Video Article

Isolation of Murine Lymph Node Stromal Cells

Maria A. S. Broggi*[1], Mathias Schmaler*[1], Nadège Lagarde[1], Simona W. Rossi[1]

[1]Department of Biomedicine, Immunoregulation, University of Basel and University Hospital Basel

*These authors contributed equally

Correspondence to: Simona W. Rossi at simona.rossi@unibas.ch

URL: http://www.jove.com/video/51803
DOI: doi:10.3791/51803

Keywords: Immunology, Issue 90, lymph node, lymph node stromal cells, digestion, isolation, enzymes, fibroblastic reticular cell, lymphatic endothelial cell, blood endothelial cell

Date Published: 8/19/2014

Citation: Broggi, M.A.S., Schmaler, M., Lagarde, N., Rossi, S.W. Isolation of Murine Lymph Node Stromal Cells. *J. Vis. Exp.* (90), e51803, doi:10.3791/51803 (2014).

Abstract

Secondary lymphoid organs including lymph nodes are composed of stromal cells that provide a structural environment for homeostasis, activation and differentiation of lymphocytes. Various stromal cell subsets have been identified by the expression of the adhesion molecule CD31 and glycoprotein podoplanin (gp38), T zone reticular cells or fibroblastic reticular cells, lymphatic endothelial cells, blood endothelial cells and FRC-like pericytes within the double negative cell population. For all populations different functions are described including, separation and lining of different compartments, attraction of and interaction with different cell types, filtration of the draining fluidics and contraction of the lymphatic vessels. In the last years, different groups have described an additional role of stromal cells in orchestrating and regulating cytotoxic T cell responses potentially dangerous for the host.

Lymph nodes are complex structures with many different cell types and therefore require a appropriate procedure for isolation of the desired cell populations. Currently, protocols for the isolation of lymph node stromal cells rely on enzymatic digestion with varying incubation times; however, stromal cells and their surface molecules are sensitive to these enzymes, which results in loss of surface marker expression and cell death. Here a short enzymatic digestion protocol combined with automated mechanical disruption to obtain viable single cells suspension of lymph node stromal cells maintaining their surface molecule expression is proposed.

Video Link

The video component of this article can be found at http://www.jove.com/video/51803/

Introduction

Lymph nodes are specialized compartments where adaptive immune responses against foreign and self-antigens are initiated and coordinated. The procedure presented here describes a short enzymatic digestion combined with automated mechanical pipetting to obtain lymph node single cell suspension and gain access to viable lymph node stromal cells that maintain the surface expression of several molecules.

Lymph node stromal cell form the scaffold of the lymph node and fulfill three major functions: first they filter body fluids to sample antigens, pathogens and their pathogen associated molecular pattern (PAMPs), as well as cytokines and danger associated molecular pattern (DAMPs) present in the body. Second, they attract and instruct antigen presenting cells (APC) and lymphocytes to interact and initiate adaptive immune responses; and third, they provide a structural environment for the homeostasis and differentiation of lymphocytes[1-3]. During inflammation lymph node stromal cells produce growth factors, cytokines and chemokines, adapt to swelling thereby organizing the interaction between dendritic cells (DCs), T-, and B- cells. The orchestration of immune responses is only possible due to the complex structural architecture formed by different stromal cell populations.

Lymph node stromal cells are CD45 negative cells and can be distinguished by the expression of CD31 or gp38 in fibroblastic and endothelial cells[1-6]. $gp38^{+}CD31^{-}$ defines T zone reticular cells (TRC, also known as FRC: fibroblastic reticular cells), $gp38^{+}CD31^{+}$ defines lymphatic endothelial cells (LEC), $gp38^{-}CD31^{+}$ defines blood endothelial cells (BEC). Further, characterization of the subpopulations revealed the existence of other lymph node stromal cells. Indeed, a small pericyte-like cell population was characterized within the $gp38^{-}CD31^{-}$ population[7]. Therefore, adaptation of the isolation procedure is advantageous for identification and characterization of the functional properties of different lymph node stromal cells.

Before the development of lymph node stromal cells digestion protocols the study of lymph node stromal cells was limited to *in situ* observations using tissue section and microscopy. Nevertheless, structural and functional studies showed important characteristics of lymph node stromal cells. Lymph node stromal cells are associated with podoplanin, collagen and extracellular matrix (ECM) proteins to form a complex 3 dimensional structure called conduit system, which transports lymph and associated-low molecular mass proteins from the subcapsular sinus of the lymph node to the high endothelial venules in the T cells zone[8]. DCs are in close contact with stroma cells and may be observed protruding into the tubular conduit structure to sample fluid and detect antigens[8]. The interaction of lymph node stromal cells (TRCs and LECs) with DCs is mediated by the release and presentation of chemokines CCL21 and CCL19[9,10]. CCL19 and CCL21 are recognized by the CCR7 receptor

facilitating DCs and T cells to migrate to the lymph node T cell zone[4,11]. Despite using similar chemokines, DCs and T cells have different migration routes into the lymph nodes[12]. Later, using enzymatic digestion of the lymph node and isolation of pure lymph node stromal cells, functional studies were performed on the role of the different lymph node stromal cells and their ability to interact with DCs and T/B cells[6,13]. First, the crosstalk between IFN-γ producing effector T cells and lymph node stromal cells induces the production of the metabolite nitric oxide shown to dampen T cell responses and proliferation in the secondary lymphoid organs[14-16]. Second, lymph node stromal cells have been reported to support the differentiation of regulatory DC subsets via the production of IL-10[17], and to modulate naïve T cell homeostasis via the production of IL-7[6,18]. Third, TLR expression in lymph node stromal cells suggests that stromal cells are susceptible to signal derived from an infection or self-molecules released during tissue injury. Indeed, the treatment of lymph node stromal cells with the ligand of TLR3 poly(I:C) induces a modest upregulation of major histocompatibility complex class I expression and upregulation of co-inhibitory molecule PD-L1, but not of costimulatory molecules, resulting in dramatic changes in peripheral tissue antigens expression[19]. Several groups have shown lymph node stromal cells express peripheral tissue antigens and induce tolerance of self-reactive T cells[19,21-27]. Therefore, understanding the interactions between lymph node stromal cells and the other migratory and resident lymph node cells will help to find new target molecules to allow activation or suppression of immune responses during inflammation. Therefore, the implementation of the published enzymatic separation of the lymph node is needed.

Previously published protocols use different combinations of collagenase-based enzymatic digestion with low mechanical stress[6,19,20]. However, long incubations with digestion enzymes or the different combination of digestion enzyme might degrade various surface molecules required to analyze the activation status and to identify new lymph node stromal cells. Depending on the type of the stromal cell analysis, the Link Protocol or Fletcher Protocol might be more suited. In the described procedure, a slightly shorter enzymatic digestion is combined with automated mechanical disaggregation to minimize surface marker degradation of viable lymph node stromal cells. This procedure enables highly reproducible isolation and distinction of lymph node stromal cell populations with low variability and more than 95% viability. The freshly isolated lymph node stromal cells can be directly used for surface marker expression, protein analysis, and transcriptional studies, as well as establishment of stromal cells lines to perform functional assays *in vitro.*

Protocol

In this video publication and protocol, all animal procedures were conducted in accordance to the animal protocol approved by the Cantonal Authority Basel-Stadt, Switzerland.

1. Lymph Nodes Preparation and Digestion

1. Pre-heat water in a beaker to 37 °C on a magnetic stirrer with heating plate.
2. Prepare Basic Medium as following: DMEM medium (without pyruvate) supplemented with 2% FCS, 1.2 mM $CaCl_2$ and Pen/Strep (100 units of penicillin, 100 µg of streptomycin).
3. Sterilize all dissection instruments before use.
4. Euthanize lymph node donor mice per CO_2 asphyxiation and aseptically dissect the lymph nodes. Do not dissect the surrounding fat. NOTE: This protocol is optimized for peripheral skin-draining lymph node (inguinal, brachial, axillary).
5. Place lymph nodes in a sterile Petri dish containing 2 ml ice cold basic medium.
6. Disrupt the lymph node capsule using two 25 G needles fixed on 1 ml syringe.
7. Transfer the disrupted lymph node tissue in a 5 ml polypropylene round-bottom tube containing 750 µl basic medium supplemented with 1 mg/ml Collagenase IV and 40 µg/ml DNAse I.
8. Add one sterile magnetic stirrer in each tube.
9. Place tube in the beaker with 37 °C preheated water and stir the tubes at a slow rate (1 round/sec) for 30 min.
10. Remove the tube from the magnetic stirrer with heating plate and let lymph node fragments settle.
11. Carefully remove the supernatant enriched in "non-stromal cell". NOTE: If the analysis of T, B, dendritic cells and $CD45^-gp38^-CD31^-$ is foreseen, save the "non-stromal cells" fraction.
12. Wash remaining lymph node tissue once with 750 µl basic medium. NOTE: This step is necessary only if working with immune-competent mice.
13. Let lymph node fragments settle.
14. Remove the non-stromal cell-floating fraction.
15. Add to the lymph node fragments 750 µl basic medium supplemented with 3.5 mg/ml Collagenase D and 40 µg/ml DNase I.
16. Place tube back in the beaker containing the 37 °C preheated water.
17. Digest lymph node tissue for 5 min while slowly stirring.
18. Disaggregate lymph node tissue fragments by pipetting and mixing 700 µl for 10 cycles at maximal speed using an automated multichannel pipette. NOTE: This disrupts lymph node tissue to improve digestion.
19. Place the tube back in the beaker with 37 °C preheated water.
20. Digest lymph node tissue fragments for another 10 min while slowly stirring.
21. Disaggregate lymph node tissue fragments by pipetting and mixing for 99 cycles at maximal speed using an automated multichannel pipette.
22. Add 7.5 µl of 0.5 M EDTA to ensure maintenance of single cell suspension.
23. Disaggregate lymph node tissue fragments by pipetting and mixing for 99 cycles at maximal speed using an automated multichannel pipette.
24. Add 750 µl basic medium and pass cells through a 70 µm nylon mesh.
25. Centrifuge cell suspension 5 min at 1,500 x g, 4 °C.
26. Stromal cells can now be used for further analysis.

2. Staining of Lymph Node Stromal Cells

1. Use a combination of anti-CD45, anti-podoplanin (gp38), anti-CD31 antibodies to successfully recognize TRC, LEC, BEC and DN cells.
2. Incubate the digested lymph node tissue with Live/Dead tracker, anti-CD45-FITC (1:200), anti-gp38-PE (1:200), anti-CD31-APC (1:200) in 100 µl HBSS containing 2% FCS for at least 20 min, 4 °C, in the dark.

3. Wash the cells adding 500 l of HBSS containing 2% FCS
4. Centrifuge cell suspension 3 min at 1500 x g, 4°C.
5. Re-suspend in 100 µl of HBSS containing 2% FCS.
6. Run the stained cells in a flow cytometer equipped with the following optics: excitation source with up to three lasers: blue (488 nm, air-cooled, 20 mW solid state), red (633 nm, 17 mW HeNe), and violet (405 nm, 30 mW solid state).
7. Gate $CD45^-$ cells to exclude hematopoietic cells.
8. Gate singlets (FSC-W) and live cells (LIVE/DEAD tracker) to exclude doublets and dead cells.
9. Plot gp38 *vs.* CD31 to visualize TRC ($gp38^+CD31^-$), LEC ($gp38^+CD31^+$), BEC ($gp38^-CD31^+$) and DN ($gp38^-CD31^-$) cells (see **Figure 1**).

Representative Results

The present protocol is a modified digestion protocol published by Link *et al*., 2007[6] with a shorter digestion time (45 min maximum) due to mechanical disaggregation with an automated multichannel pipette. In addition, the procedure is more standardized, minimizes degradation of surface markers on different lymph node stromal cells and allows the handling of more than one sample at the same time.

Collagenase IV and Collagenase D in Links protocol[6] and current protocol or Collagenase P and Dispase in Fletchers protocol[13] maintain lymph node stromal cells viability and spare the digestion of several surface markers including CD45, gp38 and CD31. To test the current protocol, axillary, brachial and inguinal lymph nodes were removed and digested to isolate their stromal cells. Analysis of CD45, gp38 and CD31 expression demonstrated the presence of TRC, LEC, BEC and DN cells isolated from C57BL/6 mice (**Figure 1**, gating strategy).

Mechanical stress might reduce the viability of lymph node stromal cells during digestion. Comparing the viability of isolated subpopulations of stromal cells using the three different protocols, it was found that the viability was higher than 95% in the Fletcher protocol, but lower for both Current protocol and Link protocol (**Figure 2A**), although Current protocol show better survival in the BEC subpopulation then Link protocol. The total cells number recovered post digestion of the lymph node stromal cell subsets was slightly higher in Current protocol compared to Link protocol and Fletcher protocol (**Figure 2A**). These results demonstrate that the Current protocol maintains viability of lymph node stromal cells similar to published protocols. The major differences between the three protocols are listed in **Table 1**.

Surface markers are required to characterize lymph node stromal cells by flow cytometry and to isolate them by cell sorting. TRCs and LECs isolated via Current, Link and Fletcher protocols were compared for $I\text{-}A^b$, CD140a, CD80, PD-L1 and CD40 expression. After isolating stromal cells by all three protocols, we found the expression of $I\text{-}A^b$, CD80, CD140a, PD-L1, and CD40 was higher upon digestion with CP and LP in both TRCs and LECs (**Figure 2B**). These results suggest that degradation of some surface molecules with Collagenase IV and D are less strong than with Collagenase P and Dispase.

Taken together, the current protocol includes a short digestion combined with automated mechanical disaggregation to minimize surface marker degradation of viable lymph node stromal cells.

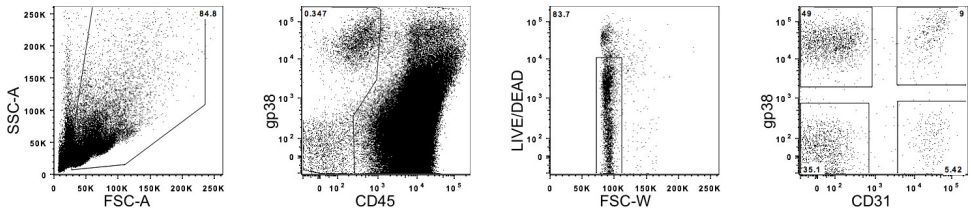

Figure 1. TRC, LEC, BEC, and DN cells staining, gating, and quantification. Lymph nodes from C57BL/6 mice were digested following the current protocol and stained with CD45, gp38 and CD31, Live/Dead to define TRC, LEC, BEC, and DN cells by flow cytometry. Data show representative results of the staining. Click here to view larger image.

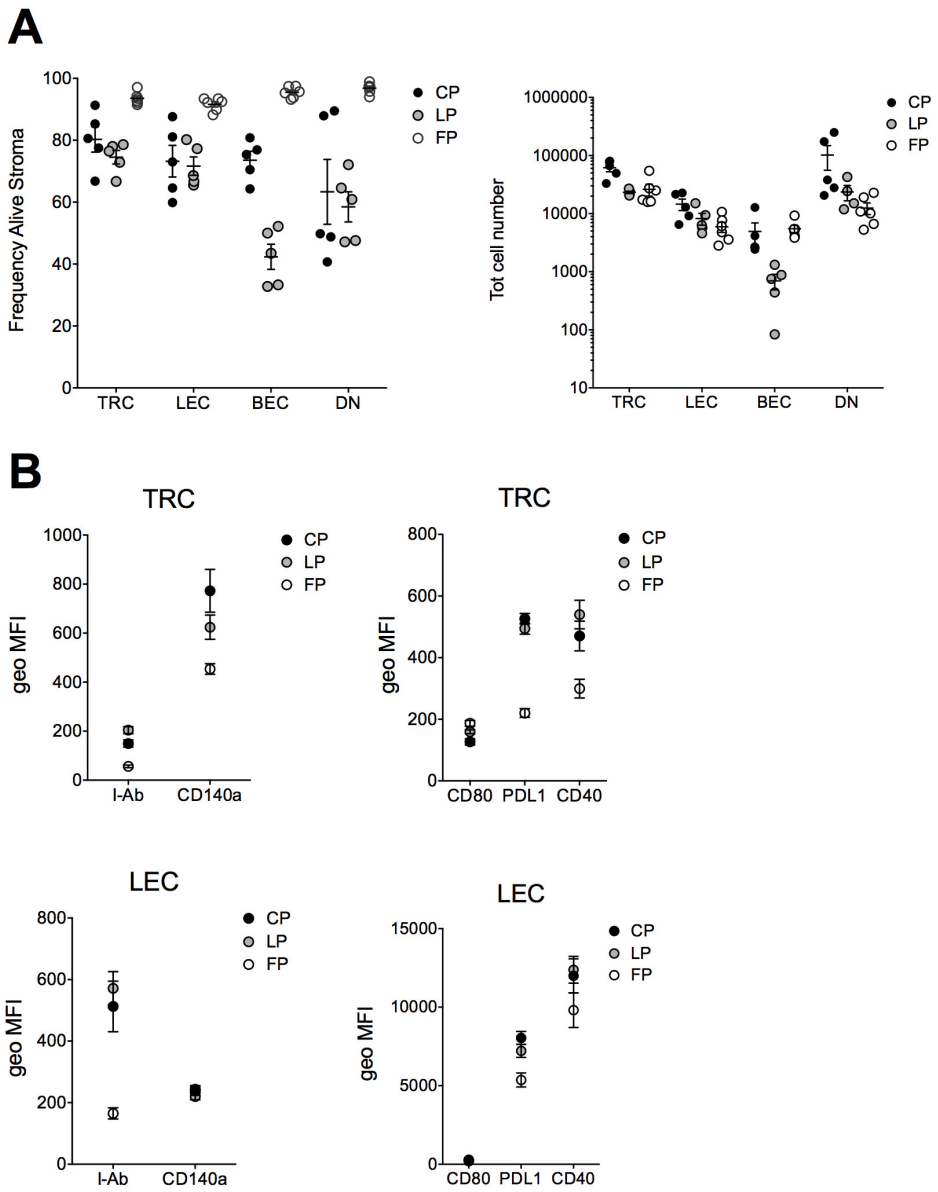

Figure 2. Viability and surface marker expression of lymph node stromal cells. Lymph nodes from C57BL/6 mice were digested following the Current (CP), Link's (LP) and Fletcher's protocol (FP) and stained with CD45, gp38 and CD31 to define TRC, LEC, BEC and DN cells by flow cytometry. **A)** Viability and total cell number of all lymph node stromal cell subpopulations after Live/Dead staining (n ≥ 5, pooled data of 2 independent experiments, bars represent SEM). **B)** Analysis of surface expression of I-A^b (APC-Cy7), CD140a (PerCP-Cy5.5), CD80 (PerCP-Cy5.5), PD-L1 (PE-Cy7) and CD40 (PE-Cy7) on TRCs and LECs (n = 6 for FP and n = 11 for LP and CP, pooled data of 2 independent experiments, bars represent SEM). Geometric MFI auto-fluorescence values for LEC-APC-Cy7: 324 ± 97, LEC-PECy7: 163 ± 82.5, LEC-PerCpCy5.5: 156 ± 36, TRC-APC-Cy7: 349 ± 152, TRC-PECy7: 117 ± 54, TRC-PerCP-Cy5.5: 121 ± 45. Click here to view larger image.

Current Protocol-Enzymes	Current-protocol	Link Protocol-enzymes	Link-protocol	Fletcher Protocol-enzymes	Fletcher-protocol
Collagenase IV, Dnase I	30 min, 37 °C, stirring	Collagenase IV, DNAse I	30 min, 37 °C	Collagenase P, Dispase, DNAse I	20 min, 37 °C, invert every 5 min
Collagenase D, Dnase I	5 min, 37 °C, stirring, resuspend	Collagenase D, DNAse I	20 min, 37 °C	Collagenase P, Dispase, DNAse I	10 min, 37 °C, resuspend 30 sec
	10 min, 37 °C, stirring	Collagenase D, DNAse I	37 °C, resuspend every 10 min until complete digestion	Collagenase P, Dispase, DNAse I	37 ° C, resuspend every 5 min until complete digestion
	automated mechanical resuspension				

Table 1. Short summaries of CP, LP, FP enzymes used and protocols.

Discussion

The study of lymph node stromal cells recently became a research focus due to the development of two published digestion protocols[6,13]. Both protocols are adequate to gain single lymph node stromal cells but differ in the use of digestion enzymes and the time of digestion. Since stromal cells and their surface markers are sensitive to enzymatic digestion and mechanical stress, an optimized protocol is required.

The isolation of viable lymph node stromal cells from freshly dissected lymph node is the first step in order to perform phenotypic and functional analysis. Therefore, it is important to carefully digest with the defined concentration of enzymes, at a stable temperature and for the indicated time. Two digestion protocols have been described[6,13], however with rather long digestion steps. To reduce the time of digestion, mechanical disaggregation was included and standardized using a multichannel pipette. One of the advantages of the current protocol is that it allows isolation of stromal cells in only 45 min, therefore minimizing their incubation time with digestion enzymes. In addition, constant stirring during the digestion allows the optimal access of the digestion enzymes to the lymph node structure. Moreover, the digestion volume was reduced to 750 µl minimizing the amount of enzyme used.

Excessive mechanical force disrupts tight junctions but might cause cell death at the same time. For this reason we tested several combinations of re-suspension cycles and different dissociation devices. We found that the use of an automated multichannel pipette allows the dissociation within 2 applications of 99 cycles; apply a constant pressure to the cells and this will result in single cell suspension with optimal viability. Four to six samples can be mixed and pipetted at the same time reducing the time of lymph node stromal cell isolation.

The digestion protocol has to maintain the surface expression of almost all molecules; only in this way the concomitant staining with several markers can reveal if a population is homogeneous or heterogeneous. For example, the use of BP-3 and CD35 in the TRC gp38^{+}CD31^{-} allows for the visualization and isolation of medullary TRC (Sanjiv Luther, personal communication). Furthermore, the poorly characterized DN fraction was further characterized as containing Aire^{+} cells and pericyte like cells positive for integrin α7 (reviewed in [7,13]). Therefore, the present protocol was compared to the published ones. As shown in the results, similar frequencies and numbers of lymph node stromal cells were obtained with the current protocol compared to the published ones. Interestingly, frequency and number of isolated TRC were highest using Fletcher protocol suggesting a better dissociation of the TRC structures than in the Current protocol and Link protocol. Moreover, the expression of various surface molecules was enhanced with the Current protocol compared to Fletcher protocol. These differences in the digestion method might be the reason for slightly different expression patterns in the lymph node stromal cell studies[6,19,21]. It might be useful to test different digestion protocols because of the advantages of each protocol. The Current protocol might be better for surface expression analysis, while Fletcher protocol might be useful for isolation of high numbers of viable cells for transcription analysis (as published in Malhotra *et al.*[7]). Therefore comparing all three digestion protocols might be important to obtain optimal results after isolation of lymph node stromal cells and should be seen as complementary.

Disclosures

The authors declare they have no competing financial interests.

Acknowledgements

The authors thank Sanjiv Luther and colleagues for helpful discussions in establishing the current lymph node digestion protocol. This work was supported by SNF grants PPOOA-_119204 and PPOOP3_144918 to S.W.R.

References

1. Mueller, S. N., & Ahmed, R. Lymphoid stroma in the initiation and control of immune responses. *Immunological reviews.* **224**, 284-294 (2008).
2. Roozendaal, R., & Mebius, R. E. Stromal Cell - Immune Cell Interactions. *Annual Review of Immunology.* **29** (1), 23-43 (2011).
3. Turley, S. J., Fletcher, A. L., & Elpek, K. G. The stromal and haematopoietic antigen-presenting cells that reside in secondary lymphoid organs. *Nature Reviews Immunology.* **10** (12) (2010).
4. Bajénoff, M. *et al.* Stromal cell networks regulate lymphocyte entry, migration, and territoriality in lymph nodes. *Immunity.* **25** (6), 989-1001 (2006).
5. Katakai, T., Hara, T., Sugai, M., Gonda, H., & Shimizu, A. Lymph node fibroblastic reticular cells construct the stromal reticulum via contact with lymphocytes. *The Journal of experimental medicine.* **200** (6), 783-795 (2004).
6. Link, A. *et al.* Fibroblastic reticular cells in lymph nodes regulate the homeostasis of naive T cells. *Nature Immunology.* **8** (11), 1255-1265 (2007).
7. Malhotra, D. *et al.* Transcriptional profiling of stroma from inflamed and resting lymph nodes defines immunological hallmarks. *Nature Immunology.* **13** (5), 499-510 (2012).
8. 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. *The Journal of experimental medicine.* **192** (10), 1425-1440 (2000).
9. 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** (1), 19-29 (2005).
10. MartIn-Fontecha, A. *et al.* Regulation of dendritic cell migration to the draining lymph node: impact on T lymphocyte traffic and priming. *The Journal of experimental medicine.* **198** (4), 615-621 (2003).
11. 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** (23), 12694-12699 (2000).

12. Braun, A. *et al.* Afferent lymph-derived T cells and DCs use different chemokine receptor CCR7-dependent routes for entry into the lymph node and intranodal migration. *Nature Immunology.* **12** (9), 879-887 (2011).
13. Fletcher, A. L. *et al.* Reproducible isolation of lymph node stromal cells reveals site-dependent differences in fibroblastic reticular cells. *Frontiers in immunology.* **2**, 35 (2011).
14. Lukacs-Kornek, V. *et al.* Regulated release of nitric oxide by nonhematopoietic stroma controls expansion of the activated T cell pool in lymph nodes. *Nature Immunology.* **12** (11), 1096-1104 (2011).
15. Siegert, S. *et al.* Fibroblastic reticular cells from lymph nodes attenuate T cell expansion by producing nitric oxide. *PLoS ONE.* **6** (11), e27618 (2011).
16. Khan, O., Headley, M., Gerard, A., Wei, W., Liu, L., & Krummel, M. F. Regulation of T cell priming by lymphoid stroma. *PLoS ONE.* **6** (11), e26138 (2011).
17. Svensson, M., Maroof, A., Ato, M., & Kaye, P. M. Stromal cells direct local differentiation of regulatory dendritic cells. *Immunity.* **21** (6), 805-816 (2004).
18. Onder, L. *et al.* Endothelial cell-specific lymphotoxin-β receptor signaling is critical for lymph node and high endothelial venule formation. *The Journal of experimental medicine.* **210** (3), 465-473 (2013).
19. Fletcher, A. L. *et al.* Lymph node fibroblastic reticular cells directly present peripheral tissue antigen under steady-state and inflammatory conditions. *The Journal of experimental medicine.* **207** (4), 689-697 (2010).
20. Fletcher, A. L., Malhotra, D., & Turley, S. J. Lymph node stroma broaden the peripheral tolerance paradigm. *Trends in Immunology.* **32** (1), 12-18 (2011).
21. Cohen, J. N. *et al.* Lymph node-resident lymphatic endothelial cells mediate peripheral tolerance via Aire-independent direct antigen presentation. *Journal of Experimental Medicine.* **207** (4), 681-688 (2010).
22. Lee, J.-W. *et al.* Peripheral antigen display by lymph node stroma promotes T cell tolerance to intestinal self. *Nat Immunol.* **8**, 181-190 (2006).
23. 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. *J Immunol.* **179,** 993-1003 (2007).
24. Gardner, J. M. *et al.* Deletional tolerance mediated by extrathymic Aire-expressing cells. *Science.* **321,** 843-847 (2008).
25. Gardner, J. M. *et al.* Extrathymic Aire-expressing cells are a distinct bone marrow-derived population that induce functional inactivation of CD4# T cells. *Immunity.* **39,** 560-572 (2013).
26. Magnusson, F. C. *et al.* Direct presentation of antigen by lymph node stromal cells protects against CD8 T-cell-mediated intestinal autoimmunity. *Gastroenterology.* **134,** 1028-1037 (2008).
27. Tewalt, E. F. *et al.* Lymphatic endothelial cells induce tolerance via PD-L1 and lack of costimulation leading to high-level PD-1 expression on CD8 T cells. *Blood.* **120** (24), 4772-4782 (2012).

