Multi-tier mechanics control stromal adaptations in swelling lymph nodes

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Abstract

Lymph nodes (LNs) comprise two main structural elements: Fibroblastic reticular cells (FRCs) that form dedicated niches for immune cell interaction and capsular fibroblasts that build a shell around the organ. While LNs are fairly stable in size during homeostatic conditions, immunological challenge causes more than 10-fold increase in size within only a few days. How a solid organ can accommodate such extreme volumetric changes is poorly understood. Here, we characterize the biomechanics of LN swelling on the cellular and organ scale. We identify lymphocyte trapping by influx and proliferation as drivers of an outward pressure force, causing FRCs and their associated conduits to stretch. After an initial phase of relaxation, FRCs sense the resulting strain via cell matrix adhesions, which coordinates local growth and remodeling of the stromal network. While the expanded FRC network adopts its typical configuration, a massive fibrotic reaction of the organ capsule sets in and counters further organ expansion. Thus, different fibroblast populations mechanically control LN swelling in a multi-tier fashion.

Introduction

Lymph nodes (LNs) are the macroscopic organs of the adaptive immune system. As opposed to other organs, the LN parenchyma only contains few resident cells, while the bulk of lymphocytes is in transit. Lymphocytes circulate through the bodies' vascular systems, and from there they enter LNs via high endothelial venules (HEVs). Within the LN parenchyma, lymphocytes actively migrate to scan large numbers of potentially antigen-bearing dendritic cells (DCs). After several hours of unsuccessful search, lymphocytes exit the organ again via efferent lymphatic vessels that eventually drain back into the blood circulation and the cycle starts again[1,2]. Lymphocytes are densely packed within LNs and make up about 95% of its total cellularity. Despite dynamic cellular exchange, homeostatic LN size remains relatively stable. Known modulators of homeostatic LN cellularity (e.g., during circadian rhythms[3,4]) are mainly chemoattractants and adhesion molecules[2,5–8], which serve as entry and exit signals for lymphocytes, as well as stromal cell-derived survival factors[9–12] and adrenergic signals[13,14]. The main stromal cells found in LNs are FRCs. These form the non-hematopoietic backbone of the organ and deposit bundled fibers of extracellular matrix (ECM) that assemble an intricate 3D network termed conduits. FRCs enwrap these ECM conduits and form an interface with the immune cells[15,16]. They provide a multitude of signals that serve to compartmentalize the organ and support migration, cellular interactions and expansion of immune cell subsets[17–19].

Upon immunological challenge, reactive LNs swell rapidly by recruiting large numbers of naïve lymphocytes via HEVs, while lymphocyte egress via efferent lymphatics is initially blocked[20,21]. LNs can swell up to 10-fold in size in the order of days, imposing an internal structural problem on the FRC network that has to cope with this volumetric challenge. It has been demonstrated that FRCs are able to relax and expand upon interaction with activated DCs[22,23]. This has been proposed as one mechanism that allows the LN to create additional space during the swelling phase. In addition, FRC numbers expand during inflammation and various redundant mechanisms that drive this expansion in the early and late phase of LN swelling have been described[20,22–26]. The ratio of FRC- to lymphocyte numbers remains fairly constant in the swelling LN and trapping of naïve lymphocytes in the absence of inflammatory stimuli has been demonstrated as a sufficient stimulus for the FRC network to expand[20]. How network expansion is coordinated to prevent under- or

63   overgrowth of the FRC network is unknown, and although mechanical forces are obvious

64   feedback parameters, these aspects of LN swelling have not been measured until to date.

65   We measure the cellular and mechanical changes accompanying LN swelling and show that

66   mechanical load on the conduit network and subsequent FRC mechanosensing are central

67   to expansion of the FRC network and LN growth.

68

69   ## Results

70   ### The reactive lymph node resists swelling

71   To understand the global mechanical behavior of LNs while expanding, we started out with

72   a quantitative characterization of bulk tissue properties of the reactive organ. Upon

73   immunization with keyhole limpet hemocyanin in complete Freund's adjuvant (KLH/CFA) in

74   footpads of wild-type (wt) mice, we observed a more than 10-fold increase in volume of

75   draining popliteal LNs after 14 days, when the organ reached its maximum size. The volume

76   was calculated from 2D side-view images, which correlated well with the weight of the

77   organ and showed that LNs swell on average about 0.75 mm^3 per day (Figure 1A,B & Figure

78   S1A,B). We measured tissue mechanics by compressing explanted popliteal LNs between

79   two parallel plates at 75% of their original height (25% strain), while the resisting force

80   exerted by the LN on the top plate was measured over a time period of 20-60 minutes

81   (Figure 1C). During this time the LN underwent a viscoelastic relaxation behavior and

82   reached a new force-equilibrium, which is described by the stress-relaxation curve (Figure

83   1D & Figure S1C,D). Together with the geometrical parameters of LNs measured before

84   compression and at the new force-equilibrium, the *effective resistance* (σ, *surface tension*),

85   the *viscosity* (μ_2, fluidic resistance to deformation by an applied force), and the *elastic*

86   *modulus* (Young's Modulus, or elastic resistance to deformation by an applied force) of the

87   tissue were derived by modelling the parameters to a generalized Kelvin model (Figure

88   S1E)[27]. At equilibrium (long time scale), the LN resisted the external force exerted by the

89   plate, which sets the *effective resistance* (given in Newton/m). This parameter describes the

90   collective forces resisting organ expansion and is a measure of how much force is necessary

91   to drive the swelling of the LN by a certain length scale.

92   During the course of organ expansion (D0-D14), we observed a ~4-fold increase of effective

93   resistance and values remained elevated until the endpoint at D14 (Figure 1E). Viscosity –

94   the resistance to deformation on the medium time scale – only increased in the last phase

95   of swelling, while elasticity – the resistance to deformation on the short time scale – was

96   selectively increased from the homeostatic condition at D2 and D14 of inflammation (Figure

97   1F,G).

98   These data demonstrate that tissue properties of LNs show complex adaptive dynamics

99   upon swelling, and suggest that the mechanical features of the organ resist the forces

100   driving expansion.

101

102   **Lymphocyte numbers generate pressure and drive lymph node swelling**

103   Having defined that mechanical properties of the LN resist swelling, we next asked what are

104   the internal forces driving organ expansion. Lymphocytes constantly recirculate through LNs

105   where they are densely packed within the FRC scaffold (Figure S1F). Entry via HEVs,

106   blocking of exit in the early inflammation phase, and proliferation upon activation are the

107   main factors that increase cellularity of the node, making these potential factors driving

108   expansion[28–30]. Hence, we manipulated entry and activation and tested the impact on LN

109   bulk tissue properties.

110   We first perturbed lymphocyte entry under homeostatic conditions using an L-selecting

111   antagonizing antibody; anti-CD62L (α-CD62L). L-selectin is expressed on naïve lymphocytes

112   and rate-limiting for transmigration via HEVs[31]. After 24h of either α-CD62L or PBS

113   intravenous (i.v.) injection, popliteal LNs from wt mice were harvested and used for parallel

114   plate compression experiments. We found that blocking of lymphocyte entry significantly

115   reduced LN volume, effective resistance and viscosity, while the Young's Modulus remained

116   unchanged (Figure 2A-E). These data suggest that lymphocyte influx represents an internal

117   force, balancing the effective resistance of the homeostatic LN.

118   Next, we asked how lymphocyte cellularity affects bulk tissue properties of LNs during

119   inflammatory swelling. In order to distinguish between contribution of recirculating and

120   locally proliferating lymphocytes, we treated wt or OT-II mice with either α-CD62L or PBS,

121   immunized with KLH/CFA and measured tissue properties after four days (Figure 2F-J). Mice

122   carrying the OT-II TCR transgene suppress their natural TCRs [31], thereby creating a situation

123   where lymphocyte homing is maintained but the majority of B and T cell responses against

124   the mismatched KLH antigen eliminated. Homing, and to a lesser extent proliferation,

125   contributed significantly to LN swelling, while elimination of both parameters reduced LN

126   volumes even further (Figure 2G). In agreement with the findings under homeostatic

127   conditions, α-CD62L administration reduced the effective resistance in both wt and OT-II

128   mice, with blockade of homing showing the dominant effect (Figure 2H). While the viscosity

129   in the tested conditions did not show a clear trend with lymphocyte cellularity, the Young's

130 Modulus was reduced when lymphocyte trafficking was blocked in inflammation (Figure
131 2I,J).

132 Thus, lymphocyte trapping is not a consequence of LN swelling but drives the process as it
133 generates an outward pressure force which is countered by the organ's effective resistance.

134

135 **The FRC network stretches upon lymph node swelling**

136 Next, we investigated which mechanical features of the LN are resisting it's expansion. The
137 two candidate structures mediating the LNs effective resistance to swelling are the organ
138 capsule, and the FRC- and conduit network. To understand how the FRC network adapts to
139 volumetric changes upon swelling we quantified FRC spacing (gaps) within the T zone over
140 the time course of inflammation in Ccl19-Cre; mTmG (FRC-mGFP) (membrane Tomato,
141 membrane Green Fluorescent Protein (GFP)) mice that selectively express membrane GFP
142 (mGFP) in FRCs[32,33]. To this end, a circle-fitting algorithm was used to quantify the
143 distribution of gaps in FRC networks from two-dimensional (2D) tissue sections (Figure
144 3A,B). While we found no obvious disruptions of network integrity, the FRC network
145 dynamically adapted over time. In the first four days of inflammation the FRC network
146 widened, as indicated by larger gaps, and in the following days returned to homeostatic
147 levels (Figure 3C,D).

148 These results suggest that the intact FRC network initially stretches upon swelling and
149 subsequently remodels to accommodate the increased numbers of proliferating and
150 immigrating lymphocytes.

151

152 **Conduits are stretched in the swelling lymph node**

153 The FRC network comprises two principal structural components: the FRC itself and the ECM
154 conduit, a complex fibrillar structure, which the myofibroblastic FRC both produces and
155 ensheathes (Figure 4A). Both components have the potential to bear load and confer
156 mechanical resistance to swelling. In the following, we devised strategies to quantitatively
157 measure if and to what extent the two structures experience mechanical forces. We started
158 out with the ECM component and as a proxy for mechanical strain, we chose to investigate
159 the structural organization of the conduit's fibrillar collagen. Like in tendons and other
160 elastic ECM structures, fibrillar alignment should increase with strain.

161   We fixed homeostatic and reactive LNs and removed all cellular components by alkali-
162   maceration (Figure S2A,B). To resolve the 3-dimensional (3D) organization of individual
163   collagen fibrils, scanning transmission electron microscopy (STEM) tomograms of T zone
164   conduits were acquired at 2 degrees differential tilting angles (Figure 4A). 3D images were
165   reconstructed using computed weight back projection (Figure S2C,D). We quantified the
166   extent of conduit stretching by 3D manual tracking of individual collagen fibril segments and
167   by computationally calculating the centerline of each conduit based on the average
168   direction of its collagen fibrils. This allowed us to determine the angle of misalignment of
169   individual fibril segments relative to the centerline (Figure 4C,D). We found that compared
170   to homeostasis (D0), early in inflammation (D2, D4) conduit collagen fibrils become
171   progressively aligned, whereas later in inflammation (D14) they again adopted a misaligned
172   configuration (Figure 4D).
173   These results suggest that conduits stretch and bear an increased mechanical load early
174   upon LN swelling. At later time points they revert to the homeostatic state. Initial stretching
175   and later adaptation are well in line with our previous findings on network configuration.
176
177   **FRC network tension increases upon lymph node swelling**
178   We next addressed the cellular component of the FRC stretching-response. To study if the
179   previously observed change in conduit conformation is mirrored by the tension-state of the
180   FRC network, we directly measured FRC network tension by intravital laser ablation and
181   recoil analysis[34,35]. To this end, inguinal LNs of FRC-mGFP mice were surgically exposed and
182   the FRC network was imaged around 20 µm below the capsule. Individual strands of the 3D
183   network were cut using a high-power ultra-violet (UV)-laser (Figure 5A). FRC network
184   tension was subsequently determined in kymographs by calculating local recoil velocities of
185   the region around the site of ablation (Figure 5B,C and Supplemental Movie 1). We found
186   that at D4 and D8 of inflammation the tension on the FRC network almost doubled and was
187   restored again to homeostatic levels at D14 of inflammation.
188   As a proxy for the cellular mechano-response, we next measured the nuclear vs. cytoplasmic
189   intensity of the transcription factors Yes-associated protein (YAP), and transcriptional co-
190   activator with PDZ binding motif (TAZ), which are well-established downstream responses of
191   cytoskeletal tension[36]. FRCs and endothelial cells stained positive for YAP/TAZ, while no
192   signal could be observed in leukocytes (Figure 5D,E). The nuclear:cytoplasmic ratio of

193   YAP/TAZ (N:C YAP/TAZ ratio) in FRCs remained stable from D0 to D2 of inflammation but

194   increased at D4 and D8 of inflammation, confirming that FRCs experience increased

195   cytoskeletal tension during LN swelling. The N:C ratio decreased after FRC tension peaked

196   (Figure 5C,F), thereby faithfully recapitulating the tension as measured by laser cutting.

197   Interestingly, after two weeks of inflammation we observed a large population of FRCs that

198   had a negative N:C ratio, suggesting that those cells were completely shielded from active

199   tension.

200   Together, these data demonstrate that FRC tension increases upon LN swelling. However,

201   compared with the ECM conduit (Figure 4), tension at the cellular level increases with a two

202   day time delay. This kinetic is well in line with previous observations, suggesting that early in

203   inflammation FRCs experience a relaxation of actomyosin contractility[22,23]. This relaxation is

204   transient and followed by an increase in cytoskeletal tension. At later time points when

205   network geometry adopts its homeostatic configuration, tension drops again.

206

207   **FRCs in the swelling lymph node undergo distributed clonal expansion**

208   The above data suggest that lymphocyte influx drives LN expansion, which initially stretches

209   the FRC network and elicits a mechanosensitive response by the cells, once their initial

210   relaxation[22,23] ceases. We next wanted to understand how the FRC network expands,

211   remodels and re-establishes its typical configuration and mechanical state, while

212   maintaining structural integrity.

213   To this end, we devised an approach to map the spatio-temporal expansion of the FRC

214   network in the swelling LN *in situ*. We used a sparse clonal labeling approach named Mosaic

215   Analysis with Double Markers (MADM)[37–39]. MADM labeling depends on rare

216   interchromosomal mitotic recombination driven by Cre-loxP sites (Figure S3A,B). Two

217   reciprocally split GFP and tdTomato genes (GT and TG) on identical loci of homologous

218   chromosomes are used to create transheterozygous offspring (GT/TG). Interchromosomal

219   recombination can take place in G_2 phase and restores functional GFP and tdTomato

220   expression. To trigger rare recombination specifically in FRCs we used the Ccl19-*Cre*

221   transgene[32]. When followed by X-segregation of chromosomes in mitosis, two daughter

222   cells are formed in which one expresses GFP (green lineage) and the other tdTomato (red

223   lineage). Recombination is irreversible and all subsequent progeny cells become part of the

224   lineage.

225   Homeostatic and reactive LNs were cleared and imaged by 3D light-sheet fluorescence
226   microscopy (LSFM). In reactive nodes, prominent clusters of FRCs emerged at D4 and D8 of
227   inflammation, while such clusters were rarely observed in homeostasis (Figure 6A,B & Figure
228   S3C). This suggested that individual FRC clones expanded following immunization and that
229   daughter-FRCs stay close to their precursor. No substantial bias in red vs. green lineage was
230   apparent, justifying combined analyses (Figure S3D). FRC clusters were quantitatively
231   analyzed using a density-based spatial clustering of applications with noise (DBSCAN)[40] for
232   which FRC bodies were semi-automatically mapped in 3D as spheres with a 12 µm diameter
233   (Figure S3E). A cluster was defined as at least three FRCs from the same lineage within a
234   radius of 20 µm. We found that both the number of labeled FRCs, number of clusters and
235   number of FRCs in clusters were significantly increased in reactive nodes (Figure 6C-F). Since
236   clusters can arise by chance, the cluster results were compared to the average of 10
237   simulated distributions in which the same number of FRCs were randomly distributed in the
238   same volume. Although clusters arise by chance in random distributions, FRCs in
239   inflammation were less uniformly distributed and formed more and larger clusters with FRC
240   numbers ranging from 3-14 (Figure 6G, Figure S3F). Clone size distribution was exponential
241   as is expected from an equipotent population of precursors that are dividing
242   stochastically[41]. To quantify the extent of FRC clustering within LNs, we defined a cluster
243   factor (CF) as the ratio of the number of FRCs found in clusters between the observed and
244   simulated distributions (Figure S3G). Hence, a CF of 100 indicates that 100x more cells are
245   found in clusters as by chance alone. We found an average CF of 5 in homeostatic conditions
246   and 117 in inflammation (respectively, 70 and 165 for D4 and D8), confirming that FRCs
247   form clusters in the swelling LN (Figure 6H).
248   We next investigated if FRC clusters are found at the vicinity of HEVs in the swelling LN as it
249   has been shown that *de novo* FRCs can derive from perivascular fibroblasts in the
250   developing spleen[42,43]. To this end, the CF was plotted as a function of the distance from
251   HEVs. No enrichment of clusters was found at these specific perivascular areas (Figure 6I).
252   To better understand the relationships between the measured parameters a correlation-
253   matrix was created (Figure 6J). As expected, the number of labeled FRCs correlated with the
254   number of observed clusters and the number of FRCs found in clusters, while the number of
255   clusters correlated well with the number of FRCs found in clusters. In the reactive LN *de*
256   *novo* MADM-events can occur, but our data indicate that these do not affect the CF as the

257 CF is independent of the number of labeled FRCs, and the relative numbers of FRC in

258 clusters remains unchanged in inflammation (Figure 6J, Figure S3H). LN volume was the best

259 determinant for the CF and also correlated well with the number of labeled FRCs, number of

260 clusters and FRCs in clusters (Figure 6J,K).

261 Together, these data show that FRCs expand in randomly distributed clusters and that FRC

262 growth correlates with LN volume (Figure 6K). This suggests that FRCs can expand in

263 response to local signals independent of their localization.

264

265 **Talin1 is required for FRC mechanosensing.**

266 Given our previous observations, mechanical forces appeared as an attractive feedback

267 parameter regulating FRC network growth. Mechano-coupling of fibroblasts to their

268 underlying matrix is mediated by integrins and their associated intracellular force-sensitive

269 adaptor molecule talin. FRCs express both Talin1 and Talin2 isoforms which play non-

270 redundant roles in integrin activation and force transduction[44]. We selectively deleted

271 Talin1 in FRCs by generating Ccl19-*Cre; Talin1fl* (FRC$^{\Delta TLN1}$) mice.

272 Peripheral LNs of FRC$^{\Delta TLN1}$ mice were reduced in size and had lowered levels of the T-zone

273 chemokine CCL21 in the paracortex and the T cell zone was significantly reduced in size in

274 FRC$^{\Delta TLN1}$ LNs (FigureS4A-C). Within the T cell zone podoplanin (PDPN)+ FRCs$^{\Delta TLN1}$ still formed

275 a regularly interconnected network and expressed intercellular adhesion molecule 1 (ICAM-

276 1) and vascular cell adhesion molecule 1 (VCAM-1), arguing for regular differentiation

277 (Figure S4D). Within HEVs, CCL21 levels were unaffected (Figure S4B). Hence, despite the

278 reduced size, the basic organization and differentiation of the Talin1 deficient FRC network

279 was maintained. When FRC$^{\Delta TLN1}$ mice were immunized with KLH, LN swelling was substantial

280 (Figure7A) and during the initial four days after immunization, relative weight gain was

281 comparable to control mice. A mild reduction was only seen at D14. This demonstrates that

282 lymphocyte influx and proliferation were still occurring within the Talin deficient FRC

283 network, emphasizing the suitability of the genetic model to ask the principal question, if

284 FRC mechanosensing is required for network adaptation.

285 When we analyzed N:C YAP/TAZ ratio almost no FRCs$^{\Delta TLN1}$ showed a nuclear localization

286 both under homeostatic and reactive conditions (Figure 7B,C). This finding demonstrates

287 that Talin1 dependent mechanotransduction in FRCs is rate-limiting for YAP/TAZ nuclear

288 translocation and strongly indicate that FRCs$^{\Delta TLN1}$ lose their mechanosensitivity. To assess

289 the functional consequences for FRC network integrity we performed *in situ* network

290 analysis. To this end, 3D volumes of FRC networks were acquired from cleared thick

291 vibratome slices and network spacing was quantified using a 3D sphere-filling algorithm

292 (Figure 7D,E). In agreement with the previous 2D GAP analysis (Figure 2) the network of

293 control mice widened and remained intact at D4 of inflammation. The $FRC^{\Delta TLN1}$ network

294 under homeostatic conditions was widened compared to controls, but structurally intact.

295 Upon immunization the $FRC^{\Delta TLN1}$ network integrity became severely compromised, showing

296 large FRC-free gaps (Figure 7D,E & S4E). These defects were most apparent at D4 and partly

297 recovered by D14 of inflammation.

298 These data suggest that the FRC network in $FRC^{\Delta TLN1}$ mice failed to adapt to organ swelling

299 and partly disintegrated or ruptured. To investigate the fate of FRCs under these conditions

300 thick vibratome sections were stained for cleaved caspase 3 (cC3) and Ki67, to identify

301 apoptotic and proliferating FRCs, respectively (Figure 7F,G). These analyses showed little

302 apoptotic and proliferating FRCs in $FRC^{\Delta TLN1}$ and control mice under homeostatic conditions.

303 At D4 after immunization, the number of apoptotic FRCs per volume increased significantly

304 in $FRC^{\Delta TLN1}$ mice as compared to controls, while proliferation was similar in both control and

305 knockout. At D8, apoptotic FRCs per volume unit were still larger in $FRC^{\Delta TLN1}$ compared to

306 controls and proliferating FRCs were increased (Figure 7F,G).

307 These data indicate that compromised mechanosensing causes a severe dysregulation in

308 survival and proliferation of the FRC compartment, which leads to a loss of network

309 integrity. This finding is line with the idea that FRC remodeling is locally controlled by

310 mechanical feedback.

311

312 **Capsule fibrosis constrains lymph nodes at late time points**

313 Network analysis and tension measurements indicated that the FRC network reached a

314 "new equilibrium" two weeks after immunization, because it adopted its homeostatic

315 configuration. Nevertheless, effective resistance remained high at these late time points,

316 raising the question if another structure contributes to the force balance.

317 The LN capsule can be divided into two components: a floor that includes floor lymphatic

318 endothelial cells (fLECs) along with a basement membrane that connects to conduits, and a

319 roof that consists of ECM with embedded lymphoid fibroblasts to which ceiling lymphatic

320   endothelial cells (cLECs) adhere. We investigated the structural and mechanical properties

321   of the two capsule components in homeostatic and inflammatory conditions.

322   fLECs are sparsely labeled in FRC-mTmG mice, as is demonstrated by their double positivity

323   for mGFP and LYVE-1, and their location in the floor of the subcapsular sinus (SCS) (Figure

324   S5A). Since the SCS floor is densely populated by fLECs, we used this sparse labeling to our

325   advantage as it enabled the measurement of active tension on single fLECs *in vivo* using UV-

326   laser ablation (Figure 8A). We found that fLECs had high levels of basal tension that

327   exceeded those of FRCs (Figure 8B). Interestingly, after two days of inflammation fLECs

328   showed reduced tension that was reverted to homeostatic levels in the further swelling

329   phase. Likewise, no rise in active tension on the long time scale (D8-D14) was found on the

330   capsule ECM following UV laser ablation of explanted popliteal LNs (Figure S5B,C).

331   The absence of a continuous rise in active tension on the capsule floor and roof entails that

332   these components are being remodeled to keep up with the volumetric increase of the

333   swelling LN. The capsule was therefore assessed by histology in Prospero homeobox protein

334   1 (Prox1)- GFP mice in which the cytoplasm of all LECs is labeled with GFP. Additional 4$'$,6-

335   diamidino-2-phenylindole (DAPI) and platelet-derived growth factor receptor- β (PDGFR-β)

336   staining allowed the measurement of capsule thickness and mesenchymal cell layers of the

337   capsule above the SCS in homeostasis and inflammation. We found that the capsule

338   thickness remained unchanged in the first four days of inflammation. Strikingly, the capsule

339   thickness increased ~14-fold from D8 to D14 of inflammation, forming a dense fibrotic layer

340   between the parenchyma and surrounding adipose and muscle tissue, while the SCS

341   remained intact (Figure 8C,D & Figure S5D).

342   We asked if such significant remodeling of the capsule resulted in changes in its mechanical

343   properties. To this end a micro-pipette with a small diameter was used to locally aspirate

344   ERTR7 labeled capsules of popliteal LN explants. Using Laplace's law, an effective Young's

345   modulus of the capsule was derived (Figure 8E). We found that the elastic modulus of the

346   capsule remained stable over the first eight days of inflammation but was doubled at D14

347   (Figure 8F). By multiplying the capsule thickness and Young's modulus of the capsule, we

348   derived the passive capsule tension; a measure of the amount of force necessary to enlarge

349   the whole thickness of the capsule by a certain length, i.e. the force needed to swell the LN.

350   The passive tension showed a substantive increase from D0 to D8 of inflammation and kept

351   rising to a massive 150-fold increase at D14 of inflammation (Figure 8G).

352   These data indicate that during a sustained immune response, tension dissipates from the

353   remodeling FRC network at the intermediary time scale (D4-D14), while the capsule

354   remodels and becomes thicker, stiffer and more resistant to swelling at the long time scale

355   (D8-D14), establishing a new force-equilibrium within the organ that resists further swelling

356   (Figure 8H).

357

Discussion

Like glia cells of the nervous system, lymphatic stroma cells were long considered the passive structural elements of the immune system. Only the last two decades revealed that the stromal compartment decisively orchestrates immune cell encounters by providing trophic and tactic cues and that in turn stromal cells dynamically respond to signals provided by the immune cells[18,19]. Despite palpation of reactive LNs being part of every physical exam and LNs being unmatched in their ability to change volume, the mechanical aspects of LN swelling have never been directly addressed. By measuring organ mechanics across different time and size scales we establish that reactive swelling of the LN is a multi-tier process controlled by mechanical feedback. This allows the organ to expand in a step-wise controlled fashion, without compromising its delicate architecture.

We demonstrate that upon inflammation, accumulating lymphocytes inflate the node and initially stretch the FRC network. This is in line with previous findings that FRCs show an early and transient increase in cell size[20]. Stretching puts tension on the ECM conduit as revealed by a straightened configuration of its ECM fibrils. Although our ultrastructural analysis did not show obvious ruptures of fibrillar collagen, Martinez et al.[45] detected gaps in the ECM (but not the cellular) compartment of swelling LNs, raising the possibility that in some areas of the LN, flexibility of the ECM cannot keep up with cellular deformation. Interestingly, both our laser cutting experiments and the kinetics of nuclear shuttling of YAP/TAZ revealed that compared to their ECM conduit, FRCs experience cytoskeletal tension only with a time delay of two days. This is well in line with findings of the labs of Turley and Reis e Sousa, who showed that early LN swelling is accompanied by a relaxation of the FRC system, allowing the network to stretch[22,23]. Mechanistically, they demonstrated that CLEC-2 ligand expressed on activated DCs binds podoplanin on FRCs and that this interaction relaxes actomyosin contractility within the FRC myofibroblastic network. Such a transient FRC relaxation explains why tension-increase of the ECM conduit precedes tension-increase of FRCs and implies that the FRC cytoskeleton only experiences significant tension once the DC-mediated relaxation signals fade after three to four days. Accordingly, FRCs show increased αSMA expression only after the initial days of inflammation[20].

While the FRC network uses its intrinsic pliability to accommodate short-term volumetric changes, sustained strain on the FRC cytoskeleton triggers the next stage of LN swelling, which is characterized by actual growth and structural remodeling of the network. Our

390   results on genetic ablation of Talin1 in FRCs strongly support the idea that adhesion-
391   dependent mechanosensing is a critical feedback parameter that locally controls growth and
392   survival of the network, so that it reverts to its typical geometry, while being increased in
393   size. In line with these results, mice with a gain of function mutation in the
394   mechanosensitive YAP/TAZ pathway show fibrotic LNs with impaired FRC differentiation[46]
395   and blockade of β1 integrin triggered FRC apoptosis in swelling (but not in homeostatic)
396   LNs[47]. A critical prerequisite of a model where FRC mechanosensing locally controls network
397   remodeling is that the FRC responsiveness is not restricted to specific niches but rather
398   distributed throughout the organ. Our clonal analyses show that this is indeed the case and
399   match results in follicular dendritic cells and marginal reticular cells that were also shown to
400   undergo clonal expansion[48].
401   Beyond a week of structural adaptation the FRC network of the now massively enlarged LN
402   seemed to reach a new "mechanical equilibrium" as indicated by gap analysis, ECM
403   alignment, tension measurements and YAP/TAZ translocation. Nevertheless, bulk
404   mechanical properties of the LN did not return to homeostatic levels. They rather showed
405   an elevated effective resistance, indicating that another structure now countered the
406   internal pressure generated by the trapped lymphocytes. We thus turned to the capsule as
407   the second major stromal element and found that during these late stages, thickness and
408   mechanical strength of the capsule were massively increased, explaining resistance to
409   further organ expansion. Although capsule fibrosis is a characteristic histopathological
410   descriptor of reactive LNs[49], its mechanistic contributions are completely unexplored and
411   future studies will hopefully show how it contributes to sustained or chronic immune
412   responses.
413   As the multi-tier model of LN swelling moves through a succession of check-points, it has
414   the advantage of being adaptable to very different types of swelling-scenarios. Transient
415   swelling, as it occurs e.g. during circadian fluctuations, might stretch the network, but is
416   unlikely to cause structural remodeling. In contrary, sustained immune responses with
417   massive lymphocyte trapping and germinal center reactions, might rely on a fibrotic
418   strengthening of the capsule in order to limit excessive expansion of the organ.
419

420   **Methods**

421

422   **Animals**

423   All animal experiments are in accordance with the Austrian law for animal experiments.
424   Permission was granted by the Austrian Federal Ministry of Science, Research and Economy
425   (identification code: BMWFW 66.018/0010-WF/V/3b/2016). Mice were bred and
426   maintained at the local animal facility in accordance IST Austria Ethical Committee or
427   purchased from Charles River and maintained at the local animal facility in accordance with
428   IST Austria Ethical Committee. OT-II (Stock No: 004194) was bought from JAX. Ccl19-Cre
429   mice have been described previously[32]. MADM-7[39] and Talin1floxed[50] mice were provided
430   by Simon Hippenmeyer and David Critchley, respectively. All mice are on a C57BL/6J
431   background, with exception of MADM-7 which have a CD-1 background. Mice of both sexes
432   between the age of 6 to 20 weeks were used for experiments. For immunization, KLH
433   protein was dissolved in PBS to 5 mg/mL of and then mixed 1:1 with CFA (both Sigma-
434   Aldrich) upon which 40 µL of the immunization mixture was injected in footpads and flanks
435   of draining popliteal and inguinal LNS. LNs were harvested after various timepoint up to
436   two weeks of induction of immunization to be used for histology or explant experiments, or
437   used for *in vivo* imaging experiments. For LN cellularity manipulation experiments, mice
438   were i.v. injected with 100 µg α-CD62L (MEL14) (BioXCell), and control mice with PBS alone.
439   For steady-state evaluation, LNs were harvested 24h after injections were given, and for
440   inflammation conditions injections were given at onset of inflammation. Mice were
441   anesthetized by isoflurane inhalation (IsoFlo, Abbott) for all injection-based experiments, or
442   anaesthetized with a ketamine/xylazine/acepromazine mixture for *in vivo* imaging
443   experiments.

444

445   **Histology and Imaging**
446   LNs were fixed in 4% paraformaldehyde (Electron Microscopy Sciences) in PB (0.1 M, pH 7.4)
447   at 4°C overnight. For cryosections, tissues were additionally embedded for 24h in a solution
448   of 30% glucose in PB (0.1 M, pH 7.4) before embedding and freezing in Tissue-Tek optimum
449   cutting temperature (OCT) compound (Sakura). Cryostat section (10-12 µm) were collected
450   on Superfrost/Plus glass slides (Thermo Fisher Scientific). Alternatively fixed tissues were

451   embedded in 4% low melting temperature agarose (Invitrogen) after fixation and 100-400

452   μm sections were cut using a vibratome (VT1200S, Leica Microsystems).

453

454   Cryostat sections were air-dried for 2h at room temperature (RT) and washed in PBS.

455   Sections were blocked in SEABLOCK blocking buffer (Thermo Fisher Scientific) or in 5%

456   bovine serum albumin (BSA) (Thermo Fisher Scientific) in PBS for 1h, followed by incubation

457   of primary antibody solution diluted in 1% BSA/PBS for 1.5h at RT, 3 washing steps in PBS

458   and subsequent incubation of secondary antibody solution diluted in 1%BSA/PBS for 30 min

459   at RT. Finally, sections were washed three times in PBS, air-dried and mounted using

460   Fluoromount-G with DAPI (Thermo Fisher Scientific). Vibratome sections were blocked in 5%

461   BSA/0.3%Triton- x/PBS for 2h at RT under agitation followed by primary antibody incubation

462   in 1% BSA/0.3%Triton-x/PBS overnight at 4°C under agitation or for 2 days at RT in some

463   cases. The following day sections were washed three times in PBS. In case primary

464   antibodies were not conjugated with a fluorescent dye, samples were incubated with a

465   secondary antibody in 1% BSA/0.3%Triton-x/PBS for 4h at RT under mild agitation and

466   subsequently washed three times in PBS. All samples were then incubated in DAPI solution

467   for 15 min and mounted on a glass slide using Fluoromount-G (Thermo Fisher Scientific).

468   The following primary antibodies were used: α-CD3ε AF488 (17A2)(eBioscience), α-B220-

469   Biotin (RA3-6B2) (eBioscience), α-Collagen IV-Biotin (Abcam), α-CCL21-Biotin (R&D Systems),

470   α-PDPN-Biotin (8.1.1) (eBioscience), α-PDGFR-β (R&D Systems), α-YAP/TAZ (D24E4) (Cell

471   Signal), α-cleaved Caspase 3-AF647 (Asp175) (Cell Signal), α-Ki67-APC (SolA15)

472   (eBioscience), α-ICAM-1 (YN1/1.7.4) (BioXCell), α-VCAM-1 (Phe25-Glu698) (R&D Systems),

473   α-Fibroblast Marker-AF647 (ERTR7) (Santacruz Biotech). α-PNAd (MECA-79) was derived

474   from a concentrated hybridoma supernatant (kind gift from Christine Moussion). The

475   following secondary antibodies were used: Streptavidin-Cy3 (Sigma-Aldrich), Streptavidin-

476   AF647 (Jackson ImmunoResearch), chicken α-goat AF488 (Invitrogen), donkey α-rat AF647

477   (Jackson ImmunoResearch), donkey α-rabbit AF647 (Jackson ImmunoResearch), goat α-

478   mouse IgM AF647 (Invitrogen). Images were acquired on a Zeiss LSM800 inverted confocal

479   laser scanning microscope (CLSM) with the following objectives: 10x/NA0.45, 20x/NA0.8,

480   40x/NA1.2 Water and 63x/NA1.4 Oil Plan-APOCHROMAT.

481   Thick vibratome sections were in some cases cleared using the Ce3D protocol as described

482   previously[51]. Briefly, following antibody-staining samples were washed at RT on a shaker for

483   8h in washing buffer (PBS/0.3% Triton X-100, 0.5% 1-thioglycerol) which was refreshed after

484   4h. Next samples were cleared in freshly prepared Ce3D solution for 2x 1h, mounted in a μ-

485   dishes (Ibidi) and submerged in Ce3D solution. A cover-glass was placed on top to mount

486   cleared samples to the bottom of the well and the dish sealed with parafilm. Large 3D

487   volumes (xy:306x306 μm, z: 50-300 μm) were acquired from Ce3D cleared thick vibratome

488   sections using a spinning-disc microscope (Dragonfly, Andor) with a Apochromat LWD λS

489   40x/1.15 Water 0.60 mm WD objective.

490   **3D LSFM Sample Preparation and Imaging**

491   Terminally anesthetized Ccl19-cre hem; MADM-7$^{GT/TG}$ mice (mix C57BL/6J and CD1

492   background) were *in vivo* stained by retro-orbital injection of 40 μg/PBS mouse α-peripheral

493   node addressin (PNAd) concentrated hybridoma supernatant labeled with Atto-647N-NHS

494   (Atto-Tec). After 10 min, popliteal LNs were harvested and fixed in 4% paraformaldehyde

495   (Electron Microscopy Sciences) overnight at 4°C. Samples were washed in PBS, cleaned

496   under a stereomicroscope and cleared with the CUBIC protocol[52]. Briefly, samples were

497   incubated in CUBIC reagent 1 for 3 days at 37°C, which was replaced every 24h. Samples

498   were then washed with PBS, embedded in 2% low-meting temperature agarose (Sigma-

499   Aldrich), and sequentially dehydrated in 30% sucrose (Sigma-Aldrich) (1 day at 4°C) and 50%

500   sucrose (2 days at 4°C). Finally, samples were incubated in CUBIC reagent 2 for 2 days at RT.

501   Cleared samples were imaged using a custom LSFM setup[53]. Acquired images were stitched

502   using the FIJI Grid/Collection-stitching plugin (Preibitsch Laboratory), despeckled and

503   manually registered using a custom-alignment-tool in Matlab (developed by Ekaterina

504   Papusheva).

505

506   **FRC Cluster Analysis**

507   MADM-labeled cells were detected in 3D by employing a spot detection algorithm (Bitplane

508   Imaris) for each channel (tdTomato & GFP) separately. Chromatic aberrations and the

509   sequential nature of the image acquisition led to a channel misalignment which was

510   corrected for in the following way: the spot coordinates were exported from Imaris and

511   treated as a point cloud for each channel. These point clouds were then registered onto

512   each other using the Iterative closest point algorithm which corrected the shift and the

513   rotation of the spectral channels. Cells were then sorted into color classes (green (GFP), red

514   (tdTomato) or yellow (double positive) lineage). Red or green, if the spot existed solely in

515   one of the point clouds, or yellow if there were two corresponding spots in both channels

516   that are closer than the typical cell radius.

517

518   For the cluster analysis the LN outline and the HEVs were segmented in Imaris using the

519   surface detection feature. To correct errors in the cell detection, falsely detected spots from

520   autofluorescent structures outside of the LN volume were excluded from further analysis. In

521   order to avoid edge effects cells in a region 100 μm from the surface of the analyzed LNs

522   were excluded. FRC clusters were analyzed using a custom Matlab script utilizing a density-

523   based spatial clustering of applications with noise (DBSCAN) algorithm[40] in which FRCs were

524   represented as 3D spheres with a 12 μm diameter. An FRC cluster was defined as a

525   minimum of three FRCs of the same lineage (green or red) within a search-radius of 20 μm

526   from each FRC-sphere's surface.

527

528   To generate a random distribution (simulated FRCs), FRC-spheres were placed into the same

529   volume occupied by the real cells and excluded from HEV volumes. For each timepoint the

530   average of 10 distributions was used. The *cluster factor (CF)* was defined as *FRCs in*

531   *clusters/Total number of FRCs* divided by *Simulated FRCs in clusters/Total number of*

532   *simulated FRCs.*

533

534   **FRC Network GAP Analysis**

535   2D: A confocal laser scanning microscope with a 40x 1.2 water-objective (LSM800, Zeiss)

536   was used to acquire image stacks (range 10-30 μm and spaced at 1μm) with a field of view

537   of 240x240 μm and a pixel size of 0.5 μm from T zones in which FRCs were labeled by mGFP

538   (Ccl19-Cre; mTmG mice). These were subsequently segmented using Ilastik software, the

539   result was then transformed into a binary image and noise was removed using a custom FIJI

540   script that utilized the particle detection algorithm. Binarized 3D image-stacks were then

541   used to measure the spacing (gaps) in the network by analyzing the pore-size distribution on

542   individual z-sections. The pore-size distribution was obtained analogously to the pore-size

543   analysis described in *Acton et al.*[22]. Starting with a circle size which corresponded to the

544   maximum gap of the network, circles were consecutively positioned into fitting

545   corresponding gaps of the network. The maximum circle size was determined from a

546   distance transform of the segmented network. Once no more circles of the maximum size

547   could be placed into gaps of the network, the disk size was reduced by one unit and the

548   placement of the disks of reduced sized commenced. This way, the gaps in the network

549   were consecutively filled with circles of decreasing size until the entirety of the gap area was

550   filled. The results were then averaged over the

551   3D: Large 3D volumes (xy:306x306 µm, z:50-500 µm) were acquired from Ce3D[51] cleared

552   thick vibratome sections using a Apochromat LWD λS 40x/1.15 Water 0.60 mm WD

553   objective on a spinning disc microscope (Dragonfly, Andor). Acquired 3D stacks were

554   corrected for fluorescent intensity in z-axis using 'bleach correction (histogram matching)'

555   function in Fiji. Imaris was then used to generate a 3D binary image of the FRC network by

556   utilizing a surface detection feature from the FRC-network fluorescent channel. A custom

557   Matlab script was subsequently used to fit 3D spheres in the 3D gaps of the network analog

558   to the 2D approach.

559

560   **Parallel Plate Compression Experiments**

561   Explanted popliteal LNs were cleaned from adipose tissue under a stereomicroscope and

562   placed on a glass plate within the 37°C, RPMI 1640 (Invitrogen) filled incubation chamber of

563   a MicroSquisher device (CellScale). LNs were oriented to have their long axis along the field

564   of view of the camera. Compression was performed with a glass plate glued onto either a

565   0.304 or 0.408 mm diameter 40 GPa tungsten filament with a length of 60 mm. The glass

566   slide on the compression probe was coated with Poly-HEMA (Sigma-Aldrich) to reduce

567   sticking of the samples. LNs were then compressed by 25% of the initial height by lowering

568   the upper plate down in a timespan of 30 seconds. Lateral side-views of LNs were recorded

569   up to 20-60 min after onset of the experiment, while resistant forces were measured on the

570   upper plate. Compression protocols, images and force acquisition were realized with the

571   SquisherJoy software (CellScale). Length, height, contact area and curvature of LNs were

572   manually measured before compression, and at the equilibrium timepoints using Fiji

573   software. The recorded compression force together with the measured geometrical

574   parameters were used to calculate volumes, Young's modulus, effective resistance and

575   viscosity using a generalized Kelvin model[27]. This was done as following:

576   The force required to maintain a constant strain of 25% on a LN was measured over time

577   (F(t)). The force initially peaks and then follows a relaxation curve which is fitted by a double

578   exponential decay curve. The simplest way to describe this bimodal dynamic is to

579   incorporate two dashpots with constants μ_1 and μ_2, and two springs with k1 and k2

580   constants. After 20-60 min, the system reaches an equilibrium where the exerted force by

581   the plate equals the effective resistance of the LN (σ). Therefore, we have:

$$\sigma = \frac{F_{eq}/\pi R_3{}^2}{\left(1/_{R1} + 1/_{R2}\right)}$$

582   where F_{eq} is the equilibrium force at steady state and R1, R2 and R3 are derived from the

583   geometry of the LN.

584   To obtain the elastic modulus, the stress and strain need to be acquired:

585   Stress (s) is calculated from the force at equilibrium divided by plate contact area:

$$s = F_{eq}/\pi R_3{}^2$$

586   and the strain (ε) from:

$$\varepsilon = 1 - \frac{h_{eq}}{h_0}$$

587   where h_0 and h_{eq} correspond to the initial and equilibrium height of the compressed LN.

588   From here the elastic modulus (E) can be derived:

$$E = s/\varepsilon$$

589   Next, by fitting a double exponential decay to the force curve we obtain two time-scales, τ_1

590   and τ_2, where:

$$\tau_i = {}^{\mu_i}/_{k_i} \quad , i = 1, 2$$

591   Following up on the derivations of the equations as in *Forgacs et al.*[27], μ_1 and μ_2 can be

592   acquired readily, where μ_1 corresponds to the initial fast response in the order of seconds

593   and μ_2 to the slower response in the order of minutes, of which the latter one becomes

594   relevant for the rearrangements of the cells within LNs. Hence, we use μ_2 as our viscosity.

595   Measurements in which the LN was damaged during preparation (lymphocytes leaking out)

596   or moved/rolled during compression were excluded. In a few cases the viscosity could not

597   be determined (infinitely small) and were also excluded.

598   LN volumes were calculated from side-view images at *t=0* with the following formula:

$$V = \frac{4}{3} \pi R1 \left(\frac{h_0}{2}\right)^2$$

599

600 **Micropipette Assay**

601 Popliteal LN explants were cleared from fat and incubated for 10 min in a 2 µg/mL ERTR7-

602 AF647 (Santa-Cruz) in RPMI 1640 (Invitrogen) to label the capsule. LNs were subsequently

603 placed on 3% methylcellulose coated glass bottom Petri dishes (MatTek) in RPMI and kept

604 at 37°C, while imaged on an inverted Leica SP5 microscope using a 20x, 0.7NA objective

605 (Leica Microsystems). The local Young's Modulus of the capsule was measured with a glass

606 micropipette connected to a Microfluidic Flow Control System (Fluigent, Fluiwell), with

607 negative pressure ranging from 7-750 Pa, a pressure accuracy of 7 Pa and change rate of

608 200 Pa*s^{-1}. The micropipette equipment was mounted on a motorized micromanipulator

609 (Eppendorf, Transferman Nk2). Both systems were controlled by Dikeria software, Labview

610 (National Instruments). A fire polished micropipette with an inner diameter of 15 µm and

611 flat end (BioMedical instruments) was used for aspiration. The chosen diameter ensured

612 that mainly the capsule was probed and not the underlying parenchyma. While localizing

613 the LN capsule with the micropipette, the pressure inside the micropipette was kept at 0 Pa.

614 For measurements, a negative pressure of 750 Pa was applied, which resulted in the

615 instantaneous aspiration of the capsule. This pressure was chosen as lower pressure

616 regimes did not result in proper aspiration of the capsule. The tongue length of the capsule

617 in the micropipette upon aspiration was manually measured in Fiji from acquired movies.

618 The elasticity was subsequently calculated using the Laplace law:

$$E = \frac{\varDelta P}{\left(\frac{h}{d}\right)^2}$$

619 With $\varDelta P$ being the pressure difference between micropipette and atmosphere, h the height

620 of the measured tongue, and d the micropipette diameter.

621

622 **SEM Sample Preparation and Imaging**

623 Terminally ketamine/xylazine/acepromazine anesthetized mice were transcardially perfused

624 with PB (0.1 M, pH 7.4) and subsequently fixed with 2.5% glutaraldehyde and 2%

625 paraformaldehyde (Science Services) in PB (0.1 M, pH 7.4). LN samples were then dissected

626 and post-fixed in the same buffer for another hour at RT. They were dehydrated in a graded

627 ethanol series of 50%, 70%, 90%, 96%, 100% in H$_2$O for a minimum of 10 min per step and

628 subsequently kept overnight in fresh 100% ethanol at 4°C. Once in 100% ethanol, samples

629 were dried with a critical point dryer (EM-CPD300, Leica Microsystems), cut in half and

630 coated with a 4nm layer of platinum using a sputter coater (EM-ACE600, Leica

631 Microsystems). The samples were imaged with a field emission scanning electron

632 microscope Merlin compact VP (Carl Zeiss) at 3 kV. The signal was detected by an Everhart-

633 Thornley type secondary electron detector.

634

635 **STEM Tomography Sample Preparation**

636 Alkali maceration of LNs was performed as previously described[54,55]. Briefly, popliteal LNs

637 were isolated from 8–12 weeks old wt C57BL/6 mice and directly fixed in a 2.5%

638 glutaraldehyde and 2% paraformaldehyde in PB (0.1 M, pH 7.4) for a minimum of two weeks

639 at 4°C. Samples were then macerated in aqueous 2.5 M (10% w/v) NaOH solution for 5 days

640 at RT under mild agitation. Next sections were rinsed in H_2O under mild agitation for one to

641 two days until samples became pale. If results were not sufficient, the maceration step was

642 repeated.

643

644 Samples were then treated with 0.5% tannic acid (w/v) in PB (0.1 M, pH 7.4) two times 1h

645 each with freshly prepared solutions, washed in PB and treated with aqueous 1% osmium

646 tetroxide (w/v) for 30 min at 4°C. Samples were contrast enhanced with aqueous 1% uranyl

647 acetate (w/v) overnight at 4°C and Walton´s lead aspartate for 30 min at 60°C. Samples

648 were then dehydrated in graded ethanol, infiltrated with anhydrous propylene oxide and

649 embedded in hard-grade epoxy resin (Durcupan® ACM, Fluca). Samples were consecutively

650 infiltrated with a 3:1 mixture of anhydrous acetone and Durcupan® for 1h at 4°C, 1:1

651 aceton/Durcupan® for 1.5h at 4°C, 1:3 aceton/Durcupan® for 2h at 4°C and mere Durcupan®

652 overnight at RT. Samples were transferred to BEEM capsules (Electron Microscopy

653 Sciences), filled with freshly prepared Durcupan® and cured for 48h at 60°C.

654

655 **STEM Tomography Imaging**

656 Semi-thin sections were cut at 450 nm using an UC7 ultramicrotome (Leica Microsystems)

657 and collected onto formvar-coated 200-line bar grids + 1C/bar (Science Services, G200PB)

658 and coated with evaporated carbon to a thickness of 8nm. Grids were cut in half, mounted

659 on a Half-Mesh High Tilt holder (Jeol, EM-21010/Z09291THTR) and observed under a JEM

660 2800 scanning transmission electron microscope (Jeol) operated at 200 kV in STEM mode.

661 To compensate for focus, contrast and brightness, and stage shift during image tilt series

662 recording, an automated system was used comprising the STEM Recorder V3 Vers. 3.2.8.0

663 and the STEM Magica Controller Vers. 0.9.8.1 (both System In Frontier Inc.). Images were

664 collected at 2° intervals between +/-76° of single tilt axis. Magnification was x80k – x600k,

665 image size 512x512 pixels giving a pixel sizes from 6.749463 nm/px to 0.899928 nm/px.

666

667 **Conduit Stretching Quantification**

668 STEM Tomography images were aligned by cross-correlation and 3D structure of area of

669 interest computed by weighted back-projection using Composer software Vers. 3.0 (System

670 In Frontier Inc.). A 3D Gaussian blur filter and background subtraction (rolling ball algorithm)

671 pre-processing step were performed on the images using FIJI. The 3D image stacks were

672 subsequently loaded into Imaris, and fibrils of conduits were manually traced using the

673 Filament Tracer tool and exported to Matlab format using the Object Exporter (exported

674 from Imaris as filaments). The overall orientation and curvature of the centerline of the

675 entire conduit was approximated by fitting a cubic spline curve with four support points

676 which minimized a hand-crafted cost function through all fibril track data. This cost function

677 penalizes the distance of the desired centerline to the tracks, the total curvature of the

678 centerline and the difference in length between it and the fibril bundles, and ensures that

679 the support points are spaced evenly. In a few cases this spline curve was corrected by hand

680 if it was found to not adequately represent the center line of the bundle. The alignment of

681 the individual fibrils with respect to the centerline of the conduit was calculated as follows:

682 The spline centerline was interpolated in a continuous fashion and the 3D orientation was

683 calculated. Likewise, the tracks of individual fibrils were first smoothed to reduce tracing

684 errors and the 3D orientation of each segment of the trace was calculated. The alignment

685 angle A between the fibril is then given by the angle between the orientation of the

686 segment and the orientation of the centerline at the point that is closest to the segment:

687

$$A = \text{acos}\left(abs\left(\frac{\vec{v}_{fibril} \cdot \vec{v}_{centerline}}{\left|\vec{v}_{fibril}\right| \times \left|\vec{v}_{centerline}\right|} \right) \right)$$

688

689 **UV-Laser Cutter Setup**

690 The UV-laser cutter setup is based on a previously described layout[35,56]. In brief, a passively

691 Q-switched solid-state 355 nm UV-A laser (Powerchip, Teem Photonics) with a repetition

692 rate of 1 kHz, pulse energy of 15 µJ, pulse-length of <350 ps and peak-power of 40 kW was

693 used in conjugation with a spinning disc microscope (Axio Observer Z1, Zeiss). The system is

694 controlled using custom-built software (LabView, National Instruments) enabling cutting in

695 3D. Typically 5% of the power is used to cut tissues.

696

697 **UV-Laser Ablation Experiments**

698 Tension on FRCs, fLECs and capsule ECM was measured by conducting laser ablations on an

699 inverted UV-laser ablation setup with a manufacturer 40x 1.2NA water immersion lens in

700 homeostatic and inflamed LNs. For all experiments 25 UV pulses at 1000 Hz to 40

701 equidistant sites using 200 ms exposure time and frame rate were used to ablate and

702 capture tissue recoil. For FRC and LEC ablation, we established an intravital setup where

703 Ccl19-Cre hem; mTmG hom mice were anesthetized and intact inguinal LNs exposed using a

704 skin flap surgery. The paracortical site of the LN was mounted on a custom-made stage

705 which allowed the LN temperature to be regulated at 37°C. For capsule ECM ablation,

706 popliteal LNs were harvested and incubated in 100 µM TAMRA in RPMI 1640 solution (both

707 Invitrogen) for 15 min at RT and directly used for experiments. Explanted LNs were mounted

708 at room temperature in a glass bottom Petri dish (MatTek) in RPMI and prevented from

709 floating using a 22x22 mm cover glass topping.

710 Cuts were performed in either three z-planes spaced 1 µm apart along a length of either 10

711 µm for FRCs, or in one z-plane along 20 µm for fLECs and capsule ECM. FRC cuts were

712 typically performed at 10-20 µm depth underneath the capsule. Recoil of FRCs and LECs was

713 quantified from kymographs made in FIJI, while capsule ECM recoil was quantified using

714 PIVlab in Matlab. In the latter case, temporal recoil velocities were measured between

715 bandpass filtered pre- and consecutive post-cut frames by averaging the component of the

716 calculated velocity in the perpendicular direction to the cut, within an area of the

717 surrounding cut site.

718

719 **YAP/TAZ Quantification**

720   The nuclear to cytoplasmic ration of stained YAP/TAZ in FRCs were measured from 3D

721   sections of peripheral LNs. In FIJI, FRCs were identified by the mGFP labeling and for each

722   FRC the average YAP/TAZ fluorescent intensity of the nucleus (identified by DAPI) was

723   divided by the average intensity of the adjacent cytoplasm of the cell body. In other cases,

724   YAP/TAZ localization was qualitatively assessed to contain to either contain a higher nuclear

725   or cytoplasmic YAP/TAZ intensity.

726

727   **CCL21 Quantification**

728   Cryosections containing both a control and FRC$^{\Delta TLN1}$ peripheral LN in a single section were

729   stained for CCL21 and pair-wise imaged using similar settings. The average fluorescent

730   intensities of CCL21 were then measured from paracortical areas and normalized to the

731   control.

732

733   **Proliferation and Apoptosis Measurements of FRCs**

734   Large 3D volumes (xy:306x306 μm, z: 50-150 μm) stained for either cleaved Caspase 3 or

735   Ki67 were acquired from Ce3D cleared thick vibratome sections were corrected for

736   fluorescent intensity in z-axis using 'bleach correction (histogram matching)' function in Fiji.

737   Imaris was then used to generate a 3D isosurface of the FRC network by utilizing a surface

738   detection feature from the FRC-network fluorescent channel. The isosurface was then used

739   to mask the cleaved Caspase 3 and Ki67 channels so only the fluorescent signal within the

740   FRC network remained. Positive nuclei were then manually counted from 2D slice views and

741   normalized for per volume unit.

742

743   **Capsule Thickness Measurements**

744   The thickness of capsules was measured in vibratome sections of PROX1-GFP or wt mice,

745   stained for PDGFR-β and DAPI. The size of the capsule was then manually measured in FIJI

746   from the SCS to the surrounding adipose or muscular tissue from at least 3 locations and

747   were averaged per LN.

748

749   **Statistical Analysis**

750   All statistical analyses were performed in GraphPad Prism 8. P-values <0.05 were considered

751   significant.

752

753   **Software**

FIJI	Schindelin et al., 2012	https://fiji.sc/
Imaris	Bitplane	https://imaris.oxinst.com/packages
Excel	Microsoft	https://products.office.com/en-us/?rtc=1
Prism	GraphPad Software	https://www.graphpad.com/scientific-software/prism/
MATLAB	MATLAB Software	https://www.mathworks.com/products/matlab.html
Ilastik	Sommer et al., 2011	https://www.ilastik.org/
SquisherJoy	Cell Scale	https://www.cellscale.com/products/microtester/
Composer software	System Frontier Inc.	In https://temography.com/en/composer-en/
PIVlab	Thielicke and Stamhuis 2014	https://pivlab.blogspot.com/

754

755   **Data availability**

756   Data supporting the findings of this study are available from the corresponding

757   authors upon.

758

759   **Code availability**

760   Code used for various analyses in this study are available from the

761   corresponding authors upon request.

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762

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889

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Contributions

897 F.P.A. designed experiments, and performed all *in vivo* and *ex vivo* experiments with
898 assistance of M.H., M.B., S.S and G.K.; J.A. cleared samples and did light-sheet imaging,
899 F.P.A. did all other fluorescent imaging. W.A.K. processed samples for electron tomography,
900 and W.A.K., T.C. and F.P.A. acquired tomograms. F.P.A. did all data processing and analysis
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902 the interpretation of MADM and mechanical data, respectively. S.L discussed data. M.S.
903 directed the study, F.P.A. and M.S. wrote the manuscript, and all authors critically reviewed
904 the manuscript.

Ethics declarations

The authors declare no competing interests.

905

906   **Figure 1.** The reactive lymph node resists swelling. (**a**) Side-view focus-stack of popliteal LNs

907   in homeostasis (D0) and inflammation (D14). Scale bar = 500 µm. (**b**) Volumes of swelling

908   LNs calculated from 2D-side views over the course of two-week following onset of

909   immunization. Means±SEM are connected (blue line) and a linear regression line (dashed)

910   has been fitted to the data. (**c-d**) Overview of the stress-relaxation experiment on explanted

911   popliteal LNs (D2 of inflammation depicted). (**c**) Measured geometrical parameters are

912   annotated on 2D-side images acquired during a measurement. Scale bar = 300 µm. (**d**) The

913   stress relaxation curve is given by the measured force over time from which the equilibrium

914   force (long timescale) parameter), viscosity (medium timescale) and Young's Modulus (short

915   timescale) relaxation parameters are derived. (**e-g**) Quantification of tissue properties in

916   homeostasis (D0) and inflammation (D2, D4, D8, D14) derived from the stress relaxation

917   curve and geometrical properties. Each datapoint represents a single measured LN.

918   Measurements are pooled from LNs harvested from at least 5 mice and multiple

919   experiments. Mean±SEM. (**e**) Quantification of the effective resistance. (**f**) Quantification of

920   the viscosity. (**g**) Quantification of the Young's Modulus. For statistical analysis see

921   Supplementary Information, table 1. $*P<0.05$, $**P<0.01$, $****P<0.0001$.

922

923   **Figure 2**. Lymphocyte numbers generate pressure and drive lymph node swelling. (**a-j**)

924   Analysis of stress-relaxation experiments on explanted LNs. Each datapoint represents a

925   single measured LN. Measurements are pooled from LNs harvested from at least 5 mice and

926   multiple experiments. Mean±SEM are given. (**a**) Side-view of explanted homeostatic LNs

927   from mice treated with anti-CD62L or PBS. Scale bars = 300 µm. Dashed lines depict the

928   outline of LNs. (**b**) Quantification of LN volume. (**c**) Quantification of the effective resistance.

929   (**d**) Quantification of the viscosity. (**e**) Quantification of the Young's Modulus. (**f**) Side-view of

930   explanted D4 inflamed LNs from wt and OTII mice treated with anti-CD62L or PBS. Scale bars

931   = 400 µm. Dashed lines depict the outline of LNs. (**g**) Quantification of LN volumes. (**h**)

932   Quantification of the effective resistance. (**i**) Quantification of the tissue viscosity. (**j**)

933   Quantification of the Young's Modulus. For statistical analysis see Supplementary

934   Information, table 1. ns, not significant. ns, not significant. $*P<0.05$, $**P<0.01$, $***P<0.001$,

935     $****P<0.0001$.

936

937   **Figure 3.** The FRC network stretches upon lymph node swelling. (**a**) Overview of the FRC

938   network gap analysis. Left: mGFP labeled FRCs (green), middle: segmented FRC network

939   (white), right: gaps (randomly colored circles) in the FRC network. Scale bar = 20 µm. (**b**)

940   Representative images of FRC networks gaps in homeostasis (D0) and inflammation (D2, D4,

941   D8 and D14). (**c**) Averaged and smoothed distribution of FRC network fitted circle

942   distribution plotted as the Weighted Area Fraction as a function of the fitted circle diameter.

943   (**d**) Quantification of the mean fitted-circle diameter. Each datapoint represents the average

944   value of 10-30 analyzed consecutive sections of an acquired 3D volume. Means for each

945   timepoint are depicted as a black line. Data are for each timepoint pooled from 5 popliteal

946   or inguinal LNs from at least 3 mice and 2 separate experiments. For statistical analysis see

947   Supplementary Information, table 1. ***$P<0.001$, ****$P<0.0001$.

948

949   **Figure 4.** Conduits are stretched in the swelling lymph node. (**a**) Overview of STEM

950   tomography acquisition of macerated LN samples. Images show the fibrillar collagen of T

951   zone conduits at a single tilt angle, and a maximum intensity projection crop of a 3D conduit

952   reconstructed from multiple tilting angles. (**b**) Representative 3D reconstructions of fibrillar

953   collagen (blue) from macerated conduits at homeostasis (day 0) and inflammation (D2, D4

954   and D14) in which the conduit center line (yellow) and tracked fibril segments (grey) are

955   annotated. (**c**) Visual representation of the conduit fibril alignment analysis of an imaged 3D

956   conduit volume. Angles of individual fibril segments (thick colored lines) with the center line

957   of the conduit (dashed black line) are measured at multiple points along the fibril segment

958   (thin colored lines) and averaged per fibril segment. (**d**) Quantification of conduit fibril

959   alignment with center line. Each datapoint represents an individual fibril segment. Means

960   are depicted as black lines. For statistical analysis see Supplementary Information, table 1.

961   ns, not significant. ***$P<0.001$, ****$P<0.0001$.

962

963   **Figure 5.** FRC network tension increases upon lymph node swelling. (**a**) Overview of *in vivo*

964   UV-laser cut experiment of the FRC network. Inguinal LNs from FRC-mGFP mice are

965   surgically exposed and kept hydrated at 37°C. A high UV-laser cuts the FRC network along 10

966   µm at 3 different planes after which the local recoil of the FRC network is imaged. Scissor

967   and line indicate cutting location and arrows the recoiling FRC network. Scale bars = 20 µm.

968   (**b**) Representative example of FRC network recoil. Images depict stills from before (t=-1s),

969   directly after (t=0s) and late after (t=6.2s) cutting (scale bars = 5 μm), with corresponding

970   kymograph along the recoil axis (scale bar x-axis = 1s, y-axis = 2 μm). Scissor and line

971   indicate cutting location and arrows the recoiling FRC network. Dashed lines in kymograph

972   indicate slopes used to calculate the recoil velocity, and vertical white line the cut. (**c**)

973   Quantification of recoil velocity from kymographs. Each datapoint represents a single FRC

974   network cut. Data is pooled from 3-4 animals and at least 2 experiments per analyzed

975   timepoint. Means±SEM are depicted and connected with a line. (**d**) 3D view of the FRC

976   network and stained for YAP/TAZ. Stack size 20 μm. Scale bar = 20 μm. (**e**) Representative

977   examples of YAP/TAZ nuclear and cytoplasmic localization. N:C = nuclear to cytoplasmic

978   fluorescent intensity ratio. (**f**) Quantification of YAP/TAZ N:C ratio. Dashed line indicates an

979   equal ratio. Each datapoint corresponds to a single measured FRC. Measurements are taken

980   from a total of 3 LNs per analyzed timepoint from at least two separate experiments.

981   Means±SEM are depicted and connected with a line. For statistical analysis see

982   Supplementary Information, table 1. ns, not significant. *P<0.05, **P<0.001, ****P<0.0001.

983

984   **Figure 6**. FRCs in the swelling lymph node undergo distributed clonal expansion. (**a**)

985   Schematic overview of the sparse mosaic analysis with double markers (MADM) labeling

986   approach for FRC cluster imaging. (**b-k**) Quantification of light sheet images from cleared

987   LNs in homeostasis (D0) and inflammation (D4 and D8). All graphs depict the mean±SD. Each

988   datapoint represents a single popliteal LN. LNs are retrieved from at least 3 mice and 2

989   separate experiments for each condition. (**b**) LN volumes. (**c**) Numbers of MADM labeled

990   FRCs. (**d**) Numbers of FRCs found in clusters. (**e**) Number of clusters. (**f**) 3D view of HEVs

991   (grey) and smoothed convex-hull of cluster volumes (randomly colored) in a homeostatic

992   (D0) and inflamed (D4) LN, scale bar = 200 μm (**g**) Frequency distribution of FRC cluster sizes

993   found in observed and simulated data in homeostasis (D0) and inflammation (D4 and D8).

994   (**h**) Quantification of the cluster factor (CF) between homeostatic (D0) and inflamed LNs (D4

995   and D8). (**i**) Number of FRC clusters in homeostatic (D0) and inflamed LNs (D4 and D8)

996   plotted as a function of the distance from the nearest HEV. (**j**) Correlation-matrix of paired

997   variables assessed in the cluster analysis. Spearman correlation: *p*-values are given and the

998   correlation-coefficients are color-coded. (**k**) CF plotted as a function of the LN volume. A

999   spline-fit is plotted through the data-points. For statistical analysis see Supplementary

1000   Information, table 1. ns, not significant. **P<0.01, ***P<0.001, ****P<0.0001.

1001

1002

1003 **Figure 7.** Talin1 is required for FRC mechanosensing. (**a**) Quantification of LN weights in

1004 homeostasis and following inflammation (D4 and D14) in control and FRC$^{\Delta Talin1}$ mice. Each

1005 datapoint represents a single LN. Data is pooled from at least 5 mice from multiple

1006 experiments per condition. Insert depicts the grow curves of the plotted data fitted with a

1007 non-linear regression function. Mean±SEM. (**b**) T zone FRC network of FRC-mGFP (control)

1008 and FRC$^{\Delta Talin1}$-GFP mice stained for YAP/TAZ. Enlargements depict representative examples

1009 of the N:C of YAP/TAZ. Scale bars = 10 μm. (**c**) Quantification of YAP/TAZ localization in FRCs

1010 of control and FRC$^{\Delta Talin1}$ mice in homeostasis (D0) and at inflammation (D4). Mean±SEM. (**d**)

1011 Representative 3D images of 3D network analysis of control and FRC$^{\Delta Talin1}$ mice in

1012 homeostasis (D0) and following inflammation (D4 and D14). Imaged stack size: 100-300 μm.

1013 Scale bars = 50 μm. (**e**) Quantification of FRC network analysis as shown in **d**. Left: average

1014 weighted volume fraction plotted as function of the sphere diameter. Right: average sphere

1015 diameter for all conditions. Each datapoint represents an individual LN from at least 3 mice

1016 and 2 experiments per condition. Means are depicted by black lines. (**f**) Quantification of

1017 cleaved Caspase3+ FRCs from FRC-mGFP (control) and FRC$^{\Delta Talin1}$-GFP mice in homeostasis

1018 (D0) and inflammation (D4 and D8). Each datapoint represents a single LN. Data pooled

1019 from 3 mice per timepoint. Images depict the identification of an apoptotic FRC. Scale bar =

1020 3 μm. (**g**) Quantification of Ki67+ FRCs from FRC-mGFP (control) and FRC$^{\Delta Talin1}$-GFP mice in

1021 homeostasis (D0) and inflammation (D4 and D8). Each datapoint represents a single LN.

1022 Images depict the identification of a proliferating FRC. Scale bar = 3 μm. Data pooled from 3

1023 mice per timepoint. Mean±SD. For statistical analysis see Supplementary Information, table

1024 1. ns, not significant. *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001.

1025

1026 **Figure 8.** Capsule fibrosis constrains lymph nodes at late timepoints. (**a**) Overview of

1027 intravital UV-laser cut experiments of the SCS fLECs. Inguinal LNs from FRC-mGFP mice, in

1028 which SCS fLECs are sparsely labeled with a mGFP are surgically exposed. A high UV-laser

1029 cuts the cell along 20 μm in one plane after which the local recoil is imaged. Images depict

1030 stills from before (t=-1s), directly after (t=0s) and late after (t=10.2s) cutting (scale bars = 5

1031 μm), with corresponding kymograph along the recoil axis (scale bar x-axis = 1s, y-axis = 2

1032 μm). Scissor and line indicate location of cut and arrows the recoiling cell. Dashed lines in

1033   kymograph indicate slopes used to calculate the recoil velocity, and vertical white line the

1034   cut. (**b**) Quantification of experiments as described in **a** for homeostatic (D0) and inflamed

1035   (D2, D4, D8, D14) LNs. Each datapoint represents a recoil-measurement of a single fLEC.

1036   Data pooled from 3 mice and at least two experiments. Mean±SEM are depicted and

1037   connected with a line. (**c**) Representative confocal images of LN capsules from PROX1-GFP

1038   mice in homeostasis (D0) and inflammation (D4, D8 and D14), in which LECs are labeled by a

1039   cytoplasmic GFP. Mesenchymal cells are stained for PDGFR-β, and nuclei are counterstained

1040   with DAPI. Scale bars = 20 μm. (**d**) Quantification of the capsule thickness measured from

1041   the SCS as depicted in **c.** Each datapoint represents the average of 3 measurements per LN.

1042   Mean±SEM are connected with a line. Data pooled from 5 LNs derived from 3 mice per

1043   timepoint. (**e**) Overview of micro-pipette assay for capsule stiffness measurements.

1044   Capsules of explanted popliteal LNs are labeled by brief incubation with Alexa Fluor 647-

1045   conjugated anti-ERTR7 antibody, upon which a small diameter glass pipette is placed on the

1046   capsule and a defined negative pressure applied. The ECM 'tongue' entering the pipette is

1047   subsequently measured. The local effective Young's Modulus of the capsule is derived using

1048   Laplace's law. Scale bar = 50 μm. (**f**) Quantification of measurements as described in **e** for

1049   homeostatic (D0) and inflamed (D2, D4, D8 and D14) LNs. Each datapoint represents the

1050   average of 3 measurements per LN. Mean±SEM are connected with a line. Data pooled from

1051   5 LNs derived from 3 mice per timepoint. (**g**) Quantification of the Capsule swelling

1052   resistance as measured by the Passive Tension, given by multiplying the capsule thickness

1053   and Young's modulus from **d** and **f**, respectively. Each datapoint represents a single LN.

1054   Mean±SEM are connected with a line. (**h**) Schematic of the mechanical dynamics of the

1055   swelling LN. For statistical analysis see Supplementary Information, table 1. ns, not

1056   significant. **P<0.01, ***P<0.001.

1057   **Supplementary Figure 1.** (**a**) Quantification of popliteal LN volumes calculated from 2D side

1058   views in homeostasis (D0) and inflammation (D2, D4, D8, D14) acquired from parallel plate

1059   compression experiments. Mean±SEM. (**b**) Relation between calculated volumes and

1060   measured weights of popliteal LNs in homeostasis and inflammation. A regression line is

1061   fitted. (**c**) Example of a double-exponential force fit (red line) on a stress-relaxation

1062   measurement. Arrow indicates the force at equilibrium (F_{eq}). (**d**) Average of stress-relaxation

1063   curves for all measured conditions. Mean±SEM. (**e**) Schematic representation of the

1064   generalized Kelvin model and formulas used to calculate tissue properties from parallel

1065   plate stress-relaxation experiments. Adapted from *Forgacs et al.*. (**f**) SEM image of packed

1066   lymphocytes in the LN paracortex. Scale bar = 2 µm. For statistical analysis see

1067   Supplementary Information, table 1. **P<0.01, ****P<0.0001.

1068   **Supplementary Figure 2.** (**a**) Schematic of LN processing for STEM tomography of T zone

1069   conduits. (**b**) Example of a macerated conduit imaged by STEM in which the characteristic D-

1070   period of collagen fibrils can be observed. Scale bar = 500 µm. (**c**) Examples of a T zone

1071   conduit at different tilting angles acquired by STEM tomography. (**d**) Schematic of a

1072   computed weight back projection to reconstruct a 3D volume of a conduit from differential

1073   tilting angles.

1074   **Supplementary Figure 3.** (**a**) Schematic of the MADM labeling principle. Rare

1075   interchromosomal recombination in the G2 cell cycle phase following x-segregation of

1076   chromosomes labels FRCs with either a cytoplasmic tdTomato or GFP. (**b**) Sparse labeling of

1077   FRCs. Scale bar = 200 µm. (**c**) 3D fluorescent intensity images of GFP labeled FRCs and *in situ*

1078   labeled HEVs by anti-PNAd-ATTO647n antibody. (**d**) Quantification of the MADM labeling

1079   distribution in homeostasis (D0) and inflammation (D4 and D8). Each datapoint represents a

1080   single LN. Mean±SD. (**e**) Overview of the labeling of HEVs and mapping of labeled FRCs (only

1081   tdTomato+FRCs are shown) at D4 of inflammation. The enlarged image depicts the mapping

1082   of individual FRCs by a grey sphere at the center of each cell. Scale bars = 200 µm. (**f**)

1083   Formula for the calculation of the cluster factor (CF) per LN. (**g**) Quantification of the

1084   average distance to the nearest neighbor (NN) of both mapped FRCs and randomly localized

1085   FRCs from simulations in homeostasis (D0) and inflammation (D4 and D8). Means are

1086   depicted by black lines. (**h**) Quantification of relative numbers of MADM-labeled FRCs found

1087   in clusters in both homeostasis (D0) and inflammation (D4 and D8). Mean±SD. For statistical

1088   analysis see Supplementary Information, table 1. ns, not significant. **P<0.01, ***P<0.001.

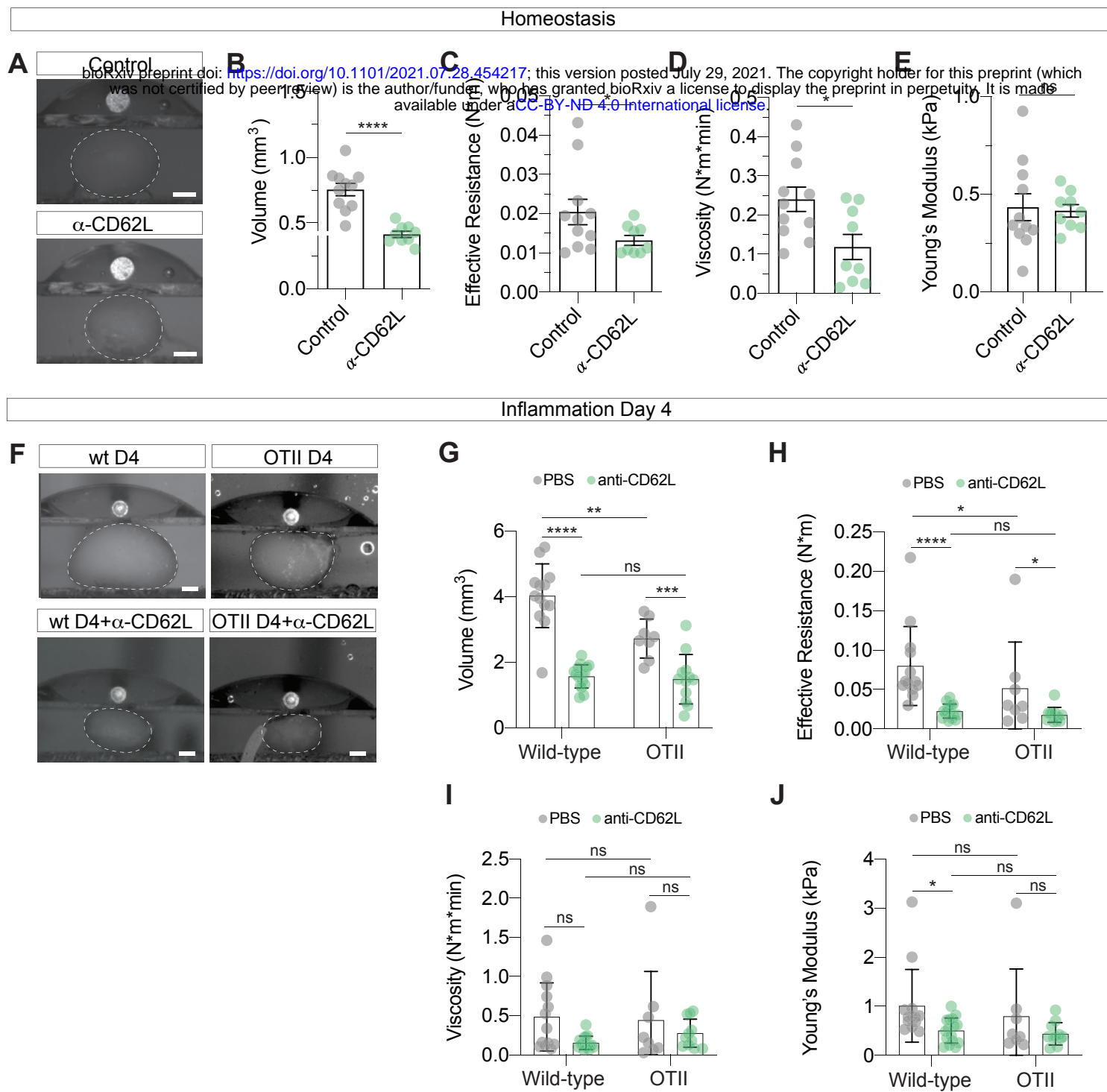1089   **Supplementary Figure 4.** (**a**) Quantification of steady-state popliteal and inguinal LN weights

1090   from control and FRC$^{\Delta Talin1}$ mice. Each datapoint represents a measured LN. Images show a

1091   representative inguinal LN for each condition. Scale bars = 1 mm. (**b**) Quantification of

1092   steady-state T zone CCL21 protein as measured *in situ* by fluorescent intensity following

1093   staining for CCL21. Measurements are taken from histological sections from 5 LNs of 3 mice
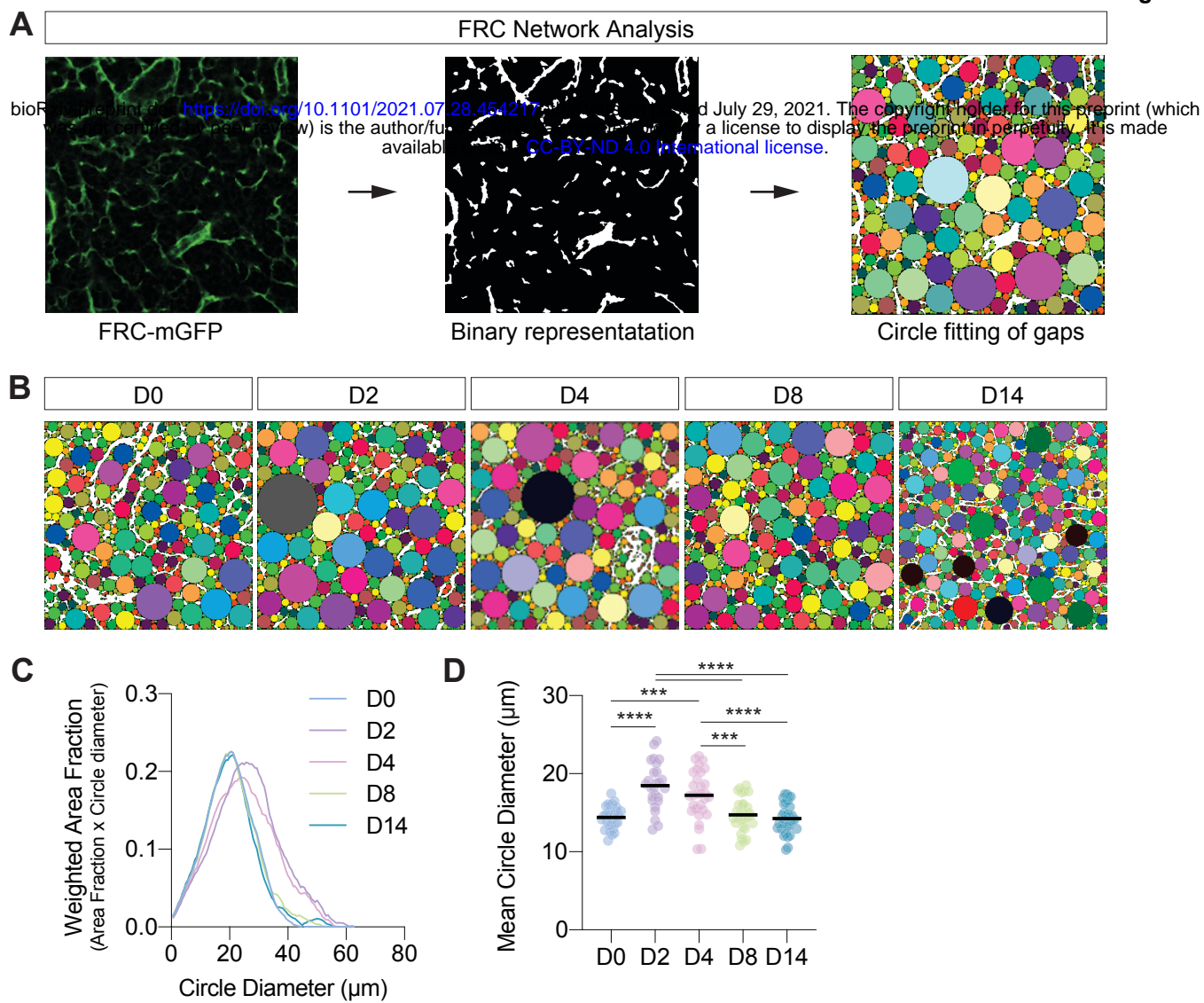
1094   for each condition. Mean±SEM. Scale bars = 20 μm. (**c**) T zone and B-cell follicles in steady-

1095   state popliteal LNs from control and FRC$^{\Delta Talin1}$ mice stained for CD3 (T cells) and B220 (B

1096   cells). Scale bars = 200 μm. (**d**) ICAM-1, PDPN, VCAM and merged staining from steady-state

1097   popliteal LN T zones from control and FRC$^{\Delta Talin1}$ mice. Scale bars = 20 μm. (**e**) Overview of

1098   the T zone FRC network in homeostasis (D0) and inflammation (D4 and D14) between FRC-

1099   mGFP (control) and FRC$^{\Delta Talin1}$-mGFP mice stained for collagen IV. Scale bar = 50 μm. For

1100   statistical analysis see Supplementary Information, table 1. *P<0.05, ****P<0.0001.

1101   **Supplementary Figure 5.** (**a**) Ccl19-Cre; mTmG mice sparsely label fLECs of the SCS. Zoomed

1102   window depict the SCS with LECs brightly labeled with tdTomato (mTdT) and the sparsely

1103   labeled fLEC (mGFP), given by the double positive labeling with the fLEC-marker LYVE-1

1104   (gray). (**b**) Overview of the UV-laser cut experiment on TAMRA labeled capsule ECM of

1105   explanted LNs in homeostasis (D0) and inflammation (D2, D4, D8, D14), which faithfully

1106   represents capsule ECM as observed in alkali macerated LNs imaged by STEM and SEM.

1107   Particle imaging velocimetry (PIV) was used to measure displacement of structures above

1108   and below the cut site from band-pass filtered images. Recoil displacement is depicted by

1109   orange vectors. Scale bar STEM image = 5 μm, SEM image = 1 μm, fluorescent TAMRA-

1110   labeled image = 20 μm. (**c**) Quantification of recoil velocities of capsule cuts from

1111   experiments on homeostatic (D0) and inflamed (D2, D4, D8, D14) LNs as described in **b**. Each

1112   datapoint represents a single cut. Between 14 and 24 cuts were taken per measured

1113   timepoint from at least 5 LNs from two animals and at least two separate experiments.

1114   Mean+SEM connected by a line. (**d**) Representative histological examples of LN capsules

1115   from PROX1-GFP mice in homeostasis and D14 of inflammation, stained for PDGFR-β and

1116   counterstained with DAPI. Scale bars = 200 μm. For statistical analysis see Supplementary

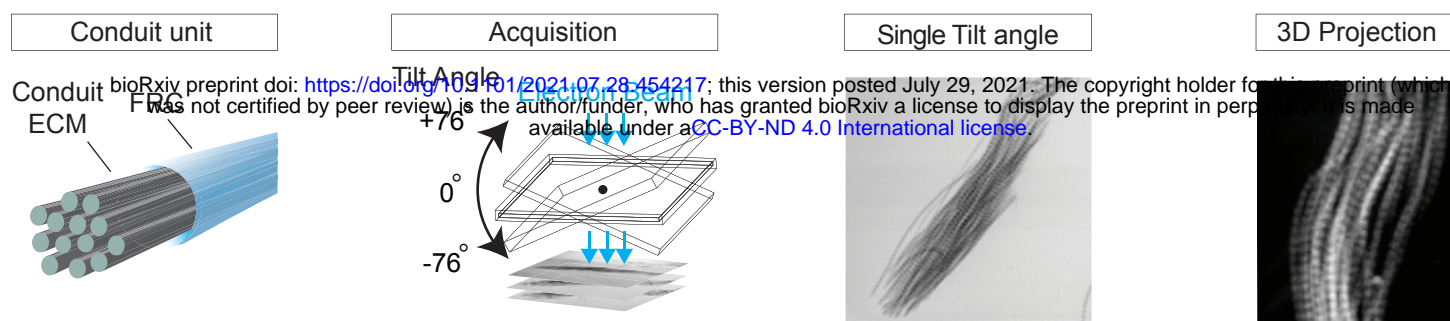1117   Information, table 1. *P<0.05, **P<0.01.

1118   **Supplementary Movie 1.** Example of low (homeostasis; D0) and high tension (Inflammation;

1119   D4) of the FRC network as given by the recoil speed following *in vivo* UV-laser cutting in

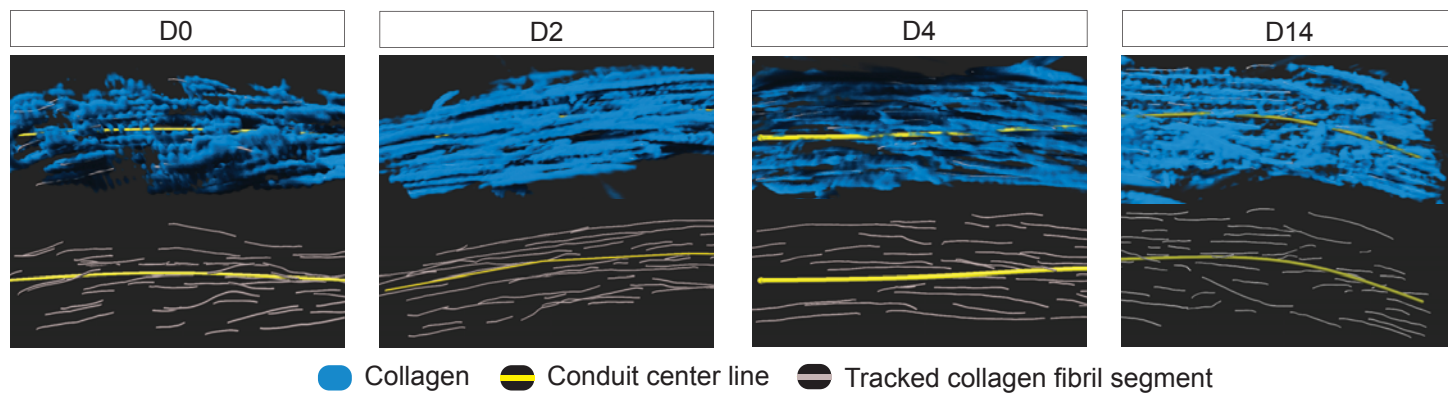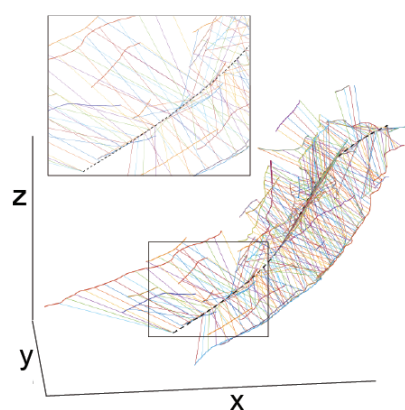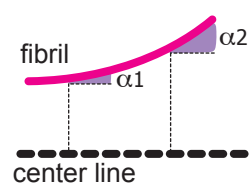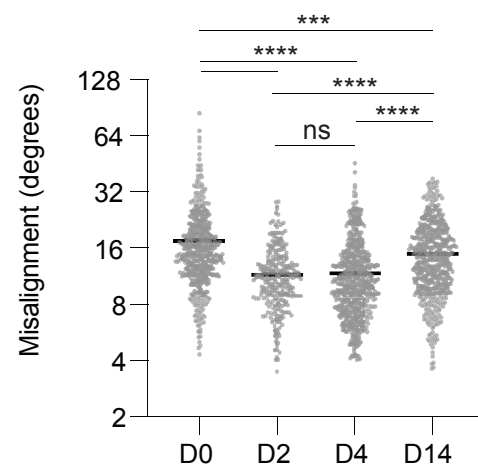1120   surgically exposed inguinal LNs of FRC-mGFP mice.

Figure 1

A

Lymph Node Swelling: D0 vs D14

B

$R^2 = 0.862$

$Y = 0.7645 \cdot X + 0.7724$

Days of Inflammation

C

Before Compression	At Equilibrium
0% Strain	25% Strain

Measured Force

H_0 L H_{eq} R2   R1   R3

D

Stress Relaxation Curve

Max Compression   Equilibrium   Release Plate

short, medium, and long term relaxation

Time (min)

E

****
**
*
**

D0 D2 D4 D8 D14

F

*

D0 D2 D4 D8 D14

G

*   *

D0 D2 D4 D8 D14

Figure 2

Homeostasis

A Control

α-CD62L

Inflammation Day 4

F wt D4 | OTII D4

wt D4+α-CD62L | OTII D4+α-CD62L

Figure 3

A

FRC Network Analysis

FRC-mGFP → Binary representatation → Circle fitting of gaps

B

| D0 | D2 | D4 | D8 | D14 |

C

Weighted Area Fraction (Area Fraction x Circle diameter)

Circle Diameter (μm)

D0
D2
D4
D8
D14

D

Mean Circle Diameter (μm)

D0 D2 D4 D8 D14

***
****
****
****
***

Figure 4

A

| Conduit unit | Acquisition | Single Tilt angle | 3D Projection |

Conduit
ECM

Fibril

Tilt Angle

Electron Beam

+76°

0°

-76°

B

| D0 | D2 | D4 | D14 |

🔵 Collagen   ━ Conduit center line   ━ Tracked collagen fibril segment

C

| Collagen fibril alignment analysis | Angle measurements |

z

y

x

fibril

α1   α2

center line

D

***

****

****

****

ns

128
64
32
16
8
4
2

Misalignment (degrees)

D0   D2   D4   D14

Figure 5

A

Before cut | After cut

FRC-mGFP

Skin flap
Inguinal lymph node
Cover glass
40X
488nm imaging laser
high power UV-laser

B

t=-1.0s | t= 0.0s | t= 6.2s | Kymograph

FRC-mGFP

C

FRC Network Tension

ns
**
****
ns

Recoil Velocity (μm/min)
D0 D2 D4 D8 D14

D

3D: FRC mGFP YAP/TAZ | 3D: YAP/TAZ

E

Low N:C YAP/TAZ | Medium N:C YAP/TAZ | High N:C YAP/TAZ

N:C = 0.32 | N:C = 1.85 | N:C = 2.74

FRC-mGFP   YAP/TAZ   DAPI

F

FRC Mechanosensing

*
****
****
ns

Ratio Nuclear:Cytoplasmic Intensity
D0 D2 D4 D8 D14

Figure 6

A

Ccl19-Cre hem; MADM-7$^{GT/TG}$
Sparse MADM labeling of FRCs

○ GFP+ FRC
○ tdTomato+ FRC

Homeostasis   **Inflammation**

Footpad injection KLH/CFA → D0   D4   D8

Endpoint analyses

Figure 6

Figure 7

Figure 8

A

t= -1.0s t= 0.0s t= 10.2s Kymograph

fLEC-mGFP

Skin flap
Inguinal Lymph Node
Glass
40X

B fLEC Tension

ns

**

Recoil Velocity (μm/min) — D0 D2 D4 D8 D14

C D0 D4 D8 D14

PROX1-GFP PDGFR-β DAPI

D Capsule Thickness

***
**

Capsule Thickness (μm) — D0 D2 D4 D8 D14

E Capsule Stiffness Measurement

micro-pipette φ: 15 μm

Lymph node

P= 0 mBar   P = -750 mBar

P_{atm} $P_{pipette}$
d
h
Lymph Node

$$E\ (Pa) = \frac{\Delta P}{\left(\frac{h}{d}\right)^2}$$

ERTR7

F Capsule Stiffness

**

Young's Modulus (kPa) — D0 D2 D4 D8 D14

G Capsule Resistance

***
**

Passive Tension (kPa*μm) — D0 D2 D4 D8 D14

H Mechanical Dynamics of the Swelling Lymph Node

LN Volume
Capsule Thickness

FRC Network Configuration

Conduit Allignment

FRC Tension & Mechanosensing

FRC Clonal Expansion

Capsule Resistance

Effective Resistance

Day 0 Day 2 Day 4 Day 8 Day 14

Phase I FRC relaxation and stretching

Phase II Increasing FRC tension and proliferation

Phase III Decreasing FRC tension, and growth

Phase IV Growth with capsule thickening and further strong resistance to growth

A Lymph Node Volumes

Volume (mm^3)

20

15

10

5

0

D0 D2 D4 D8 D14

****
****
****

B Volume-Weight Correlation

Volume (mm^3)

15

10

5

0

0 5 10 15

Weight (mg)

$R^2 = 0.9779$

C Force Fit

Force (µN)

150

100

50

0 5 10 15 20 25 30

Time (min)

Force (t) = $A_1 * e^{-t/\tau 1} + A_2 * e^{-t/\tau 2}$

F_{eq}

D Stress Relaxation Curves

Force (mN)

4

2

0

0 5 10 15 20

Time (min)

— D0
— D2
— D4
— D8
— D14

E

Generalized Kelvin Model

Viscous Components   Elastic Components
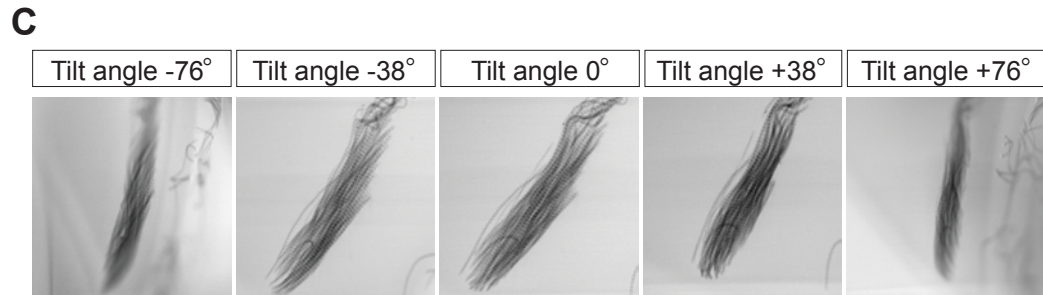
μ_1 k1

μ_2 k2

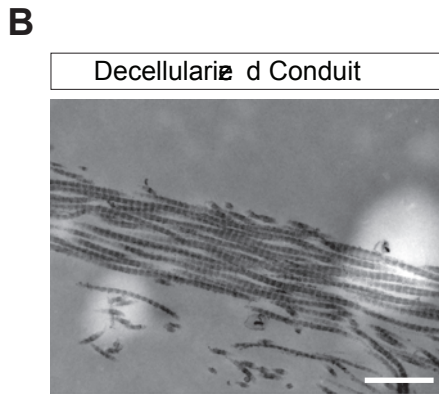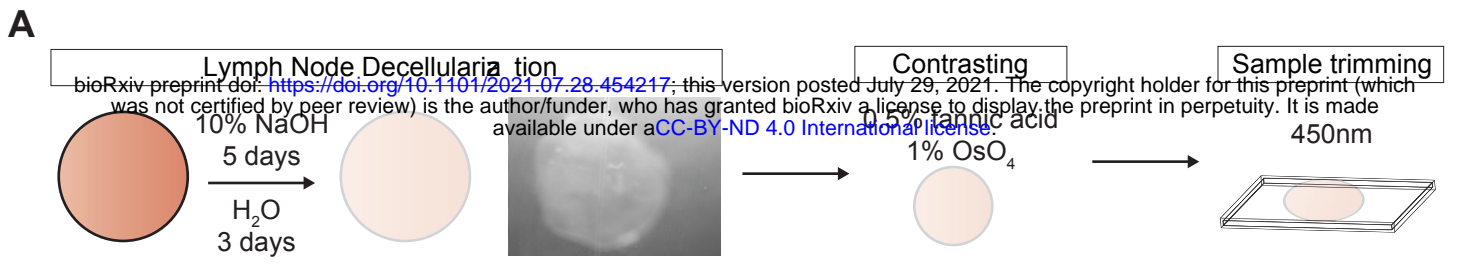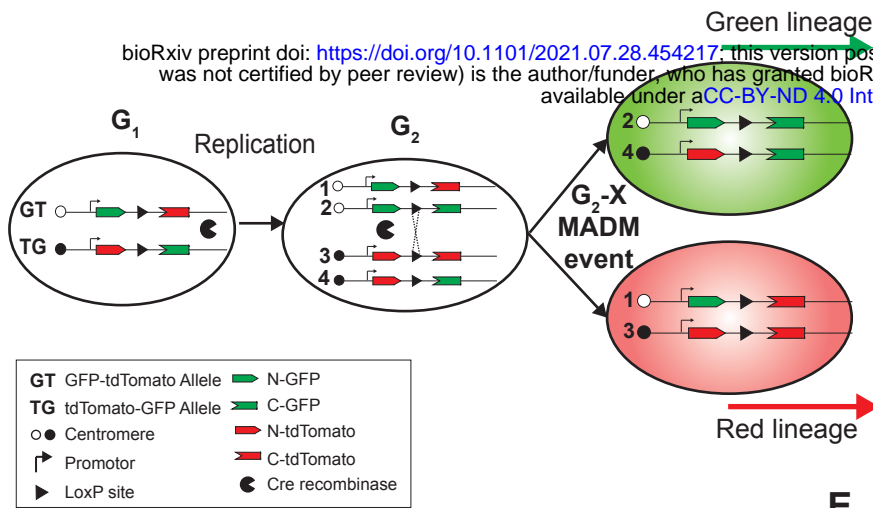Effective Resistance

σ

μ_0

1) **Volume** = (4/3) π R1 $(H_0/2)^2$

2) **Contact Area** = π R3^2

3) **Effective Resistance** = σ
   is derived from

   $$\frac{F_{eq}}{\pi R3^2} = \sigma \left(\frac{1}{R1} + \frac{1}{R2} \right)$$

4) **Strain** = 1 - (H_{eq} / H_0)

5) **Stress** = F_{eq} / Contact Area

6) **Elastic Modulus** = Stress / Strain

7) $\mu_0 = F_{eq} / \sigma$

8) $K_1 = A_1 / \mu_0$ $K_2 = A_2 / \mu_0$
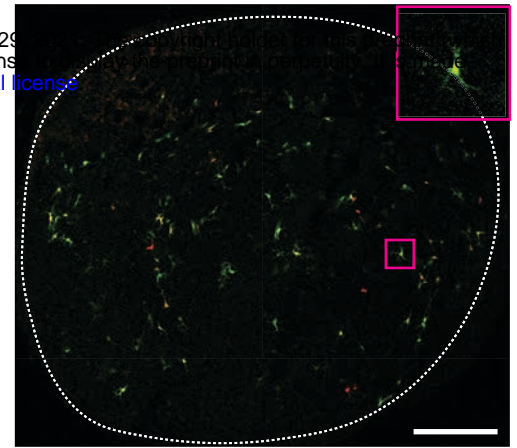
9) **Viscosity** = $\mu_2 = \tau_2 K_2$

F

A

Lymph Node Decellularization

Contrasting

Sample trimming

10% NaOH
5 days
H_2O
3 days

0.5% tannic acid
1% OsO_4

450nm

B

Decellularized Conduit

C

| Tilt angle -76° | Tilt angle -38° | Tilt angle 0° | Tilt angle +38° | Tilt angle +76° |

D

Computed weight back projection
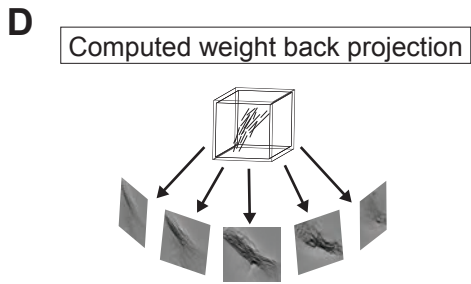
A

Mosaic Analysis with Double Markers (MADM) Principle

Green lineage

Red lineage

G_1 Replication G_2 G_2-X MADM event

GT	GFP-tdTomato Allele		N-GFP
TG	tdTomato-GFP Allele		C-GFP
○ ●	Centromere		N-tdTomato
⌐	Promotor		C-tdTomato
▶	LoxP site		Cre recombinase

B

Sparse Labeling of FRCs

C

Homeostasis   Inflammation

FRCs-GFP+   HEVs

D

● tdTomato+GFP-   ● tdTomato-GFP+

Number of labeled FRCs

ns   ns

**   D0   D4   D8

E

3D: HEV network   3D: tdTomato+FRCs

Slice view FRCs   Mapping of FRCs
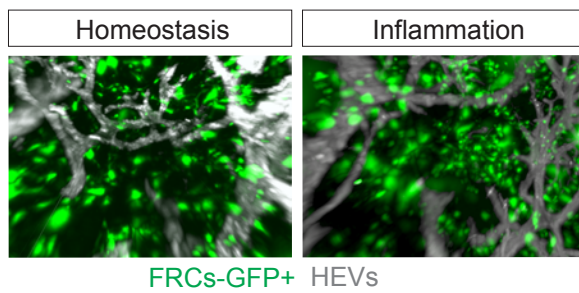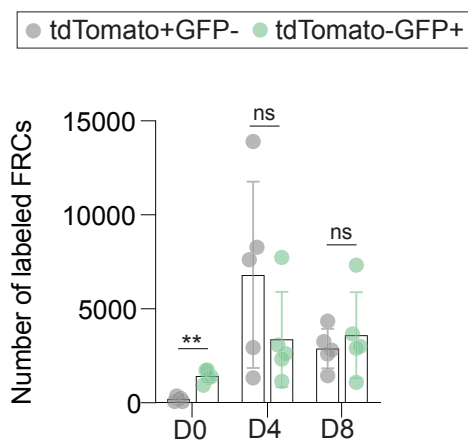
F
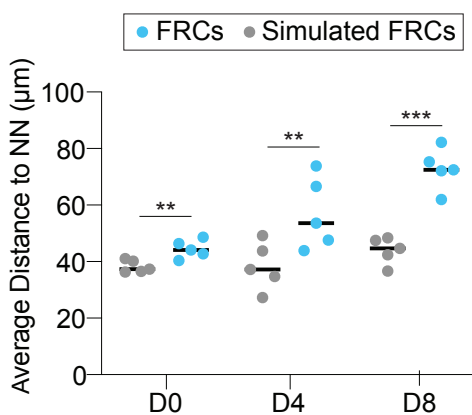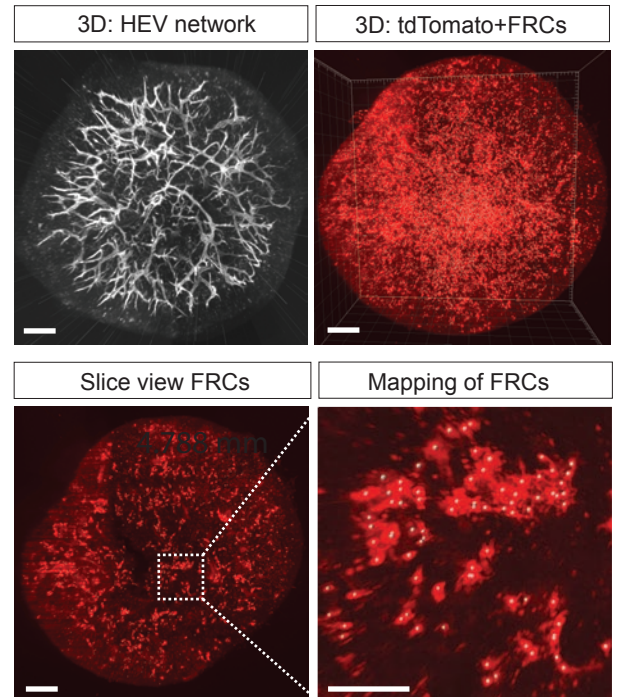
Cluster Factor Calculation

$$CF = \dfrac{\dfrac{\#\ FRCs\ Clustered}{\#\ FRCs\ Total}}{\dfrac{\#\ Simulated\ FRCs\ Clustered}{\#\ Simulated\ FRCs\ Total}}$$

G

● FRCs   ● Simulated FRCs

Average Distance to NN (µm)

**   **   ***

D0   D4   D8

H

● D0   ● D4   ● D8

FRCs in clusters (% of labeled)

ns

Homeostasis   Inflammation

A Lymph Node Weights

A Ccl19-Cre; mTmG

SCS	mTdT	mGFP	LYVE-1	MERGE

B

Capsule ECM (STEM)

Capsule ECM (SEM)

Explant

popliteal Lymph Node

Glass

40X

561nm imaging laser
High Power UV-laser

Capsule ECM (TAMRA labeled)

Pre Cut | Post Cut

bandpass filtered

C

Capsule ECM Tension

Recoil Velocity (um/s)

D0 D2 D4 D8 D14

Vectors only | PIV

D

Homeostasis	D14	

Muscle

Capsule

Parenchyma

PROX1-GFP PDGFR-β DAPI