Tumor-induced stromal reprogramming drives lymph node transformation

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Lymph node (LN) stromal cells, particularly fibroblastic reticular cells (FRCs), provide critical structural support and regulate immunity, tolerance and the transport properties of LNs. For many tumors, metastasis to the LNs is predictive of poor prognosis. However, the stromal contribution to the evolving microenvironment of tumor-draining LNs (TDLNs) remains poorly understood. Here we found that FRCs specifically of TDLNs proliferated in response to tumor-derived cues and that the network they formed was remodelled. Comparative transcriptional analysis of FRCs from non-draining LNs and TDLNs demonstrated reprogramming of key pathways, including matrix remodeling, chemokine and/or cytokine signaling, and immunological functions such as the recruitment, migration and activation of leukocytes. In particular, downregulation of the expression of FRC-derived chemokines CCL21 and cytokine IL-7 were accompanied by altered composition and aberrant localization of immune-cell populations. Our data indicate that following exposure to tumor-derived factors, the stroma of TDLNs adapts on multiple levels to exhibit features typically associated with immunosuppression.

RESULTS

Expansion and remodeling of TDLN stromal networks
To study the role of stromal cells of TDLNs before the onset of metastasis, we used a well-established B16.F10 melanoma model in which we injected B16.F10 cells into mice and assessed TDLNs over 14 d. Brachial TDLNs were significantly larger at pre-metastatic time points relative to the size of their counterparts in control mice that received PBS (non-draining lymph nodes (NDLNs)) (Fig. 1a). Flow cytometry confirmed that the enlargement resulted from an increase in LN cellularity over time (Fig. 1b,c and Supplementary Fig. 1a). In contrast, NDLNs remained at a constant size (Fig. 1c). The pre-metastatic state of TDLNs was confirmed by quantitative RT-PCR analysis of mRNA encoding melanoma markers (Tyrp1 and Dct), which was not detectable in TDLNs (Supplementary Fig. 1b). Moreover, the enlargement and corresponding cellularity were confined to sentinel LNs, as adjacent LNs did not increase in size (Supplementary Fig. 1c).

Stromal populations within TDLNs were identified by differential expression of the mucin-type glycoprotein podoplanin and the adhesion molecule CD31 (PECAM-1) among CD45− cells (gating strategy, Fig. 1b). Over the period of analysis, significant population expansion
Figure 1 LN expansion and FRC remodeling. (a) Confocal microscopy of CD31, podoplanin and the lymphatic endothelial cell marker Lyve-1 in stromal populations of NDLNs (left) and TDLNs at day 11 (right). (b) Gating strategy for flow cytometry of NDLNs (top) and TDLNs (bottom). Stromal subtypes are gated on CD45−, live singlets (far left and middle), followed by analysis of podoplanin and CD31 (far right). Numbers in or adjacent to outlined areas indicate percent CD45− cells (far left), singlets among those CD45− cells (middle left) and live cells among those CD45− singlets (middle right), or (far right) percent podoplanin-positive CD31− FRCs (top left), podoplanin-positive CD31+ LECs (top right) or podoplanin-negative CD31− BECs (bottom right). FSC, forward scatter; SSC, side scatter. (c–f) Total LN cells (c) or BECs, LECs and FRCs (d) in NDLNs and TDLNs from female C57BL/6 mice, assessed by flow cytometry over 14 d. (e,f) Total LN cells (e) and stromal cells (BECs, LECs and FRCs) (f) in brachial NDLNs (braND), brachial TDLNs (braTD), inguinal NDLNs (iND) or inguinal TDLNs (iTD) (key) from B16.F10 mice (female C57BL/6) or CreERloxPtenBrafµmice (equal ratio, male and female) (horizontal axis). (g,h) Microscopy (left) and 'skeleton analysis' (g, right) or gap analysis (h, right) of collagen I networks in NDLNs and TDLNs (at day 11) of female C57BL/6 mice, presented as branches per field of view (FDV) (g, right) or gap area, measured by circles fitted between branches (h, right). Outlined areas (top right corners at left) indicate 2.5× enlargement of a portion of the main image. (i) High-power confocal laser-scanning microscopy of conduit end and side views in NDLNs and TDLNs (at day 11) of female C57BL/6 mice (left), and quantification of results at left, presented as the ellipse area of the end-view collagen core (right). Scale bars, 200 µm (a), 50 µm (g,h), or 0.256 µm (i, NDLN side), 0.441 (i, NDLN end), 0.363 µm (i, TDLN side) or 0.549 µm (i, TDLN end). Each symbol (e–i) represents an individual LN (e,f) or conduit cross-section (g,h); small horizontal lines indicate the mean (± s.e.m.). *P < 0.05, **P < 0.01 and ***P < 0.001 (two-way analysis of variance (ANOVA) (c–f) or two-tailed unpaired t-test (e–i)). Data are representative of three experiments (a), five experiments (b), two independent experiments with n = 3 NDLNs and n = 5 TDLNs (c,d; mean ± s.e.m.), n = 6 brachial NDLNs and n = 10 brachial TDLNs (B16.F10) or n = 7 inguinal NDLNs, n = 4 inguinal TDLNs, n = 4 brachial NDLNs and n = 4 brachial TDLNs (Lyve-1CreERloxPtenlox) (e,f), or three independent experiments (g–i) with n = 6 NDLNs and n = 4 TDLNs with three fields of view per LN (g,h), or n = 3 NDLNs and n = 4 TDLNs with five conduits per LN and three measurements per conduit (i).
of blood endothelial cells (BECs), lymphatic endothelial cells (LECs) and FRCs was recorded specifically within TDLNs (Fig. 1d). Next we studied \( \text{Tyr}^{\text{CreER}} \text{Braf}^{\text{C aptenlox}} \) mice, which develop melanoma at multiple sites following activation of a constitutively active (CA) mutant of the serine-threonine kinase B Raf(B Raf(V600E)) and deletion of a \( \text{loxP} \)-flanked allele encoding the tumor suppressor PTEN (\( \text{Ptenlox} \)), both mediated by melanocyte-specific tamoxifen-inducible Cre recombinase expressed under the control of the promoter of the gene encoding tyrosinase (\( \text{Tyr}^{\text{CreER}} \)). Consistent with the results obtained for B16.F10 tumors above, TDLNs from mice bearing induced tumors were also enhanced (Fig. 1e), with the enlargement supported by increased cellularity of stromal populations (Fig. 1f and Supplementary Fig. 1d). Proliferation of FRCs was confirmed by \textit{in vivo} labeling with the thymidine analog Edu, whereas turnover of LECs and BECs remained in line with that of their NDLN counterparts (Supplementary Fig. 1e). In contrast, we noted a significantly higher ratio of FRCs to whole-node cells for TDLNs than for NDLNs after 11 d (\( P <0.05 \) (two-tailed unpaired \( t \)-test); Supplementary Fig. 2a), which led us to assess changes in the FRC network in more detail. Networks remained intact in TDLNs, with \( \text{Supplementary Fig. 2a} \), which showed expression of the top deregulated gene probes, with cutoff of a change in expression of 1.5-fold and a \( P \) value of <0.05. Data are representative of one experiment with \( n = 3 \) mice per group.

Transcriptional modification of TDLN FRCs

Given the structural alterations reported above, we next sought to identify how FRCs of TDLNs adapt to the evolving microenvironment, as well as the consequences of such changes. We sorted FRCs from LNs at days 4 and 11 after inoculation of B16.F10 cells and subjected the FRCs to whole-genome transcriptional profiling. At day 4, tumors were barely palpable (data not shown); thus, this time point was chosen to represent an early stage of development at which communication between lymphatics and TDLNs was probably at its earliest stages. In contrast, large tumors at day 11, with established stromal and LN connections, represented the late stage of LN transformation. Inter-replicate coefficients of variation confirmed consistency between samples, with means of 0.036 (NDLNs), 0.037 (TDLNs at day 4) and 0.035 (TDLNs at day 11). Application of a cutoff of 1.5-fold for change in expression in the gene array revealed distinct differences between FRCs from TDLNs and those from NDLNs (Supplementary Fig. 2f; b). We initially calculated principal components. When plotted, eigenvalues of these showed that the majority of variance (88.9% and 4.5%, respectively) was contained within principal component 1 (PC1) and PC2 (Supplementary Fig. 2i), which partitioned into their respective sample types: PC1 effectively separated NDLNs and TDLNs at day 4 and TDLNs at day 11 were distinct and replicable. That result was further confirmed by hierarchical clustering, which showed strong association within all data sets (Fig. 2b). Correlation matrix plots supported the conclusion of this relationship and showed strong association within all data sets (Fig. 2c). A heat map of the data linked to hierarchical clusters revealed that clusters of samples (NDLNs, TDLNs at day 4 and TDLNs at day 11) exhibited the same pattern of gene-expression changes within their groups (Fig. 2c, bottom). These data demonstrated that the gene-expression profiles for TDLNs at day 4 and TDLNs at day 11 were distinct and replicable and indicated that the FRCs underwent a gradual reprogramming response after exposure to tumor factors, with day 4 representing a distinct and transitional state.
Identification of pathways deregulated in FRCs

Significant overall differences in expression of 1.5-fold were observed for the conditions (P < 0.05; Supplementary Fig. 3a). Differential gene expression between day 4 and day 11 illustrated transient increases or decreases, which probably represented early activation or repression of FRC signaling pathways. This expression either returned to control levels by 11 d or continued to be further upregulated or downregulated. While expression within the array differed over time, the number of altered genes remained similar: 106 genes were downregulated at day 4, and 81 genes were downregulated at day 11, with an overlap (i.e., genes similarly upregulated in both conditions) of 39 genes, whereas 117 genes were upregulated at day 4, and 131 genes were upregulated at day 11, with an overlap of 25 genes (Fig. 3a). We determined the genes with the greatest deregulation between day 4 and day 11 in TDLNs relative to that in NDLNs, as ranked by their expression (Fig. 3b). Aqp1 was among the genes most upregulated after 4 d, but by 11 d its expression returned to baseline (Fig. 3b). In contrast, Fxyd6, Ig/h4, Thy1 and Ptx3 were among the genes most upregulated at day 11 (Fig. 3b), which indicated a unique late-stage signature. Clustering of the genes with the greatest deregulation into functional groups (according to the products encoded) highlighted differences in proliferation, metabolism, mitochondrial function, movement and migration, and cell-junction pathways (Fig. 3c). Associated gene-product functions were collated by common pathways of the products of deregulated genes in TDLNs at day 4 and TDLNs at day 11, generated by

Figure 3 Identification of specific genes and pathways deregulated in TDLN FRCs. (a) Overlap of genes significantly downregulated (blue) or upregulated (red) in various comparisons (periphery) of NDLNs, TDLNs at day 4, and TDLNs at day 11. (b) Expression of genes significantly downregulated (blue) or upregulated (red) (cutoff (vertical lines) as in a) in various comparisons (above plots) of NDLNs, TDLNs at day 4, and TDLNs at day 11, plotted against P values (horizontal lines indicate P < 0.05 or P < 0.01). Each symbol represents the transcriptome of FRCs from two brachial LNs pooled per female mouse. (c) Expression of genes encoding products in key pathways, among the genes most deregulated in TDLNs at day 4 versus NDLNs or in TDLNs at day 11 versus NDLNs; functional groups (left margins) assigned by GSEA and IPA. Data are representative of one experiment with three biological replicates per condition.
Figure 4 Perturbation in chemokine and cytokine signaling alters the composition and localization of immune cells.

(a) Expression of genes encoding signaling cytokines and chemokines in NDLNs and TDLNs at day 4 or day 11 (above plot; as in Fig. 2a). (b) Quantitative RT-PCR analysis of Ifi7 and Ccr21 in NDLNs and TDLNs at day 0 or day 11 (key) from B16.F10 mice (female C57BL/6) and TyCreER{T2}Braf^{CA Ptenlox} mice (male and female) (horizontal axis), presented relative to that of NDLNs, set as 1. (c) Confocal microscopy of NDLNs (top) and TDLNs at day 11 (bottom) stained for podoplanin (green), ER-TR7 (blue) and CCL21 (red). (d) Frequency of T cells (CD45+CD3ε+) among singlets in NDLNs and TDLNs (day 11) from B16.F10 mice (female C57BL/6) and TyCreER{T2}Braf^{CA Ptenlox} mice (male and female) (horizontal axis), presented relative to that of NDLNs, set as 1. (e) Confocal images of NDLNs and TDLNs at day 11 stained for CD3ε (green), CD45R (red) and PNAd (blue; left), and quantification of clustering (right).

FRC chemokine-cytokine signaling affects immune composition

FRCs provide chemical cues necessary for immunological homeostasis and leukocyte trafficking and survival within the LN20. Both GSEA and IPA identified significant deregulation in the expression of genes encoding chemokines and cytokines by TDLN FRCs. While
genes encoding signaling molecules, such as *Limk2*, *Kras*, *Tgfrb2* and *Src*, were upregulated in TDLNs, genes encoding cytokines and chemokines, including *Il11*, *Il7*, *Cd4* and *Ccl21*, were downregulated in TDLNs, relative to their expression in NDLNs (Fig. 4a). As FRCs produce the bulk of CCL21 and IL-7 (refs. 2,8,12), we verified the expression of Ccl21 and Il7 mRNA in independent sample sets by quantitative RT-PCR; this confirmed significant downregulation of these mRNAs in TDLNs in both tumor models assessed (Fig. 4b). Confocal imaging further showed downregulation of CCL21 expression at the protein level (Fig. 4c). We measured smaller T cell areas and concurrently larger B cell follicles per TDLN relative to that of their NDLN counterparts (Supplementary Fig. 4a), and we quantified reductions in the cellularity of CD3ε⁺ cells by flow cytometry (Fig. 4d). Although we measured no difference in the frequency of CD8α⁺ T cells in TDLNs at day 4 versus day 11 (Supplementary Fig. 4b), a significantly lower frequency of CD4⁺ T cells was observed in day-11 TDLNs than in NDLNs (Fig. 4e). Within this population, the frequency of naive CD4⁺CD62L⁺CD44⁻ T cells was lower in TDLNs at day 11 than in NDLNs (Fig. 4f). This was accompanied by significantly greater frequency of memory (CD62L⁻CD44⁺) CD4⁺ T cells, activated (CD62L⁻CD44⁺) CD4⁺ T cells (Fig. 4f) and CD4⁺Foxp3⁺ regulatory T cells (Fig. 4g and Supplementary Fig. 4c) in TDLNs than in NDLNs, at day 11. Moreover, we observed impaired efficiency in the homing of CD4⁺ T cells into TDLNs at day 11 (Fig. 4h). Upon examining the cellular architecture of TDLNs, we observed mislocalization and disorganization of major immune-cell populations. In contrast to NDLNs, in which T cell and B cell zones were clearly delineated (Supplementary Fig. 4d, left), TDLNs exhibited integration and loss of this margin (Fig. 4i and Supplementary Fig. 4d, right). We observed a transitional stage in TDLNs at day 4 (Supplementary Fig. 4d, middle). Furthermore, B cells clustered around high endothelial venules (HEVs) of TDLNs (Fig. 4j), but we measured no difference in the capacity of B cells to home to NDLNs or to day-11 TDLNs at 18 h after the adoptive transfer of splenocytes expressing green fluorescent protein (Fig. 4k). The accumulation of B cells around HEVs specifically within TDLNs in the absence of homing defects suggested that the migration and localization of B cells was disrupted once they exited from HEVs. Moreover, consistent with the lower expression of Il7 mRNA (Fig. 4b), *in vitro* labeling with EdU showed diminished proliferation of T cells and B cells in TDLNs relative to that in NDLNs (Supplementary Fig. 4e.f).

Other factors deregulated at the mRNA level included CXCL14 (which is chemotactic to monocytes and DCs), CCL25 (which is chemotactic to DCs) and CCL7 (which is chemotactic to monocytes), all of which were upregulated in TDLNs at day 11 (Fig. 4a). Consistent with that, following a transient dip at day 4, the number of CD11c⁺
DCs and CD11b+ macrophages (Supplementary Fig. 5a) was significantly increased at day 11 (Supplementary Fig. 5b,c). Together these data suggested that tumor-driven perturbation of FRC-derived guidance cues modified not only the composition but also the localization of key populations of immune cells within TDLNs.

**Activation of TDLN FRCs**

Fibrosis at the primary tumor21, mediated by hyper-activated fibroblasts (cancer-associated fibroblasts (CAFs)) recruited and educated in the local microenvironment, provides pro-tumor support22. We therefore sought to determine if fibroblasts of pre-metastatic TDLNs underwent similar changes. Transcriptional profiling showed expression of genes encoding typical activation markers of the Pdpn, Fn1, Cd248, Acta2, S100a4, Vim, Myl and Col families (Fig. 5a), indicative of greater activation of FRCs in TDLNs than in their NDLN counterparts. The upregulated expression of Pdpn, S100a4, Thy1 and Cd248 was further verified with independent data sets (Fig. 5b and Supplementary Fig. 5d). These trends were largely supported by the genetic model, and expression of podoplanin protein was significantly higher in TDLNs than in NDLNs of either tumor model (Fig. 5c). Moreover, flow cytometry showed more granularity in FRCs of TDLNs than in their NDLN counterparts. To investigate the activation status further in vitro, we compared cultured FRCs treated with tumor-conditioned medium (TCM) obtained from B16.F10 cells with cells treated with control conditioned medium (CCM) from non-tumor cells. Podoplanin was upregulated at the level of both mRNA (Fig. 5e) and protein (Fig. 5f), and treatment with TCM enhanced the capacity...
of FRCs to contract collagen gels (Fig. 5g and Supplementary Fig. 5e). Thus, exposure to tumor-derived factors at the TDLN might have pushed the FRCs toward an activated, pro-fibrotic ‘CAF-like’ state.

**TDLN conduits allow enhanced solute transport**

Profiling of TDLN FRCs also hinted at previously undocumented activity; in particular, the expression of genes encoding many channels and ion transporters was deregulated in TDLNs at day 4 or TDLNs at day 11 relative to their expression in NDLNs (Fig. 6a). For example, *Aqp1* was substantially upregulated in the FRCs of TDLNs at day 4, before undergoing downregulation by day 11 (as verified by quantitative RT-PCR; Supplementary Fig. 5f). To investigate if changes to channels or ion transporters were able to affect fluid transport through the conduit system, we first used an FRC culture model to measure dextran passage *in vitro*. While we found no difference between cells treated with CCM and those treated with TCM in their transport of 10-kDa dextran, we observed significantly greater transit of 70-kDa and 500-kDa dextran following exposure to TCM (Fig. 6b), indicative of a less-selective barrier. We analyzed the transport of dextran into size-restricted conduits *in vivo* in control (PBS-treated) or tumor-bearing mice. Quantification of 70-kDa dextran revealed its transport further into paracortical areas of TDLNs at day 11 (whereas ER-TR7 coverage did not change; Fig. 6c), where it was restricted to the FRC-lined conduits (Fig. 6d,e). In contrast, 10-kDa dextran freely entered into conduits of both NDLNs and TDLNs (Fig. 6d). 500-kDa dextran was observed beyond the subcapsular sinus in TDLNs but was undetectable in the paracortical area (data not shown).

As a result of the altered TDLN environment, substantial changes in the cell-assembly machinery would be expected to underlie the restructuring and enlargement of the FRC networks, along with a need for enhanced interaction between FRCs and associated matrix proteins essential to the conduit. The thickened collagen cores but reduced branches that we noted (Fig. 1g–i) suggested that the increased number of FRCs in TDLNs supported the increased diameter of the conduit. In doing so, FRCs would form contacts with a larger number of neighboring cells and encounter a greater area of neighboring cells. That hypothesis was confirmed by network analysis. Such analyses cluster sets of genes based on their products’ functions and locations and highlight families of genes whose products are significantly deregulated and either are involved in the same biological pathways or are expressed together, or physically interact. In TDLNs, four significantly relevant gene groups encoding products heavily involved in cell structure, shape and extracellular matrix were interlinked (Fig. 6f; interaction networks, Supplementary Fig. 6). Together with the data above showing larger diameter conduits at day 11 (Fig. 1g), such altered transporter repertoires would indicate a perturbation of conduit capacity, whereby conduits of TDLNs were more permissive to fluid entry and transit, which would potentially enable greater penetration of soluble tumor-derived factors into deeper areas of the LN.

**DISCUSSION**

LN functions as a major immunological hub essential for immunological homeostasis and the generation of appropriate immune responses, yet LNs are also the first site of metastasis for many cancers that manage to avoid immune-system-mediated clearance. It is increasingly accepted that LNs receive and respond to tumor-derived signals to generate a pro-tumor niche, but how these responses manifest and what in the LN drives them remains unclear. The stromal populations of the LN not only provide structural support but also are essential for its maintenance and physiological function.1–3,6,8–12,16,23–28.

While studies have shown that tumor-derived factors contribute to LN lymphangiogenesis,29–31, the LN fibroblasts and FRCs and the conduit network they form have not been thoroughly investigated in the context of the tumor. Here we found that proliferation, remodeling and transcriptional reprogramming of FRCs occurred in TDLNs. That in turn affected FRC-driven chemokine signaling, trafficking events, localization of immune cells and transport, all of which have the potential to contribute to impaired function within TDLNs.

Consistent with published studies,29,31,32 TDLNs enlarged and were supported by the expansion and structural reorganization of stromal compartments. Beyond gross network remodeling, FRCs specifically within TDLNs displayed strikingly altered transcriptional profiles. The expression of two FRC-derived factors key to LN function, CCL21 and IL-7, was downregulated. Perturbation of these essential guidance cues can contribute to abnormal homing, localization and survival of immune cells. It has been reported that TDLNs exhibit diminished CCL21 expression33,34. Consistent with those findings, we observed smaller T cell zones in TDLNs than in NDLNs, accumulation of B cells around HEVs and loss of the B cell–T cell zone boundary, as well as frequent T cells within B cell follicles, in TDLNs. Those features are a phenocopy of plt/plt mice, in which spontaneous loss of LN-specific isoforms of CCL19 and CCL21 ‘translates’ into fewer T cells and impaired immune responses.3,12,26,35. Our results also draw parallels with other pathological states such as infection, in which lower LN expression of CCL21 and disruption of FRC networks underpin aberrant homing and mislocalization of leukocytes to support evasion of the immune system by *Salmonella* species or viral particles.16,27,36. Thus, we speculate that similar functional impairments to immune responses might exist in TDLNs in which FRC networks and guidance cues are disrupted. This, however, remains to be determined. Moreover, our observations about B cells are consistent with reports demonstrating accumulation of B cells in TDLNs. Although the activation status or subtypes of the B cells that occupy TDLNs remains to be determined, it is possible that they might function as regulatory B cells, which would add a further dimension to the local immunosuppressive environment. We also note that pathogen-related inflammation did not underlie our observations. First, stromal modifications were consistent in two independent melanoma models, one of which was genetically driven rather than the result of an allograft. Second, a comparison of our array with data from published results characterizing the responses of FRCs to lipopolysaccharide-mediated inflammation20 shows that the response of the FRCs in TDLNs is tumor dependent. In particular, key factors such as CCL21 and IL-7 were inversely regulated in the two pathological settings (data not shown).

Consistent with published studies of LN stroma,37,38 the FRCs of TDLNs acquired a gene signature indicative of a more activated status. This gene signature in particular is reminiscent of that of fibroblasts found within the tumor microenvironment. These hyper-activated fibroblasts modulate the extracellular matrix to support a tumor and are now emerging as key immunomodulatory intermediates. Cancer-associated fibroblasts produce a host of factors that recruit populations of immune cells, including myeloid-cell-derived cells and regulatory T cells, to the tumor but also promote their polarization into a more suppressive phenotype.39–42. Having observed the accumulation of similar populations in TDLNs, we cannot exclude the possibility that following sustained exposure to tumor-derived factors, the FRCs of TDLNs adopt a more CAF-like state to generate a supportive, immunosuppressive niche.

Within TDLNs, wider conduits and enhanced collagen deposition indicate increased stiffness of the node,32 but remodeling of the collagen core might also contribute to the size-exclusion properties...
of the conduits, that in TDLNs is disrupted. That, combined with deregulated junction properties and protein pores of the FRCs lining these channels, would suggest altered integrity of the conduit network. Such 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, which would upset the functional status quo. Moreover, the process of lymphangiogenesis both in primary tumors and in connected LNs enhances the drainage capacity, and consequently these stromal populations experience enhanced fluid flow and shear stresses. Mechanical cues such as those, rather than chemical, tumor-derived signals (data not shown), might also act as a stimulus for FRC proliferation or act in synergy to drive the transcriptional reprogramming. We have not excluded this possibility in our study here, but this avenue warrants more in-depth investigation by 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 NDLNs and TDLNs from multiple tumor models, we have demonstrated that FRCs immediately downstream of tumors acquired unique transcriptional programs. Together with structural remodeling, these deregulated pathways and adapted FRC traits contributed to modified composition and aberrant localization of immune cells that might ultimately translate to a more suppressive, pro-tumor environment.

METHODS

Methods and any associated references are available in the online version of the paper.

Accession codes. GEO: microarray data, GSE73728.

Note: Any Supplementary Information and Source Data files are available in the online version of the paper.

ACKNOWLEDGMENTS

We thank members of the Shields Group for comments and discussions; members of the Ares Facility QU, E23 and T29 staff for animal husbandry and technical assistance; R. Butler for support with image analysis and algorithm development; Cambridge Genomic Services for microarray services and post-analysis advice; and the CIMR flow cytometry core facility for advice and support in flow cytometry and cell-sorting applications. Supported by Medical Research Council core funding (J.S.) and the Royal Society (UF130039 to B.A.H.).

AUTHOR CONTRIBUTIONS

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

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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ONLINE METHODS

Animal experiments. All experiments involving mice were performed in accordance with UK Home Office regulations under Project License PPL 80/2574. For mouse models, G"Power was used to estimate samples sizes required to achieve 80% power with 5% threshold. For syngeneic tumors, 2.5 × 10⁶ B16.F10 melanoma cells were inoculated subcutaneously into the shoulder region of immune competent 8- to 9-week-old female C57BL/6 mice assigned to control or tumor groups randomly. Animals were excluded only if tumors failed to form or if health concerns were reported. Control mice were injected with 50 µl PBS. Tumor size was monitored with calipers and the volume was calculated based on the ellipsoid formula \(V = \frac{4}{3} \pi R^2 \times (length \times width)^2\). Animals were culled after 4, 7, 11 or 14 days and tumor- or PBS-draining (brachial) and non-draining (axillary) lymph nodes were isolated for subsequent analysis. For Edu in vivo proliferation assays, mice received 200 µl of 500 µg/ml 5-ethyl-2'-deoxyuridine (EdU, Thermo Scientific) in PBS via intra peritoneal injection 24 h before tumor cell injection and every 48 h thereafter. LNs were isolated at days 4, 7 and 11 after tumor cell injection and analyzed by flow cytometry. For dextran transport assays, day-11 tumor-bearing mice received lysine fixable dextran 10,000 MW (Texas Red) 70,000 MW (biontin) and 500,000 MW (FITC; all Life Technologies; at a final concentration of 5 mg/ml in PBS) via subcutaneous injection into the forelimb. 10 min later brachial lymph node was harvested and fixed in 4% PFA overnight before transferring into a 30% sucrose medium. Lymph nodes were isolated and suspended in 0.05% EDTA/PBS (Jackson Laboratory) and 5 × 10⁶ cells were injected into the tail vein of wild-type C57BL/6 mice. After 18 h, LNs were isolated and immune cell contents were analyzed based on GEP', CD8α, CD4, CD3e, CD45 and CD45R staining (antibody inventory, Supplementary Table 1). For spontaneous murine melanoma, tumors were induced in 5- to 10-week-old TyrCreER\beta2F64Prelox (B6.Cg-BrafLm1Mmcm Ptentm1Hwu Tg[Tyr-cre/ERT2]13Bos/J, Jackson Laboratory) and 5 × 10⁶ mice were injected into the tail vein of wild-type C57BL/6 mice. After 18 h, LNs were isolated and immune cell contents were analyzed based on GEP', CD8α, CD4, CD3e, CD45 and CD45R staining (antibody inventory, Supplementary Table 1). For spontaneous murine melanoma, tumors were induced in 5- to 10-week-old TyrCreER\beta2F64Prelox (B6.Cg-BrafLm1Mmcm Ptentm1Hwu Tg[Tyr-cre/ERT2]13Bos/J, Jackson Laboratory) mice⁶⁸ following topical application of 4-hydroxy tamoxifen in DMSO (25 mg/ml) for 4 consecutive days to either shoulder or flank. Non-carrier littersmates were used as controls. As for injected tumors, draining lymph nodes (inguinal and brachial LNs) were harvested for subsequent analysis.

Flow cytometry. Lymph nodes were mechanically disrupted and digested in a 500 µl mixture of 1 mg/ml collagenase A (Roche) and 0.4 mg/ml DNase I (Roche) in PBS at 37 °C for 30 min with 600 rpm rotation. Following centrifugation at 1,000 r.p.m. for 5 min, the supernatant was discarded and replaced with 500 µl of PBS containing 1 mg/ml Collagenase D (Roche) and 0.4 mg/ml DNase I. The mixture returned to 37 °C for 20 min with 600 rpm rotation before addition of EDTA (final concentration 10 mM). Suspensions were passed through a 70 µm mesh before immunostaining with fixable viability dye live/dead viral (Molecular Probes) and combinations of fluorescently conjugated antibodies (Supplementary Table 1). For intracellular staining Foxp3/Transcription Factor Fixation/Permeabilization Kit (eBioscience) guidelines were followed. For Edu proliferation assays, the Click-IT Flow Cytometry Assay Kit Alexa Fluor 488 (Thermo Scientific) was followed as per manufacturers guidelines. Flow cytometry was performed on CyAn ADP (Beckman Coulter) and LS Fortessa (BD Biosciences) analyzers. Offline analysis was performed with FlowJo software (Treestar).

Cell processing after sorting for RNA analysis. For RNA processing, LN cell suspensions were sorted on a High speed Influx Cell Sorter (100 µm nozzle, BD Biosciences) into RNA protect Cell Reagent (QIAGEN). RNA was isolated with the RNeasy plus micro Kit (QIAGEN) and RNA quality and quantity was analyzed with a Bioanalyzer (Agilent Technologies). Only RNA samples with a RIN value above 8 and total concentration of 100 µg/ml were further processed for whole transcriptome amplification via the Ovation Pico SL WTA V2 Kit (NuGEN). Quantitative RT-PCR was performed using TaqMan assays (Pdpn Mm01348912_g1, Il7 Mm01295803_m1, Cc21a Mm03646971_gH, S100a4 (FSP1) Mm00803372_g1, Thy1 Mm00493682_g1, Agrp Mm00431834_m1, Tyrp1 Mm00453201_m1, Dct Mm01225584_m1) and a StepOne Real Time PCR System instrument (both Life Technologies). Microarray hybridization and data normalization. RNA was assessed for concentration and quality using a SpectroStar (BMG Labtechn) and a Bioanalyzer (Agilent Technologies). Microarray experiments were performed at Cambridge Genomic Services, University of Cambridge, using the MouseWG-6 v2 Expression BeadChip (Illumina) according to the manufacturer’s instructions. Total RNA was amplified using the Ovation Pico WTA V2 Kit (NuGEN) and subsequently labeled using the Biotin Il kit (NuGEN). The concentration, purity and integrity of the resulting cRNA were measured using the Nanodrop ND-1000 (Thermo Scientific) and by Bioanalyzer. cRNA was then hybridized to the MouseWG-6 v2 Beadchip overnight following by washing, staining and scanning using the Bead Array Reader (Illumina). Raw microarray data was preprocessed using the ‘lumi’ bioconductor⁴⁹ in R. After filtering for significance, data were transformed using the Variance Stabilization Transformation⁵⁰ from lumi, and normalized using quantile normalization.

Assessment of metastasis. RNA was isolated from whole TDLNs as described above and quantitative RT-PCR was performed using TaqMan assays (Tyrp1 Mm00453201_m1 and Dct Mm01225584_m1) and a StepOne Real Time PCR System instrument (both Life Technologies). A standard curve was performed in parallel in which 1 × 10⁶ lymph node cells in suspension were spiked with exact numbers of B16.F10 tumor cells (titrated by index flow-cytometry sorting from 100,000 down to a single tumor cell).

Principal-component analysis. Principal-component analysis (PCA) was performed using the R ‘stats’ package. Log2 transformed data was used as input for the analysis. PCA was performed on all probes with a P value of <0.05 and a change in expression of >1.5-fold at either day 4 or day 11. Many-dimensional data from the gene array was reduced into linearly uncorrelated variables (Principal Components). The three largest principal components were then plotted against one another to assess the relationships between different biological repeats of each sample. PCA was visualized with the python library ‘matplotlib’ (‘matplotlib: v1.4.3’, and can be found on zenodo.org).

Correlational analysis. Correlational analysis was performed using the R ‘stats’ package on all probes with a P value of >0.05 and a change in expression of >1.5-fold at either day 4 or day 11.

Hierarchical clustering. Hierarchical clustering was performed for the probes with the greatest differences in expression (all of those with a P value of <0.05 and a change in expression of >1.5-fold at either day 4 or day 11). Clustering was performed with the R ‘stats’ package and utilizes the Euclidean distance between each sample, which are then clustered using the Ward algorithm.

Heat map analysis. For the presentation of differential probe expression values in heat maps, the TIGR MultiExperiment Viewer (TIGR MeV)⁵³ tool for microarray data analysis was used. The data were row centered and normalized.

Analysis of functional enrichment in gene signatures. Microarray data were analyzed and interpreted with the help of Ingenuity Pathway Analysis (Ipa, QIAGEN) and GSEA⁵²,⁵³ (Broad Institute), focusing on canonical pathways and disease and biofunction (IPA) and KEGG canonical pathways (GSEA). Restricting the analysis for probes at a change in expression of ≥1.5-fold and P value 0.05 obtained after raw data processing. Results were collated and presented in heat maps.

Cell culture. B16.F10 (CRL 6475, ATCC) cells were maintained in DMEM with 10% FBS (Sigma-Aldrich) and 100 µ/ml penicillin-streptomycin (both Life Technologies). FRCs were maintained in RPMI (R875, Sigma-Aldrich) with 10% FBS (Sigma-Aldrich), 10 mM HEPES, 100 U/ml penicillin-streptomycin (both Life Technologies), 15 µM beta-mercaptoethanol (Sigma-Aldrich). For tumor cell conditioned medium (TCM) or control conditioned medium (CCM) production; B16.F10 cells or FRCs were grown until 50% confluent in full growth medium, subsequently the medium was exchanged for RPMI-1640 containing 2% FBS and medium was harvested after 24 h. After sterile filtration, medium was stored at -80 °C. For treatments TCM or CCM were mixed with 50% full growth medium and performed over 7 days. RNA extraction.
was performed using RNase Mini Kit (QIAGEN). 1 µg of RNA was used for reverse transcription using First Strand cDNA synthesis Kit (Thermo Scientific) and quantitative RT-PCR was performed as mentioned above.

Isolation and culture of FRCs. FRCs were isolated from murine lymph nodes of C57BL/6 as previously described.13,54. Cells were characterized based on their expression of PDLP and VCAM-1 and their lack of expression of CD45 and CD31.

In vitro contraction assay. After 7 days of preconditioning in either TCM or CCM, 1.5 × 10^5 FRCs were seeded into collagen gels at a final concentration of 2 mg/ml (Rat tail collagen, BD Biosciences) into 24 well plates. Following 20 min of polymerization at 37 °C, 500 µl of full medium was added and the gel was detached from culture dish using a pipette tip. Gels were allowed to contract for 24 h and the difference in size was quantified using ImageJ.

In vitro permeability assay. After 7 d of preconditioning in TCM or CCM, 6.5 × 10^4 FRCs were seeded onto PCF 0.4 µm pore transwell inserts (Millipore) and cultured overnight in full growth media. To ensure the presence of confluent monolayers, inserts were stained with CellMask plasma membrane stain (Molecular Probes). Inserts were transferred to a fresh 24-well plate and 500 µl basal media was added to the lower chamber. 200 µl media containing 2% FBS and dextrans at 10,000 MW (Cascade Blue) 70,000 MW (TRITC) and 500,000 MW (FITC) all Life technologies) at a final concentration of 1 mg/ml were added to the upper chamber and fluorescence of the lower chambers’ media was measured after 24 h.

Immunofluorescence. Lymph nodes were embedded in OCT medium (TissueTek). 10 µm frozen sections were fixed in ice-cold acetone for 2 min, blocked with 5% chicken serum, 2% BSA in PBS, and incubated with the primary anti-mouse antibodies at 4 °C overnight (antibody information, Supplementary Table 2). For all staining, Alexa Fluor secondary antibodies (Thermo Scientific) were used at a dilution of 1:300. Sections were mounted in ProLong Gold (Thermo Scientific) and confocal images were taken using a Leica SP5 or Zeiss LSM 880 confocal microscope and processed with Velocity (Perkin Elmer) or FIJI (ImageJ). Skeleton and gap analyses are based on collagen I staining and confined to the T cell paracortical area of the LN. The analysis was performed using a macro for FIJI (ImageJ). Condit thickness was measured by 0.1 µm z-stack Airyscans of the collagen I core rotated to display ellipse area of cross section for NDLNs and TDLNs and measured in Velocity (Perkin Elmer). For dextran studies, fluorescence intensity quantifications were performed in FIJI (ImageJ). 70,000 MW dextran (biotin, SA-647 Alexa Fluor (Thermo Scientific)) was quantified over total T cell paracortical area and back normalized on area size. Following FCs were calculated to the average of all NDLNs (Ctrl). For calculating area fraction in FIJI, the Otsu threshold was used and adjusted according to ER-TR7 area coverage.

Statistical analyses. Statistical analyses were performed using GraphPad Prism 6 software (GraphPad). For comparisons of three or more groups, data were subjected to one-way or two-way ANOVA analysis, followed by post hoc test (Dunnett’s when comparing every mean to a control mean, or Tukey’s multiple comparisons test when comparing every mean to every other mean). When two groups were compared, a two-tailed unpaired Student’s t-test was applied. P ≤ 0.05 was considered significant.

Network analysis. In order to study the relatedness of the top expressed genes, a network analysis was performed using the Multiple Association Network Integration Algorithm (MANIA).33 A large data set of roughly 300 gene association networks are used to correlate genes, and create gene networks from a list of highly deregulated probes. The 599 most deregulated 11-day TDLN probes were used as an input for the algorithm, and connections between probes visualized using Cytoscape. The networks generated using the MANIA algorithm include three edge types between nodes: Predicted Edges, whereby functional relationships are implied due to orthology with other organisms; Co-localization Edges, whereby linked gene products are expressed in the same cellular location; and Co-expression edges, where the expression of gene products is similar across conditions in a previously published gene-expression study. Genes are linked through calculated edges, and networks of linked genes generated.

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