

1 **TUMOR INDUCED STROMAL REPROGRAMMING DRIVES**
2 **LYMPH NODE TRANSFORMATION**

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20 For submission to Nature Immunology

21 Short title:

22 **Stromal reprogramming drives lymph node transformation**

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1 **ABSTRACT**

2 Lymph node (LN) stromal cells, particularly fibroblastic reticular cells (FRCs),
3 provide critical structural support and regulate immunity, tolerance and
4 transport properties of LNs. In many tumors, LN metastasis is predictive of
5 poor prognosis. However, stromal contribution to the evolving
6 microenvironment of tumor draining LNs (TDLN) remains poorly understood.
7 Here we show that FRCs of TDLNs expand but also significantly remodel.
8 Comparative transcriptional analysis of resting and TDLN FRCs demonstrated
9 reprogramming of key pathways including matrix remodeling,
10 chemokine/cytokine signaling and immune functions including leukocyte
11 recruitment, migration and activation. Stromal-mediated structural and
12 transcriptional adaptations, including downregulation of CCL21 and IL7, were
13 accompanied by altered cellular composition and aberrant localization, both
14 characteristics typical of immune dysfunction and the generation of a
15 suppressive niche.

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17 **INTRODUCTION**

18 LNs form an integral part of both our lymphatic and immune systems, acting
19 as “filters” to surveil potential lymph borne pathogens, and as an
20 immunological hub maintaining homeostasis or eliciting effective immune
21 responses. To enable these specialized functions, the LN is highly organized
22 into discrete cellular compartments. The supporting stromal cells are central
23 to organization and function^{1, 2, 3} and the major stromal subsets, lymphatic
24 endothelial cells (LEC), blood endothelial cells (BEC) and fibroblastic reticular
25 cells (FRC) can be distinguished by their relative expression of surface
26 markers podoplanin and CD31².

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28 Lymph draining from peripheral tissues enters via LEC-lined afferent
29 lymphatic vessels and along lymphatics that line the subcapsular and
30 medullary sinuses before exiting in efferent lymphatics⁴. However, smaller
31 constituents such as chemokines and soluble antigen below 70kDa can cross
32 the lymphatic sinus floor and penetrate deeper into the LN, along narrow
33 conduit channels formed by collagen fibrils and FRCs^{5, 6, 7}. While the conduit

1 network provides underlying structural support⁴, FRCs have additional
2 properties vital for proper lymph node function. They produce a number of
3 chemical cues that are critical for immune cell migration, localization and
4 survival such as homeostatic chemokines CCL19 and 21⁸, whose receptor
5 CCR7 is present on naïve T cells, B cells and Dendritic Cells (DCs)⁹.
6 Gradients of these chemokines direct intranodal migration and survival during
7 homeostasis and infection^{3, 10, 11}, lymphocyte homing to LNs¹² and mediate
8 interactions between T cells and DCs⁸. FRCs are a major source of IL7,
9 essential for naïve T cell survival². Those resident in follicles also contribute to
10 B cell homeostasis and follicle identity via the production of the cytokine
11 BAFF¹³. Moreover, specific destruction of FRCs is a method employed by
12 viruses to avoid detection during infection¹⁴. More recently, evidence indicates
13 that FRCs not only contribute to the onset of effective immune responses, but
14 conversely to immunological tolerance, switching off an immune response via
15 deletion of self-reactive T cells^{15, 16, 17}.

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17 LNs also feature in numerous pathologies. In cancer, they represent the first
18 site of metastasis for many tumor types and are independently predictive of
19 poor prognosis^{18, 19}. Yet, despite our increasing efforts to understand the
20 processes of lymphatic metastasis and LN colonization²⁰, the mechanisms
21 underlying the failure of effective anti-tumor immune responses in the LN, and
22 the relationship of both to poor outcome remain poorly characterized. Tumor-
23 derived interstitial fluid and its constituents drain to downstream LNs, bathing
24 the cells it encounters en route. Therefore, the potential exists for tumors to
25 exploit this means of communication to remotely control responses in tissues
26 such as the LN to its survival advantage.

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28 Given our current knowledge pertaining to stromal cell contribution in LN
29 function, and the importance of stromal cells within the tumor
30 microenvironment, we characterized the response and potential
31 consequences of changes to FRCs in TDLNs. Here we use transcriptomic
32 analysis of stromal populations isolated from LNs to demonstrate that FRCs,
33 specifically within TDLNs, undergo structural remodeling and transcriptional
34 modifications, and that these correlate with gross modifications in cellular

1 composition and localization *prior* to the arrival of tumor cells. These
2 observations imply that aberrant stromal cues impact the downstream
3 structure and function of tumor draining lymph nodes.

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1 RESULTS

2 **Enlargement of TDLNs is supported by expansion of stromal cells and** 3 **FRC network remodeling.**

4 The role of stromal cells within tumor draining lymph nodes (TDLNs) and their
5 contribution to the evolving microenvironment has yet to be established.
6 Therefore to study stromal cells during tumor development, prior to the
7 establishment of LN metastases, we utilized a well-established B16.F10
8 melanoma model in which individual draining LNs were assessed over a
9 period of 14 days. Confocal imaging of whole nodes illustrated significant
10 enlargement of tumor-draining brachial LNs (**Fig. 1a** and **Supplementary Fig.**
11 **1a**). Quantification of LN cellularity by flow cytometry further confirmed the
12 expansion of LNs downstream of tumors (**Fig. 1b** and **c**) at pre-metastatic
13 time points. The absence of tumor cells in TDLNs was confirmed by qRT-PCR
14 of *Tyr1* and *Dct* mRNA expression in total LNs (**Supplementary Fig 1b**). In
15 contrast, LNs of PBS-injected control mice (non-draining lymph nodes;
16 NDNLNs) remained at a constant size over the period examined (**Fig. 1c**). The
17 observed increases in cellularity were confined specifically to sentinel LNs, as
18 adjacent, but not primary draining LNs, did not expand (**Supplementary Fig.**
19 **1ci** total LN and stromal cells **cii**).

20 To examine stromal cells within TDLNs, populations were identified based on
21 the differential expression of PDPN and CD31 among CD45⁻ non-
22 hematopoietic cells (gating strategy **Fig. 1b**). Using this approach, significant
23 expansion in BECs, LECs and FRCs was recorded over the course of tumor
24 development specifically within TDLNs (**Fig. 1d**). Consistent with B16.F10
25 allografted tumors, TDLNs of tumor-bearing *Tyr::CreER, Braf^{CA}, Pten^{lox}* mice,
26 which develop melanoma after induction of melanocyte-specific *Braf^{V600E}*
27 expression and *Pten* silencing, were also enlarged (**Fig. 1e** and
28 **Supplementary Fig. 2a**). In these animals, tumors developed at multiple
29 independent sites; primarily the shoulder and lower flank. Quantification of
30 cellularity in draining inguinal LNs (iLNs) and brachial LNs (braLNs) confirmed
31 enlargement, which was supported by expansion of all stromal populations
32 (**Fig. 1f** and **Supplementary Fig. 2a**). Proliferation of expanding FRCs was
33 confirmed by *in vivo* EdU labeling. Surprisingly, turnover of LECs and BECs

1 remained in line with ND counterparts (**Supplementary Fig. 2b**). Indicative of
2 a requirement beyond the provision of structural support to an enlarging node,
3 a significant increase in the ratio of FRCs to whole node cell counts after 11
4 days was measured (**Supplementary Fig. 2c**) leading us to examine FRC
5 network changes after tumor induction in more detail. FRC networks and
6 conduits remained intact in TDLNs, with collagen I cores surrounded by ER-
7 TR7 matrix and PDPN⁺ FRCs (**Supplementary Fig.3a-c**). Skeleton analysis
8 of FRC network complexity revealed less branches per field of view in FRCs
9 of TDLNs compared to ND LNs (**Fig. 1g**), whereas branch length
10 (**Supplementary Fig. 3d**) and FRC cell size (data not shown) were
11 unchanged. Gap analysis demonstrated significant greater distances between
12 adjacent FRC networks in TDLNs (**Fig. 1h**), and further evaluation of PDPN
13 lined conduits using high power Airyscans revealed that conduit thickness as
14 measured by ellipse area of the central collagen I core was significantly
15 enlarged in TDLNs (**Fig. 1i**). Moreover, such detailed end-on scans highlight
16 changes to the architecture of individual TDLN conduits compared with ND
17 counterparts (**Fig. 1i** and **Supplementary Fig.3c**). Together, the data imply
18 that conduits of TDLNs enlarge rather than increase in frequency, and hence
19 increasing FRC numbers might be required to provide cellular coverage to
20 support the growing conduit diameter.

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22 **Transcriptional profiling identifies alterations in TDLN FRCs**

23 Considering observations of both TDLN enlargement and FRC network
24 remodeling and frequent reports of immune dysfunction in tumors²¹⁻²⁶ we
25 sought to identify how FRCs of expanding TDLNs adapt to the evolving
26 microenvironment, and whether these changes ultimately translate to pro-
27 tumor structural and functional modifications in the LN. To do this, FRCs from
28 LNs at days 4 and 11 post B16.F10 inoculation were freshly sorted and
29 subjected to whole genome transcriptional profiling. At day 4, tumors were
30 barely palpable thus this time point was chosen to represent an early stage of
31 tumor development, where the tumor microenvironment is not yet fully
32 established and communications with draining lymph nodes are likely at their
33 earliest stages via resident dermal lymphatic vessels. In contrast, large day 11

1 tumors with established stroma and LN connections represent the late stage
2 of LN transformation. Inter-replicate coefficients of variation confirmed
3 consistency between samples, with means of 0.036, 0.037, and 0.035 for
4 NDLN, day 4 (4d) and day 11 (11d) TDLNs respectively. Analysis of the gene
5 array data revealed distinct transcriptomes between FRCs from TDLNs and
6 NDLNs. By plotting probe expression level in order of highest change to
7 lowest change for day 4 (4d), day 11 (11d) and NDLN expression profiles
8 (**Supplementary Fig. 4a-c**), and applying a cutoff of probes with a fold
9 change $> \pm 1.5$, it is clear that significantly large expression changes occur
10 within TDLNs. We performed statistical analysis on these most significantly
11 altered probes from both 4d and 11d TDLN arrays resulting in a total of 244
12 significantly deregulated probes. We initially calculated principal components.
13 When plotted, eigenvalues of the principal components (**Supplementary Fig.**
14 **4d**) highlight that a majority of variance in the data (88.9% and 4.5%
15 respectively) is contained within the first two components, and the first two
16 components for these deregulated probes partition into their respective
17 sample types (**Fig. 2a**). Principal component 1 separates NDLN and day 4
18 TDLN effectively, and principal component 2 separates NDLN and day 11
19 TDLN probes. Correlation matrix plots of the same probe set reinforced this
20 relationship (**Fig. 2b**), showing strong association within all datasets. This was
21 further confirmed by hierarchical clustering (**Fig. 2c**) where all three probe
22 types were clustered into their respective groups (NDLN, 4d TDLN, and 11d
23 TDLN), and TDLN samples from the two different time points were clustered
24 closer to each other than to NDLN samples. The heatmap, however, linked to
25 the hierarchical clusters (**Fig. 2c**, bottom) reveals that clusters of samples
26 (NDLN, day 4 TDLN, and day 11 TDLN) exhibit the same pattern of changes
27 in expression levels within their groups i.e. all NDLN show the same probe
28 expression changes. These data demonstrate that expression profiles for 4d
29 TDLNs and 11d TDLNs are distinct and replicable, and implies that FRCs are
30 undergoing a gradual reprogramming response after exposure to tumor
31 factors with 4d representing a distinct and transitional state, rather than simply
32 exhibiting a weaker profile of 11d TDLNs.

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34 **Identification of specific genes and pathways deregulated in TDLN FRCs**

1 Initially, FRCs from TDLNs after 4 or 11 days of tumor exposure were
2 compared to ND FRCs using probes exhibiting over 1.5 fold difference and a
3 p-value below 0.05 (**Supplementary Fig. 5a**). Differential deregulation of
4 probe expression between days 4 and 11 highlighted transient increases or
5 vice versa, which likely represent early activation or repression of FRC
6 signaling pathways, and either return to control levels by day 11 or continue to
7 be further up or downregulated. Whilst the expression levels of probes within
8 the array clearly differ over time, the number of probes with altered expression
9 values is comparable. 106 probes were downregulated with a fold change
10 greater than 1.5 at day 4, and 81 at day 11 with an overlap (i.e. probes that
11 similarly upregulated in both) of 39 (**Fig. 3a i**), whereas 117 probes were
12 upregulated at day 4, 131 at day 11 with an overlap of 25 probes (**Fig. 3a ii**).
13 Volcano plots illustrate the top deregulated probes between 4d/11d versus ND
14 and 11d versus 4d as ranked by their expression levels (**Fig. 3b**). For
15 example, AQP1 is among the top upregulated genes after 4d (**Fig. 3b i**),
16 however, by 11d it returns to baseline expression levels (**Fig. 3b iii and**
17 **Supplementary Fig. 8a**). In contrast, FXYD6, IGH-4, THY1 and PTX3 (**Fig.**
18 **3b ii**) are among the top upregulated genes when comparing 11d and 4d (**Fig.**
19 **3b iii**), indicating that these represent a unique late stage signature.
20 Clustering the top deregulated genes into functional groups, clear differences
21 in genes key to cell proliferation, protein metabolism, mitochondrial function,
22 movement and migration, and junction molecules were observed (**Fig. 3c**).
23 Functional annotations were collated using GSEA and ingenuity (IPA)
24 analysis according to the overlapping deregulation of probes in 4d and 11d
25 TD from KEGG (GSEA, normalized enrichment score, **Supplementary Fig.**
26 **5b**), canonical pathways (IPA, displayed ordered by z score or P value,
27 **Supplementary Fig. 5c**) and from disease and biofunctions (IPA, displayed
28 ordered by z score or P value, **Supplementary Fig. 5d**).

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30 **Perturbation of FRC-derived chemokine/cytokine signaling modifies** 31 **immune composition of TDLNs**

32 FRCs are an essential source of chemokines and cytokines necessary for
33 immune homeostasis, leukocyte trafficking and survival within the LN²⁷. Both
34 GSEA and Ingenuity (IPA) analyses identified these pathways to be

1 significantly deregulated in FRCs of TDLNs (**Fig. 4a**). While signaling
2 molecules such as LIMK2, KRAS, TGFBR2 and SRC were upregulated,
3 cytokines and chemokines including IL19, IL7, CCL4 and CCL21 were
4 downregulated after 4 and 11d. As FRCs provide the bulk of CCL21 and IL7²,
5 ^{8, 12}, directly contributing to lymphocyte localization and survival, mRNA levels
6 were verified in independent sample sets by qRT-PCR confirming significant
7 downregulation of IL7 and CCL21 mRNA in TDLN FRCs in both tumor models
8 examined (**Fig. 4b**). Confocal imaging further confirmed a reduction in CCL21
9 expression at the protein level (**Fig. 4c**). Focusing on the B16.F10 model, a
10 reduction in T cell area and concurrent increase in the B cell follicle size per
11 node was measured in imaged TDLNs (**Supplementary Fig. 6a**) and
12 corresponding reductions in CD3e⁺ cellularity were confirmed by flow
13 cytometry (**Fig. 4d**). Although no change in CD8a⁺ T cells were measured
14 (**Supplementary Fig. 6b**), a significant reduction in the percentage of CD4⁺ T
15 cells was observed in TDLNs after 11 days of tumor drainage (**Fig. 4e**). Within
16 this population, the percentage of naïve CD4⁺CD62L⁺CD44⁻ T cells dropped
17 (**Fig. 4f**). This was accompanied by increases in memory (CD62L⁺CD44⁺) and
18 activated (CD62L⁻CD44⁺) CD4⁺ T cells (**Fig. 4f**) as well as a significant
19 increase in CD4⁺FoxP3⁺ regulatory T cells (**Fig. 4g** and **Supplementary Fig.**
20 **6c**). Moreover, we observed impaired homing efficiency of CD4⁺ T cells into
21 11d TDLNs (**Fig. 4h**). Considering the observed FRC network remodeling,
22 altered chemokine profiles and immune composition into account, we
23 examined the cellular architecture of LNs and observed mislocalization and
24 disorganization of major immune cell populations in TDLNs. In contrast to
25 ND LNs where T and B cell zones were clearly delineated (**Supplementary**
26 **Fig. 6c**, left panel), TDLNs exhibited integration of the 2 populations with loss
27 of delineation between T/B cell borders (**Supplementary Fig. 6d** right panel,
28 and **Fig. 4i**). A transitional stage was observed in 4d TDLNs (**Supplementary**
29 **Fig. 6c** middle panel). Furthermore, in TDLNs, B cells were frequently
30 clustered around high endothelial venules (HEVs, **Fig. 4j i**, quantified in **Fig.**
31 **4j ii**). As no differences in B cell homing capacity were measured between ND
32 and TDLNs (**Fig. 4k**), this, together with FRC-derived cytokine changes would
33 imply that in TDLNs, once exited HEVs, B cells are not able to sense the
34 appropriate cues responsible for directing them to the B cell follicle. Moreover,

1 staining with EdU indicated a reduced proliferation of T cells and B cell
2 compartments from TDLNs (**Supplementary Fig. 6e and f**). Beyond CCL21
3 and IL7, the gene array also highlighted other factors deregulated in FRCs of
4 TDLNs. These included CXCL14, chemotactic to monocytes and DCs;
5 CCL25, chemotactic to DCs; and CCL7, chemotactic to monocytes; all of
6 which were upregulated at day 11. Consistent with gene array trends, and
7 following a transient dip at day 4, CD11c⁺ dendritic cells (DCs) and CD11b⁺
8 Macrophages (MΦ, **Supplementary Fig. 7a**) significantly increased in
9 numbers in 11d TDLNs (**Supplementary Fig. 7b and c**).

11 **FRCs of TDLNs are more activated**

12 Disruption of ECM homeostasis and “cancer-associated fibrosis” is commonly
13 observed at the primary tumor²⁸, and is mediated by hyper-activated
14 fibroblasts (cancer-associated fibroblasts, CAFs) within the local
15 microenvironment²⁹. Therefore, adaptation of the pre-metastatic lymph node,
16 reliant of fibroblast remodeling is also likely to be reminiscent of fibrosis^{30, 31}.
17 Microarray data highlighted elevated expression levels of genes encoding
18 typical fibroblast activation markers including podoplanin, fibronectin, CD248,
19 α-smooth muscle actin, FSP1, vimentin, myosin light chains and collagens
20 (**Fig. 5a**) indicating the heightened activation status of FRCs in nodes draining
21 tumors. Levels of PDPN, FSP1, THY1 and CD248 were further verified on
22 independent data sets by qRT-PCR (**Fig. 5b and Supplementary Fig. 7d**)
23 and PDPN at the protein level by flow cytometry (**Fig. 5c**). Although not
24 significant, trends for PDPN, FSP1 and THY1 in the genetic model largely
25 supported that of the B16 at the mRNA level, however, at the protein level
26 podoplanin was significantly increased in both models. Moreover, flow
27 cytometry indicated that tumor draining FRCs increase in granularity, which is
28 indicative of increased internal complexity and corresponding increased
29 activation status (**Fig. 5d**). To investigate the activation status further, cultured
30 FRCs treated with tumor conditioned medium (TCM) obtained from B16.F10
31 cells for 7 days were compared to control conditioned medium (CCM) treated
32 cells. *In vitro*, PDPN was upregulated at both mRNA (**Fig. 5e**) and protein
33 levels (**Fig. 5f**), and TCM treatment enhanced the capacity of FRCs to
34 contract collagen gels (**Fig. 5g**).

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TDLN conduits are more permissive for large molecular weight solute transport

Profiling of TDLN FRCs also hinted at previously undocumented behavior, in particular, a significant number of channels/ion transporters are deregulated in either 4d or 11d TDLNs (**Fig. 6a**). For example, Aquaporin 1 (AQP1) is highly upregulated in FRCs of 4d TDLNs, before subsequent downregulation by 11d (verified by qRT-PCR in **Supplementary Fig. 8a**). *In vitro*, cultured FRC monolayers exhibited a less selective barrier, allowing greater trans-monolayer transport of 500 kDa dextran following exposure to TCM (**Fig. 6b**). To investigate if changes to levels of these channels and transporters can impact fluid transport through the conduit system *in vivo*, dextran transport studies were performed and quantified. The capacity of conjugated dextran of different molecular weights to transit into the normally size-restricted conduits was analyzed and measured. In both resting and TDLNs, 10 kDa dextran freely entered into FRC lined conduits, but in contrast to resting nodes, 70 kDa dextran permeated further into paracortical area of 11d TDLNs (**Fig. 6c and d**), where it was restricted to the FRC lined conduits (**Fig. 6e**). Taken together with earlier data showing larger diameter conduits at 11d (**Fig. 1g**), altered transporter repertoires point to a perturbation of conduit capacity, whereby in TDLNs conduits are more permissive for fluid to enter and transit, potentially enabling greater penetration of soluble tumor-derived factors to deeper areas of the LN. As a result of the altered environment of TDLNs, significant changes in cell assembly machinery would be expected to underlie the restructuring and enlargement of the FRCs, as would the need for interaction with associated matrix proteins essential to the conduit. The observation of thickened collagen cores but reduced branches implies that additional FRCs go to support the increased diameter of the conduit. In doing so, FRCs will form contacts with a larger number of neighbors, and encounter a greater area of their neighboring cells. As predicted, network analysis (**Fig. 6f**, with interaction networks shown in **Supplementary Fig. 8b**), highlights four significantly relevant probe groups heavily involved in cell structure, shape and extracellular matrix. Such analyses link probe sets into functionally and spatially linked networks, highlighting families of genes that are both

1 significantly deregulated *and* involved in the same biological pathways,
2 expressed together, or have physical interactions. A schematic model shown
3 in **Fig. 6g** illustrates the conduit profile in a resting state (ND) and at an
4 advanced pre-metastatic stage (TDLN day 11). Gene array expression data
5 for day 4 suggests that cells proliferate and conduits begin to reorganize,
6 potentially driven by increased drainage from the tumor or exposure to tumor-
7 derived factors. At the same time, deregulation of ion channels and
8 aquaporins result in changes to the cells capacity to deal with fluid and the
9 immunological profile of the cell changes. By day 11, imaging studies,
10 combined with gene expression analysis show that a new altered state is
11 reached, characterized by transcriptional signatures and structural
12 adaptations that drive modulation of a) scaffolding proteins involved in the
13 cytoskeleton, cell junctions and extracellular matrix remodelling, b) cytokines
14 (CCL21 and IL7) and other biochemical cues, and c) conduit integrity,
15 permeability and consequently transport properties throughout the node.

1 **DISCUSSION**

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3 Lymph nodes function as a major immunological hub, essential for immune
4 homeostasis and generation of appropriate immune responses, yet LNs are
5 also the first site of metastasis for many cancers that manage to avoid
6 immune-mediated clearance. It is increasingly accepted that LNs receive and
7 respond to tumor-derived signals generating a pro-tumor niche, but it remains
8 unclear as to how these responses manifest and who in the LN drives them.
9 The stromal populations of the LN not only provide structural support but are
10 essential to its maintenance and physiological function^{1-3, 6, 8-14, 32-36}. While
11 studies have shown that tumor-derived VEGF and VEGF-C contribute to LN
12 lymphangiogenesis and vascular reorganization³⁷⁻³⁹, the fibroblasts of the LN,
13 FRCs, and the conduit network they form have not been thoroughly
14 investigated in the context of the tumor and subsequent modulation of LN
15 behavior. Here we describe that expansion, remodeling and transcriptional
16 reprogramming of FRCs occurs in TDLNs. This in turn impacts FRC-driven
17 chemokine signaling, trafficking events, immune localization and transport, all
18 of which have the potential to contribute to impaired lymph node function, that
19 in the context of a tumor may provide a pro-tumor environment.

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21 We demonstrate that TDLNs enlarge, consistent with previous studies^{37, 39, 40}.
22 Integral to this enlargement is structural reorganization of the node supported
23 by expansion and adaptation of the stromal compartments. In particular, we
24 observed that the FRCs not only increased in number, but the resulting
25 network exhibited fewer FRC branches that were further apart, and conduit
26 diameters were significantly greater. To understand the potential ramifications
27 of such changes within the stromal compartment we then analyzed
28 transcriptomes of FRCs isolated from resting and tumor draining LNs. This
29 analysis revealed striking transcriptional reprogramming events restricted to
30 the node immediately downstream of a tumor, and identified a transitional
31 process with early response genes and deregulation of key pathways
32 including fibrosis, chemokine and cytokine signaling, immune cell migration,
33 activation and trafficking.

1 Key to LN function, FRC-derived CCL21 and IL-7 were deregulated.
2 Significant downregulation was verified in two independent murine models of
3 melanoma. Decreased expression of both can contribute to abnormal immune
4 cell homing, localization and survival. It has been previously reported that
5 TDLNs exhibit reduced CCL21^{41, 42}. Consistent with these findings we
6 observed gross architectural aberrations, with loss of the clear demarcation
7 between B and T cells, T cells frequently located within B cell zones, and
8 reduced T cell area. These features phenocopy *plt/plt* mice, where
9 spontaneous loss of LN-specific CCL19 and CCL21 isoforms translate to
10 fewer T cells and impaired immune responses^{2, 12, 34, 43}. Moreover, CCL21
11 produced by FRCs surrounding high endothelial venules (HEVs) is essential
12 for egress of B cells and T cells from the circulation towards their respective
13 compartments. As observed in TDLNs, B cells displayed no impairment in LN
14 homing, accumulated around HEVs indicating disruption of their normal
15 guidance cues.

16 Our results also draw parallels with other pathological states such as
17 infection, where reduced nodal CCL21 underpins the aberrant homing and
18 mislocalization of key immune populations required for immune evasion by
19 *Salmonella* or virus particles^{14, 35, 44}. It should be noted however, that
20 pathogen-related inflammation was not underlying our observations. Firstly,
21 stromal modifications were consistent in two independent melanoma models,
22 one of which is genetically driven rather than allografted. Secondly, a
23 comparison of our array with data from Malhotra et al., in which responses of
24 lymph node stroma to LPS-mediated inflammation were characterized²⁷,
25 shows that the response of the FRCs downstream of a tumor is tumor-
26 dependent. In particular, key factors such as CCL21 and IL7 were inversely
27 regulated between the two pathological settings (data not shown).
28 Furthermore, upregulation of several other chemotactic factors were recorded
29 indicating changes to other immune populations in the LN; CXCL14,
30 chemotactic to monocytes and DCs, CCL25, chemotactic to DCs, and CCL7,
31 chemotactic to monocytes. Additionally, CCL4 also known as MIP-1 β was
32 found to be downregulated. CCL4-responsive antigen-naïve CD8 T cells have
33 been reported to chemotact to sites rich in stimulated DCs, which is implicated

1 in optimal activation of CD8⁺ T cells and long term memory⁴⁵. Since FRCs
2 have been shown to be the source of CCL4¹¹ in the LN, a downregulation in
3 TDLNs may result in a reduction of this T cell population. Beyond the
4 disruption of immune compartmentalization, we also present evidence to
5 implicate FRC transcriptional modulation in the altered immune composition of
6 TDLNs. Within draining nodes, we observed fewer naïve CD4⁺ T cells and
7 accumulation of Tregs consistent with previous studies correlating their
8 presence with immune suppression and disease progression⁴⁶. We detected
9 enlarged B cell follicles within TDLNs, and although the activation status or
10 subtypes of the B cells occupying TDLNs remains to be determined, recent
11 reports have demonstrated that B cells do indeed accumulate in TDLNs⁴⁷,
12 and that these may function as regulatory B cells⁴⁸ adding a further dimension
13 to the local immune suppressive environment.

14 Transcripts of TDLN FRCs also indicated the acquisition of a more activated
15 status evident from upregulation of Thy1, podoplanin, FSP1, CD248, vimentin,
16 collagens, fibronectin, α smooth muscle actin, and the capacity to contract
17 collagen gels more efficiently upon receipt of tumor conditioned media,
18 consistent with previous work^{49, 50}. This signature in particular is reminiscent
19 of fibroblasts found within the tumor microenvironment, and that also possess
20 immune suppressive attributes⁵¹⁻⁵⁵, leading us to speculate that in LNs
21 downstream of tumors, FRCs adopt a more CAF-like state to provide a
22 supportive niche^{28, 30, 31}.

23

24 Within TDLNs, wider conduits and enhanced collagen deposition point to
25 increased stiffness of the node⁴⁰, but the remodeling of the collagen core may
26 also contribute to the size exclusion properties of the conduits^{5, 6}, that in
27 TDLNs was disrupted with large MW dextran reaching deeper into the conduit
28 network than in resting nodes. This, combined with deregulated junction
29 properties and protein pores of the FRCs lining these channels, suggests an
30 altered integrity of the conduit network. These changes have the potential to
31 lead to rapid, but poorly controlled delivery of tumor-derived factors, debris
32 and antigen to the deeper areas of the LN upsetting the functional status quo.
33 Moreover, the process of lymphangiogenesis both at primary tumors and

1 connected LNs enhances the drainage capacity, and consequently these
2 stromal populations experience raised fluid flux and shear stresses.
3 Mechanical cues such as these rather than chemical, tumor-derived signals
4 (data not shown) may also act as a stimulus for FRC proliferation⁵⁶⁻⁵⁹ or
5 synergize to drive the transcriptional reprogramming. We have not excluded
6 this in the present study, but this avenue warrants more in depth investigation
7 using *in vitro* studies in which the effects of biophysical stimuli i.e. flow can be
8 isolated.

9

10 In summary, using functional assays and comparative transcriptome analysis
11 of FRCs in resting and TDLNs in multiple tumor models, we demonstrate that
12 FRCs immediately downstream of tumors acquire unique transcriptional
13 programs. Together with structural remodeling, these deregulated pathways
14 and adapted FRC traits contribute to modified immune composition and
15 aberrant localization that may ultimately translate to a more suppressive, pro-
16 tumor environment.

17

18 **METHODS**

19 Methods and any associated references are available in the supplementary
20 information of the paper.

21

22 **AUTHOR CONTRIBUTIONS**

23 A.R. planned and performed majority of experiments and associated analysis;
24 L.H. performed *in vitro* experiments; D.S. performed *in silico* analysis; B.A.H.
25 contributed to *in silico* analysis and data interpretation; J.S. conceived project,
26 planned and performed experiments and contributed to data interpretation.
27 A.R. J.S. and D.S. co-wrote the paper. All authors contributed to editing of
28 manuscript and critical review.

29

30 **ACKNOWLEDGEMENTS**

31 The authors would like to thank members of the Shields Group for
32 constructive comments and continued scientific discussion, Ares Facility QU,
33 E23 and T29 staff members for animal husbandry and technical assistance,

1 Richard Butler of the Gurdon Institute for support with image analysis and
2 algorithm development, and Cambridge Genomic Services for microarray
3 services and post-analysis advice. This research was supported by the CIMR
4 Flow Cytometry Core Facility. We wish to thank all FCCF staff members for
5 their advice and support in flow cytometry and cell sorting applications. Work
6 was supported by Medical Research Council funding. BAH is supported by
7 the Royal Society (University Research Fellowship).

9 COMPETING FINANCIAL INTEREST

10 The authors declare no competing financial interest.

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11

1 **Figure legends**

2 **Figure 1:** LN expansion and FRC network remodeling in TDLNs. (a)
3 Representative confocal images of ND (left) and 11d TD (right) brachial LNs
4 showing stromal cell populations and the increased size of TDLNs. (b) Flow
5 cytometry gating strategy for isolation of stromal cells from ND (top) and 11d
6 TD (bottom) LNs. Stromal subtypes: FRC, PDPN⁺CD31⁻; LEC, PDPN⁺CD31⁺;
7 BEC, PDPN⁻CD31⁺. Numbers in boxes represent percentages of parent
8 population in gate. (c and d) Expansion of TD (red) compared to ND (PBS
9 control, cyan) LNs over a time course of 14 days measured by flow cytometry.
10 Total LN cells (c), BECs, LECs, and FRCs (d) were quantified. Quantification
11 of total LN cells (e) and stromal cells (f) of TDLNs and ND LNs measured by
12 flow cytometry in shoulder B16.F10 or induced *Tyr::CreER, Braf^{CA}, Pten^{lox}*
13 tumors on either the shoulder or lower flank. For shoulder tumors the brachial
14 LN (braLN) and for flank tumors the inguinal LN (iLN) were identified to be the
15 primary draining LNs. (g) Skeleton analysis of collagen I networks in T cell
16 areas determined the number of conduit branches per Field Of View (FOV) in
17 ND and TDLNs. (h) Gap analysis of collagen I networks determined the
18 distance between conduit branches in ND and TDLNs. (i) Confocal Airyscans
19 of conduit end and side views stained for PDPN, collagen I and ERTR7 (left
20 panel). Conduit thickness measured by 0.1 μ m z-stacks of the conduit
21 collagen I core rotated to display ellipse area of cross section for ND and
22 TDLNs (right panel). (c and d) Data are representative of two independent
23 experiments with each 2 (Ctrl) and 3 (Tumor) LNs from independent mice per
24 replicate. (e and f) Data are representative of 2 independent experiments with
25 each 3 (ND) and 5 (TD) LNs (B16.F10) and 3/4 (iND) and 2 (iTDL, braND,
26 braTD) (*Tyr::CreER, Braf^{CA}, Pten^{lox}*) from independent mice per replicate. (g
27 and h) Data are from 3-6 individual LNs from independent mice with 3 FOV
28 analyzed per LN, (i) 3-4 individual LNs from different mice with 5 conduits per
29 LN imaged and 3 measurements per conduit performed in shortest and
30 longest axis. Data points indicate the mean \pm s.e.m. *P <0.05, **P <0.01 and
31 ***P <0.001. For time courses, data were subjected to two-way ANOVA,
32 followed by post hoc analysis. When two groups were compared, a two-tailed

1 unpaired Student's t-test was applied. Scale bars (a) 200 μm , (g and h) 50
2 μm .

3

4 **Figure 2:** Statistical analysis of microarray results. (a) Principle Component
5 Analysis of samples from ND, 4d TD, and 11d TD LNs. Analysis was
6 performed on the most significantly deregulated samples ($\text{FC} > \pm 1.5$), and
7 those with a p value of greater than 0.05, leaving a total of 244 probes. The
8 top two most significant eigenvectors are illustrated, accounting for 93.4% of
9 the total variability between samples. Expression data was \log_2 transformed
10 and normalized by row before principal component analysis was performed.
11 (b) Heatmap of coefficients of correlation for most significantly deregulated
12 probes (the same dataset as used in part a). Red indicates the highest
13 correlation. (c) Hierarchical clustering analysis of all samples with heatmap of
14 the top deregulated probes. All analyses were performed on probes with an
15 expression fold change of over 1.5 and a P value <0.05 . Each data point
16 representing transcriptomes of FRCs of 2 brachial LNs, pooled per mouse.

17

18 **Figure 3:** Identification of specific genes and pathways deregulated in TDLN
19 FRCs. (a) Venn diagrams displaying overlap between significantly
20 downregulated (i), and significantly upregulated (ii) probes in ND, 4d TD, and
21 11d TD samples. (b) Significantly deregulated probes represented on volcano
22 plots for 4d TD vs. ND (i), 11d TD vs. ND (ii), and 11d TD vs. 4d TD (iii).
23 Probes displayed with the most significantly deregulated ($\text{FC} > \pm 1.5$)
24 represented as blue (downregulated) or red (upregulated). (c) Heatmaps of
25 key pathways involving the top deregulated genes with a FC of over 1.5 and a
26 $P < 0.05$ compared between 4d TD vs. ND and 11d TD vs. ND. Functional
27 groups were assigned with GSEA and IPA.

28

29 **Figure 4:** Perturbation in LN critical chemokine/cytokine signaling pathways
30 correlates with changes in immune cell composition and localization. (a)
31 Heatmap of significantly deregulated probes ($P < 0.05$) falling into the category
32 of cytokine and chemokine signaling molecules. Pathway analyses were
33 performed with GSEA and IPA. (b) mRNA expression levels of IL7 and
34 CCL21 measured by qRT-PCR in an independent FRC sample set from

1 B16.F10 ND, 4d TD and 11d TD LNs, and ND and TD LNs obtained from
2 *Tyr::CreER, Braf^{CA}, Pten^{lox}* tumor-bearing mice. (c) Representative confocal
3 images of LN paracortical areas of ND (top panel) and 11d TD (bottom panel)
4 LNs stained for PDPN (green), ERTR7 (blue) and CCL21 (red). (d) Flow
5 cytometric quantification of T cells (CD45⁺CD3e⁺) as percentage of singlets
6 within ND and 11d TD (B16.F10) LNs. (e) Quantification of CD4⁺ T cells
7 (CD45⁺CD3e⁺CD4⁺) in ND and 11d TD (B16.F10) LNs. (f) Quantification of
8 CD4⁺ T cell populations; naïve CD62L⁺CD44⁻, memory CD62L⁺CD44⁺ and
9 activated CD62L⁻CD44⁺. (g) Flow cytometric measurement of regulatory T
10 cells (CD45⁺CD3e⁺CD4⁺FoxP3⁺). (h) LN homing assay: Splenocytes were
11 isolated from GFP⁺ C57bl/6 mice and injected into wt mice. After 18h LNs
12 were isolated and immune cell contents were analyzed by flow cytometry.
13 Quantification of homed CD4⁺ T cells is expressed as ratio of
14 CD4⁺GFP⁺:GFP⁺ within ND and 11d TD (B16.F10) LNs. (i) Representative
15 confocal images of B cell follicles of ND and 11d TDLNs (B16.F10) stained for
16 CD3e (green), CD45R (red) and Collagen I (blue). (j) Representative confocal
17 images (i) of ND and 11d TD LNs (B16.F10) stained for CD3e (green) CD45R
18 (red) and PNAd (blue). Quantification of B cell clustered HEVs on LN sections
19 (ii). (k) LN homing assay as described in (h): Flow cytometric quantification of
20 CD45R⁺ expressed as ratio CD45R⁺GFP⁺:GFP⁺ within ND and 11d TD LNs
21 (B16.F10). Each data point represents whole transcriptome amplified (WTA)
22 mRNA samples of brachial LNs pooled per mouse, with 3 mice per condition
23 and technical duplicates of WTA (B16.F10) and with 5 mice (ND) and 7 mice
24 (TD) (*Tyr::CreER, Braf^{CA}, Pten^{lox}*) (b). Data collected from 5-6 individual ND or
25 TD LNs from different mice (d). Data representative of 8-10 individual LNs per
26 condition from two independent experiments (e and f). Data are from 6 ND
27 and 9 TD LNs from different mice (g). Data collected from two independent
28 experiments with each 4 (ND) and 3 (TD) LNs (h and k). Data representative
29 of 6 (ND) and 5 (TD) LNs obtained from independent mice (jii). Data points
30 indicate the mean ± s.e.m. *P <0.05, **P <0.01 and ***P <0.001. For
31 comparisons of three or more groups, data were subjected to one-way
32 ANOVA, followed by post hoc analysis. When two groups were compared, a
33 two-tailed unpaired Student's t-test was applied. Scales bars (c and j) 50 µm,
34 (i) 51 µm (ND) and 38 µm (TD).

1
2 **Figure 5:** FRCs in TDLNs become more activated. (a) Heatmap of
3 significantly deregulated probes ($P < 0.05$) falling into the category of fibroblast
4 activation status. (b) mRNA expression levels of PDPN, FSP1 and THY1
5 measured by qRT-PCR in an independent FRC sample set from ND (cyan) 4d
6 TD (black) and 11d TD (red) LNs from B16.F10, and ND (cyan) and TD (red)
7 LNs obtained from the *Tyr::CreER, Braf^{CA}, Pten^{lox}* model. (c) Flow cytometric
8 analysis of PDPN surface expression as measured by relative mean
9 fluorescence intensity of the geometric mean in FRCs isolated from B16.F10
10 ND, 4d and 11d TDLNs, or ND and TDLNs from the *Tyr::CreER, Braf^{CA}, Pten^{lox}*
11 mice. (di) Representative scatter profile of alive FRCs from ND (left) and 11d
12 TDLN (right) and (dii) geometric mean of the side scatter of FRCs sorted from
13 B16.F10 ND (cyan), 4d TD (black) and 11d TD (red) LNs, or ND (cyan) and
14 TD (red) LNs from *Tyr::CreER, Braf^{CA}, Pten^{lox}* mice. (e) mRNA and (f) protein
15 expression of PDPN by *in vitro* cultured FRCs treated with control conditioned
16 medium (CCM) or tumor conditioned medium (TCM) for 7 days as measured
17 by qRT-PCR or flow cytometry. (g) Comparison of the collagen gel contractile
18 activity of *in vitro* FRCs pretreated with CCM or TCM. Each data point
19 represents WTA mRNA samples of brachial LNs pooled per mouse, with 3
20 mice per condition and technical duplicates of WTA (B16.F10) and with 5
21 mice (ND) and 7 mice (TD) (*Tyr::CreEr, Braf^{CA}, Pten^{lox}*) LNs. (b). Data are from
22 4-7 (B16.F10) or 4-12 (*Tyr::CreER, Braf^{CA}, Pten^{lox}*) individual LNs taken from
23 different mice (c and d). Data are representative of three independent
24 experiments (e - g) performed in triplicate per condition. Data points indicate
25 the mean \pm s.e.m. * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$. For comparisons of
26 three or more groups, data were subjected to one-way ANOVA followed by
27 post hoc analysis. When two groups were compared, a two-tailed unpaired
28 Student's t-test was applied

29
30 **Figure 6:** Modified transporter repertoires within TDLN FRCs translate to
31 altered solute transport throughout the conduit system of the node. (a)
32 Heatmap for significantly ($P < 0.05$) deregulated probes involved in ion/solute
33 conduction or membrane permeability. (b) *In vitro* measurement of relative
34 permeability of 10 70 and 500 kDa dextran transport through an FRC

1 monolayer pretreated for 7 days with CCM or TCM and measured after 22 h.
2 **(c)** Filling of the conduit network in ND and 11d TD LNs 10 min after
3 subcutaneous injection of Texas Red-labeled 10 kDa dextran and biotin-
4 labeled 70 kDa dextran. Quantification of fluorescence signals of 70kDa
5 dextran and ERTR7 per paracortical area displayed as area fraction **(ci** and
6 **cii)**. **(d)** Quantification of 70 kDa dextran as relative fluorescence intensity (FI)
7 per area and representative high-magnification micrograph of the paracortical
8 region from ND and 11d TDLNs counterstained with PDPN. Each channel is
9 gray scaled **(e)** Close up of dextran filled conduit from a 11d TDLN, staining
10 for 70 kDa dextran (magenta) within a PDPN⁺ FRC (green) lined conduit. **(f)**
11 Network analysis of the top deregulated probes within gene arrays for 11d
12 TDLN samples. Top networks calculated with the MANIA algorithm are shown
13 with heatmaps of the probes for each time point. Sets of probes are related
14 through either function, regulation, or physical space. **(g)** Schematic of the
15 proposed changes in FRC conduits. In response to tumor factors ND (left)
16 FRCs proliferate leading to an increase in the size of the conduit by 11d
17 (right), and matrix deposition manifesting as increased diameter of the
18 collagen core. Subsequent to this there is an increase in the amount of
19 solute/ion transporters expressed within the cells leading to a potential
20 increase in fluid movement within the conduits. By 11d TD, the increased
21 expression of solute/ion channels leads to a potential increase in the ability for
22 fluid to pass through the conduit. The concurrent upregulation of extracellular
23 matrix components and altered cell-cell interactions indicate a potential
24 thickening of the matrix of the conduit core. Furthermore, two important FRC
25 factors, CCL21 and IL7 are downregulated over time leading to changes in
26 immune cell localization and composition. Data are representative of two
27 independent experiments **(b)** performed in five replicates per condition. Data
28 are from 4 (ND) or 3 (TD) (B16.F10) individual LNs taken from different mice
29 **(c** and **d)**. Data points indicate the mean \pm s.e.m. *P <0.05, **P <0.01 and
30 ***P <0.001. For comparisons, a two-tailed unpaired Student's t-test was
31 applied. Scale bars **(d)** 50 μ m **(e)** 3.4 μ m.

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