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Fluid Flow Regulates Stromal Cell Organization and CCL21 Expression in a Tissue-Engineered Lymph Node Microenvironment

Alice A. Tomei,* Stefanie Siegert,† Mirjam R. Britschgi,† Sanjiv A. Luther,3,4† and Melody A. Swartz3,4,*

In the paracortex of the lymph node (LN), T zone fibroblastic reticular cells (TRCs) orchestrate an immune response by guiding lymphocyte migration both physically, by creating three-dimensional (3D) cell networks, and chemically, by secreting the chemokines CCL19 and CCL21 that direct interactions between CCR7-expressing cells, including mature dendritic cells and naive T cells. TRCs also enwrap matrix-based conduits that transport fluid from the subcapsular sinus to high endothelial venules, and fluid flow through the draining LN rapidly increases upon tissue injury or inflammation. To determine whether fluid flow affects TRC organization or function within a 3D network, we regenerated the 3D LN T zone stromal network by culturing murine TRC clones within a macroporous polyurethane scaffold containing type I collagen and Matrigel and applying slow interstitial flow (1–23 μm/min). We show that the 3D environment and slow interstitial flow are important regulators of TRC morphology, organization, and CCL21 secretion. Without flow, CCL21 expression could not be detected. Furthermore, when flow through the LN was blocked in mice in vivo, CCL21 gene expression was down-regulated within 2 h. These results highlight the importance of lymph flow as a homeostatic regulator of constitutive TRC activity and introduce the concept that increased lymph flow may act as an early inflammatory cue to enhance CCL21 expression by TRCs, thereby ensuring efficient immune cell trafficking, lymph sampling, and immune response induction. The Journal of Immunology, 2009, 183: 4273–4283.

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†Abbreviations used in this paper: LN, lymph node; 2D, two dimension(al); 3D, three dimension(al); dyn, dyne; ECM, extracellular matrix; HEV, high endothelial venule; LT, lymphoxygen; PDGF-R, platelet-derived growth factor receptor; PU, polyurethane; TRC, T zone fibroblastic reticular cell.

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tissue injury or infection, we hypothesized that TRCs are sensitive and responsive to fluid flow, particularly with regard to CCL21 expression that regulates immune response induction in the LN.

In this study, we present a tissue-engineered model of the stromal cell network found in LN T zones and demonstrate the effects of 3D culture and flow on regulating its morphology and CCL21 expression. This implicates fluid flow as an important modulator of LN stromal cell function.

Materials and Methods

Generation of murine TRC clones

Peripheral LNs (pool of axillary, brachial, and inguinal) were isolated from adult 53/-/- mice (mix of C57BL/6 and 129 background) (28), digested as previously described (6), and cultured at 37°C with 4.5% CO2 in RPMI 1640 plus GlutMAX-I (Invitrogen) containing 10 mM HEPES, 50 U/ml penicillin, 50 μg/ml streptomycin, 10% FBS, and 50 μg M-2 ME (Sigma-Aldrich). After 24 h, nonadherent cells were removed and the adherent fibroblastic cells were cultured for several weeks and then subconflned using limited dilution. Outgrowth over 2–3 mo yielded only CD45 / podoplanin clones. For this study the p53/-/- TRC clone H7.2A10 was selected based on its rapid growth and a surface phenotype comparable to that of ex vivo cells. A TRC clone derived from p19arf/-/- LNs gave comparable results for surface phenotype, extensive network formation in 3D culture, and lack of CCL19/21 expression in two dimensions (2D) (data not shown). Both p19arf/-/- and p53/-/- mice were chosen because they lack important tumor suppressor genes typically expressed in fibroblasts; LN fibroblasts isolated from these mice were therefore prone to immortalization due to additional genetic modifications.

TRC transduction with pgk-GFP or pgk-tomato lentivirus

Lentiviral vectors pseudotyped with the vesicular stomatitis virus G protein were generated by transfection into 293T cells of the packaging construct pCMVΔR8.74, a plasmid producing the vesicular stomatitis virus G envelope (pMD2.G), and either the vector pRRL.SIN.PPT.PGK.GFP.WPRE for GFP transduction as previously described (29). The plasmids were a gift from D. Trono (Ecole Polytechnique Federale de Lausanne, Lausanne, Switzerland). Culture medium was collected at 48 h, pooled, filtered at 0.2 μm, concentrated −1000-fold by ultracentrifugation, and stored at −80°C until used. TRCs (105 cells) were transduced with 10 μl of concentrated virus, sorted for high titer or GFP expression, and kept in a P2-level laboratory until the HIV-1 p24 capsid protein of the virus reached an undetectable level by ELISA (RETROtek; ZepetoMetrix) before proceeding to any experiments. 3D polyurethane scaffold (“sponge”) fabrication

Polyurethane sponges were prepared as described (30). Briefly, alginate beads, with diameters of 50–500 μm, were prepared by extruding a solution of 2% sodium alginate (Fluka) in distilled water through a 25-gauge needle fitted with a syringe into a solution of 102 mM CaCl2 in 0.9% NaCl solution. The resulting PU scaffold or sponge was rinsed over 3 days in 2% sodium alginate (Fluka) in distilled water through a 25-gauge needle before proceeding to the RNA isolation and purification step. To ensure that effects were independent of strain, sex, or age, in each mouse we compared CCL21 expression in one “blocked flow” LN with a sham-operated control “normal flow” LN. All animal experiments were approved by the Office Vétérinaire Cantonale Vaud, Lausanne, Switzerland (authorization no.1996).

In vivo flow disruption through the LN

To determine whether total CCL21 expression in the LN could be affected by flow, we compared CCL21 gene expression in the inguinal LNs after 2 h of blocked vs normal flow. A total of seven male and female 5- to 14-wk-old C57BL/6 and B6SJL F1 mice were used (Charles River Laboratories); to ensure that effects were independent of strain, sex, or age, in each mouse we compared CCL21 expression in one “blocked flow” LN with a sham-operated control “normal flow” LN. All animal experiments were performed in accordance with institutional guidelines and had been approved by the Office Vétérinaire Cantonale Vaud, Lausanne, Switzerland (authorization no.1996).

TRCs (106 cells) were transduced with 10 μl of concentrated virus, sorted for high titer or GFP expression, and kept in a P2-level laboratory until the HIV-1 p24 capsid protein of the virus reached an undetectable level by ELISA (RETROtek; ZepetoMetrix) before proceeding to any experiments.
25-gauge needle before proceeding to the RNA isolation and purification step. For TRCs from 3D culture, the matrix was first digested in a solution of 2.25 mg/ml collagenase D (Roche) in RPMI 1640 at 37°C for 1 h and then the cells were collected by centrifugation (2000 × g) at 4°C for 5 min. The cell pellet was then lysed in RNA lysis-binding solution and passed through a 25-gauge needle before proceeding to the RNA isolation and purification step.

Total RNA was extracted according to the manufacturer’s protocol (RNAqueous or RNAqueous-Micro; Ambion), and cDNA synthesis was performed with the iScript cDNA synthesis kit (Bio-Rad Laboratories) on 0.5–1 μg of RNA in a reaction total volume of 20 μl at 55°C for 5 min, 42°C for 30 min, and 85°C for 5 min.

Quantitative real-time PCR for CCL21ser and CCL19atg, the LN-specific isoform of CCL21 (33) and the functional transcript for CCL19 (5), respectively, was performed with the iQ SYBR Green SuperMix (Bio-Rad) protocol with 1 μg of cDNA in a 25-μl reaction with 200 nM forward and reverse primers on an iCycler iQ (Bio-Rad Laboratories). Analytical PCR conditions were as follows: one cycle at 95°C for 15 s, 60°C for 1 min, 95°C for 15 s, 55°C for 1 min, and 55°C for 10 s. Gene expression was normalized to the expression of the housekeeping gene P0. Primer sequences are shown in Table I.

ELISA

Secretion of CCL19 and CCL21 by TRCs cultured in 3D flow conditions was determined by ELISA. Briefly, the matrix was first digested in a solution of 2.25 mg/ml collagenase D (Roche) in RPMI 1640 at 37°C for 1 h and then the matrix was separated from the cells by centrifugation (2000 × g) at 4°C and for 5 min. Protein concentration in the digested matrix solution was determined by ELISA (DuoSet; R&D Biosystems) according to the manufacturer’s instructions.

Immunohistochemistry and fluorescence and confocal microscopy

TRCs were stained and analyzed as previously described (6). Mouse LNs were isolated, cryosectioned (10- or 20-μm thick), and fixed in acetone for 5 min at −20°C. Whole 3D samples (collagen or collagen in PU scaffold) were fixed in 2% paraformaldehyde in PBS at 4°C for 2 h and washed in PBS. TRCs in ibidi chips were fixed in 2% paraformaldehyde in PBS at 4°C for 20 min and washed in PBS.

Standard immunostaining protocols were used with overnight incubations in primary Abs at 4°C and 1-h incubations in secondary Abs at room temperature. The following Abs were used at the recommended concentrations: Syrian hamster anti-mouse gp38/podoplanin (clone 8.1.1); rabbit anti-mouse LYEVE-1 (Reliatech); rat anti-mouse ETRE7 (Hycult Biotechnology); and goat anti-mouse CCL21 (R&D Systems). Respective secondary Abs were from Molecular Probes (Invitrogen) except for Cy3-conjugated goat anti-sheep hamster and biotinylated donkey anti-goat IgGs (both from Jackson ImmunoResearch Laboratories). For the latter, Alexa Fluor 488-conjugated streptavidin (Molecular Probes) was used for detection. All sections and samples were counterstained with DAPI (Invitrogen) and mounted in Vectashield mounting medium (Vector Labs).

Images were acquired using an Axiovert 200M fluorescence microscope with a AxioCam MRm camera or a LSM 510 Meta confocal laser scanning microscope (all from Zeiss). The 3D matrix was imaged by confocal reflectance microscopy as described earlier (33). NIH ImageJ was used for image analysis to quantify cell numbers, gel areas, fluorescence intensities, and positively stained pixels. Imaris (Bitplane) was used to make 3D reconstructions and sections of confocal serial images of LN sections and TRCs in 3D matrices.

Fibrous ECM was imaged by confocal reflectance microscopy using an LSM 510 Meta Zeiss Axiosvert 200M inverted confocal laser scanning microscope, a Zeiss Plan Neofluar 25 × 0.8 numerical aperture or a Pl-Apochromat 40 × 0.1 numerical aperture oil immersion objective, and 514-nm light from an argon laser as previously described (35–37).

### Results

**Isolated TRC clone exhibits similar surface phenotype as ex vivo TRCs**

LN-resident TRCs are podoplanin^+Lyve1^− cells that form a reticular network throughout the T zone and wrap around conduits expressing ERTR-7 (Fig. 1A) (6). They secrete CCL19 and CCL21, but due to differences in expression levels and binding affinity to sulfated proteoglycans (38, 39), only CCL21 can be imaged by immunostaining and is associated with TRCs and ERTR-7^+ conduits (Fig. 1A).

To create an abundant source of LN TRCs for in vitro studies, several immortalized TRC clones were generated by long-term culture of adherent ex vivo cells from peripheral LNs of p53^−/− mice. They displayed fibroblastic morphology and retained expression of podoplanin (Fig. 1B) while being negative for the lymphatic endothelial cell marker Lyve-1 and the general endothelial marker CD31 (data not shown). Although CCL21 expression was readily detected in podoplanin^+Lyve1^− TRCs 3 days after isolation from peripheral LNs, it was largely lost in TRC clones after clonal selection and expansion in static 2D cultures (Fig. 1B and data not shown), as previously reported (40). Other than CCL21 expression, however, TRC clones resembled ex vivo TRCs in their surface phenotype, including uniform presence of podoplanin, ICAM-1, VCAM-1, PDGF-Ra, PDGF-Rb, and lymphoxygen (LT) β receptor (LTβR) while lacking expression of CD31, the hematopoietic marker CD45, the macrophage marker CD11b, and the follicular dendritic cell markers CD16/32 and CD35 (Fig. 1C and data not shown). These results validate the identity and purity of the generated TRC clones.

### 3D culture of TRCs recapitulates in vivo morphology

The structural organization of TRCs within the LN is important for guiding lymphocyte interactions and for directing flow pathways for Ag and cytokine trafficking from the subcapsular sinus to the HEV area in the paracortex. We thus optimized our model of the LN stroma to recapitulate some of this architecture. When isolated and placed in 2D cultures, GFP^+ TRCs could not form the 3D reticular networks observed in vivo (Fig. 2A). Culture in 3D collagen-only matrices allowed the cells to organize into 3D structures; but because TRCs possess a myofibroblast phenotype (6), they contracted the matrix to 20% of its original size within 3 days (Fig. 2B), making long-term culture difficult. To address this, we used a composite matrix that incorporated the collagen in a PU scaffold with very large interconnected pores (200–600 μm) (30) to provide macroscale structural integrity and anchoring for the

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**Table I. Forward and reverse primer sequences used for quantitative PCR**

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<tr>
<th>Primer Target</th>
<th>Primer Name</th>
<th>Forward Sequence (5’→3’)</th>
<th>Reverse Sequence (5’→3’)</th>
<th>Ref.</th>
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</thead>
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<tr>
<td>HKG, P0^*</td>
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<td>CGAAAATCTCCAGAGCCACCATGG</td>
<td>GTCAGCATGCTTCAGAGGTGG</td>
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<tr>
<td>Murine CCL21serine</td>
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<tr>
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<td>mCCL19atg</td>
<td>CTGCCCTCAGATTATTCGCCAT</td>
<td>AGGTAGCGGAAGGCTTTCAC</td>
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</tbody>
</table>

^* HKG, Housekeeping gene.
FIGURE 1. TRC clones cultured in 2D display a similar phenotype as ex vivo cells but lose CCL21 expression. A, Immunofluorescent labeling of 10-μm-thick mouse LN cryosections showing TRCs (bottom: red, podoplanin⁺; purple, Lyve-1⁺) associating with conduits (top: red, ERTR-7⁺) and rich in CCL21 protein (green). Scale bar, 50 μm. B, TRCs (red, podoplanin⁺; purple, Lyve-1⁺) isolated from murine LNs and cultured on plastic for 3 days (ex vivo TRCs) showed lymphatic endothelial cell (Lyve1⁺podoplanin⁺) or HEV contamination, whereas TRC clones cultured in 2D showed a pure TRC phenotype (Lyve1⁺podoplanin⁺); however, only the ex vivo TRCs and not the clones stained strongly for CCL21 protein (green). Nuclei are in blue. Scale bars, 50 μm. C. The surface phenotype of ex vivo TRCs and the TRC clone, analyzed by flow cytometry and pregated on CD45⁺CD31⁻ cells, showed positive expression of podoplanin, ICAM-1, VCAM-1, PDGF-Rα, and PDGF-Rβ, confirming that the TRC clone maintained a TRC phenotype. Shown are representative data from 3–5 experiments.
collagen gel without compromising the collagen microenvironment on the microscale for the TRCs. The choice of an elastomeric PU scaffold as a support for mechanically weak collagen matrices comes from the hypothesis that myofibroblast-like cells are able to exert a greater force on their ECM if the stiffness of their substrate is increased (41). PU matrices have been widely used as biomaterials due to their high biocompatibility and tunable mechanical properties (42). Indeed, the combination of a PU support with a collagen matrix prevented matrix contraction by TRCs and provided overall stiffness while allowing cell adhesion to the collagen, which is critical for integrin-mediated adhesion and proliferation.

After 1 wk of culture in these composite matrices, TRCs formed 3D reticular networks resembling more closely those in the LN than those in collagen-only cultures (Fig. 2C). In composite matrices, TRCs spread and reorganized their surrounding matrix into heterogeneous fiber bundles much more than did TRCs cultured in collagen alone (Fig. 2C). In addition, TRCs cultured within PU-supported collagen matrices showed slightly increased transcript levels of CCL19atg (the functional transcript for CCL19 (5, 43), and CCL21ser (the isoform of CCL21 that is produced in the LN (5, 43)) compared with TRCs cultured in 2D or 3D collagen alone (data not shown).

Interstitial flow drives functional organization of TRCs and up-regulates CCL21

Fluid flow through the functional LN is an omnipresent mechanical force acting on the resident TRCs that line the permeable conduits through which lymph is continuously transported (13, 20, 44). Increased lymph flow is an immediate response to tissue injury or inflammation (21–27), and presumably there is a lack of flow through LNs that are blocked or that drain from dysfunctional lymphatic vessels. We therefore tested the effects of interstitial fluid flow, or lack thereof, on the organization and chemokine secretion by TRCs grown in the composite matrix (Fig. 3A). We found that TRC organization was enhanced with slow interstitial

![FIGURE 2. TRCs cultured in a 3D composite matrix exhibit enhanced organization and interconnectedness compared with those cultured on 2D or in 3D collagen alone. A, TRC morphology in a murine LN T zone section stained for podoplanin (left) compared with the GFP* TRC clone in a 2D culture (right). Scale bar: 100 μm. B, Consistent with its myofibroblast phenotype, TRC culture in “collagen only” matrices induced drastic collagen contraction. By culturing the cells in a composite matrix made by polymerizing collagen gels into the macropores of a porous PU sponge, cell contraction of the matrix was prevented (n = 4). C, TRCs (top row) cultured in 3D composite matrices vs 3D collagen alone induced more matrix reorganization, as evidenced by confocal reflectance (bottom row). Scale bars: 50 μm.](http://www.jimmunol.org/)

by guest on April 21, 2017
flow, as evidenced by channel-like structures (Fig. 3B) and partially aligned TRCs and matrix structures (Fig. 3C). This is consistent with earlier findings that slow interstitial flow can induce fibroblasts to align themselves and their ECM (35, 45, 46).

Cells expressing high CCL21 levels were also markedly enriched by slow interstitial flow and were mostly associated with channels and the aligned cell-matrix structures (Fig. 4). Therefore we hypothesized that fluid shear stress might be directly mechanostimulating TRCs to up-regulate CCL21. To address this, we used two additional flow models. The first included interstitial flow through a cell-embedded collagen matrix (Fig. 5A), with a porous polyethylene cylinder anchoring the matrix around the edges only. This alternative 3D flow model was used to precisely control flow velocity through the 3D matrix, which was not achievable with the flow model using the composite matrix because it was highly heterogeneous throughout the sample by design (i.e., the outlet was through a needle to mimic flow collecting into an efferent lymphatic vessel).

In this alternative flow model (Fig. 5A), interstitial flow was approximately uniform and we could readily estimate the average shear stress on cells for fixed average bulk flow velocities through the matrix. The pressure head was varied to obtain three different flow velocities through the matrix: 1, 10, and 23 \( \mu \text{m/min} \), with estimated average and peak shear stresses on the 3D embedded TRCs as 0.008 and 0.02 dyne/cm\(^2\), 0.04 and 0.1 dyne/cm\(^2\), and 0.07 and 0.2 dyne/cm\(^2\), respectively. Shear stresses experienced by TRCs under the three different flow conditions were estimated based on Brinkman’s equation for flow around spheres in porous matrices (31, 47) and accounted for the effects of ECM fibers on the flow heterogeneity around the cell, which showed that the average and peak stresses can be two and 10 times greater than those predicted by Brinkman (32). Although it is difficult to estimate physiologically relevant flow velocities in the LN, the flow rates tested were representative of the range of interstitial flow velocities measured in normal and tumor-associated inflamed skin (48, 49).

After 7 days of culture, only the highest flow velocity tested, 23 \( \mu \text{m/min} \), caused a statistically significant increase in cell density (8-fold) compared with the static controls, where cell density increased 2-fold over 7 days of culture (Fig. 5B). The reason for this cell

**FIGURE 3.** Interstitial flow enhances TRC organization in the composite matrix. A, Schematic of the flow chamber for TRC cultures in 3D collagen/Matrigel within macroporous polyurethane (PU) scaffolds. The TRC matrix is cultured within a poly(dimethylsiloxane) (PDMS) chamber attached to a coverslip, whereas a 25-gauge needle inserted through the middle of the culture allows medium to flow out via a small pressure head. B, In the presence of slow interstitial flow (right), TRCs (red) organized more extensively than those cultured in static conditions (left). Scale bar, 200 \( \mu \text{m} \). C, Under static conditions, TRCs (red) were generally spread throughout the composite matrix uniformly, but when cultured in the presence of slow interstitial flow (right), TRCs formed channel-like structures with associated matrix bundling (right, cells + matrix). Scale bars: cells, 200 \( \mu \text{m} \); cells + matrix, 50 \( \mu \text{m} \).
density increase was not determined, but could have possibly resulted from mechanical stimulation or enhanced nutrient delivery (50).

Interstitial flow also induced an increased secretion of both CCL19 and CCL21 into the matrix surrounding TRCs as measured by ELISA on the digested matrix (Fig. 5C). The lowest flow rate (1 μm/min) induced the highest secretion of CCL21 and CCL19, but there were no statistically significant differences between the three flow rates tested. This flow effect observed in 3D occurred at least in part at the transcriptional level, as CCL21ser mRNA was generally increased (Fig. 5D).

Fluid shear stress as a mechanism for flow-induced CCL21 up-regulation

To explore the direct effects of fluid shear stress on cell response, a second model was used in which TRCs were plated on a 2D monolayer and exposed to low levels of laminar shear stress. This differed from the forces imposed by fluid flow in 3D in a number of ways; in 3D, cells are spread and attached to the matrix in all directions, and the matrix fibers around the cell strongly buffer the shear stress imposed on the cell surface, which is highly heterogeneous (32). Furthermore, cells in 3D under flow may experience pressure, drag, and shear forces, as well as gradient skewing of secreted chemokines and other proteins (51). In 2D, cells are only attached to a planar surface and thus directly exposed, only on the apical surface, to the fluid shear stress.

We found that shear stresses as low as 0.005 and 0.01 dyne/cm² directly induced CCL21 protein production (Fig. 5, E and F). Higher shear stress (0.05 dyne/cm² and above) caused some cell detachment and more variability in the expression of cell-associated CCL21, yielding an increase that was not statistically different from that of static (Fig. 5F). The CCL19 protein could not be detected in any 2D conditions (data not shown). These findings are consistent with the CCL21 secretion seen mainly in the channel-like structures under flow conditions in our composite matrix (Fig. 4) and suggest that TRC lining conduits could be sensitive to shear stress and up-regulate CCL21 accordingly to increase immune cell trafficking in the affected draining LN when increased lymph flow is present, as is the case during acute inflammation.

Flow through the LN regulates CCL21 expression in vivo

To explore whether flow affects CCL21 in the LN in vivo, we examined the effects of blocked flow on CCL21 gene expression by surgically cutting the afferent lymphatics of the inguinal LN after 2 h. Although longer term studies would have allowed protein expression to be observed, we chose this time point to avoid complicating factors such as inflammatory cytokines induced by surgical disruption. First, we tracked intradermally injected FITC-dextran that was taken up into the lymph to demonstrate the flow through the inguinal nodes and to visualize the afferent and efferent lymphatics on each side of the mouse (Fig. 6A). Two hours after cutting the afferent and the efferent lymphatics on one side to effectively block lymph flow through the node (and sham treating the other side), LNs were excised and RNA extracted. CCL21ser expression was significantly decreased in the “blocked flow” compared with the “normal flow” LNs (Fig. 6B). Thus, the CCL21 expression level appeared to be sensitive to the flow conditions through the LN in vivo.

Discussion

The biology of LN TRCs has been mainly studied in vivo (52, 53) and, as a consequence, our knowledge is limited about the factors that specifically control TRC proliferation, organization, and function. Our tissue-engineered in vitro model of the LN T zone stroma presented in this article demonstrates that 3D organization and the forces associated with interstitial fluid flow are important regulators of TRC morphology and CCL21 expression.

Previously, Shimizu and colleagues reported the characterization of TRC lines generated from BALB/c LNs and grown on either 2D surfaces or on a nylon mesh to generate ERTR7⁺ fibers (40); but these cultures were not in a true 3D setting and did not surround the cells with a matrix-rich environment with which the cells could interact and could remodel. To this end, we generated
presumably immortalized TRC clones from p53−/− B6/129 mouse LNs that resemble ex vivo TRCs in their fibroblastic morphology, surface phenotype, and contractile properties. Culture in natural 3D biological matrices, which serve as a substrate for cell adhesion, growth, and differentiation as well as a mechanical support for cell tension (54), proved to be critical for the formation of long dendrites and reticular cell networks as seen in vivo, rather than the flattened and lamellar appearance observed in 2D cultures. This finding is reminiscent of several other cell types, including fibroblasts, epithelial cells, and endothelial cells, which need an appropriate 3D environment to take on the physiologically relevant shape and multicellular organization (50). However, consistent with the myofibroblastic phenotype of TRCs, excessive matrix contraction is a problem for 3D culture in soft biomatrices like collagen (55). To overcome this, we used a macroporous scaffold of relatively stiff (but linearly elastic) porous polyurethane (30) that counteracted matrix contraction by TRCs for long-term culture and, importantly, triggered matrix remodeling. In addition, it appeared to stabilize the interactions between TRCs, leading to a more extended cellular network. Finally, we observed chemokine expression almost exclusively in the context of the composite matrix. Together, these data suggest that although a 3D environment is important for TRC culture, it is not sufficient for TRCs to acquire in vivo-like properties; the matrix also has to be stiff enough to allow cell extension and withstand contractile forces by the TRCs. In vivo, TRCs not only line the conduits, but those particular cells connect to the capsule and the vessels that provide a highly rigid endpoint to this myofibroblast network (13, 52).

In addition to being sensitive to a 3D organization and a rigid structure, TRCs were also responsive to the physical force of interstitial flow by increasing their organization and CCL21 expression. In LNs, lymph fluid that drains from the periphery through

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**FIGURE 5.** Fluid flow increases TRC proliferation and CCL19 and CCL21 secretion. A, Schematic of the simplified 3D flow chamber. TRCs were cultured in a collagen matrix within a porous polyethylene (PE) ring to prevent cell contraction over time, and fluid flow rates through the TRC matrix were controlled by the pressure head applied. B, Cell density was seen to increase with the highest flow velocity tested (23 μm/min) after 7 days of culture. Data (n = 3) were normalized to the original cell densities (10⁶ cells/ml). C, Flow increased secretion of CCL21 and CCL19 protein in the matrix surrounding TRCs, compared with static controls, as measured by ELISA on digested 3D cultures (n = 12). D, Flow in 3D induced an increase in the gene expression of CCL21ser as represented by quantitative real-time PCR (n = 12). ND, Not detectable. E and F, Immunostaining and quantification of TRC-associated CCL21 protein following exposure to 2D laminar flow-derived shear stresses (0.005, 0.01, and 0.05 dyne/cm²) showed that CCL21 production (green) by TRCs (red) was increased directly by 0.005 and 0.01 dyn/cm² shear stress (p < 0.05). Relative CCL21 area represents the number of green (CCL21⁺) pixels per DAPI⁺ cell (n = 3; where DAPI is 4',6'-diamidino-2-phenylindole). Scale bars, 100 μm.
difficult to directly compare because of their fundamentally differ-
changes in CCL21 expression, although we note that the actual
flows (as opposed to pressure drag forces, for example) drives the
stress experiments suggested that shear stress resulting from such
vitro culture systems were much larger. Our 2D laminar shear
in the absence of such nanoscale conduits; the channels in our in
studies indicated that the dimensions of the conduits are extremely small, sig-
ificant shear forces can be present even with extremely small flow
velocities (56). We also note that small hydrostatic pressures are
present in the conduits and that the fibers of the conduits may
shield the cells from shear stress. We cannot experimentally rule
out that pressure forces rather than shear stresses are present on
TRCs in vivo. However, we addressed the question of whether
out that pressure forces rather than shear stresses are present on
TRCs in vivo. Afferent lymphatics pass through conduits containing a perme-
able basal lamina. TRCs adhering to this lamina are therefore con-
tantly exposed to slow lymph flow driven by small pressure gra-
dients (and very small levels of hydrostatic pressures that were
used to induce the flow, on the order of 0.5 cm of water) and,
because the dimensions of the conduits are extremely small, sig-
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out that pressure forces rather than shear stresses are present on
TRCs in vivo. However, we addressed the question of whether
shear stress can directly affect cells by applying flow onto the cells
in the absence of such nanoscale conduits; the channels in our in
vitro culture systems were much larger. Our 2D laminar shear
stress experiments suggested that shear stress resulting from such
flows (as opposed to pressure drag forces, for example) drives the
changes in CCL21 expression, although we note that the actual
stresses imposed on cells in 2D vs 3D cultures under flow are
difficult to directly compare because of their fundamentally differ-
ent nature, including the fact that stromal cells never exist physi-
ologically in 2D conditions. Together, our data suggest that even
during homeostasis when lymph flow rates are low, these shear
forces could maintain TRC activity, and when lymph flow is
blocked, CCL21 expression declines. In this way, circulating naive
T cells would be less chemoattracted to blocked LNs and more
chemoattracted to LNs draining injured or inflamed tissues (ac-
knowledging that the actual flow values within the conduits of the
LN are unknown).

Interestingly, higher flow (23 μm/min) induced TRC prolifera-
tion in vitro. To date, only the two cytokines, LT and TNF-α, have
been described as increasing the proliferation of TRC lines, es-
pecially when added together (40). An increased T zone stromal cell
network is a likely prerequisite for accommodating the largely in-
creased number of T cells found in the draining LN early during the
immune response (40). Our finding that flow alone may also
enhance TRC proliferation implies that TRC proliferation may be
activated even before inflammatory cytokines reach the draining
LN, with the later signals of TNF-α and LT helping to maintain that
proliferation.

Our evidence that flow induced the transcription and secretion of
CCL21 by LN TRCs suggests that flow is an important cue to
maintaining LN function and adds to previous findings that tran-
scriptional events can be triggered by fluid flow in bone marrow
stromal cells (57). Although transcription was triggered more
strongly at higher flow rates, ECM-associated chemokine was in-
creased even at the lowest flow rate tested, indicating that secretion
may be triggered at a certain threshold of flow rate, which is con-
sistent with the heterogeneity of the flow patterns likely to be
found within the T zone of a typical LN. To date, LT and TNF-α
have been considered the principal regulators of CCL21 expres-
sion based on observations of reduced transcripts and proteins in
the spleens of mice deficient in LT or TNF-α (2). Interestingly,
these stimuli were not sufficient to trigger the expression of CCL21
in TRC lines cultured in 2D (40), similar to the findings with our
TRC clones (data not shown). In contrast, 3D culture in the com-
posite matrix under flow conditions stimulated CCL21ser expres-
sion. These findings highlight the notion that naturally occurring
biophysical conditions are necessary for these cells to be main-
tained in an activated and functional state. The partial dedifferen-
tiation seen with TRCs cultured in 2D is reminiscent of observa-
tions with other cells isolated ex vivo, e.g., high endothelial venule
cells from LNs and cutaneous endothelial cells (58, 59). Thus, the
chemokine environment in the LN is likely to be cooperatively
regulated and perhaps fine tuned by the combination of biophysical
as well as biochemical signals.

Consistent with this, in vivo CCL21 transcription was also de-
pendent on continuous lymph flow, as suggested by the rapid
down-regulation of CCL21 mRNA 2 h after ligating the affer-
ent and efferent lymphatic vessels of naive LNs. Based on our in vitro
data, we propose that lack of fluid flow is involved in this partial
loss of chemokine transcription. However, we cannot exclude po-
tential contributions from chemical components contained within
the lymph. Furthermore, in the murine LN, CCL21ser is expressed
by both TRCs and HEVs (60), and thus our observed reduction
could have occurred in either of these cell types. Of note, func-
tional afferent lymph vessels are known to be critical for the main-
tenance of the differentiated state of HEVs in LNs, and blocking
afferent lymph flow in mice led to morphological and phenotypical
alterations of HEVs within 4–7 days (61); however, this was as-
associated with strongly decreased lymphocyte recruitment. Because
CCL21 gene expression levels were down-regulated after only 2 h
in our experiments, it is probable that this effect was a direct result

![FIGURE 6. Fluid flow through the LN regulates CCL21 expression in vivo. A, Lymph flow through the inguinal LN was blocked by cutting the afferent and efferent lymphatic vessels. FITC dextran uptake was used as a marker of lymphatic transport and flow. LN dextran uptake by the “blocked flow” node was reduced by blocking the lymph flow draining to that node, compared with the control LN “normal flow”. Scale bars, 1 mm (left) and 2.5 mm (right). B, After 2 h, expression of CCL21ser and CCL19atg transcripts were assessed and the former was found to be significantly de-
creased in the blocked LNs (p < 0.05) compared with their corresponding control LNs (n = 7). Quantitative real-time PCR results are displayed as the ratio between normal flow and blocked flow.](http://www.jimmunol.org/)

A

<table>
<thead>
<tr>
<th>blocked flow</th>
<th>normal flow</th>
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<tr>
<td>before block</td>
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<td>1min after block</td>
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<td>2h after block</td>
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B

<table>
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<th>normal flow / blocked flow gene expression</th>
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<tbody>
<tr>
<td>CCL21ser</td>
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<td>CCL19atg</td>
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of flow change rather than an indirect effect related to subsequent changes in lymphocyte trafficking.

Because TRCs are an important source of CCR7 ligands in the LN that regulate lymphocyte trafficking and promote the interactions between mature APCs and naive T cells, and because they line the conduits through which lymph flows from the subcapsular sinus to the T cell zone (13–15), their sensitivity to flow is a very efficient way to sense changes in the peripheral tissue; increases in lymph flow through peripheral tissue occur within seconds to minutes after tissue injury, whereas chemokine production by peripheral immune cells and the trafficking of these cells to the LN occur on a time scale of hours to days. These findings are also consistent with previous reports showing that early during immune response there is an increase in CCL21 expression in lymphatic vessels of inflamed skin and in draining LNs (12, 62–64) This is in contrast to later phases of the immune response when down-regulation of CCL21 and CCL21 expression often occurs (12, 65). Part of the early increase in CCL21 protein accumulation may be at the level of HEVs, leading to increased T cell recruitment during the initial swelling of the LN (66). This CCL21 may be expressed by HEVs themselves (in mice only), come from the inflamed site via lymph and conduits, or derive from increased production by TRCs. The latter two processes would be strongly accelerated by increased fluid flow from the capsule via the conduits to the HEVs. Furthermore, given the similarities between the reticular stroma observed in the spleen compared with the LN, it is possible that similar mechanisms of CCL21 regulation by flow might act on the splenic fibroblastic reticular cells that are found in the white pulp, with flow in this case percolating through splenic conduits fed by the blood circulation (67, 68).

In conclusion, by implicating the physical microenvironment as a regulator of TRC function, these findings further expand the complex picture of biochemical cues and signaling events that collectively regulate immune responses. Because increased lymph drainage is an immediate response to increased capillary permeability, arguably the first physiological response to injury or inflammation, TRC sensitivity to lymph flow may help optimize the immune response by preparing the LN for increased immune cell trafficking before peripheral APCs reach the node.

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Disclosures

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References


