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Supplementary information

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Supplementary Notes

A single-cell atlas of non-haematopoietic cells in human lymph nodes and lymphoma reveals a landscape of stromal remodelling

Yoshiaki Abe[1], Mamiko Sakata-Yanagimoto[2,3,4]*, Manabu Fujisawa[2], Hiroaki Miyoshi[5], Yasuhito Suehara[3], Keiichiro Hattori[3], Manabu Kusakabe[2,3], Tatsuhiro Sakamoto[2,3], Hidekazu Nishikii[2,3], Tran B. Nguyen[2], Yohei Owada[6], Tsuyoshi Enomoto[6], Aya Sawa[7], Hiroko Bando[8], Chikashi Yoshida[9], Rikako Tabata[10], Toshiki Terao[10], Masahiro Nakayama[11], Koichi Ohshima[5], Kensuke Usuki[12], Tatsuya Oda[6], Kosei Matsue[10], and Shigeru Chiba[2,3]*

[1] Department of Hematology, Graduate School of Comprehensive Human Sciences, University of Tsukuba.

[2] Department of Hematology, Faculty of Medicine, University of Tsukuba.

[3] Department of Hematology, University of Tsukuba Hospital.

[4] Division of Advanced Hemato-Oncology, Transborder Medical Research Center, University of Tsukuba.

[5] Department of Pathology, School of Medicine, Kurume University.

[6] Department of Gastrointestinal and Hepato-Biliary-Pancreatic Surgery, Faculty of Medicine, University of Tsukuba.

[7] Department of Breast-Thyroid-Endocrine Surgery, University of Tsukuba Hospital.

[8] Department of Breast and Endocrine Surgery, Faculty of Medicine, University of Tsukuba.

[9] Department of Hematology, National Hospital Organization, Mito Medical Center.

[10] Division of Hematology/Oncology, Department of Internal Medicine, Kameda Medical Center.

[11] Department of Otolaryngology, Head and Neck Surgery, University of Tsukuba.

[12] Department of Hematology, NTT Medical Center Tokyo.

** Correspondence*

1 **Related to "Human LN BECs: ten subclusters"**

2 The following is a comment regarding BEC subcluster-specific marker genes.

3 ABECs are characterized by selective expression of *GJA5* (Fig. 2h,i; Extended Data Fig. 2a),
4 defined as a marker of larger arteries in murine tissues, including LNs[1,2]. By contrast, caBECs
5 specifically express *SSUH2* (Fig. 2h,i; Extended Data Fig. 2a), a marker of arteries of varying size in
6 different mouse tissues[1]. aBECs are characterized by expression of *INSR* (Fig. 2h; Extended Data
7 Fig. 2a) and *EDNRB* (Fig. 2i), both capillary markers in mouse livers, lungs, and colon[1]. tBECs
8 belong to a small but highly distinct subcluster that selectively expresses *PGF* and *LY6H* (Fig. 2h,i;
9 Extended Data Fig. 2a). *PGF* is described as an endothelial tip cell marker in human lung cancer[3],
10 whereas *LY6H* has not been shown to be a specific marker in mice or humans[1]. Other human tip cell
11 markers, including *CXCL4*, *PXDN*, and *LXN*[3], were identified as DEGs in tBECs (Fig. 2h;
12 Supplementary Table 5). cBECs are characterized by abundant *PLVAP* and *CA4* expression (Fig.
13 2h,i; Extended Data Fig. 2a), in agreement with mouse data describing them both as capillary
14 markers in multiple tissues[1]. C-aHEVs express DEGs of cBECs (including *CA4*) and aHEVs (Fig.
15 2h,i; Supplementary Table 5), indicative of the transitioning nature of C-aHEVs. Also, *HES1*, a
16 capillary marker in several mouse tissues, was highly expressed in C-aHEVs (Fig. 2h)[1]. C-aHEVs
17 and aHEVs express genes encoding heat shock proteins (*HSPA1A*, *HSP90AA1*, and *HSPA1B*), as
18 well as those involved in NFkB (*NFKBIA* and *NFKBIZ*) and JNK (*JUN*, *JUNB*, *JUND*, *FOS*, and

19 *FOSB*) activation. They also express genes associated with oscillatory shear stress (*EGR1*, *ICAM1*,

20 *MT2A*, *MT2X*, and *ADM*) at higher levels than do cBECs and hHEVs (Extended Data Fig. 2b;

21 Supplementary Tables 5,6)[4]. aHEVs, hHEVs, and CXCL10-HEVs all express genes specific to

22 HEVs, including *ACKR1*, *C7*, and *CH25H* (Supplementary Table 5)[5]. CXCL10-HEVs are unique in

23 their expression of the chemokines *CXCL9*, *CXCL10*, and *CXCL11* (Fig. 2h,i). Some suggest that a

24 subset of HEVs express *CXCL10*, which facilitates trafficking of activated T cells and monocytes

25 across HEVs[6,7], though others have not detected *CXCL10* mRNA in HEVs[8]. Therefore, the CXCL10

26 function in HEVs remains unclear[9]. Mouse LNs have a BEC subpopulation with highly similar

27 phenotypes to CXCL10-HEVs that exhibit a prominent interferon signalling signature, although they

28 were classified as capillary BECs[2]. VBECs are characterized by high expression of *CPE* and

29 *VCAM1* (Fig. 2h; Extended Data Fig. 2a; Supplementary Table 5), which are markers of large

30 veins[1,2].

31 We examined the expression of marker genes for mouse LN BEC subclusters[2] in our human

32 BEC data and summarized an assumed correspondence relationship between mouse and human LN

33 (Extended Data Fig. 2k,l).

34

35 **Related to "Human LN LECs: eight subclusters"**

36 The following discussion addresses results of unsupervised clustering in MFLN LECs, DEG

37 analysis across subclusters, trajectory analysis, and IF staining.

38 Putative cLECs (LEC I), particular SCS ceiling LECs that cover medullary regions (III),

39 capillary LECs in surrounding tissues (IV), and valve LECs (V) were observed in our clustering, as

40 suggested by Takeda et al and Xiang et al[10,11], though we annotated LECs III and IV as collectLECs

41 and msLECs, respectively (Fig. 3a). We observed several slight differences between our analysis and

42 previous analyses[10,11]. First, our analysis detected a subcluster in which gene expression patterns

43 matched those seen in bLECs, a cell type proposed by Xiang et al (e.g. intermediate *BMP2*

44 expression between cLECs and fLECs) (Fig. 3c; Extended Data Fig. 3a)[11], although bLECs were not

45 detected by Takeda et al (Fig. 3e)[10]. Second, we initially could not identify fLECs and pfsLECs as

46 distinct subclusters[10,11]. Lastly, two valve LEC subclusters (Up- and Down-valves) were identified as

47 distinct, in contrast to a previous study that identified them after applying sub-clustering analysis to

48 an initially-identified valve cluster (LEC V) (Fig. 3e)[10]. We speculate that these inconsistencies are

49 due to the fact that previous studies collected LEC-enriched cells based on PDPN positivity in flow

50 cytometric analysis, and thus LECs with relatively low PDPN expression may have been

51 overlooked. Indeed, *PDPN* expression was particularly low in bLECs and down-valves (Extended

52 Data Fig. 3a).

53 DEG analysis has revealed that the “fLEC and pfsLEC” subcluster is unique in harbouring

54 more than twice as many DEGs as other LEC subclusters (Extended Data Fig. 3b). These DEGs

55 include chemokine genes such as *CXCL1*, *CXCL5*, and *CCL20*, and genes associated with immune
56 cell adhesion, including *ICAM1*, *VCAM1*, and *SELE* (Extended Data Fig. 3a,c; Supplementary Table
57 8), supporting the immunological function of fLECs in cell trafficking and migration. Genes
58 reportedly specific to pfsLECs (e.g. *MARCO* and *CLEC4G*[11]) were also detected as top DEGs for the
59 "fLEC and pfsLEC" subcluster (Extended Data Fig. 3a,c; Supplementary Table 8)[10,11]. *ACKR4*,
60 *MFAP4*, *CCL21*, *CLDN11*, and *GJA4* were also consistently identified as top DEGs for cLECs,
61 collectLECs, msLEC, valve LECs (both Up- and Down-valves), and down-valves, respectively (Fig.
62 3c; Extended Data Fig. 3a,c)[10].
63 To verify that a single "fLEC and pfsLEC" subcluster consists of both fLECs and pfsLECs,
64 we applied unbiased sub-clustering analysis to the "fLEC and pfsLEC" subcluster. As expected, it
65 comprised two transcriptionally distinct populations (Extended Data Fig. 3d). In agreement with the
66 findings of Takeda et al, the proportion of pfsLECs was smaller than that of fLECs (Extended Data
67 Fig. 3e)[10]. Notably, in DEG analysis between fLECs and pfsLECs, genes described as specific to
68 LEC II and VI[10] were separately detected as top DEGs for fLECs and pfsLECs, respectively
69 (Extended Data Fig. 3f; Supplementary Table 9).
70 Next, we performed trajectory analysis on integrated LEC data. We identified all LEC
71 subclusters in a *Monocle 3*-generated cell object (Extended Data Fig. 3g). As expected, bLECs and
72 collectLECs were adjacent to cLECs (Extended Data Fig. 3h). Remarkably, a trajectory from fLEC

73 and pfsLEC subclusters connected with remaining LEC subclusters in the middle region of bLECs

74 and msLECs, and a direct trajectory connection was observed between pfsLECs and msLECs

75 (Extended Data Fig. 3h), supporting positional relationships inferred across these subclusters[11]. Both

76 Up- and Down-valves were connected to collectLECs (Extended Data Fig. 3h), supporting the

77 annotation of collectLECs (Fig. 3d).

78 IF staining showed that PAI1- (encoded by *SERPINE1*) positive bLECs were exclusively

79 embedded in the intermediate area of the SCS (Extended Data Fig. 3i). PTX3-positive LECs

80 (msLECs) were preferentially located at the medulla (Extended Data Fig. 3j), although a small

81 proportion of cLECs also showed PTX3 positivity (Fig. 3c; Extended Data Fig. 3j). MARCO-

82 positive LECs (pfsLECs) were preferentially situated at perifollicular sinuses (Extended Data Fig.

83 3k), as shown in mice[11]. MFAP4-positive LECs (collectLECs) were observed only in afferent and

84 efferent collecting vessels (Extended Data Fig. 3l).

85

86 **Related to "Human LN NESCs: twelve subclusters"**

87 The following discussion addresses results regarding the annotation of TRCs, PCs, MRCs, and

88 FDCs.

89 DEG analysis showed that TRCs were marked by high *CCL19* and *CCL21* expression (Fig.

90 4d,e; Supplementary Table 10)[12], and PCs by specific expression of *NDUFA4L2* and *HIGD1B* (Fig.

91 4d,e; Supplementary Table 10)[13]. Approximately half of MRC DEGs were seen in FDC DEGs
92 (Supplementary Table 10), consistent with the idea that MRCs differentiate into FDCs[14]. FDC
93 markers *FDCSP*, *CR2*, and *FCER2* were correctly detected as FDC-specific DEGs (Fig. 4d,e;
94 Supplementary Table 10).

95 GO analysis revealed that TRCs were enriched with GOs related to leukocyte and dendritic
96 cell migration (Fig. 4i; Supplementary Table 12), in accordance with their reported function in
97 recruiting naive T cells and dendritic cells to the T-cell zone to facilitate interaction[12]. Genes
98 associated with smooth muscle structure or contraction were enriched in PCs and in SMC
99 subclusters, whereas PCs abundantly expressed genes associated with blood vessel development
100 (Fig. 4i; Supplementary Table 12). MRCs and FDCs were highly enriched with GOs relevant to
101 cytokine- and chemokine-mediated signalling and antigen processing and presentation (Fig. 4i;
102 Supplementary Table 12), consistent with their role in positioning and presenting antigens to B
103 cells[12]. Finally, FDCs expressed genes involved in immunoglobulin-mediated immune response and
104 B-cell-mediated immunity (Fig. 4i; Supplementary Table 12)[12].

105 IF staining showed HIGD1B-positive cells (PCs) were observed around BECs, including
106 arteries and HEVs (Extended Data Fig. 4n), and were more prevalent around HEVs in IFRs (aHEVs)
107 than around hHEVs (Extended Data Fig. 4n), indicating that they may support lymphocyte
108 recruitment and maintain endothelial cell integrity[12]. MRCs and FDCs were identified as BAFF- and

109 CR2 (also known as CD21)-positive, respectively (Extended Data Fig. 4o).

110 We examined the expression of key genes for mouse LN NESC subclusters[15] in our human

111 NESC data and assumed a correspondence relationship between mouse and human LNs. Although

112 some degree of correlation was detected between mouse and human LN NESCs, we observed more

113 complicated heterogeneity in human LN NESCs than in mouse LNs and found that mouse NESC

114 markers were not necessarily specific to human NESC counterparts (Extended Data Fig. 4q,r).

115

116 **Additional basic information of the single-cell LNNHC atlas**

117 All subclusters identified in the single-cell LNNHC atlas included cells from all MFLN samples

118 with some variations in cell proportion (Extended Data Fig. 5a). Notably, NHCs from a patient with

119 a benign tumour (MFLN 8) were distributed uniformly among NHCs from all MFLN samples

120 (Extended Data Fig. 5b). Furthermore, in NHC subclusters harbouring at least 50 cells from the

121 sample MFLN 8, 80–100% of the top DEGs for all MFLN sample subclusters matched MFLN 8

122 DEGs (Extended Data Fig. 5c).

123 We also profiled DEGs between mLN and pLN NHC subclusters using MFLN data

124 (Supplementary Table 13). In this dataset, we observed markedly higher expression of *MADCAM1*

125 in mLN HEV subclusters (aHEVs and hHEVs) compared with pLN counterparts (Extended Data

126 Fig. 6a; Supplementary Table 13), in agreement with MADCAM1-dependent manners of

127 lymphocyte homing in mLN HEVs[7]. Consistently, GO analysis of DEGs between mLN and pLN
128 subclusters showed that mLN HEV subclusters were enriched with *MADCAM1*-containing GO
129 terms associated with cell adhesion and interactions with integrin (Extended Data Fig. 6b;
130 Supplementary Table 14). We also found that multiple mLN NHC subclusters (particularly, NESC
131 subclusters) highly expressed genes associated with inflammatory response including *PTX3*, *CCL2*,
132 and *TNFAIP6* relative to pLN counterparts (Extended Data Fig. 6a,b; Supplementary Table 13).
133 Consistent with the constant exposure of mLNs to enterobacteria-derived factors, GO terms
134 associated with inflammatory response, TNF-signalling, and response to molecules of bacterial
135 origin were enriched in mLN LEC and NESC subclusters (Extended Data Fig. 6b; Supplementary
136 Table 14). Additionally, pLN LEC and NESC subclusters abundantly expressed genes associated
137 with the maintenance of LN homeostasis and structures (Extended Data Fig. 6a,b; Supplementary
138 Tables 13,14).
139
140 **Additional prognostic analyses**
141 We tested the prognostic value of the markers proposed in our analyses (*LY6H*, *LOX*, *TDO2*, and
142 *REM1*) in the intermediate prognosis group of FL. Notably, we found that elevated *LOX* and *TDO2*
143 expressions were prognostic even in the intermediate prognosis group, while the prognostic

144 efficiency of *LY6H* expression was slightly not significant and that of *REM1* expression was not

145 prognostic (Extended Data Fig. 9f).

146 Since these prognostic markers were derived primarily from TRCs or tBECs, we developed a

147 FL TRC signature (Supplementary Table 19) and evaluated its prognostic proficiency. However, we

148 could not extract sufficient number of genes for developing a FL tBEC signature. As a result, we

149 observed no significant difference in the overall survival of patients with high and low FL TRC

150 signature (data not shown).

151 The upregulation of tip cell markers (*LY6H* and *LOX*) was prognostic in FL (Fig. 7a,b),

152 suggesting that enhanced tumour angiogenesis might affect FL prognosis, hence we also examined

153 the prognostic impact of conventional pan-BEC markers, *PECAM1* and *CDH5*[16]. However, we did

154 not detect any adverse prognostic impact of these markers in the microarray dataset[17] (Extended

155 Data Fig. 9g).

156

157 **Related to "Observation of NHC subclusters across lymphomas"**

158 The following addresses findings observed in scRNA-seq analysis of PTCL and tDLBCL stroma.

159 Unsupervised clustering analysis of integrated MFLN and lymphoma NHC data detected

160 almost all NHC subclusters in both PTCL and tDLBCL (Extended Data Fig. 10a–d). Furthermore,

161 we found that BECs and LECs in these lymphomas consistently increased and decreased,

162 respectively, as observed in FL (Extended Data Fig. 10b,d). tDLBCL BECs contained abundant

163 arterial subclusters and CXCL10-HEVs (Extended Data Fig. 10d), suggesting accelerated

164 angiogenesis and elevated HEV activities. Furthermore, we observed considerable alterations in the

165 tDLBCL NESC proportions compared with the MFLN counterparts: the proportion of advSCs,

166 medullary SCs, TNF-SCs, C7-SCs, AGT-SCs, and TRCs decreased considerably, whereas that of

167 FSCs (MRCs and FDCs) increased drastically in tDLBCL, and the perivascular cell subcluster

168 compositions remained relatively comparable (Extended Data Fig. 10d). Representative DEGs

169 upregulated in FL subclusters, including *LY6H* and *LOX* at tBECs, *SELE* at HEV subclusters, and

170 *VCAM1* at FSCs, were also observed in tDLBCL stroma, although some key DEGs were not

171 validated, probably because of the small sample size of tDLBCL cohort (Extended Data Fig. 10e). In

172 line with the findings of analyses on MFLN and FL NESCs (Fig. 4g,h, 5a), trajectories in tDLBCL

173 NESCs indicated that non-FSC subclusters had differentiated into FSCs (Extended Data Fig. 10f–h),

174 likely representing a terminal form of stromal remodelling in FL.

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