Multitier mechanics control stromal adaptations in the swelling lymph node

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Lymph nodes (LNs) comprise two main structural elements: fibroblastic reticular cells that form dedicated niches for immune cell interaction and capsular fibroblasts that build a shell around the organ. Immunological challenge causes LNs to increase more than tenfold in size within a few days. Here, we characterized the biomechanics of LN swelling on the cellular and organ scale. We identified lymphocyte trapping by influx and proliferation as drivers of an outward pressure force, causing fibroblastic reticular cells of the T-zone (TRCs) and their associated conduits to stretch. After an initial phase of relaxation, TRCs sensed the resulting strain through cell matrix adhesions, which coordinated local growth and remodeling of the stromal network. While the expanded TRC network readopted its typical configuration, a massive fibrotic reaction of the organ capsule set in and countered further organ expansion. Thus, different fibroblast populations mechanically control LN swelling in a multitier fashion.

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pposed to other organs, the LN parenchyma contains few resident cells, while the bulk of lymphocytes is in constant transit and millions of them pass through the organ every day1–3. Despite this dynamic cellular exchange, homeostatic LN size remains relatively stable. Known modulators of homeostatic LN cellularity (for example, during circadian rhythms4,5) are mainly chemoattractants and adhesion molecules6–9, which serve as entry and exit signals for lymphocytes, as well as survival factors10–12 and adrenergic signals13,14. The main stromal cells of LNs are fibroblastic reticular cells (FRCs), a heterogeneous group of cells that form the non-haematopoietic backbone of the organ15. Like glial cells of the nervous system, FRCs were long considered passive structural elements. Only in the last two decades has it been revealed that the stromal compartment decisively orchestrates immune cell encounters by providing trophic and tactic cues and that, in turn, FRCs dynamically respond to signals provided by the immune cells13,16,17. TRCs are the largest FR subset and deposit bundled fibers of extracellular matrix (ECM) that assemble an intricate three-dimensional (3D) network termed conduits. TRCs enwrap these ECM conduits and form an interface with the immune cells, while conduits associated with other FRCs are scarce18,19.

Upon immunological challenge, reactive LNs swell rapidly by recruiting large numbers of naïve lymphocytes via high endothelial venules (HEVs), while lymphocyte egress via efferent lymphatics is initially blocked20,21. LNs can swell up to tenfold in size in the order of days, imposing a structural problem on the stromal network that has to cope with this volumetric challenge. TRCs are able to relax and expand upon interaction with activated DCs22,23, potentially creating additional space during the swelling phase. In addition, TRCs increase in number and various redundant mechanisms that drive this expansion in the early and late phase of LN swelling have been described21,23–25. The ratio of TRC-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 TRC network to grow26. How network expansion is coordinated to prevent undergrowth or overgrowth of the TRC network is unknown, and, although mechanical forces are obvious feedback parameters, these aspects of LN swelling have not been measured to date.

Here we investigated the cellular and mechanical changes accompanying LN swelling and show that mechanical load on the conduit network and subsequent TRC mechanosensing are central to expansion of the TRC network and LN growth.

Results

The reactive lymph node resists swelling. To understand the global mechanical behavior of LNs while expanding, we quantitatively characterized bulk tissue properties of the reactive LN. Upon immunization of wild-type mice with keyhole limpet hemocyanin in complete Freund's adjuvant (KLH/CFA), we observed a more than tenfold increase in volume of draining LNs at day 14 after immunization, when the organ reached its maximum size. LN volume was calculated from two-dimensional (2D) side view images and showed a volumetric increase of 0.75 mm³ per day and a tripled volume by day 2 of inflammation (Fig. 1a and Extended Data Fig. 1a–c). We measured tissue mechanics by compressing explanted popliteal LNs between two parallel plates at 75% of their original height (25% strain), while the resisting force exerted by the LN on the top plate was measured over a period of 20–60 min (Fig. 1b). During this time, the LN underwent a viscoelastic relaxation behavior and reached a new force equilibrium, which is described by the stress relaxation curve (Fig. 1c and Extended Data Fig. 1d).

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**Fig. 1 | The reactive lymph node resists swelling.**

**a.** Volumes of swelling LNs calculated from 2D side views over the course of 2 weeks after immunization (n = 46). Means are connected (blue line) and a linear regression line (dashed) has been fitted to the data. **b.** Measured geometrical parameters annotated on 2D side images during a measurement (25% strain). Force is measured on the top plate. Scale bar, 300 µm. H₂, LN height before compression; L₁, LN length before compression; Hₑq, LN height at equilibrium. R₁, R₂ and R₃ indicate measured radii. **c.** Stress relaxation curve from the measured force over time (left) and the corresponding force fit (right). Colored arrows indicate short-, medium- and long-term relaxations. Force is fitted with a double exponential equation (blue line). The arrow (black) indicates force at equilibrium (Fₑq). **d-f.** Quantification of the effective resistance (d, n = 8, 11, 8 and 10), viscosity (e, n = 8, 11, 7, 6 and 10) and Young’s modulus (f, n = 8, 11, 8, 9 and 10). **g-j.** Stress relaxation measurements in LNs of wild-type (WT) mice during homeostasis (day 0; g and h) and in LNs of wild-type or OT-II mice during inflammation (day 4; i and j) following treatment with PBS or CD62L antibody intravenously injected 24 h before measurements at day 0 or injected at immunization for measurements at day 4. **g, i.** Representative side views of explanted and measured LNs. Scale bars, 300 µm (g) and 400 µm (i). **h.** Quantification of LN volume (left, n = 11 and 9) and quantification of effective resistance (right, n = 11 and 9). **j.** Quantification of LN volume (n = 13, 16, 8 and 12) and effective resistance (n = 13, 16, 8 and 11). Data from a, d-f, h and j are shown as the mean ± s.e.m. and individual datapoints represent independent measurements of single popliteal LNs. Statistical analysis was performed using Kruskal–Wallis test (d-f, unpaired two-tailed t-test (h, left), two-tailed Mann–Whitney test (j, left; y = (y²−1)/0.8 transformed) and two-way analysis of variance (ANOVA; h, right, j, right; y = ln(y) transformed). All experiments were repeated independently (≥5 mice and ≥3 experiments). For statistical details, see Supplementary Table 1. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001.

Together with the geometrical parameters of LNs measured before compression and at the new equilibrium force, the effective resistance (σ, surface tension), the viscosity (µv, fluidic resistance to deformation by an applied force) and the Young’s modulus (E, elastic resistance to deformation by an applied force) of the tissue were derived by modeling the parameters to a generalized Kelvin model (Extended Data Fig. 1e,f). At equilibrium, the LN resisted the external force exerted by the plate, which, together with LN geometry, sets the effective resistance (given in Newton per meter; Fig. 1c). This parameter describes the collective forces resisting organ expansion and is a measure of how much force is necessary to drive organ swelling by a certain length scale. During expansion (day 0 to day 14), we observed a ~fourfold increase of effective resistance and values remained elevated until the endpoint at day 14 (Fig. 1d). Viscosity only increased in the last phase of swelling, while elasticity was selectively increased from the homeostatic condition at day 2 and day 14 of inflammation (Fig. 1e,f). These data demonstrate that tissue properties of LNs show adaptive dynamics upon swelling and suggest that the mechanical features of the organ resist the forces driving expansion.

Lymphocyte numbers drive lymph node swelling. We next asked what are the internal forces driving organ expansion. Increased entry, blocked exit and proliferation of lymphocytes are the main
factors that increase cellularity within the densely packed node (Extended Data Fig. 1g)\textsuperscript{27,28}. We first perturbed lymphocyte entry through the HEVs under homeostatic conditions using an l-selectin antagonizing antibody (CD62L)\textsuperscript{29}. At 24 h after CD62L antibody administration, LN s were used for parallel-plate compression experiments. Blocking of lymphocyte entry significantly reduced LN volume, effective resistance and viscosity, while the Young’s modulus remained unchanged (Fig. 1g,h and Extended Data Fig. 1h). Thus, lymphocyte trapping is not a consequence but a cause of LN swelling. It generates an outward pressure force which is countered by the organ’s effective resistance.

**The stromal network stretches upon lymph node swelling.** Next, we investigated which mechanical features of the LN resisted expansion. The candidate structures mediating effective resistance to swelling are the organ capsule, the FRC network and its associated ECM. We first measured how the FRC compartment adapted. Using 3D light-sheet fluorescence microscopy (LSFM), we measured the growth of T-zone, B cell follicles and lymphatics upon inflammation (Fig. 2a–d). In homeostasis, T-zone, B cell follicles and lymphatics had relative volume fractions of 0.38, 0.34 and 0.28, respectively. These fractions remained fairly stable at day 4 of inflammation (Fig. 2b–d). At day 14, the relative volume fraction of follicles grew an additional 10%, mainly at the expense of the T-zone (Fig. 2b–d). At the sinus interface, B cell follicles bulged into the capsule and also impressed the surrounding T-zone, suggesting that these largely ECM-devoid structures are stiffer and mechanically
Fig. 3 | Conduits are stretched in the swelling lymph node. a, Schematic of STEM tomography acquisition of macerated popliteal lymph node samples (left) and images of the fibrillar collagen of T-zone conduits at a single tilt angle (middle) and a maximum intensity projection crop of a 3D conduit reconstructed from multiple tilting angles (right). b, Representative cropped 3D reconstructions of fibrillar collagen (blue) from macerated conduits at homeostasis (day 0) and inflammation (days 2, 4 and 14) in which the conduit centerline (yellow) and traced fibril segments (gray) are depicted. c, Visual representation of the conduit fibril alignment analysis of an imaged 3D conduit volume. Angles of individual fibril segments (thick colored lines) with the centerline of the conduit (dashed black line) are measured at multiple points along the fibril segment (thin colored lines) and averaged per fibril segment. \( \alpha_1 \) and \( \alpha_2 \) indicate measured angles. d, Quantification of conduit fibril alignment with centerline (n = 437, 244, 502 and 478). Data are shown as the mean. Datapoints represent an individual fibril segment. Statistical analysis was performed using the Kruskal-Wallis test. All experiments were repeated independently (three lymph nodes from two mice and two experiments) and data were pooled for each time point. For statistical details, see Supplementary Table 1. NS, not significant. ***\( P < 0.001 \), ****\( P < 0.0001 \).

Conduits are stretched in the swelling lymph node. The TRC network has two principal structural components: the TRCs and the ECM conduits (Extended Data Fig. 3a). Both components have the potential to bear load and confer mechanical resistance to swelling. We quantitatively measured if and to what extent the two structures experienced mechanical forces. We started out with the ECM component and analyzed the structural organization of the conduit’s fibrillar collagen as a proxy for mechanical strain. Like in tendons and other elastic ECM structures, fibrillar alignment should increase with strain. We fixed homeostatic and reactive LNs and removed all cellular components by alkali maceration (Extended Data Fig. 3b,c). To resolve the 3D organization of individual collagen fibrils, scanning transmission electron microscopy (STEM) tomograms of T-zone conduits were acquired (Fig. 3a and Extended Data Fig. 3d,e). Measuring the misalignment of individual collagen fibrils relative to the conduit centerline reflects the extent of conduit stretching (Fig. 3b,c). We found that, compared to homeostasis (day 0), early in inflammation (day 2 to day 4) conduit collagen fibrils become progressively aligned, whereas later in inflammation (day 14) they again adopted a misaligned configuration (Fig. 3d). These results suggested that conduits stretched and bore an increased mechanical load early upon LN swelling, while at later time points, they reverted to the homeostatic state.

T-zone reticular cell network tension increases upon lymph node swelling. We next investigated the role of TRCs in the stretching response. To study if the change in conduit conformation was mirrored by the tension state of the TRC network, we directly measured TRC network tension by in situ laser ablation and recoil analysis. To this end, the TRC network in FRC-mGFP mice was imaged under the capsule at interfollicular (IF) sites where network dynamics were similar to the deep paracortex (Extended Data Fig. 4a–d, Fig. 2g and Supplementary Movie 2). Cutting individual strands of the 3D network caused immediate recoil of TRCs, followed by a local repositioning of the adjacent network (Fig. 4a,d, Extended Data Fig. 4e and Supplementary Movie 3). At days 4 and 8 of inflammation, tension within the TRC network had almost doubled compared to that at homeostasis (day 0) but was restored to homeostatic levels at day 14 (Fig. 4c and Supplementary Movie 4).

As a proxy for the cellular mechanosensing response, we next measured nuclear versus cytoplasmic localization of the transcription factors YAP and TAZ, which are well-established downstream responses of cytoskeletal tension. TRCs and endothelial cells stained positive for YAP and TAZ, while leukocytes were devoid of signal (Fig. 4d,e). The nuclear/cytoplasmic ratio of YAP and TAZ (YAP/TAZ NC ratio) in TRCs remained stable from day 0 to day 2 of inflammation but increased at day 4 and day 8 (Fig. 4f), indicating that TRCs experience increased cytoskeletal tension during LN swelling. The YAP/TAZ NC ratio decreased after TRC tension peaked (Fig. 4c,f), thereby faithfully recapitulating the tension as measured by laser cutting. At week 2 after immunization, we observed a large population of TRCs that had a negative YAP/TAZ NC ratio. The TRC network initially stretched upon swelling and subsequently recoiled by day 14 (Fig. 2d–g). At day 4 of inflammation, the TRC, but not the CRC network integrity were observed, we found that TRC and CRC network tension had almost doubled compared to that at homeostasis (day 0) but was restored to homeostatic levels at day 14 (Fig. 4c). These data suggest that conduits stretched and bore an increased mechanical load early upon LN swelling, while at later time points, they reverted to the homeostatic state.
TAZ NC ratio (Fig. 4f), suggesting that those cells were completely shielded from active tension. These data suggested that TRC tension increased upon LN swelling and restored to homeostatic conditions 2 weeks after immunization.34,35

T-zone reticular cells undergo distributed clonal expansion. To test how the TRC network expanded, remodeled and reestablished its typical configuration, we devised an approach to map the spatiotemporal expansion of the TRC network in situ. We used a sparse clonal labeling approach named mosaic analysis with double markers (MADM)36–38. MADM labeling results from rare interchromosomal mitotic recombination driven by Cre-loxP sites (Extended Data Fig. 5a). Two reciprocally split GFP and tdTomato genes (GT and TG) on identical loci of homologous chromosomes are used to create trans-heterozygous offspring (GT/TG). Interchromosomal recombination can take place in the G2 phase, restores functional GFP and/or tdTomato expression and thereby irreversibly labels the lineage. To trigger recombination specifically in TRCs, we used the Ccl19-Cre transgene31 and generated Ccl19-Cre MADM-7GT/TG mice, which were immunized by KLH/CFA footpad injection (Extended Data Fig. 5b). Homeostatic (day 0) and reactive LNs (day 4 and 8) were cleared and imaged by 3D LSFM. Prominent clusters of TRCs emerged in reactive LNs, while such clusters were rarely observed at day 0 (Fig. 5a, Extended Data Fig. 5c and Supplementary Movie 5), suggesting that individual TRC clones expanded following immunization and that daughter TRCs stay close to their precursor. Quantitative analysis of TRCs using a density-based spatial clustering of applications with noise39 (Extended Data Fig. 5d) indicated the number and size of TRC clusters were significantly increased in reactive LNs (day 4 and day 8) compared to homeostasis (day 0; Fig. 5b, Extended Data Fig. 5e–g). We defined the cluster factor (CF) as the number of TRCs in observed clusters versus the in silico gener-
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We found an average CF of 6 in homeostatic conditions (day 0) and TRC clusters in inflammation (day 8). Scale bars, 200 μm. **P < 0.01, ***P < 0.001, ****P < 0.0001. For statistical details, see Supplementary Table 1. Two-tailed Spearman correlation was used for correlation matrix of paired variables assessed in the cluster analysis. A spline fit was plotted through the datapoints.

Fig. 5 | T-zone reticular cells undergo distributed clonal expansion. a, High-resolution confocal volumes of MADM sparse labeled TRCs in homeostasis (day 0) and TRC clusters in inflammation (day 8). Scale bars, 40 μm.

b, Quantification of LN volume (left; n = 5, 5 and 5) and number of clusters (right; n = 5, 5 and 5) and number of clusters (right; n = 5, 5 and 5) and number of clusters (right; n = 5, 5 and 5) of light-sheet images from cleared popliteal lymph nodes of Ccl19-Cre MADM-ΔTLN1 mice in homeostasis (day 0) and inflammation (days 4 and 8). c, Representative images of TRC cluster volumes (randomly colored) and HEVs of an entire LN in homeostasis (day 0) and inflammation (day 4) from experiments as in b. Scale bar, 200 μm. d, Frequency distribution in percentages of TRC per cluster found in observed and simulated data from experiments as in b. Data are depicted as the mean (n = 5, 5 and 5). e, Quantification of the CF per LN from experiments as in b (n = 5, 5 and 5). f, CF plotted as a function of the distance from the nearest HEV from experiments as in b. Data are depicted as the mean ± s.e.m. (n = 5, 5 and 5). g, Correlation matrix of paired variables assessed in the cluster analysis from experiments as in b (n = 15). P values are given and the correlation coefficients are color coded. h, CF plotted as a function of the LN volume from experiments as in b (n = 15). A spline fit was plotted through the datapoints. Data from b and e are depicted as the mean ± s.d. Datapoints from b and e represent a single analyzed LN. Statistical analysis was performed using one-way ANOVA (b, left), Kruskal-Wallis test (b, right, e) and two-tailed Spearman correlation (g). All experiments were repeated independently (≥3 mice and ≥2 experiments). For statistical details, see Supplementary Table 1. **P < 0.01, ***P < 0.001, ****P < 0.0001.

Talin1 is required for T-zone reticular cell mechanosensing. Mechanical forces could be a feedback parameter regulating the TRC network growth. Mechano-coupling of fibroblasts to their underlying matrix is mediated by integrins and their associated intracellular force-sensitive adaptor Talin1. FRCs express both TLN1 (Talin1) and TLN2 (Talin2) isoforms, which play nonredundant roles in integrin activation and force transduction. We generated Ccl19-Cre Talin1fl/fl (FRCΔTLN1) mice, in which Talin1 was selectively deleted in FRCs, and also crossed them with mTmG mice, to obtain FRCΔTLN1-mGFP mice, which expressed mGFP specifically in FRCs. Peripheral LNs of nonimmunized FRCΔTLN1 mice were smaller compared to Ccl19-Cre Talin1fl/+ littermate controls (Extended Data Fig. 6a) and the expression and secretion of the chemokine CCL21 by TRCs, but not HEVs, were decreased compared to littermate controls (Extended Data Fig. 6b). While the T cell zone of the FRCΔTLN1 LNs appeared smaller compared to littermate controls (Extended Data Fig. 6c), the podoplanin+ TRCs of FRCΔTLN1 mice formed a regularly interconnected network and expressed the
adhesion molecules ICAM-1 and VCAM-1 (Extended Data Fig. 6d), suggesting they differentiated normally and that the basic organization and differentiation of the Talin1-deficient TRC network in the FRC\textsuperscript{ATL1N} mice was maintained. The swelling of LNs in immunized FRC\textsuperscript{ATL1N} mice at days 1–4 after immunization was comparable to that seen in littermate controls (Fig. 6a), with a ~tenfold increase in LN weight at day 14 after immunization compared to day 0 (Fig. 6a), suggesting that lymphocyte influx and proliferation still occurred in the Talin1-deficient TRC network and that the FRC\textsuperscript{ATL1N} mice were suitable to test whether mechanosensing in TRCs was required for network adaptation. In the FRC\textsuperscript{ATL1N}, mGFP mice, based on the YAP/TAZ NC ratio, almost no TRCs showed nuclear localization.
Fig. 7 | Capsule fibrosis constrains late lymph node expansion. a, Representative images from in vivo laser cut experiments of subcapsular sinus LECs before (t = −1 s), directly after (t = 0 s) and late after (t = 10.2 s) cutting (scale bars, 5 μm), with corresponding kymograph along the recoil axis (scale bar, x = 1 s and y = 2 μm). The scissor and line indicate the location of the cut and arrows indicate the recoiling cell. Dashed lines in the kymograph indicate slopes and the vertical white line shows the cut. b, Quantification of experiments as in a during homeostasis (day 0) and inflammation (days 2, 4, 8 and 14; n = 11, 12, 20, 25 and 22). c, UV laser cut experiment on TAMRA-labeled capsule ECM of explanted LNs in homeostasis (day 0) and inflammation (day 2, 4, 8 and 14), which recapitulate ECM from scanning electron microscopy (SEM) imaging. Recoil displacement is depicted by orange vectors. Scale bars, SEM image = 1 μm and fluorescence image = 20 μm. d, Quantification of recoil velocities in e (n = 20, 14, 18, 24 and 14). e, Representative examples of LN capsules from Prox1-GFP mice in homeostasis (day 0) and inflammation (day 14), stained for PDGFR-β (only shown on right) and counterstained with DAPI. Scale bars, 200 μm. f, Quantification of capsule thickness as in e (n = 6, 6, 8, 7 and 5). g, Capsule stiffness measurements of homeostatic (day 0) and inflamed (days 2, 4, 8 and 14) explanted popliteal LNs labeled with ERT-R7 antibody. Scale bar, 50 μm. h, Quantification of capsule stiffness as in g (n = 8, 9, 4 and 6). i, Quantification of passive tension (n = 6, 6, 8, 7 and 5). Data from b, d, f and h are shown as the mean ± s.e.m. Datapoints in b and d represent independent cuts, and data in f, h and i show independently measured LNs. Statistical analysis was performed using the Kruskal-Wallis test (b, f, h and i) and one-way ANOVA (d). All experiments were repeated independently (≥4 lymph nodes from ≥4 mice and ≥2 experiments) and data from b and d was pooled for each condition. For statistical details, see Supplementary Table 1. *P < 0.05, **P < 0.01, ***P < 0.001.
(Fig. 6f,g). At day 8, apoptotic cCasp-3+ TRCs per unit volume were still larger in FRC\textsuperscript{TN}}-mGFP compared to FRC-mGFP controls, while proliferating K667+ TRCs were increased (Fig. 6f,g). These data indicate that compromised mechanosensing caused a severe dysregulation in survival and proliferation of the TRC compartment, leading to a loss of network integrity.

**Capsule fibrosis constrains late lymph node expansion.** Although the TRC network reached a ‘new equilibrium’ at week 2 after immunization, when it readopted its homeostatic configuration, effective resistance remained high at this late time point. To test if another structure contributed to the force balance, we used in situ laser ablation to investigate the structural and mechanical properties of the LN capsule, which can be divided into two components: a floor that includes floor lymphatic endothelial cells (ILECs), which are sparsely labeled in FRC-mGFP mice (Extended Data Fig. 7a), and a roof that consists of ECM with embedded fibroblasts (Extended Data Fig. 7b–d). In vivo laser cutting of ILECs showed high basal tension during homeostasis (Fig. 7a,b), which transiently dropped at day 2 after immunization and reverted to homeostatic levels at day 4 (Fig. 7b), while roof tension showed a single increase at day 2 after immunization compared to homeostasis (day 0; Fig. 7c,d). These observations, indicating the absence of a continuous rise in active tension on the capsule floor and roof after immunization, suggested that these components were being continuously remodeled to keep up with the ongoing volumetric increase of the swelling LN. Histology of the LN capsule in Prox1-GFP mice, in which the cytoplasm of all LECs is embedded fibroblasts (Extended Data Fig. 7b–d). In vivo laser cutting of ILECs showed high basal tension during homeostasis (Fig. 7a,b), which transiently dropped at day 2 after immunization and reverted to homeostatic levels at day 4 (Fig. 7b), while roof tension showed a single increase at day 2 after immunization compared to homeostasis (day 0; Fig. 7c,d). These observations, indicating the absence of a continuous rise in active tension on the capsule floor and roof after immunization, suggested that these components were being continuously remodeled to keep up with the ongoing volumetric increase of the swelling LN. Histology of the LN capsule in Prox1-GFP mice, in which the cytoplasm of all LECs is labeled with GFP (Fig. 7e and Extended Data Fig. 7e), indicated that the capsule thickness remained unchanged at days 1–4 after immunization, but increased ~14-fold at days 8–14, forming a dense fibrotic layer between the parenchyma and surrounding adipose and muscle tissue (Fig. 7e,f). Further histological characterization showed that the fibroblasts in the thickened capsule were not labeled in FRC-mGFP mice, were positive for CD34 (ref. 10), had occasional YAP/TAZ– nuclei and did not express α-smooth muscle actin (αSMA; Extended Data Fig. 7d), indicating that these fibroblasts were phenotypically distinct from FRCs.

To ask if the remodeling of the capsule resulted in changes in its mechanical properties, wild-type LN capsules labeled with an antibody to the fibroblast marker ER-TR7 were aspirated with a micropipette and an effective Young's modulus (stiffness) of the capsule was derived from the aspiration depth measurements using Laplace's law (Fig. 7g). The Young's modulus of the capsule remained stable over days 1–8 after immunization, but doubled at day 14 (Fig. 7h). By multiplying the capsule thickness and Young's modulus of the capsule, we derived the passive capsule tension, which is a measure of the amount of force necessary to enlarge the whole thickness of the capsule by a certain length. The passive tension of the capsule floor and roof after immunization, suggested that these components were being continuously remodeled to keep up with the ongoing volumetric increase of the swelling LN. Histology of the LN capsule in Prox1-GFP mice, in which the cytoplasm of all LECs is labeled with GFP (Fig. 7a,b) and podoplanin on TRCs relaxes actomyosin contractility of TRCs and thereby allows the stromal network to stretch\textsuperscript{14,15}. Such a transient relaxation explains why the tension increase of the ECM conduit preceded the tension increase of TRCs. It also implies that the TRC cytoskeleton only experiences significant tension once the DC-mediated relaxation signals fade after 3 to 4 days, which is the time window in which TRCs increase their expression of αSMA\textsuperscript{11}. We found a similar drop in tension around day 2 in PDPN\textsuperscript{+} LECs of the subcapsular sinus floor, indicating that transmigrating DCs might also influence the mechanical state of this stromal population via the CLEC-2–PDPN axis\textsuperscript{14,16}.

While the TRC network used its intrinsic elasticity to accommodate short-term volumetric changes, sustained strain on the TRC cytoskeleton triggered the next stage of LN swelling, which was characterized by actual growth and structural remodeling of the network. Our results in Talin1-deficient TRCs support the idea that adhesion-dependent mechanosensing was a critical feedback parameter that locally controlled growth and survival of the network, so that it reverted to its typical geometry, while increasing in size. A critical prerequisite of a model where TRC mechanosensing locally controls network remodeling is that the TRC responsiveness is not restricted to specific niches, but rather distributed throughout the organ. Our clonal analyses showed that this was indeed the case. In line with mechanics being a critical control parameter, mice with a gain-of-function mutation in the mechanosensitive YAP/TAZ pathway showed fibrotic LNs with impaired FRC differentiation\textsuperscript{17} and blockade of β1 integrin triggered FRC apoptosis in swelling, but not in homeostatic, LNs\textsuperscript{18}.

Beyond 1 week of structural adaptation, the TRC network of the now massively enlarged LN seemed to reach a new ‘mechanical equilibrium’, as indicated by gap analysis, ECM alignment, tension measurements and YAP/TAZ translocation. Nevertheless, bulk mechanical properties did not return to homeostatic levels, but rather showed an elevated effective resistance, indicating that another structure countered further organ expansion. We identified the capsule as the responsible stromal element for elevated resistance to swelling from day 8 to day 14, during which its thickness and mechanical strength were massively increased. Although capsule fibrosis is a characteristic histopathological descriptor of reactive LNs\textsuperscript{19}, its mechanistic contributions remain to be explored. While our work sheds some light on LN swelling, it is still unknown how the expansion process is reversed. Here, our observation that a large population of TRCs lost nuclear YAP/TAZ at week 2 of inflammation might mark the beginning of an involution process, where decreasing lymphocyte numbers and a concomitant drop in TRC tension initiate a reductive network remodeling.

The proposed multtier model of LN swelling implicates a succession of checkpoints and can be adapted to very different types of swelling scenarios. Transient swelling, as occurs during circadian fluctuations, might stretch the network, but is unlikely to cause structural remodeling. On the contrary, sustained immune responses with massive lymphocyte trapping and germinal center reactions might rely on a fibrotic strengthening of the capsule to limit excessive expansion of the organ. Our findings demonstrate that mechanical forces are decisive feedback parameters orchestrating LN swelling at the cellular and organ scales.
Online content
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Methods
Mice. All animal experiments were performed in accordance with the Austrian law for animal experiments. Permission was granted by the Austrian Federal Ministry of Science, Research and Economy (identification codes: BMWFU 66.018.013-WF/1/016022-WE/V/3b/2018). Experimental plans and treatment regimens were selected in consultation with IST Austria Ethical Committee. Mice were bred and maintained at the local animal facility or purchased from Charles River and maintained at the local animal facility in accordance with IST Austria Ethical Committee taking into account national and European guidelines. GT1 (stock no. 0043, 092756), were obtained from JAX. Cc19-Cre mice have been described previously 26, MADM-7 (ref. 3), Talin1-floxed and Prox1-GFP mice were provided by S. Hippenmeyer and D. Critchley. All mice are on a C57BL/6J background, with

Methods experiments, or anesthetized with a ketamine (100 mg per kg body weight)/ and for inflammation conditions, injections were given at immunization. Mice LN cellularity manipulation experiments, mice were intravenously injected with Sigma-Aldrich) upon which 40 μl of the immunization mixture was injected into the footpads and flanks of draining popliteal and inguinal LNs. LNs were collected after various time points up to 2 weeks after immunization to be used for histology or 1.5 h at RT, three washing steps in PBS and subsequent incubation for LN cellularity manipulation experiments, mice were intravenously injected with 100 μg CD62L antibody (MEL14, BioXCell) and control mice with PBS alone. For steady-state evaluation, LNs were collected 24 h after injections were given, and for inflammation conditions, injections were given at immunization. Mice were anesthetized with ketamine (100 mg/kg) and for inflammation-based experiments, or anesthetized with a ketamine (100 mg per kg body weight), xylene (10 mg per kg body weight)/acetromazine (3 mg per kg body weight) mixture for in vivo imaging experiments.

Histology and imaging. LNs were fixed in 4% paraformaldehyde (Electron Microscopy Sciences) in PB (0.1 M, pH 7.4) before embedding and freezing in OCT (OCT) compound (Sakura). Cryostat sections (10–12 μm) were incubated in CUBIC reagent 1 for 3 days at 37 °C, which was replaced every 24 h. Samples were then washed with PBS, incubated in 2% low melting temperature agarose (Sigma-Aldrich), and sequentially dehydrated in 30% (wt/wt) sucrose (Sigma-Aldrich; 1 day at 4 °C). Samples were then washed in PBS, incubated under mild agitation and subsequently cleared with the CUBIC protocol 25. Briefly, samples were incubated in CUBIC reagent 1 for 3 days at 37 °C, which was replaced every 24 h. Samples were then washed with PBS, incubated in 2% low melting temperature agarose (Sigma-Aldrich), and sequentially dehydrated in 30% (wt/wt) sucrose (Sigma-Aldrich; 1 day at 4 °C). Finally, samples were washed in CUBIC reagent 2 for 2 d at RT.

Cleared samples were imaged using antibody staining. Antibodies were diluted in allowed for the visualisation of the lymphatic vessels. Immunofluorescent images of the lymphatic vessels were then acquired using a confocal laser scanning microscope (CLSM) with the following settings: lambda 405 nm, x/y/z resolution 0.45 μm, 0.8 μm N.A., x/y/z = 0.41 μm, x/y/z = 0.8 μm, N.A. and 0.3 μm water and x63/1.4 N.A oil Plan-APOCHROMAT. Thick vibratome sections in those same cases cleared using the Ce3D protocol as described previously 26. Briefly, following antibody staining, samples were washed at RT on a shaker for 8 h in washing buffer (PBS; 0.03% Triton X-100, 0.5% 1-thioglycollotic), which was replaced after 4 h. Next, samples were cleared in freshly prepared Ce3D solution for 2×1 h, mounted in μ-dishes (Ibidi) and submersed in Ce3D solution. A cover glass was placed on top to mount cleared samples to the bottom of the well and the dishes were sealed with parafilm. Large 3D volumes (xy: 306×306 μm, z: 50–300 μm) were acquired from Ce3D-cleared thick vibratome sections using a spinning-disk microscope (Dragonfly, Andor) with an Apochromat LWD LS x40/1.15 water 0.60-mm WD objective.

3D light-sheet fluorescence microscopy sample preparation and imaging. For compartment volume analysis, intact LNs were fixed in 4% paraformaldehyde in PBS overnight at 4 °C, washed and cleaned under a stereomicroscope. LNs were subsequently blocked for 4 h at 37 °C and stained with 1 μg ml−1 rabbit polyclonal anti-LYVE-1 (RELIATech) for 3 days at 37 °C. After two wash steps, LNs were stained with 2 μg ml−1 anti-B220-AF488 (RA3-6N2, BioLegend) and 2 μg ml−1 anti-CD3e-AF647 (145–2C11; BioLegend) for 5 days at 37 °C. All washes and staining procedures were performed in 2% BSA/0.1% Triton X-100 in PBS, under continuous rotation at 15 r.p.m. Stained LNs were embedded in 2% low melting point agarose (Sigma-Aldrich), dehydrated in methanol and optically cleared using Methylsalicylate. Clear LN cryosections and MS sections were acquired using confocal laser scanning microscopy (CLSM) with the following settings: 0.2 μm water 0.60-mm WD objective.

For TRC cluster analysis, terminally anesthetized Cc19-Cre hem MADM-7/7 (mix C57BL/6J and CD-1 background) were in vivo stained by retro-orbital injection of 40 μg mouse α-PNAd (in PBS) concentrated hybridoma supernatant labeled with Atto-647N-NHS (Atto-Tec). After 10 min, popliteal LNs were collected and fixed in 4% paraformaldehyde (Electron Microscopy Sciences) overnight at 4 °C. Samples were washed in PBS, cleaned under a stereomicroscope and cleared with the CUBIC protocol 25. Briefly, samples were incubated in CUBIC reagent 1 for 3 days at 37 °C, which was replaced every 24 h. Samples were then washed with PBS, incubated in 2% low melting temperature agarose (Sigma-Aldrich), and sequentially dehydrated in 30% (wt/wt) sucrose (Sigma-Aldrich; 1 day at 4 °C). Finaly, samples were mounted in CUBIC reagent 2 for 2 d at RT.

Lymph node compartment size analysis. Lymphatics (LYVE-1) and B cell (B220) channels were first segmented using Liastix's pixel classification function before being merged with the T cell (CD3e) channel and imported into Imaris (Bitplane). Using the surface detection feature 3D volumes of the lymphatics, B follicles and the T-zone were generated. T-zone clusters in germinal centers and B-cell clusters in marginal zones were then excluded from analysis. To refine the volume segmentation, 3D volumes were masked and sent to Fiji. Here, small segmentation defects were manually corrected (in some cases follicle outlines, or the T-zone center where antibody penetration was suboptimal) and channels subtracted from each other to eliminate overlapping volumes (lymphatics from both B cell and T cell channel, and T cell from B cell channel). The refined segmented image was then again imported into Imaris where new 3D volumes were generated using the surface detection feature. Surface volumes were exported to Excel worksheets using the statistics tab. A custom Python script combined all data and generated volume fractions for each LN's T-zone, follicles and lymphatics volume as a fraction of their sum.

T-zone reticular cell cluster analysis. MADM-labeled cells were detected using a 3D approach with a spot detection algorithm (Imaris) for each channel (tdTomato and GFP) separately. Chromatic aberrations and the sequential nature of the image acquisition led to a channel misalignment, which was corrected for using the following method: the spot coordinates were exported from Imaris and treated as a point cloud for each channel. These point clouds were then registered onto each other using the iterative closest point algorithm which corrected the shift and the rotation of the spectral channels. Cells were then sorted into color classes (green (GFP), red (tdTomato) or yellow (GFP+tdTomato) line). Red or green, if the spot existed solely in one of the point clouds, or yellow if there were two corresponding spots in both channels that are closer than the typical cell radius.

For the cluster analysis, the LN outline and the HEVs were segmented in Imaris using the surface detection feature. To correct errors in the cell detection, falsely detected spots from autofluorescence structures outside the LN volume were excluded from further analysis. To avoid edge effects, cells in a region 100 μm from the surface of the analyzed LNs were excluded. TRC clusters were analyzed using a custom MATLAB script utilizing a density-based spatial clustering of applications with noise (DBSCAN) algorithm 33 in which TRCs were represented as 3D spheres with a 1.2 μm-diameter. A TRC cluster was defined as a minimum of three TRCs of the same lineage (green or red) within a search radius of 20 μm from each TRC sphere's surface. For visualization of cluster volumes, the convex hull of individual clusters was computed.

To generate a random distribution (simulated TRCs), TRC spheres were placed into the same volume occupied by the real cells and excluded from HEV volumes. For each time point, the average of ten distributions was used. The CF was defined as TRCs in clusters/total number of TRCs divided by simulated TRCs in clusters/total number of simulated TRCs.
image z-stacks (range 10–30 µm and spaced at 1 µm) with a field of view of 240 × 240 µm and a pixel size of 0.5 µm from T-zones of FRC-mGFP mice in which TRCs are labeled, and single sections of B cell follicles of FRC-mGFP mice in which CRC stromal cells were labeled. These were subsequently segmented using Ilastik’s pixel classification feature. The result was transformed into a binary image and noise was removed using a custom Fiji script that utilized the particle detection algorithm. Binarized 3D image stacks (T-zone) and single images (B cell follicles) were then used to measure the spacing (gaps) in the network by analyzing the pore-size distribution on individual z sections. The pore-size distribution was obtained analogously to the pore-size analysis described in work by Acton et al.25. Starting with a circle size corresponding to the maximum gap of the network, circles were consecutively positioned into fitting corresponding gaps of the network. The maximum circle size was determined from a distance transform of the segmented network. Once no more circles of the maximum size could be placed into gaps of the network, the disk size was reduced by one unit and the placement of the disks of reduced sized commenced. This way, the gaps in the network were consecutively filled with circles of decreasing size until the entirety of the gap area was filled. For the T-zone, results were averaged over the image stack.

For 3D analysis, large 3D volumes (xy: 306 × 306 µm, z: 50–500 µm) were acquired from C57Bl/6−/− mice. The force initially peaks and then follows a relaxation curve, \( \tau \). The force required to maintain a constant strain of 25% on a LN was measured using a generalized Kelvin model\(^{26}\). This was done as follows:

\[
\sigma = \frac{F_{eq}}{\pi R_1^2} = \frac{F_1}{h_1} + \frac{F_2}{h_2}
\]

where \( F_{eq} \) is the equilibrium force at steady state and \( R_1, R_2 \) and \( R_3 \) are derived from the geometry of the LN. To obtain the elastic modulus, the stress and strain need to be acquired:

\[
\text{Stress (}\sigma\text{)} = \text{force at equilibrium divided by plate contact area:}
\]

\[
\sigma = \frac{F_{eq}}{\pi R_1^2}
\]

and the strain (\( \varepsilon \)) from:

\[
\varepsilon = \frac{1}{h_3} \frac{h_3}{h_2}
\]

where \( h_1 \) and \( h_2 \) correspond to the initial height and equilibrium height of the compressed LN, respectively. From here the elastic modulus (E) can be derived:

\[
E = \frac{s}{\varepsilon}
\]

Next, by fitting a double exponential decay to the force curve, we obtain two timescales, \( \tau_1 \) and \( \tau_2 \), where:

\[
\tau_i = \frac{\mu_i}{k_i}, \quad i = 1, 2
\]

Following up on the derivations of the equations as in work by Forgacs et al.\(^{26}\), \( \mu_i \) and \( \mu_i \) can be acquired readily, where \( \mu_1 \) corresponds to the initial fast response in the order of seconds and \( \mu_2 \) to the slower response in the order of minutes, of which the latter one becomes relevant for the rearrangements of the cells within LNs. Hence, we use \( \mu_2 \) as our viscosity.

Measurements in which the LN was damaged during preparation (lymphocytes leaking out) or moved/rolled during compression were excluded. In a few cases the viscosity could not be determined (infinitely small) and was excluded.

LN volumes were calculated from side view images at \( t = 0 \) with the following formula:

\[
V = \frac{4}{3} \pi R_1 \frac{h_1}{2}
\]

Analysis of the parallel-plate compression experiment data was performed blinded to the conditions of the experiment.

**Micropipette assay.** Pulpitile LN explants were cleaved from fat and incubated for 10 min in 2 mg μl\(^{-1}\) ER-TR7-AF647 (Santa Cruz) in RPMI 1640 (Hyclone) to label the capsule. LNs were subsequently placed on 3% methylcellulose-coated glass-bottom Petri dishes (MatTek) in RPMI and kept at 37 °C, while imaged on an inverted Leica SP5 microscope using a ×20, 0.7 NA objective (Leica Microsystems). The local Young’s modulus of the capsule was measured with a glass micro pipette connected to a Hydraulic Flow Control System (Fluigent, Fluwell), with negative pressure ranging from 7 to 750 Pa, a pressure accuracy of 7 Pa and change rate of 200 Pa s\(^{-1}\). The micropipette equipment was mounted on a motorized micromanipulator (Eppendorf, Transferman NK2). Both systems were controlled by Dikera software, Labview (National Instruments). A fire polished micropipette with an inner diameter of 15 µm and flat end (BioMedical instruments) was used for aspiration. The chosen diameter ensured that mainly the capsule was probed and not the underlying parenchyma. While localizing the LN capsule with the micropipette, the pressure inside the micropipette was kept at 0 Pa. For measurements, a negative pressure of 750 Pa was applied, which resulted in the instantaneous aspiration of the capsule. This pressure was chosen as lower pressure regimes did not result in proper aspiration of the capsule. The tongue length of the capsule in the micropipette upon aspiration was manually measured in Fiji from acquired movies. The elasticity was subsequently calculated using Laplace’s law:

\[
E = \frac{\Delta P}{D}\sqrt{\frac{S}{h}}
\]

With \( \Delta P \) being the pressure difference between micropipette and atmosphere, \( h \) the height of the measured tongue and \( D \) the micropipette diameter.

**Scanning electron microscope sample preparation and imaging.** Terminally ketamine/xylazine/acepromazine-anesthetized mice were transcardially perfused with PB (0.1 M, pH 7.4) and subsequently fixed with 2.5% glutaraldehyde and 2% paraformaldehyde (Science Services) in PB (0.1 M, pH 7.4). LN samples were then dissected and post-fixed in the same buffer for another hour at RT. They were dehydrated in a graded ethanol series of 50%, 70%, 90%, 96% and 100% in H\(_2\)O for a minimum of 10 min per step and subsequently kept overnight in fresh 100% ethanol at 4°C. Once in 100% ethanol, samples were dried with a critical point dryer (EM-CPD300, Leica Microsystems), cut in half and coated with a 4-nm layer of platinum using a sputter coater (EM-ACE600, Leica Microsystems). The samples were imaged with a field emission SEM Merlin compact VP (Carl Zeiss) at 3 kV. The signal was detected by an Everhart-Thornley secondary electron detector.

**Scanning transmission electron microscopy tomography imaging.** Alkaline maceration of LNs was performed as previously described\(^{26}\). Briefly, pulpitile LNs were isolated from 8- to 12-week-old wild-type C57BL/6 mice and directly fixed in 2.5% glutaraldehyde and 2% paraformaldehyde in PB (0.1 M, pH 7.4) for a minimum of 2 weeks at 4°C. Samples were then macerated in aqueous 2.5 M (10 wt/vol) sodium hydrosol dioxide solution for 5 days at RT under mild agitation. Next, sections were rinsed in H\(_2\)O under mild agitation for 1 to 2 days until samples became pale. If results were not sufficient, the maceration step was repeated.

Samples were then treated with 0.5% tannic acid (wt/vol) in PB (0.1 M, pH 7.4) twice for 1 h each with freshly prepared solutions, washed in PB and treated with aqueous 1% osmium tetroxide (wt/vol) for 30 min at 4°C. Samples were contrast enhanced with aqueous 1% uranyl acetate (wt/vol) overnight at 4°C and Walton’s lead acetate (30 min at 60°C). Samples were then dehydrated in graded ethanol, immersed in anhydrous propylene oxide and embedded in hard grade epoxy resin (Durcupan ACM, Fluka). Samples were consecutively infiltrated with a 3:1 mixture of anhydrous acetone and Durcupan for 1 h at 4°C, 1:1 acetone/Durcupan for 1.5 h at 4°C, 1:3 acetone/Durcupan for 2 h at 4°C and mere Durcupan overnight at RT. Samples were transferred to BEEM capsules (Electron Microscopy Sciences), filled with freshly prepared Durcupan and cured for 48 h at 60°C.

**Scanning transmission electron microscopy tomography imaging.** Semi-thin sections were cut at 450 nm using an UC7 ultramicrotome (Leica Microsystems)
Ultraviolet laser cutter setup. A passively Q-switched solid-state 355-nm UV-A laser recorded an automated system was used comprising the STEM Recorder V3 version 3.2.8.0 and the STEM Magica Controller version 0.9.8.1 (both System In Frontier). Images were collected at 2° intervals between ±76° of the single tilt axis. Images were captured at z=80,000–600,000 magnification, with an image size of 512×512 pixels (px) giving pixel sizes ranging from 6.749463 nm/px to 0.899928 nm/px.

Conduit stretching quantification. STEM tomography images were aligned by cross-correlation and 3D structure of the area of interest computed by weighted back-projection using Composer software version 3.0 (System In Frontier). A 3D Gaussian blur filter and background subtraction (rolling ball algorithm) pre-processing step were performed on the images using Fiji. The 3D image stacks were subsequently loaded into Imaris, and fibrils of conduits were manually traced using the filament tracer feature of Imaris and exported to MATLAB format using the Object Exporter (exported from Imaris as filaments). The overall orientation and curvature of the centerline of the entire conduit was approximated by fitting a cubic spline curve with four support points, which minimized a handicraft cost function through all fibril track data. This cost function penalizes the distance of the desired centerline to the tracks, the total curvature of the centerline and the different length of it and the fibril bundles and ensures that the support points are spaced evenly. In a few cases, this spline curve was corrected by hand if it was found to not adequately represent the centerline of the bundle. The angle of the individual fibrils with respect to the centerline of the conduit was calculated as follows: the spline centerline was interpolated in a continuous fashion and the 3D orientation of each segment of the trace was calculated. The angle between the orientation of the segment and the orientation of the centerline at the point that is closest to the segment:

\[
\theta = \cos^{-1}\left(\frac{\mathbf{f}_i \cdot \mathbf{c}_i}{|\mathbf{f}_i| |\mathbf{c}_i|}\right)
\]

Conduit stretching analysis was performed blinded to the conditions of the experiment. Ultraviolet laser ablation experiments. The UV laser cutter setup is based on a previously described layout\(^{20,21}\). In brief, a passively Q-switched solid-state 355-nm UV-A laser (Powerchop, Teem Photonics) with a repetition rate of 1 kHz, pulse energy of 15 mJ, pulse length of <350 ps and peak power of 40 kW was used in conjugation with a single tilt axis. Images were captured at z=80,000–600,000 magnification, with an image size of 512×512 pixels (px) giving pixel sizes ranging from 6.749463 nm/px to 0.899928 nm/px.

YAP/TAZ quantification. The nuclear-to-cytoplasmic ratio of stained YAP/TAZ was measured from 3D acquisitions of peripheral LNs. In Fiji, TRCs were identified by the mGFP labeling and for each TRC the average YAP/TAZ fluorescence intensity of the nucleus (identified by DAPI) was divided by the average intensity of the adjacent cytoplasm of the cell body. In other cases, YAP/TAZ localization was qualitatively assessed to contain a higher either nuclear or cytoplasmic YAP/TAZ intensity.

CCL21 quantification. Cryosections containing both a control and FRC\(^{21,23}\) peripheral LNs in a single section were stained for CCL21 and imaged using similar acquisition settings. The average fluorescence intensities of CCL21 within images were then measured from paramacrotial areas and normalized to the mean of the control samples.

Proliferation and apoptosis measurements of T-zone reticular cells. Large 3D volumes (xy: 306×306 μm, z: 50–150 μm) stained for either cCasp-3 or Ki67 were acquired from Ce3D-cleared thick vibratome sections and were corrected for fluorescence intensity in z axis using the ‘bleach correction’ (histogram matching) function in Fiji. Imaris was then used to generate a 3D isosurface of the TRC network by utilizing a surface detection feature from the TRC network fluorescence channel. The isosurface was then used to mask the C3asp-3 and Ki67 channels so only the fluorescence signal within the TRC network remained. Positive nuclei were then manually counted from 2D slice views and normalized for per unit volume.

Capsule thickness measurements. The thickness of capsules was measured in vibratome sections of Proxl-GFP or wild-type mice, stained for PDGFR-β and DAPI. The size of the capsule was then manually measured in Fiji from the subcapsular sinus to the surrounding adipose or muscle tissue at a minimum of three locations and were averaged per LN.

Statistical analysis. All statistical analyses were performed in GraphPad Prism 8. P values <0.05 were considered significant. No statistical methods were used to predict sample sizes, but our sample sizes are similar to those reported in a previous publication\(^{20}\). Normality and equal variances were formally tested to ensure the data met the assumptions of the statistical tests used.

Software.

- Fiji/ImageJ Schindelin et al.\(^\text{28}\) https://fiji.sc/
- IMaris v8.1, 9.1, 9.3, 9.8 Bitplane https://imaris.oxinstit.com/packages
- Excel (v2011–2022) Microsoft https://products.office.com/en-us/?rtc=1
- Prism v8 GraphPad https://www.graphpad.com/scientific-software/prism/
- MATLAB v2018–2021 MATLAB https://www.mathworks.com/products/matlab.html
- Ilastik, v0.5-11.5 Sommer et al.\(^\text{19}\) https://www.ilastik.org/
- SquishierJoy Cell Scale https://www.cellscale.com/products/microtester/
- Composer software System In Frontier https://temography.com/en/composer-en/
- PIvLab Thielicke and Stamhuis\(^\text{40}\) https://pivlab.blogspot.com/
- Labview (2010) National Instruments https://www.ni.com/labview.html
- ZEN blue edition v2.3 Zeiss
- Fusion v2.2 Andor
- LAS X v2.7.3.9723 Leica

Reporting summary. Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability
Source data are provided with this paper.

Code availability
All analysis code are available upon request.

References
50. Nieswandt, B. et al. Loss of talin1 in platelets abrogates integrin activation, platelet aggregation, and thrombus formation in vitro and in vivo. J. Exp. Med. 204, 3113–3118 (2007).

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- Excel (v2011–2022) Microsoft https://products.office.com/en-us/?rtc=1
- Prism v8 GraphPad https://www.graphpad.com/scientific-software/prism/
- MATLAB v2018–2021 MATLAB https://www.mathworks.com/products/matlab.html
- Ilastik, v0.5-11.5 Sommer et al.\(^\text{19}\) https://www.ilastik.org/
- SquishierJoy Cell Scale https://www.cellscale.com/products/microtester/
- Composer software System In Frontier https://temography.com/en/composer-en/
- PIvLab Thielicke and Stamhuis\(^\text{40}\) https://pivlab.blogspot.com/
- Labview (2010) National Instruments https://www.ni.com/labview.html
- ZEN blue edition v2.3 Zeiss
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- LAS X v2.7.3.9723 Leica

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Author contributions

F.P.A. designed experiments, and performed in vivo and ex vivo experiments with assistance of M.H., S.S., J.A. and G.K.; J.A. cleared samples and performed light-sheet imaging, F.P.A. carried out all other fluorescence imaging, W.A.K. processed samples for electron tomography, and W.A.K., T.C. and F.P.A. acquired tomograms. F.P.A. did all data processing and analysis with help from R.H., S.S. and G.K. R.H. wrote all custom analysis scripts. S.H. and E.H. aided in the interpretation of MADM and mechanical data, respectively. S.A.L. and J.V.S. discussed data. M.S. directed the study. F.P.A. and M.S. wrote the manuscript and all authors critically reviewed the manuscript.

Competing interests

The authors declare no competing interests.

Additional information

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Extended Data Fig. 1 | See next page for caption.
Extended Data Fig. 1 | The reactive lymph node resists swelling. (a) Side view focus-stack of a homeostatic (day 0) and inflamed (day 14) popliteal lymph node (LN) from wild-type (WT) mice. Scale bar = 500 µm. (b) Quantification of LN volumes in homeostasis (day 0) and inflammation (day 2, 4, 8 and 14) from wild-type mice (n = 8, 11, 8, 9, 10). (c) Relation between volumes and corresponding measured weights of LNs as in a. A regression line is fitted. (d) Stress relaxation curves of homeostatic (day 0) and inflamed (day 2, 4, 8 and 14) LNs (n = 6, 6, 6, 6, 6). (e) Schematic representation of the generalized Kelvin model used to derive the effective resistance, viscosity and Young’s modulus from stress relaxation experiments on explanted popliteal LNs from wild-type mice in homeostasis (day 0) and inflammation (day 2, 4, 8 and 14). Adapted from Forgacs et al.26 (f) Schematic illustration and description of the short-, medium- and long-term relaxation events in a LN during a stress relaxation experiment. (g) Scanning electron microscopy image of packed lymphocytes in the homeostatic (day 0) LN paracortex of a wild-type mouse. Scale bar = 2 µm. (h, i) Quantification of (h; left, i; left) viscosity (n = 11, 9 and n = 11, 9) and (h; right, i; right) Young’s modulus (n = 13, 16, 8, 11) and (n = 13, 16, 8, 11), respectively, of stress relaxation measurements in (h) LNs of wild-type (WT) mice in homeostasis (day 0) and in (i) LNs of wild-type or OT-II mice during inflammation (day 4) following treatment with PBS or CD62L Ab (i.v. injected 24 h before measurements for homeostasis or at immunization for inflammation). Data from b, d, h, i shown as mean ± s.e.m. Datapoints in b, d, h, i represent independently measured LNs. Statistical analysis was performed using one-way ANOVA (b), unpaired two-tailed t test (h) and two-way ANOVA (i; y=Ln(y) transformed). All experiments were repeated independently (≥5 mice and ≥3 experiments). For statistical details see Supplementary Information, table 1. NS, not significant. *P < 0.05, **P < 0.01, ****P < 0.0001.
Extended Data Fig. 2 | The stromal network stretches upon lymph node swelling. (a) 3D reconstruction of an entire homeostatic (day 0) popliteal lymph node (LN) by LSFM following clearing and staining of T-zone, follicles and lymphatic compartment volumes by CD3ε, B220 and LYVE-1 staining, respectively. LN surface and follicles are shown. Asterisks (‘’) indicate sites where follicles locally deform the LN’s capsule (left). Scale bar = 200 µm. 3D clipping of the dashed line encircled region (right). Follicle location with respect to capsule deformations are shown. (b) Example of T-zone deformation by B cell follicle (3D cropped). Asterisk (‘’) depicts the site at which underlying T-zone is indented by and curves around a follicle. Scale bar = 100 µm. (c) LN stromal network gap analysis. FRC-mGFP (top), segmented FRC network (middle), fitted circles in gaps (bottom), randomly colored, T-zone reticular cell (TRC) network in white. (d) Representative examples of the B cell follicle light zone (and for inflammation conditions also GC) FDC stromal network (within dashed line), and CXCL12+ reticular cell (CRC) stromal network (area between the dashed and solid lines) of LNs in homeostasis (day 0) and inflammation (day 4 and 14) from wild-type (WT) FRC-mGFP mice used for gap analysis as in c. Scale bars = 50 µm. (e) Representative examples of the CRC stromal network gap analysis as in d. (f) Averaged and smoothed distribution of the CRC stromal network gaps, plotted as the weighted area fraction as a function of the fitted circle diameter as in e (n = 5, 5, 5). (g) Quantification of the mean fitted circle diameter as in e (n = 5, 5, 5). Data from f, g, shown as mean. Datapoints in g represent independently measured follicles. Statistical analysis was performed using one-way ANOVA (g). All experiments were repeated independently (3 lymph nodes from ≥3 mice and ≥2 experiments). For statistical details, see Supplementary Information, table 1. ns, not significant.
Extended Data Fig. 3 | Conduits are stretched in the swelling lymph node. (a) Schematic illustration of the conduit unit from the T-zone. (b) Example of a decellularized homeostatic (day 0) popliteal lymph node (LN). (c) Conduit imaged by scanning transmissive electron microscopy (STEM) in which the characteristic D-period of collagen fibrils can be observed. Scale bar = 500 nm. (d) Examples of a T-zone conduit at different tilting angles acquired by STEM tomography. Scale bar = 500 nm. (e) Schematic of computed weight back projection to reconstruct a 3D volume of a conduit from differential tilting angles.
Extended Data Fig. 4 | TRC network tension increases upon lymph node swelling. (a) Schematic of in vivo laser cutting in inguinal lymph nodes (iLNs) of FRC-mGFP mice. (b) Subcapsular T-zone reticular cell (TRC) network is imaged at interfollicular (IF) regions (blue arrow). Homeostatic (day 0) and inflammation (day 14) are shown. mGFP positive lymphatic endothelial cells (LECs), marginal reticular cells (MRCs) and TRCs are encountered while gradually focusing into the LN, while follicular dendritic cell (FDC) and CXCL12⁺ reticular cells (CRCs) regions are avoided. The T-zone at IF sites is identified by regular network morphology and bright fluorescent reporter intensity (1), compared to the more irregular and dimmer CRC (2) and dense and bright FDC (3) stromal networks found in the largely ECM-free (ER-TR7 negative) follicles. White dashed lines indicate the outline of follicles, and white dense spaced lines indicate the subcapsular region where MRCs are found (annotated in homeostasis). The area enclosed by the blue dashed line shows a typical area targeted for TRC laser cutting in the orthogonal plane. Scale bars: top = 50 µm, bottom = 20 µm. (c) Averaged and smoothed distribution of the subcapsular TRC network as analyzed by circle fitting gap analysis of homeostatic (day 0) and inflamed (day 2, 4, 8, 14) LNs from FRC-mGFP mice, plotted as the weighted area fraction as a function of the fitted circle diameter (n = 5, 5, 5, 5, 5). (d) Quantification of the mean fitted circle diameter as in c (n = 5, 5, 5, 5, 5). (e) Example of force propagation in the TRC network following a UV laser cut. Time point directly after cutting (t = 0 s) and late after cutting (t = 22.2 s) overlapped (left). Scissor and white line indicate the laser cut location. Scale bar = 20 µm. Overlay of movement vectors (red arrows) derived from particle imaging velocimetry analysis (right). Data from c shown as mean and d shown as mean ± s.e.m. Datapoints in d represent independently analyzed TRC network images from LNs used in laser cutting experiments. Statistical analysis was performed using one-way ANOVA (d). All experiments were repeated independently (3 mice and ≥2 experiments). For statistical details see Supplementary Information, table 1. NS, not significant. *P < 0.05, **P < 0.01, ***P < 0.001.
**Extended Data Fig. 5 | TRCs undergo distributed clonal expansion.** (a) Schematic of the mosaic analysis with double markers (MADM) labeling principle. Rare interchromosomal recombination in the G2 cell cycle phase following ∆-segregation of chromosomes labels T-zone reticular cells (TRCs) with either a cytoplasmic tdTomato or GFP. (b) Schematic of the sparse mosaic analysis with double markers (MADM) labeling approach for TRC cluster analysis in popliteal lymph nodes (LNs) from Cc19-Cre MADM-7GT/ TG mice in homeostasis (day 0) and inflammation (day 4 and 8) (left). Sparse labeling of TRCs in a histological section of a homeostatic LN (right). Scale bar = 200 µm. (c) 3D fluorescent intensity cropped images from light-sheet fluorescent microscopy of entire lymph nodes for which MADM-GFP labeled TRCs and in situ labeled high endothelial venules (HEVs) by PNAd-ATTO647n Ab are shown, as in b. Scale bars = 20 µm. (d) Example of the labeling of HEVs and mapping of MADM-labeled TRCs (only tdTomato+ TRCs are shown) at inflammation (day 4) as in c. The enlarged image depicts the mapping of individual TRCs by a grey sphere at the center of each cell. Scale bars = 200 µm. (e) Quantification of number of MADM-labeled TRCs as in c (n = 5, 5, 5). (f) Quantification of number of MADM-labeled TRCs found in clusters as in c (n = 5, 5, 5). (g) Quantification of the average distance to the nearest neighbor (NN) of both observed and simulated TRCs as in c (n = 5, 5, 5, 5). Data from d, f, g shown as mean ± s.d. and h shown as mean. Datapoints in e, f, g, h represent independently analyzed LNs. Statistical analysis was performed using Kruskal-Wallis test (e, f, g) and paired two-tailed t test. All experiments were repeated independently (≥3 mice and ≥2 experiments). For statistical details see Supplementary Information, table 1. NS, not significant. *P < 0.05, **P < 0.01, ***P < 0.001.
Extended Data Fig. 6 | Talin1 is required for TRC mechanosensing. (a) Quantification of homeostatic (day 0) popliteal and inguinal lymph node (LN) weights from littermate control and FRC\(^{\Delta TLN1}\) mice. Images show representative inguinal LNs from littermate control and FRC\(^{\Delta TLN1}\) mice. Scale bars = 1 mm.

(b) Quantification of homeostatic (day 0) T-zone CCL21 protein as measured in situ by fluorescent intensity following staining for CCL21 in littermate control and FRC\(^{\Delta TLN1}\) mice. Representative images for littermate control and FRC\(^{\Delta TLN1}\) mice in which CCL21 chemokine and high endothelial venule (HEVs) stained by PNAd Ab are shown. Scale bars = 20 µm.

c) T-zone and B cell follicles of homeostatic (day 0) popliteal LNs from littermate control and FRC\(^{\Delta TLN1}\) mice stained for CD3 ε (T cells) and B220 (B cells). Scale bars = 200 µm.

d) ICAM-1, PDPN, VCAM-1 and merged staining on histological sections of homeostatic (day 0) popliteal LN T-zones from control and FRC\(^{\Delta TLN1}\) mice. Scale bars = 20 µm.

e) Representative histological images of T-zone reticular cell (TRC) networks from homeostatic (day 0) and inflamed (day 4 and 14) LNs from FRC-mGFP and FRC\(^{\Delta TLN1}\)-mGFP mice stained for collagen IV. Scale bar = 50 µm. Data from a shown as mean and b as mean ± s.e.m. Datapoints in a, b represent independently analyzed LNs. Statistical analysis was performed using unpaired two-tailed t test (a) and two-tailed Mann-Whitney test (b). All experiments were repeated independently (≥3 mice and ≥2 experiments). For statistical details see Supplementary Information, table 1. *P < 0.05, **P < 0.01.
Extended Data Fig. 7 | Capsule fibrosis constrains late lymph node expansion. (a) Crop of a representative histological image of a FRC-mGFP mouse derived homeostatic (day 0) inguinal lymph node (LN) stained for LYVE-1. mGFP is expressed specifically in cells with Cre-recombinase activity and membrane-bound tdTomato (mTdT) in all other cells. LYVE-1⁺ floor lymphatic endothelial cells (fLECs) are sparsely labeled by mGFP in the subcapsular sinus (SCS). (b) Capsule extracellular matrix (ECM) from an alkali-macerated popliteal LN from a wild-type (WT) mouse imaged by scanning transmissive electron microscopy (STEM). Scale bar = 5 µm. (c) Representative histological images of LN capsules from Prox1-GFP mice in homeostasis (day 0) and inflammation (day 4, 8 and 14) in which LECs are labeled by a cytoplasmic GFP. Mesenchymal cells are stained for PDGFR-β, and nuclei are counterstained with DAPI. Scale bars = 20 µm. (d) Characterization of capsular fibroblasts in inflamed (day 14) popliteal LNs from a FRC-mGFP (left panel; FRCs labeled with mGFP and all other cells with mTdT) or mT mG (other panels; all cells are labeled with mTdT) mouse of which the latter was stained for CD34, YAP/TAZ or αSMA and counterstained with DAPI. Areas between dashed lines indicate the capsule. Arrows indicate YAP/TAZ positive fibroblast nuclei in the capsule. Scale bars = 10 µm. (e) Schematic of mechanical dynamics and the multi-tier model of the swelling LN. Phase I: LN growth with T-zone reticular cell (TRC) relaxation and stretching. Phase II: LN growth with increasing TRC tension and TRC network expansion. Phase III: LN growth with decreasing TRC tension and further TRC network expansion. Phase IV: LN growth and TRC network expansion with decreasing TRC tension, capsule thickening and increasing strong resistance to growth.
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For all statistical analyses, confirm that the following items are present in the figure legend, table legend, main text, or Methods section.

- The exact sample size (n) for each experimental group/condition, given as a discrete number and unit of measurement
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  Give P values as exact values whenever suitable.
- For Bayesian analysis, information on the choice of priors and Markov chain Monte Carlo settings
- For hierarchical and complex designs, identification of the appropriate level for tests and full reporting of outcomes
- Estimates of effect sizes (e.g. Cohen’s d, Pearson’s r), indicating how they were calculated

Our web collection on statistics for biologists contains articles on many of the points above.

Software and code

Policy information about availability of computer code

| Data collection | ZEN blue edition version 2.3 (Zeiss), Fusion version 2.2 (Ander), LAS X version 2.7.3.9723 (LEICA), LabVIEW 2010 (National Instruments), Squashjoy 2.05 (Cellscale), STEM Recorder V3 Vers. 3.2.8.0 and STEM Magica Controller Vers. 0.9.8.1 (both System In Frontier Inc.) |
| Data analysis   | Prism v8 (Graphpad), Excel (Vers. 2011-2022), Imaris v8.10-9.8 (Bitplane), Matlab 2017a-2021a (Mathworks), Composer Software Vers. 3.0 (System in Frontier Inc.), ImageJ and Fiji (NIH), Pilab (Thielicke and Stainhans 2014), Ilastik v1.2.2-1.3.3 |

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- A description of any restrictions on data availability
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Policy information about studies involving human research participants and Sex and Gender in Research.

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Use the terms sex (biological attribute) and gender (shaped by social and cultural circumstances) carefully in order to avoid confusing both terms. Indicate if findings apply to only one sex or gender; describe whether sex and gender were considered in study design whether sex and/or gender was determined based on self-reporting or assigned and methods used. Provide in the source data disaggregated sex and gender data where this information has been collected, and consent has been obtained for sharing of individual-level data; provide overall numbers in this Reporting Summary. Please state if this information has not been collected. Report sex- and gender-based analyses where performed, justify reasons for lack of sex- and gender-based analysis.

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Describe the covariate-relevant population characteristics of the human research participants (e.g. age, genotypic information, past and current diagnosis and treatment categories). If you filled out the behavioural & social sciences study design questions and have nothing to add here, write “See above.”

Recruitment

Describe how participants were recruited. Outline any potential self-selection bias or other biases that may be present and how these are likely to impact results.

Ethics oversight

Identify the organization(s) that approved the study protocol.

Note that full information on the approval of the study protocol must also be provided in the manuscript.

Field-specific reporting

Please select the one below that is the best fit for your research. If you are not sure, read the appropriate sections before making your selection.

☑ Life sciences  ☐ Behavioural & social sciences  ☐ Ecological, evolutionary & environmental sciences

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Life sciences study design

All studies must disclose on these points even when the disclosure is negative.

Sample size

No sample-size calculations were performed beforehand. An initial sample size used in each independent were based on pilot experiments, previous experience and comparable to those reported in previous publication. For some independent experiments data was pooled to achieve statistical power (specified in figure legends). Reproducibility between independent experiments was used as a determinant for adequate sample size. Groups of at least 3 mice per group were used in independent experiments, with exception for electron microscopy experiments for which at least 2 mice per group were used.

Data exclusions

Analysis of parallel plate compression experiments resulted in a few cases (5) where the viscosity could not be determined (infinitely small). This first occurred in pilot experiments and was made a pre-determined criteria for exclusion. These datapoints were excluded, while the other measures of these measurements (volume, elastic modulus, effective resistance) were used for analysis and were in line with other measurements.

Replication

Experiments were repeated at least twice on different days, and with sufficient animals per group and measurements per animal to demonstrate statistical significance. Experiments were all reliably reproducible.

Randomization

Animals of both sex were allocated to experimental groups (homeostasis/day of inflammation, treated/control) in a random manner.

Blinding

For analysis of TKC clustering, conduit fibril alignment and parallel plate compression experiments blinding was performed. In other experiments blinding was not deemed relevant as the data obtained was not subjective or practically unfeasible.

Reporting for specific materials, systems and methods

We require information from authors about some types of materials, experimental systems and methods used in many studies. Here, indicate whether each material, system or method listed is relevant to your study. If you are not sure if a list item applies to your research, read the appropriate section before selecting a response.
### Materials & experimental systems

| n/a | Involved in the study |
|-----|------------------------|
| ☑ | Antibodies |
| ☑ | Eukaryotic cell lines |
| ☑ | Palaeontology and archaeology |
| ☑ | Animals and other organisms |
| ☑ | Clinical data |
| ☑ | Dual use research of concern |

### Methods

| n/a | Involved in the study |
|-----|------------------------|
| ☑ | ChiP-seq |
| ☑ | Flow cytometry |
| ☑ | MRI-based neuroimaging |

### Antibodies

**Antibodies used**

- CD3ε, Rat mAb; 17A2, AF488, 1/200, Biologend, 100201
- CD3ε, Armenian Hamster mAb; 145-2C11, AF647, 2ug/ml, Biologend, 100322
- B220, Rat mAb; RA3-6B2, Biotin, 1/200, Bioscience, 36-0452-85
- B220, Rat mAb; RA3-6N2, AF488, 2ug/ml, Biologend, 103225
- Collagen IV, Polyclonal rabbit, Biotin, 1/200, Abcam, ab6581
- CCL21, Polyclonal goat, Biotin, 1/100, R&D Systems, BA457
- PDGP-intrinsic, Syrian Hamster mAb; 8.1.1, 1/150, Bioscience, 13-5381-82
- PDGF-β, Polyclonal goat, Unconjugated, 1/150, R&D Systems, AF1042
- YAP/TAZ, Rabbit mAb; D2E4, Unconjugated, 1/100, Cell Signal, 84185
- Cleaved Caspase 3 (Asp175), Rabbit mAb; D3F9, AF647, 1/150, Cell Signal, 96025
- Ki67-APC, Rat mAb; SolA15, APC, 1/100, Bioscience, 71568-82
- ICAM-1, Rat mAb; YN1/1.7.4, Unconjugated, 1/200, BioXCell, BE00201
- VCAM-1 (Phe25-Glu68), Polyclonal goat, Unconjugated, 1/150, R&D Systems, AF643
- PNLAD6, Hybridoma-derived mouse mAb; MECA-79, Unconjugated, Undiluted supernatant, ATCC, HB-9479
- Fibroblast Marker, Rat mAb; ETR7, AF647, 1/100, Santa-Cruz Biotech, sc-73355
- CD34, Rat mAb; RAM34, FITC, 1/100, Thermofisher Scientific, 11-0341-85
- oSMA, Mouse mAb; 1A4, AF488, 1/200, Thermofisher Scientific, 53-9760-82
- Lyve-1, Polyclonal rabbit, Unconjugated, 1ug/ml, ReliaTech, 103-PAS0AG

**Streptavidin; Cy3, Streptomyces avidinii, 1/200-1/400, Sigma-Aldrich, S6402**
- mouse, goat, AF647, 1/200-1/400, Invitrogen, A21238
- goat; AF488, chicken, 1/200-1/400, Invitrogen, A21467
- rat: AF647, donkey, 1/200-1/400, Jackson ImmunoResearch, 712-606-150
- rabbit: AF647, donkey, 1/200-1/400, Jackson ImmunoResearch, 711-606-152

**Streptavidin; AF647, Streptomyces avidinii, 1/200-1/400, Jackson ImmunoResearch, 016-600-084**

**Validation**

Antibodies are all from commercial vendors, and were validated by the manufacturers. Antibodies were tested in the laboratory using known positive and negative controls and/or according the manufacturer’s specifications and titrated.

### Animals and other research organisms

Policy information about studies involving animals: **ARRIVE guidelines** recommended for reporting animal research, and **Sex and Gender in Research**

**Laboratory animals**

- Laboratory mice (Mus musculus) from the following backgrounds were used: C57B/6j (wild-type), Ccl19-Cre mTmG, Ccl19-Cre Talin1f/f, Cc19-Cre mTmG;Talin1f/f, Prox1-GFP, CD-1 (MADM-7), and mixed C57B/6 & CD-1 (Ccl19-Cre MADM-7). Both sexes in the age of 6-20 weeks were used for experiments.

**Wild animals**

- No wild animals were used in this study.

**Reporting on sex**

- This information has not been collected.

**Field-collected samples**

- No field-collected samples were used in this study.

**Ethics oversight**

- All animal experiments are in accordance with the Austrian law for animal experiments. Permission was granted by the Austrian Federal Ministry of Science, Research and Economy (identification code: BMWFU 66.018/0010-WF/V/3b/2016 and 66.018/0027-WF/V/3b/2014). Experimental plans and treatment regimens were selected in consultation with IST Austria Ethical Committee. Mice were bred and maintained at the local animal facility in accordance with IST Austria Ethical Committee or purchased from Charles River and maintained at the local animal facility in accordance with IST Austria Ethical Committee taking into account national and European guidelines.

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