TUMOR INDUCED STROMAL REPROGRAMMING DRIVES LYMPH NODE TRANSFORMATION

Angela Riedel[1], *David Shorthouse*[1], *Lisa Haas*[1], *Benjamin A Hall*[1]*, *Jacqueline Shields*[1]*

[1]MRC Cancer Unit,
University of Cambridge,
Hutchison/MRC Research Centre,
Box 197,
Cambridge Biomedical Campus,
Cambridge,
CB2 0XZ

*to whom correspondence should be addressed.
Email: js970@mrc-cu.cam.ac.uk or bh418@mrc-cu.cam.ac.uk
Phone: 01223 761231 (JS) or 01223 363268 (BAH)

For submission to Nature Immunology

Short title:

Stromal reprogramming drives lymph node transformation

ABSTRACT

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

INTRODUCTION

LNs form an integral part of both our lymphatic and immune systems, acting as “filters” to surveil potential lymph borne pathogens, and as an immunological hub maintaining homeostasis or eliciting effective immune responses. To enable these specialized functions, the LN is highly organized into discrete cellular compartments. The supporting stromal cells are central to organization and function[1, 2, 3] and the major stromal subsets, lymphatic endothelial cells (LEC), blood endothelial cells (BEC) and fibroblastic reticular cells (FRC) can be distinguished by their relative expression of surface markers podoplanin and CD31[2].

Lymph draining from peripheral tissues enters via LEC-lined afferent lymphatic vessels and along lymphatics that line the subcapsular and medullary sinuses before exiting in efferent lymphatics[4]. However, smaller constituents such as chemokines and soluble antigen below 70kDa can cross the lymphatic sinus floor and penetrate deeper into the LN, along narrow conduit channels formed by collagen fibrils and FRCs[5, 6, 7]. While the conduit

network provides underlying structural support[4], FRCs have additional properties vital for proper lymph node function. They produce a number of chemical cues that are critical for immune cell migration, localization and survival such as homeostatic chemokines CCL19 and 21[8], whose receptor CCR7 is present on naïve T cells, B cells and Dendritic Cells (DCs)[9]. Gradients of these chemokines direct intranodal migration and survival during homeostasis and infection[3, 10, 11], lymphocyte homing to LNs[12] and mediate interactions between T cells and DCs[8]. FRCs are a major source of IL7, essential for naïve T cell survival[2]. Those resident in follicles also contribute to B cell homeostasis and follicle identity via the production of the cytokine BAFF[13]. Moreover, specific destruction of FRCs is a method employed by viruses to avoid detection during infection[14]. More recently, evidence indicates that FRCs not only contribute to the onset of effective immune responses, but conversely to immunological tolerance, switching off an immune response via deletion of self-reactive T cells[15, 16, 17].

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

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

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

RESULTS

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

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

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

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

Transcriptional profiling identifies alterations in TDLN FRCs

Considering observations of both TDLN enlargement and FRC network remodeling and frequent reports of immune dysfunction in tumors[21-26] we sought to identify how FRCs of expanding TDLNs adapt to the evolving microenvironment, and whether these changes ultimately translate to pro-tumor structural and functional modifications in the LN. To do this, FRCs from LNs at days 4 and 11 post B16.F10 inoculation were freshly sorted and subjected to whole genome transcriptional profiling. At day 4, tumors were barely palpable thus this time point was chosen to represent an early stage of tumor development, where the tumor microenvironment is not yet fully established and communications with draining lymph nodes are likely at their earliest stages via resident dermal lymphatic vessels. In contrast, large day 11

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

Identification of specific genes and pathways deregulated in TDLN FRCs

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

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

FRCs are an essential source of chemokines and cytokines necessary for immune homeostasis, leukocyte trafficking and survival within the LN[27]. Both GSEA and Ingenuity (IPA) analyses identified these pathways to be

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

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

FRCs of TDLNs are more activated

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

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

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

DISCUSSION

Lymph nodes function as a major immunological hub, essential for immune homeostasis and generation of appropriate immune responses, yet LNs are also the first site of metastasis for many cancers that manage to avoid immune-mediated clearance. It is increasingly accepted that LNs receive and respond to tumor-derived signals generating a pro-tumor niche, but it remains unclear as to how these responses manifest and who in the LN drives them. The stromal populations of the LN not only provide structural support but are essential to its maintenance and physiological function[1-3, 6, 8-14, 32-36]. While studies have shown that tumor-derived VEGF and VEGF-C contribute to LN lymphangiogenesis and vascular reorganization[37-39], the fibroblasts of the LN, FRCs, and the conduit network they form have not been thoroughly investigated in the context of the tumor and subsequent modulation of LN behavior. Here we describe that expansion, remodeling and transcriptional reprogramming of FRCs occurs in TDLNs. This in turn impacts FRC-driven chemokine signaling, trafficking events, immune localization and transport, all of which have the potential to contribute to impaired lymph node function, that in the context of a tumor may provide a pro-tumor environment.

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

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

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

in optimal activation of $CD8^+$ T cells and long term memory[45]. Since FRCs have been shown to be the source of CCL4[11] in the LN, a downregulation in TDLNs may result in a reduction of this T cell population. Beyond the disruption of immune compartmentalization, we also present evidence to implicate FRC transcriptional modulation in the altered immune composition of TDLNs. Within draining nodes, we observed fewer naïve $CD4^+$ T cells and accumulation of Tregs consistent with previous studies correlating their presence with immune suppression and disease progression[46]. We detected enlarged B cell follicles within TDLNs, and although the activation status or subtypes of the B cells occupying TDLNs remains to be determined, recent reports have demonstrated that B cells do indeed accumulate in TDLNs[47], and that these may function as regulatory B cells[48] adding a further dimension to the local immune suppressive environment.

Transcripts of TDLN FRCs also indicated the acquisition of a more activated status evident from upregulation of Thy1, podoplanin, FSP1, CD248, vimentin, collagens, fibronectin, α smooth muscle actin, and the capacity to contract collagen gels more efficiently upon receipt of tumor conditioned media, consistent with previous work[49, 50]. This signature in particular is reminiscent of fibroblasts found within the tumor microenvironment, and that also possess immune suppressive attributes[51-55], leading us to speculate that in LNs downstream of tumors, FRCs adopt a more CAF-like state to provide a supportive niche[28, 30, 31].

Within TDLNs, wider conduits and enhanced collagen deposition point to increased stiffness of the node[40], but the remodeling of the collagen core may also contribute to the size exclusion properties of the conduits[5, 6], that in TDLNs was disrupted with large MW dextran reaching deeper into the conduit network than in resting nodes. This, combined with deregulated junction properties and protein pores of the FRCs lining these channels, suggests an altered integrity of the conduit network. These changes have the potential to lead to rapid, but poorly controlled delivery of tumor-derived factors, debris and antigen to the deeper areas of the LN upsetting the functional status quo. Moreover, the process of lymphangiogenesis both at primary tumors and

connected LNs enhances the drainage capacity, and consequently these stromal populations experience raised fluid flux and shear stresses. Mechanical cues such as these rather than chemical, tumor-derived signals (data not shown) may also act as a stimulus for FRC proliferation[56-59] or synergize to drive the transcriptional reprogramming. We have not excluded this in the present study, but this avenue warrants more in depth investigation using *in vitro* studies in which the effects of biophysical stimuli i.e. flow can be isolated.

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

METHODS

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

AUTHOR CONTRIBUTIONS

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

ACKNOWLEDGEMENTS

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

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

COMPETING FINANCIAL INTEREST

The authors declare no competing financial interest.

REFERENCES

1. Bajenoff, M. *et al.* Stromal cell networks regulate lymphocyte entry, migration, and territoriality in lymph nodes. *Immunity* **25**, 989-1001 (2006).

2. Link, A. *et al.* Fibroblastic reticular cells in lymph nodes regulate the homeostasis of naive T cells. *Nature immunology* **8**, 1255-1265 (2007).

3. Schumann, K. *et al.* Immobilized chemokine fields and soluble chemokine gradients cooperatively shape migration patterns of dendritic cells. *Immunity* **32**, 703-713 (2010).

4. Roozendaal, R., Mebius, R.E. & Kraal, G. The conduit system of the lymph node. *International immunology* **20**, 1483-1487 (2008).

5. Gretz, J.E., Norbury, C.C., Anderson, A.O., Proudfoot, A.E. & Shaw, S. Lymph-borne chemokines and other low molecular weight molecules reach high endothelial venules via specialized conduits while a functional barrier limits access to the lymphocyte microenvironments in lymph node cortex. *J Exp Med* **192**, 1425-1440 (2000).

6. Roozendaal, R. *et al.* Conduits mediate transport of low-molecular-weight antigen to lymph node follicles. *Immunity* **30**, 264-276 (2009).

7. Sixt, M. *et al.* The conduit system transports soluble antigens from the afferent lymph to resident dendritic cells in the T cell area of the lymph node. *Immunity* **22**, 19-29 (2005).

8. Luther, S.A., Tang, H.L., Hyman, P.L., Farr, A.G. & Cyster, J.G. Coexpression of the chemokines ELC and SLC by T zone stromal cells and deletion of the ELC gene in the plt/plt mouse. *Proceedings of the*

National Academy of Sciences of the United States of America **97**, 12694-12699 (2000).

9. Forster, R., Davalos-Misslitz, A.C. & Rot, A. CCR7 and its ligands: balancing immunity and tolerance. *Nat Rev Immunol* **8**, 362-371 (2008).

10. Katakai, T. *et al.* A novel reticular stromal structure in lymph node cortex: an immuno-platform for interactions among dendritic cells, T cells and B cells. *International immunology* **16**, 1133-1142 (2004).

11. Katakai, T., Hara, T., Sugai, M., Gonda, H. & Shimizu, A. Lymph node fibroblastic reticular cells construct the stromal reticulum via contact with lymphocytes. *J Exp Med* **200**, 783-795 (2004).

12. Gunn, M.D. *et al.* A chemokine expressed in lymphoid high endothelial venules promotes the adhesion and chemotaxis of naive T lymphocytes. *Proceedings of the National Academy of Sciences of the United States of America* **95**, 258-263 (1998).

13. Cremasco, V. *et al.* B cell homeostasis and follicle confines are governed by fibroblastic reticular cells. *Nature immunology* **15**, 973-981 (2014).

14. Mueller, S.N. *et al.* Viral targeting of fibroblastic reticular cells contributes to immunosuppression and persistence during chronic infection. *Proceedings of the National Academy of Sciences of the United States of America* **104**, 15430-15435 (2007).

15. Fletcher, A.L. *et al.* Lymph node fibroblastic reticular cells directly present peripheral tissue antigen under steady-state and inflammatory conditions. *J Exp Med* **207**, 689-697 (2010).

16. Lee, J.W. *et al.* Peripheral antigen display by lymph node stroma promotes T cell tolerance to intestinal self. *Nature immunology* **8**, 181-190 (2007).

17. Nichols, L.A. *et al.* Deletional self-tolerance to a melanocyte/melanoma antigen derived from tyrosinase is mediated by a radio-resistant cell in peripheral and mesenteric lymph nodes. *Journal of immunology* **179**, 993-1003 (2007).

18. de Boer, M., van Dijck, J.A., Bult, P., Borm, G.F. & Tjan-Heijnen, V.C. Breast cancer prognosis and occult lymph node metastases, isolated tumor cells, and micrometastases. *J Natl Cancer Inst* **102**, 410-425 (2010).

19. Morton, D.L. *et al.* Sentinel-node biopsy or nodal observation in melanoma. *N Engl J Med* **355**, 1307-1317 (2006).

20. Karaman, S. & Detmar, M. Mechanisms of lymphatic metastasis. *J Clin Invest* **124**, 922-928 (2014).

21. Alderton, G.K. & Bordon, Y. Tumour immunotherapy--leukocytes take up the fight. *Nat Rev Immunol* **12**, 237 (2012).

22. Chen, D.S. & Mellman, I. Oncology meets immunology: the cancer-immunity cycle. *Immunity* **39**, 1-10 (2013).

23. Gajewski, T.F., Schreiber, H. & Fu, Y.X. Innate and adaptive immune cells in the tumor microenvironment. *Nature immunology* **14**, 1014-1022 (2013).

24. Joyce, J.A. & Fearon, D.T. T cell exclusion, immune privilege, and the tumor microenvironment. *Science* **348**, 74-80 (2015).

25. Mellman, I., Coukos, G. & Dranoff, G. Cancer immunotherapy comes of age. *Nature* **480**, 480-489 (2011).

26. Zou, W. Regulatory T cells, tumour immunity and immunotherapy. *Nat Rev Immunol* **6**, 295-307 (2006).

27. Malhotra, D. *et al.* Transcriptional profiling of stroma from inflamed and resting lymph nodes defines immunological hallmarks. *Nature immunology* **13**, 499-510 (2012).

28. Cox, T.R. & Erler, J.T. Molecular pathways: connecting fibrosis and solid tumor metastasis. *Clin Cancer Res* **20**, 3637-3643 (2014).

29. Kalluri, R. & Zeisberg, M. Fibroblasts in cancer. *Nat Rev Cancer* **6**, 392-401 (2006).

30. Kaplan, R.N., Rafii, S. & Lyden, D. Preparing the "soil": the premetastatic niche. *Cancer research* **66**, 11089-11093 (2006).

31. Kaplan, R.N. *et al.* VEGFR1-positive haematopoietic bone marrow progenitors initiate the pre-metastatic niche. *Nature* **438**, 820-827 (2005).

32. Fletcher, A.L., Acton, S.E. & Knoblich, K. Lymph node fibroblastic reticular cells in health and disease. *Nat Rev Immunol* **15**, 350-361 (2015).

33. Forster, R., Braun, A. & Worbs, T. Lymph node homing of T cells and dendritic cells via afferent lymphatics. *Trends in immunology* **33**, 271-280 (2012).

34. Gunn, M.D. *et al.* Mice lacking expression of secondary lymphoid organ chemokine have defects in lymphocyte homing and dendritic cell localization. *J Exp Med* **189**, 451-460 (1999).

35. Mueller, S.N. *et al.* Regulation of homeostatic chemokine expression and cell trafficking during immune responses. *Science* **317**, 670-674 (2007).

36. Yang, C.Y. *et al.* Trapping of naive lymphocytes triggers rapid growth and remodeling of the fibroblast network in reactive murine lymph nodes. *Proceedings of the National Academy of Sciences of the United States of America* **111**, E109-118 (2014).

37. Harrell, M.I., Iritani, B.M. & Ruddell, A. Tumor-induced sentinel lymph node lymphangiogenesis and increased lymph flow precede melanoma metastasis. *The American journal of pathology* **170**, 774-786 (2007).

38. Hirakawa, S. *et al.* VEGF-C-induced lymphangiogenesis in sentinel lymph nodes promotes tumor metastasis to distant sites. *Blood* **109**, 1010-1017 (2007).

39. Hirakawa, S. *et al.* VEGF-A induces tumor and sentinel lymph node lymphangiogenesis and promotes lymphatic metastasis. *J Exp Med* **201**, 1089-1099 (2005).

40. Rohner, N.A. *et al.* Lymph node biophysical remodeling is associated with melanoma lymphatic drainage. *FASEB journal : official publication of the Federation of American Societies for Experimental Biology* (2015).

41. Carriere, V. *et al.* Cancer cells regulate lymphocyte recruitment and leukocyte-endothelium interactions in the tumor-draining lymph node. *Cancer research* **65**, 11639-11648 (2005).

42. Soudja, S.M. *et al.* Disrupted lymph node and splenic stroma in mice with induced inflammatory melanomas is associated with impaired recruitment of T and dendritic cells. *PloS one* **6**, e22639 (2011).

43. MartIn-Fontecha, A. *et al.* Regulation of dendritic cell migration to the draining lymph node: impact on T lymphocyte traffic and priming. *J Exp Med* **198**, 615-621 (2003).

44. St John, A.L. & Abraham, S.N. Salmonella disrupts lymph node architecture by TLR4-mediated suppression of homeostatic chemokines. *Nature medicine* **15**, 1259-1265 (2009).

45. Castellino, F. *et al.* Chemokines enhance immunity by guiding naive CD8+ T cells to sites of CD4+ T cell-dendritic cell interaction. *Nature* **440**, 890-895 (2006).

46. Deng, L. *et al.* Accumulation of foxp3+ T regulatory cells in draining lymph nodes correlates with disease progression and immune

suppression in colorectal cancer patients. *Clin Cancer Res* **16**, 4105-4112 (2010).

47. Ruddell, A., Harrell, M.I., Furuya, M., Kirschbaum, S.B. & Iritani, B.M. B lymphocytes promote lymphogenous metastasis of lymphoma and melanoma. *Neoplasia* **13**, 748-757 (2011).

48. Ganti, S.N., Albershardt, T.C., Iritani, B.M. & Ruddell, A. Regulatory B cells preferentially accumulate in tumor-draining lymph nodes and promote tumor growth. *Sci Rep* **5**, 12255 (2015).

49. Acton, S.E. *et al.* Podoplanin-rich stromal networks induce dendritic cell motility via activation of the C-type lectin receptor CLEC-2. *Immunity* **37**, 276-289 (2012).

50. Astarita, J.L. *et al.* The CLEC-2-podoplanin axis controls the contractility of fibroblastic reticular cells and lymph node microarchitecture. *Nature immunology* **16**, 75-84 (2015).

51. Augsten, M. *et al.* Cancer-associated fibroblasts expressing CXCL14 rely upon NOS1-derived nitric oxide signaling for their tumor-supporting properties. *Cancer research* **74**, 2999-3010 (2014).

52. Erez, N., Truitt, M., Olson, P., Arron, S.T. & Hanahan, D. Cancer-Associated Fibroblasts Are Activated in Incipient Neoplasia to Orchestrate Tumor-Promoting Inflammation in an NF-kappaB-Dependent Manner. *Cancer cell* **17**, 135-147 (2010).

53. Harper, J. & Sainson, R.C. Regulation of the anti-tumour immune response by cancer-associated fibroblasts. *Seminars in cancer biology* **25**, 69-77 (2014).

54. Kraman, M. *et al.* Suppression of Antitumor Immunity by Stromal Cells Expressing Fibroblast Activation Protein-alpha. *Science* **330**, 827-830 (2010).

55. Shields, J.D., Kourtis, I.C., Tomei, A.A., Roberts, J.M. & Swartz, M.A. Induction of lymphoidlike stroma and immune escape by tumors that express the chemokine CCL21. *Science* **328**, 749-752 (2010).

56. Baxter, L.T. & Jain, R.K. Transport of fluid and macromolecules in tumors. I. Role of interstitial pressure and convection. *Microvasc Res* **37**, 77-104 (1989).

57. Dafni, H., Landsman, L., Schechter, B., Kohen, F. & Neeman, M. MRI and fluorescence microscopy of the acute vascular response to VEGF165: vasodilation, hyper-permeability and lymphatic uptake, followed by rapid inactivation of the growth factor. *NMR Biomed* **15**, 120-131 (2002).

58. Goldman, J. *et al.* Cooperative and redundant roles of VEGFR-2 and VEGFR-3 signaling in adult lymphangiogenesis. *FASEB journal : official publication of the Federation of American Societies for Experimental Biology* **21**, 1003-1012 (2007).

59. Hompland, T., Ellingsen, C., Ovrebo, K.M. & Rofstad, E.K. Interstitial Fluid Pressure and Associated Lymph Node Metastasis Revealed in Tumors by Dynamic Contrast-Enhanced MRI. *Cancer Res* **72**, 4899-4908 (2012).

Figure legends

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

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

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

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

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

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

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

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

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