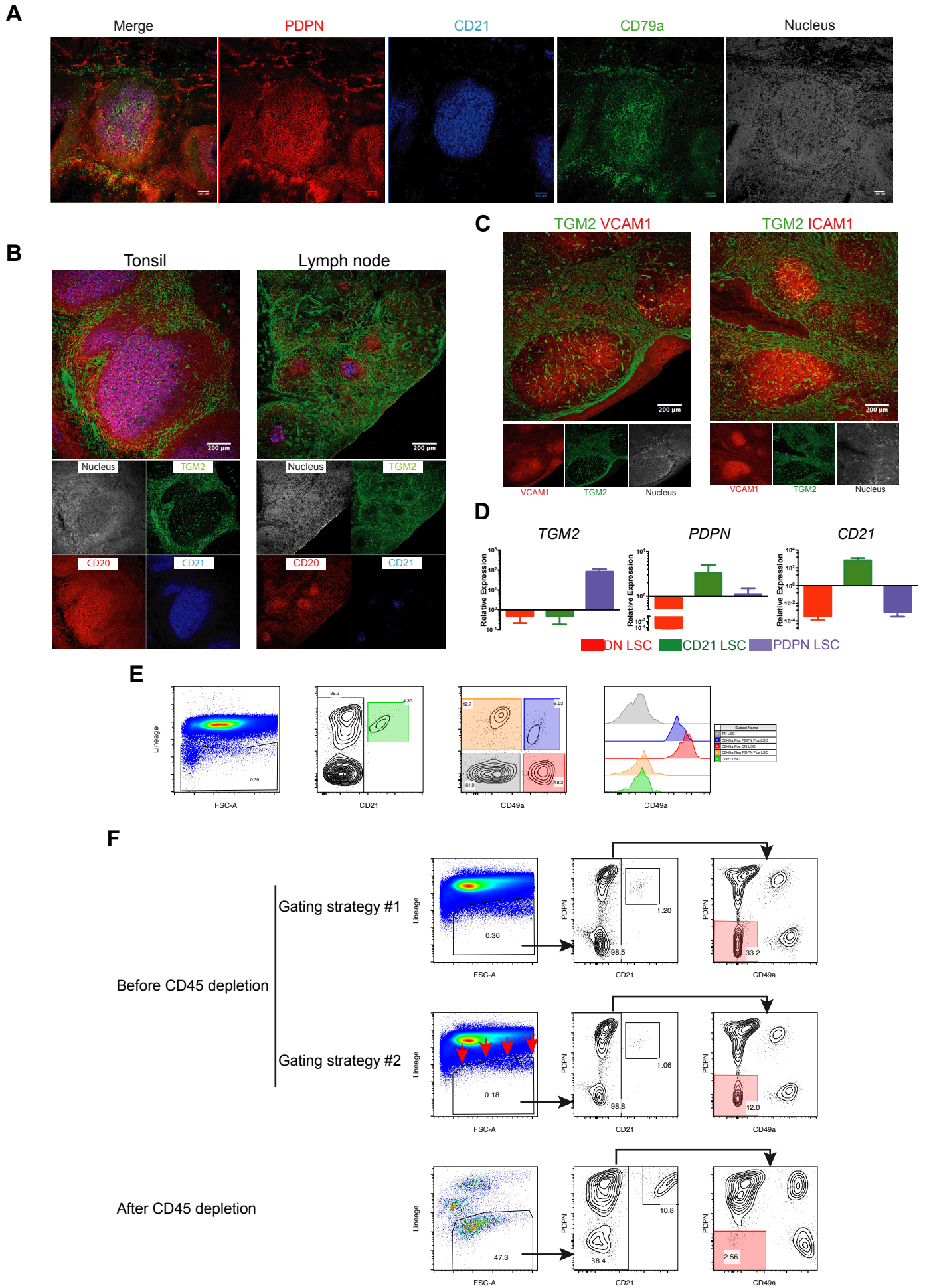
B) Comparison between tonsil and lymph node LSC organization was performed by immunofluorescence for B cells (CD20, Red), TGM2/Transglutaminase (Green), and CD21 (Blue). Nuclei were counterstained with SytoxBlue (White). Scale bars, 200 μ m.

C) Expression of adhesion molecules on tonsil sections. Left: TGM2/Transglutaminase (Green), VCAM1 (Red), and Nucleus (White). Right: TGM2/Transglutaminase (Green), ICAM1 (Red), and Nucleus (White). Scale bars, 200 μ m.

D) Expression of lymphoid stroma markers (*TGM2*, *PDPN*, and *CD21*) was studied by Q-PCR on sorted LSC subsets including DN LSCs (n=3), CD21 LSCs (n=3), and PDPN LSCs (n=3). Data represent the relative expression of corresponding genes as compared to a pool of tonsil cells.

E) Comparison of the CD49a expression on the different LSC subsets by flow cytometry.

F) Analysis of the percentage of PDPN⁻ CD49a⁻ CD21⁻ triple negative stromal cells (TN) by flow cytometry. The percentage of TNs within CD21⁻ LSC was first evaluated before depletion of CD45⁺ hematopoietic cells with non-restrictive (gating strategy # 1) or restrictive (gating strategy # 2) gating strategies to define Lineage⁻ stromal cells. TN percentage was also evaluated after previous depletion of CD45⁺ hematopoietic cells (third line).

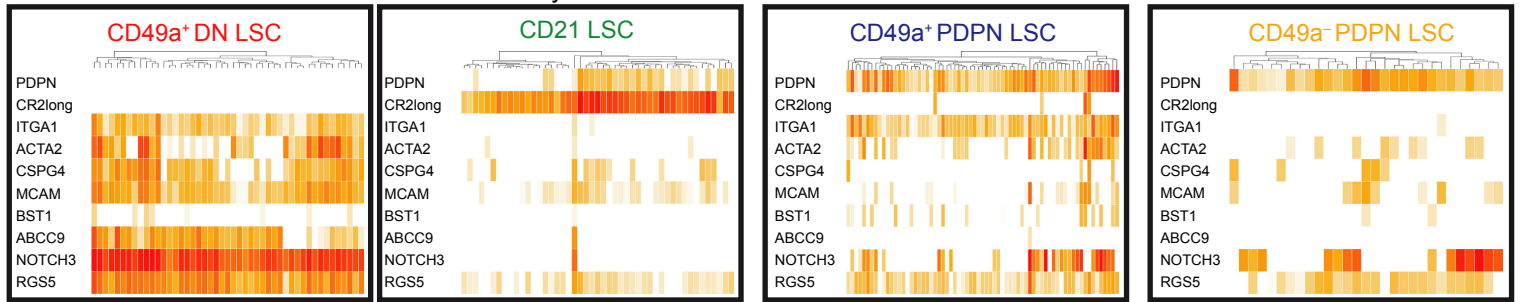
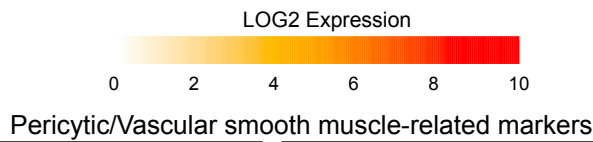


Supplemental Figure 2: Transcriptomic characterization of human LSC subsets (related to Figure 2).

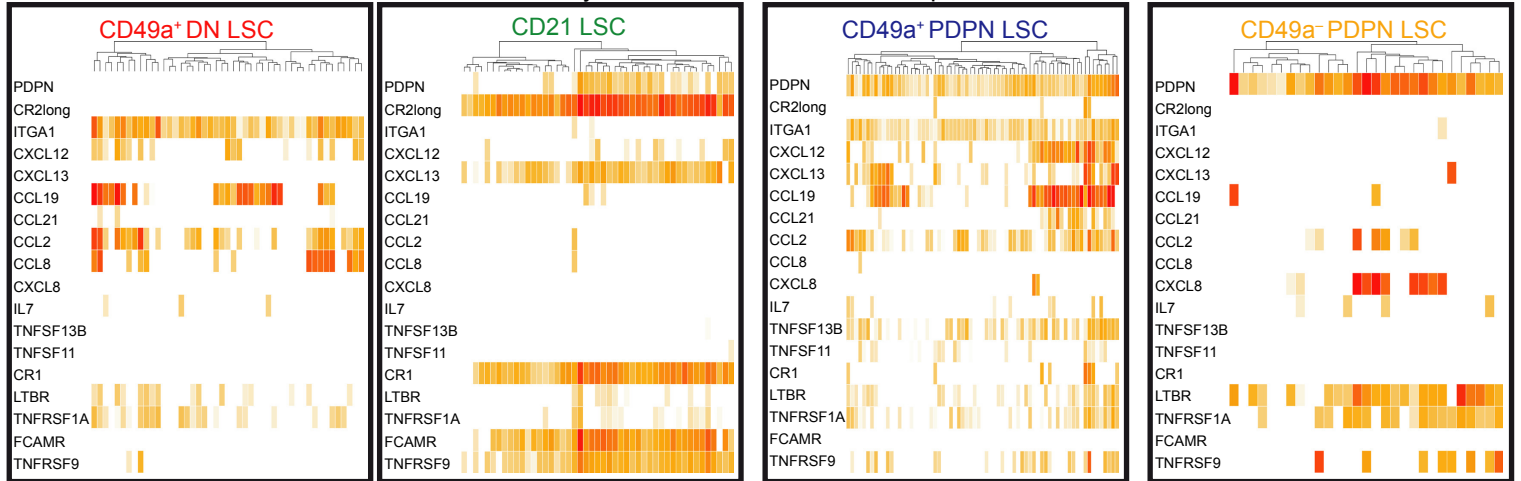
A) Heat maps of LSC subset gene expression. Shown is the Log₂-normalized expression scaled for each gene.

B) The comparison between human and mouse LSC transcriptomes was performed by GSEA. Affymetrix gene expression profiles of FRCs and DNPs from mouse skin lymph nodes were obtained from Immunological Genome Project Consortium (Malhotra et al., 2012). The graphs show the enrichment plots generated by GSEA of ranked gene expression data for the specific murine gene signatures. The gene expression data were preranked based on the wald test used to compare the human (h) samples (hFRC vs hDN and hFDC vs hDN).

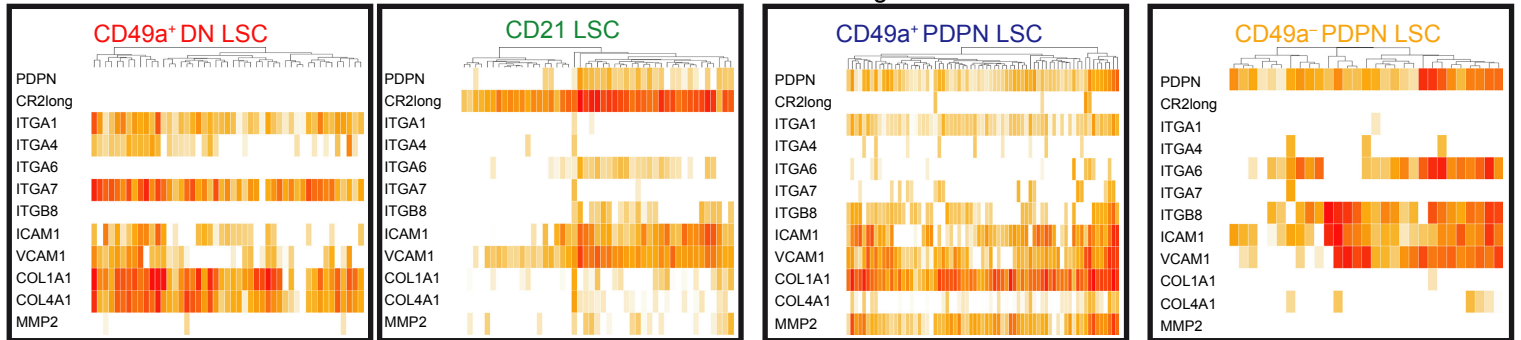
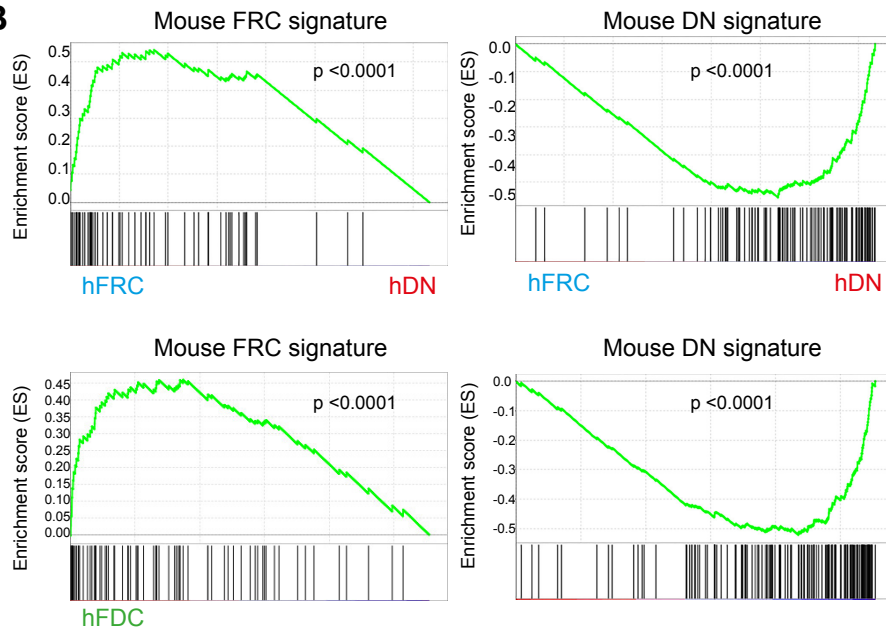
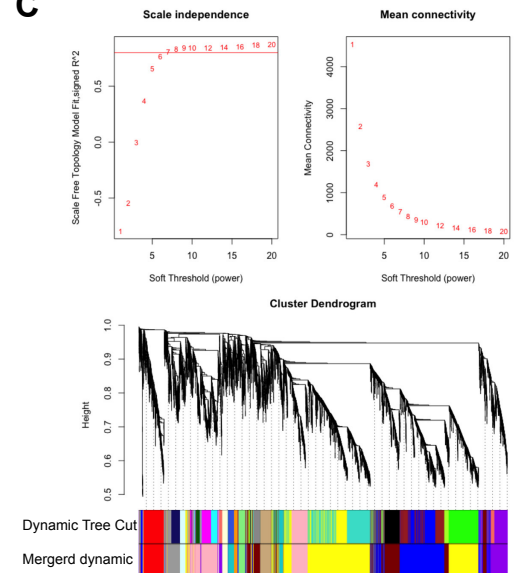
C) WGCNA analysis of human LSC subsets. The soft-thresholding power was selected (upper panels) based on the evolution of the scale free topology index in function of the soft threshold (left) and the analysis of the mean connectivity evolution in function of the soft threshold (right). Lower panel shows the gene clustering dendrogram and module delimitation based on topological overlap, with different colors representing different co-expression gene modules.

A

Cytokines / Chemokines / Receptors



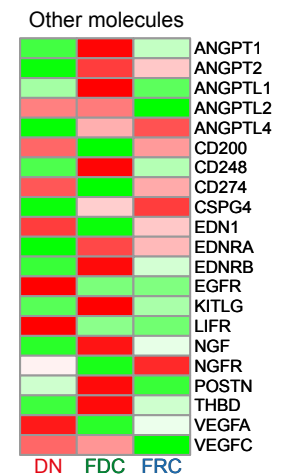
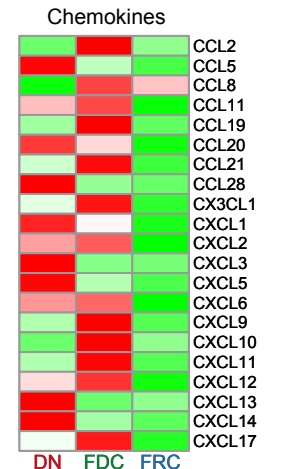
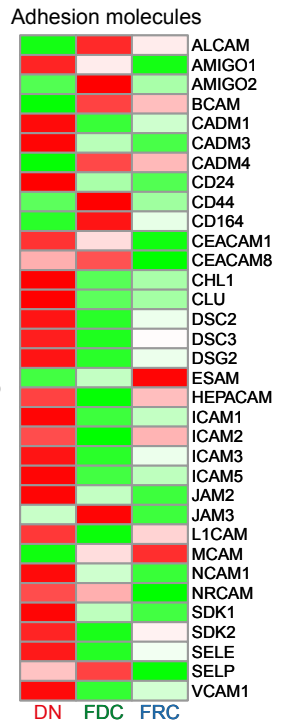
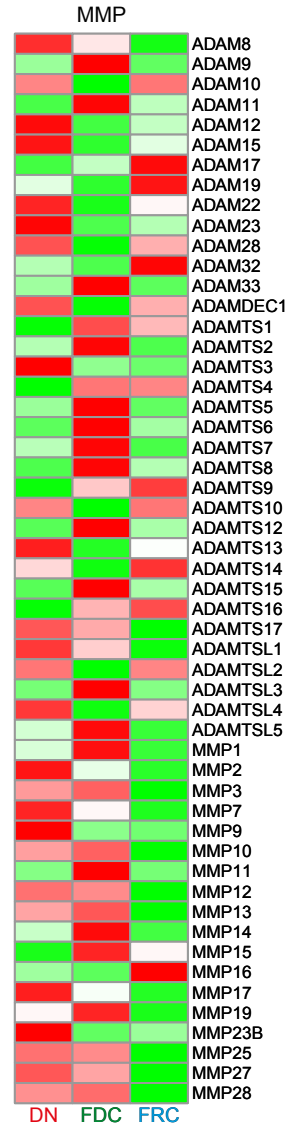
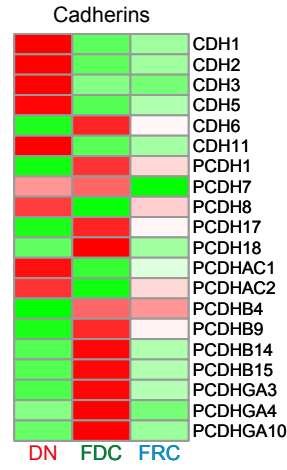
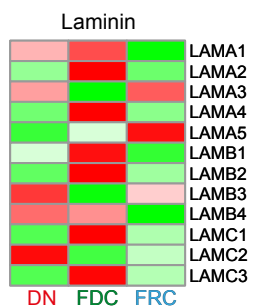
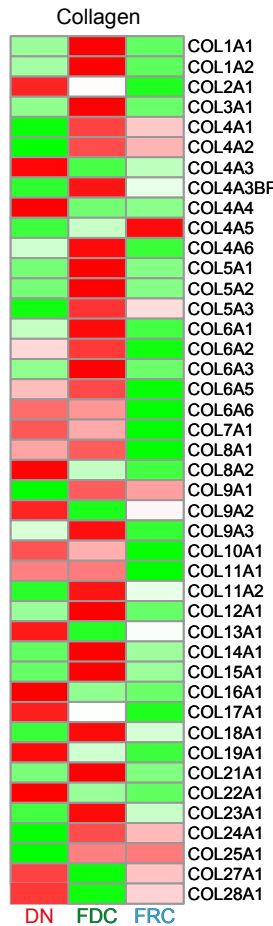
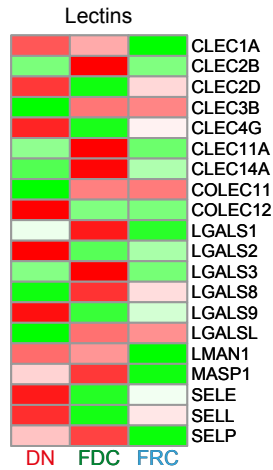
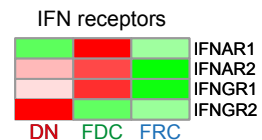
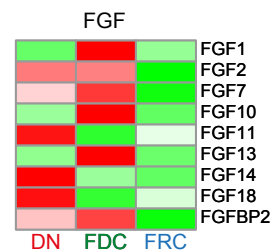
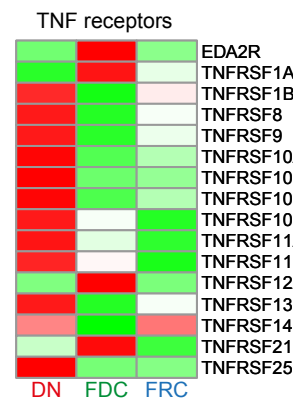
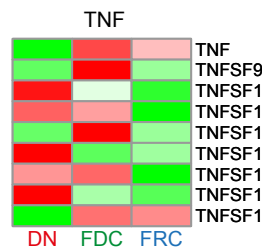
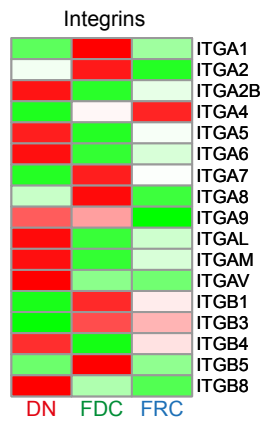
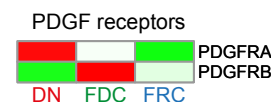
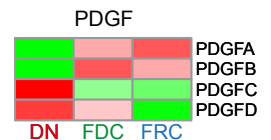
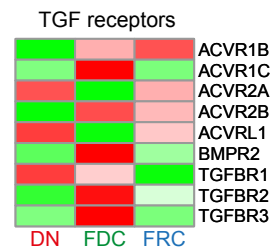
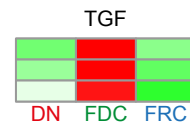
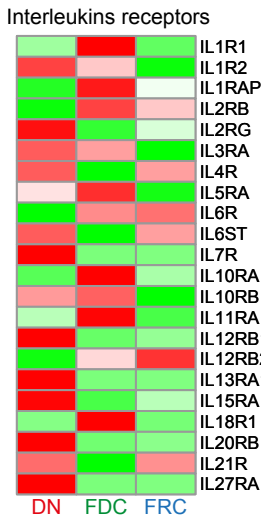
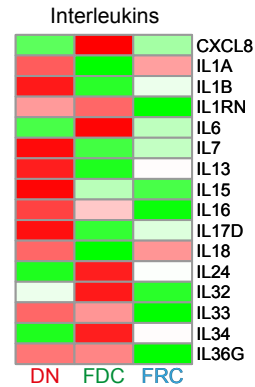
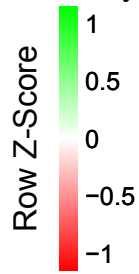
Adhesion molecules / Collagen / ECM

**B****C**

Supplemental Figure 3. Expression of immunologically relevant ligands and receptors by human LSCs (related to Figure 2).

Heat-map analysis of the expression of cytokines/cytokine receptors, growth factors/growth factor receptors, adhesion molecules, extracellular matrix components as evaluated by RNA-seq from cell-sorted DNs (n=4), FDCs (n=4), and FRCs (n=5). Shown is the mean value of log₂-transformed normalized data. Green indicated higher expression.

Color Key

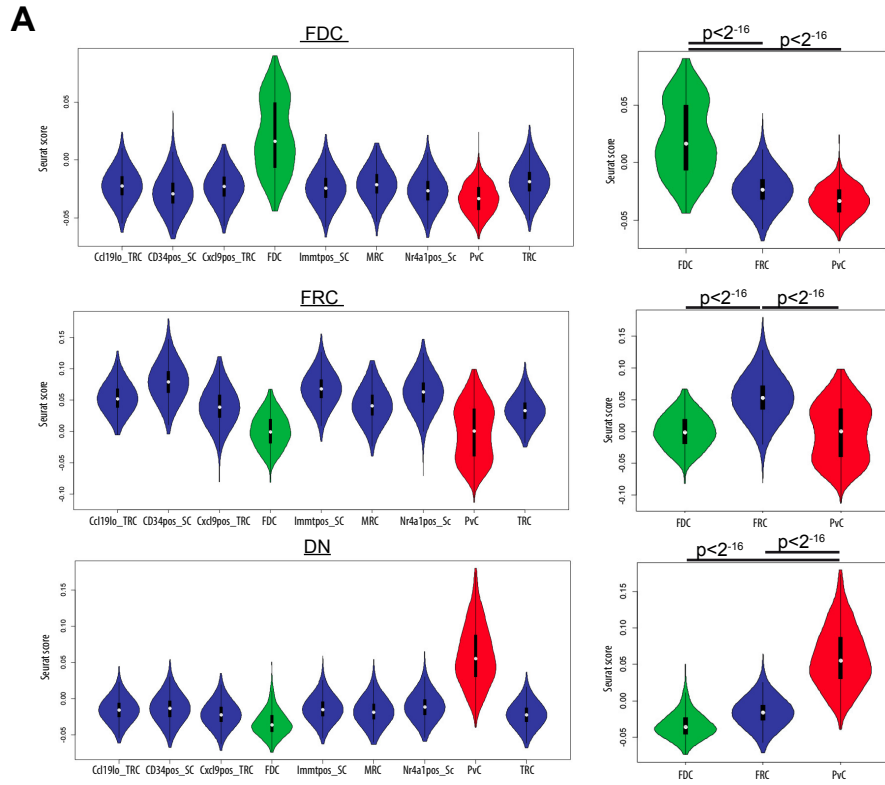


Supplemental Figure 4. Characterization of FRCs (related to Figure 4).

A) Specific gene signatures for FDCs, FRCs, and DNs were compared to signature of mouse LSC subsets previously defined by scRNAseq (Rodda et al., 2016). Seurat score indicating the proximity between human and mouse stromal cell subsets were visualized on violin plots (left panels). Gene signatures of all mouse non-FDC/non-PvC subsets were then merged and Student's t tests were performed to calculate how human FDC, FRC, and DN signatures were significantly enriched in corresponding mouse LSC signatures (right panels).

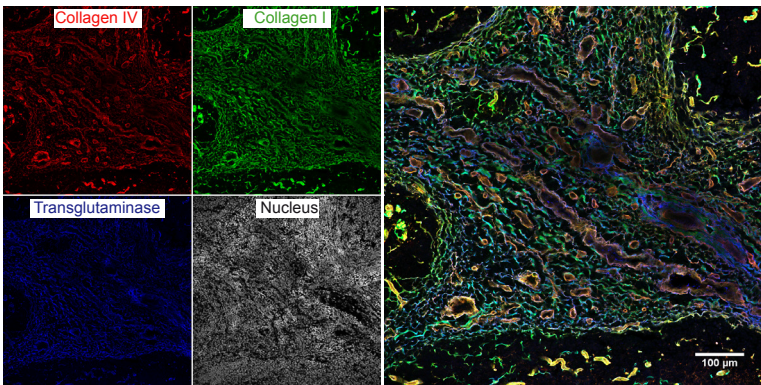
B) Immunofluorescence on tonsil section for Collagen I (Green) Collagen IV (Red), and Transglutaminase (Blue). Nuclei were counterstained with SytoxBlue (White). Left panels: lower magnification. Scale bar, 100 μ m. Right panels: higher magnification. Scale bar, 10 μ m.

C) Expression of stroma markers on LSC subsets after short-term *in vitro* expansion was evaluated by flow cytometry. CD49a⁻ PDPN⁺ LSCs and CD49a⁺ PDPN⁺ LSCs (FRCs) were seeded in culture directly after sorting and analyzed at the end of the first passage before their use for functional experiments. Red histogram shows isotype control and blue histogram the expression of the corresponding marker.

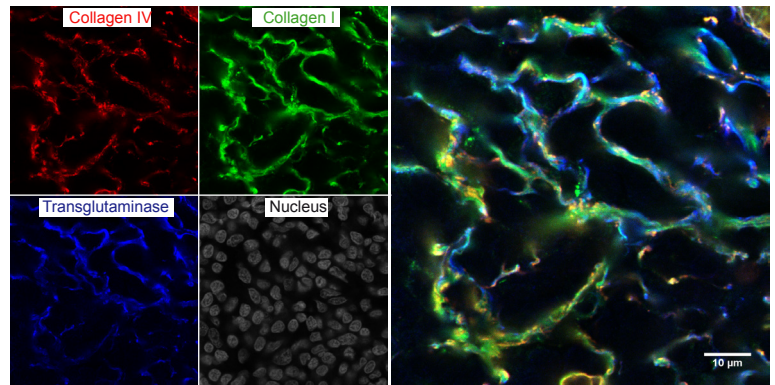


B

Collagen I Collagen IV Transglutaminase

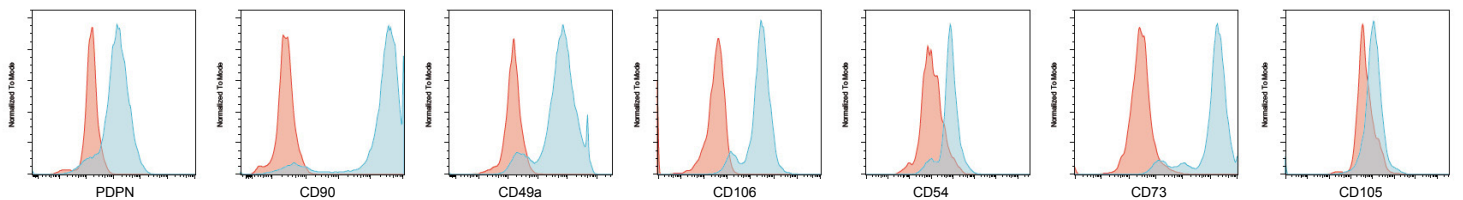


Collagen I Collagen IV Transglutaminase

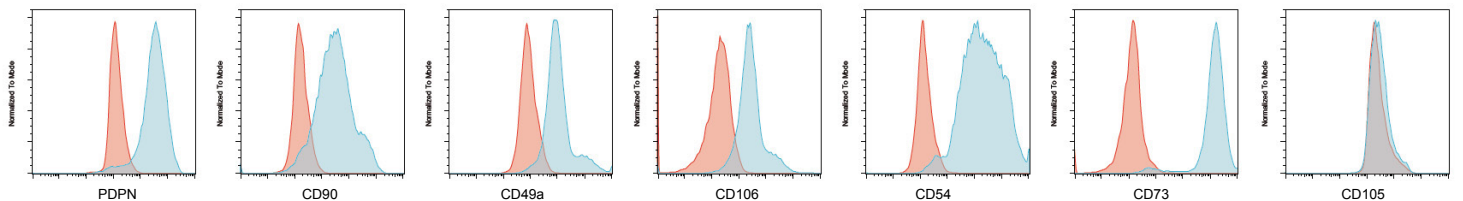


C

CD49a⁺ PDPN LSC



FRCs

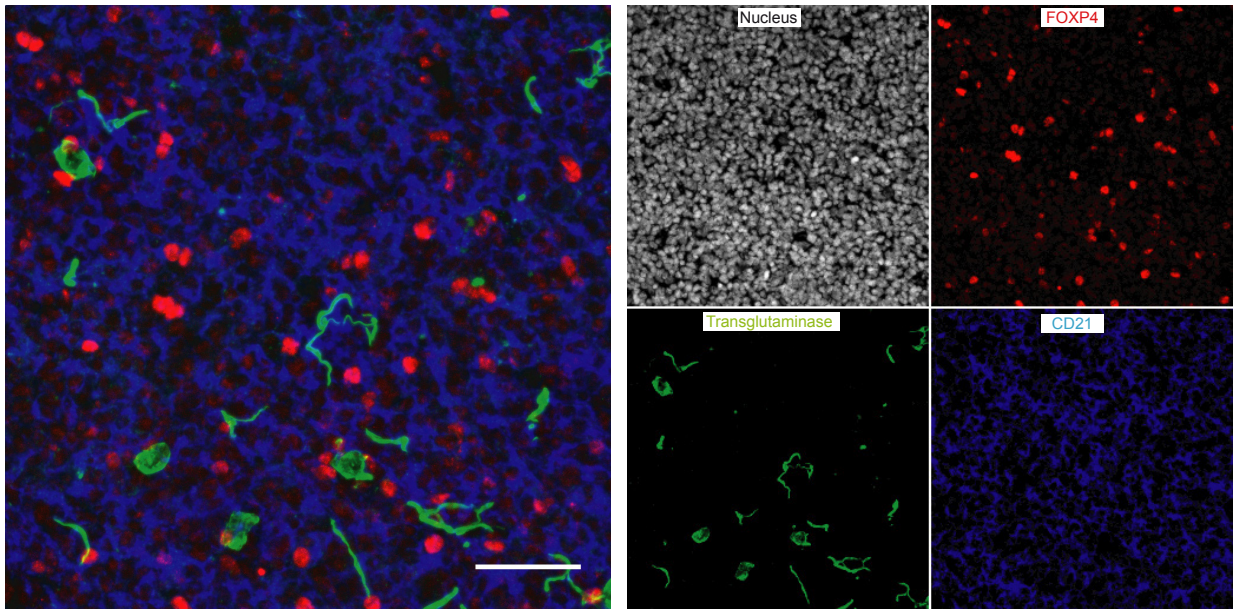


Supplemental Figure 5. FOXP4 is specifically expressed by FDCs (related to Figure 5).

A) Immunofluorescence on section of tonsil GC for TGM2/Transglutaminase (Green) FOXP4 (Red), and CD21 (Blue). Nuclei were counterstained with SytoxBlue (White). Scale bar, 50 μ m.

B) Immunofluorescence on FDC-enriched cells. After enrichment by Percoll gradient, FDCs were fixed and incubated with a control isotype (green) and CD21 (blue, left), or with FOXP4 and CD21 (right). Nuclei were counterstained with SytoxBlue.

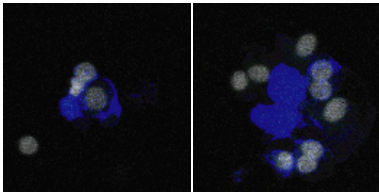
A



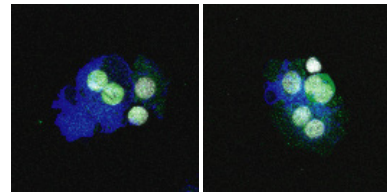
B

FDC-enriched cells

Ctrl CD21 Nucleus



FOXP4 CD21 Nucleus



Supplemental Figure 6. Analysis of FL-LSCs (related to Figure 6).

A) Comparative analysis of secondary lymphoid organs by immunohistofluorescence: reactive lymph node, tonsil, and FL lymph node. Sections were stained for CD20 (Green), CD49a (Red), CD21 (Blue), and Transglutaminase (Cyan). Scale bar, 200 μ m.

B) Chemokine expression was determined in FRCs and FDCs purified from normal *versus* FL samples by RNAseq analysis.

C) Chemokine receptor expression was determined in centrocyte (CC) *versus* FL B cells by microarrays analysis.

D) GC-confinement receptor expression was determined in CC *versus* FL B cells by microarrays analysis.

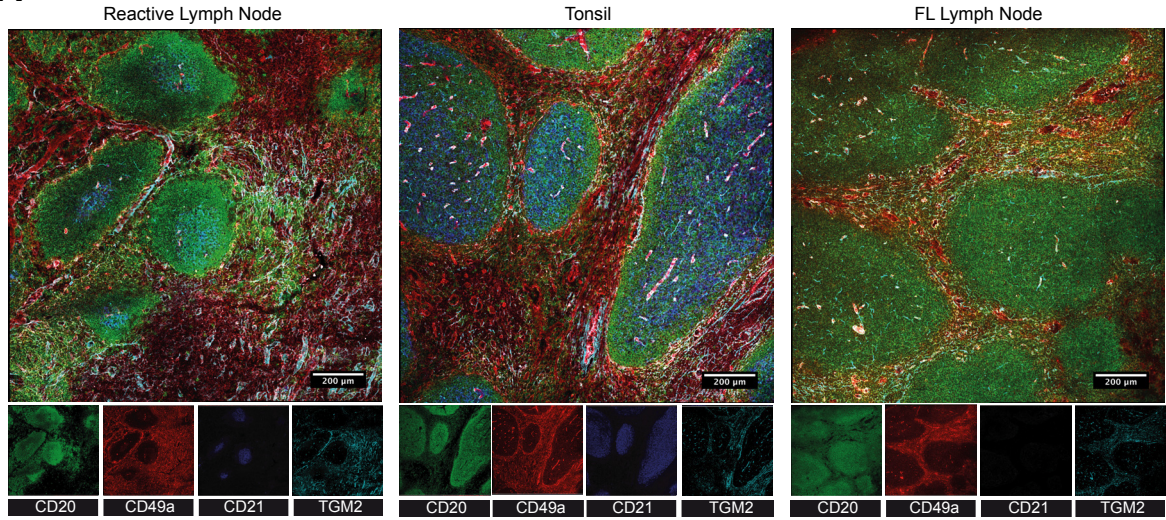
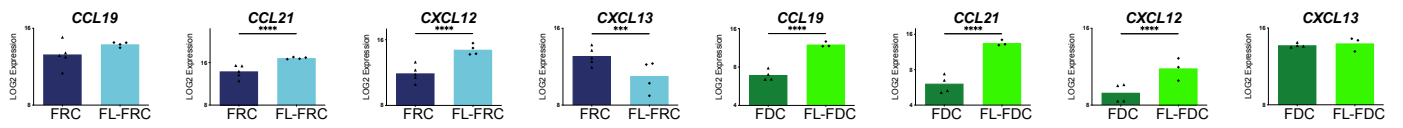
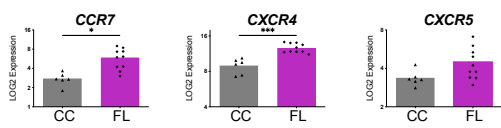
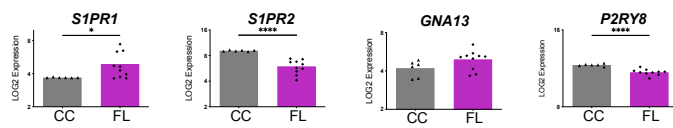
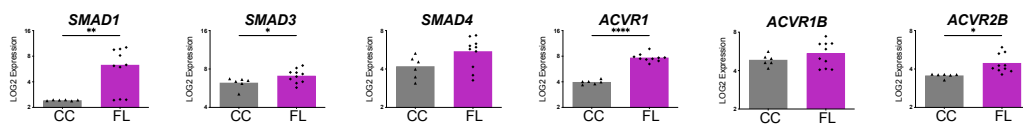
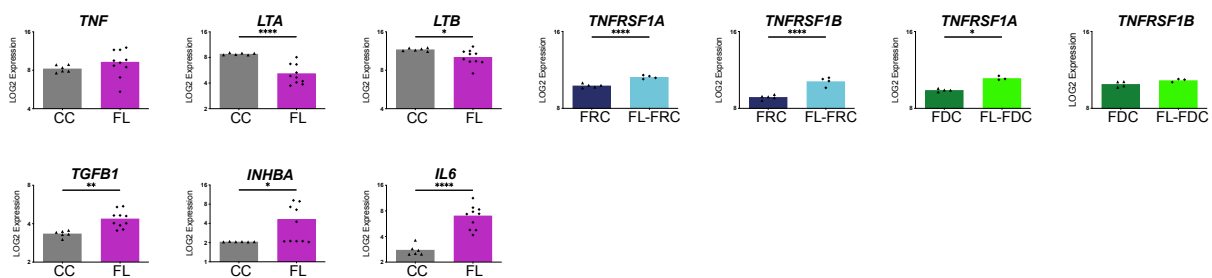
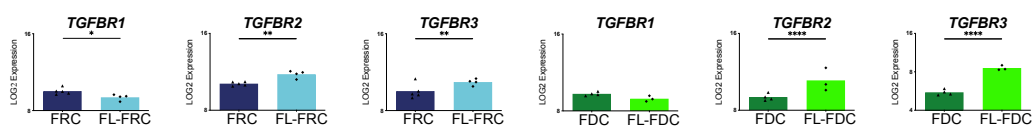
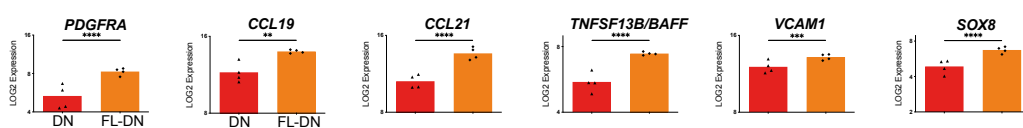
E) Expression of receptors and signaling molecules involved in the TGF β pathway was determined in CC *versus* FL B cells by microarrays analysis.

F) Expression of cytokines was determined in CC *versus* FL B cells by microarrays analysis. Expression of TNF receptors was determined in FRCs and FDCs purified from normal *versus* FL samples by RNAseq analysis.

G) TGF β receptor expression was determined in FRCs and FDCs purified from normal *versus* FL samples by RNAseq analysis.

H) Expression of mature LSC markers in DN *versus* FL-DNs cells was determined by RNAseq analysis.

B-H) * $P < .05$, ** $P < .01$, *** $P < .001$, **** $P < .0001$

A**B****C****D****E****F****G****H**

Supplemental Figure 7. Staining of marginal reticular cells in different secondary lymphoid organs (related to Figure 6).

A) Immunofluorescence on tonsil and FL lymph node (LN) sections for CD20 (Green) TRANCE (Red), and Transglutaminase (Blue), Nuclei were counterstained with SytoxBlue (White). Scale bar, 200 μ m.

B) Immunofluorescence on a reactive LN section for CD20 (Green) TRANCE (Red), and Transglutaminase (Blue). Nuclei were counterstained with SytoxBlue (White). Left panel: scale bar, 200 μ m. Right panel: scale bar, 40 μ m.

