Defining High Endothelial Venules and Tertiary Lymphoid Structures in Cancer

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Abstract

High endothelial venules (HEVs) are structurally distinct blood vessels that develop during embryonic and neonatal life in all secondary lymphoid organs except the spleen. HEVs are critical for initiating and maintaining immune responses because they extract naïve and memory lymphocytes from the bloodstream, regardless of antigen receptor specificity, and deliver them to antigen-presenting cells inside lymph nodes under homeostatic conditions. HEVs also develop post-natally in non-lymphoid organs during chronic inflammation driven by autoimmunity, infection, allografts and cancer. Extranodal HEVs are usually surrounded by dense lymphocytic infiltrates organised into lymph-node like, T- and B-cell rich areas called tertiary lymphoid structures (TLS). HEV neogenesis is thought to facilitate the generation of tissue-destroying lymphocytes inside chronically inflamed tissues and cancers.

We are studying the mechanisms underpinning HEV neogenesis in solid cancers and the role of homeostatic T cell trafficking in controlling cancer immunity. In this chapter we describe methods for identifying HEV in tissue sections of cancerous tissues in humans and mice using immunohistochemical staining for the HEV-specific marker peripheral lymph node addressin (PNAd). L-selectin binding to PNAd is a necessary first step in homeostatic lymphocyte trafficking which is the defining function of HEV. We also describe methods to measure L-selectin dependent homing of lymphocytes from the bloodstream into lymphoid tissues and tumours in preclinical cancer models.

1. Introduction

High endothelial venules (HEVs) are structurally and antigenically distinct blood vessels especially adapted for lymphocyte trafficking (1). HEV develop in every secondary lymphoid organ except the spleen (i.e. lymph nodes (LN), tonsils and Peyer's patches) during embryonic and neonatal life, and are fully integrated into the blood vasculature supplying these organs. The endothelial cells (EC) lining HEV in neonatal mice are immature and express the vascular addressin, Mucosal Addressin Cell Adhesion Molecule (MAdCAM)-1. During the first weeks of neonatal life, HEV mature into Peripheral Node Addressin (PNAd) expressing HEV (2). PNAd expression is induced by gut-derived dendritic cells in neonates (3) and maintained by Lymphotoxin-β receptor (LTβR) signalling in HEV EC stimulated by LTαβ expressing dendritic cells and lymphocytes (4, 5). HEV play a critical role in delivering naïve and memory T and B lymphocytes, regardless of antigen receptor specificity, from the bloodstream into lymphoid organs under homeostatic conditions. Here, lymphocytes scan dendritic cells and stromal networks for activating, tolerogenic and homeostatic stimuli. HEV, therefore, regulate the outcome of an immune response be it activation, tolerance or homeostatic proliferation of lymphocytes. PNAd-expressing blood vessels that resemble structurally distinct HEV in LN, develop post-natally in non-lymphoid organs during chronic inflammation driven by autoimmunity, infection and allografts (6). These extranodal HEV are characteristically surrounded by dense lymphocytic infiltrates organised into lymph-node like structures with discrete T and B-cell rich areas and germinal centres and are called tertiary lymphoid structures (TLS). Extranodal HEV facilitate entry of blood borne lymphocytes and are therefore, critical to the development and immune function of TLS. HEV-containing TLS also develop in solid, vascularised cancers and

are receiving increasing attention because of their potential role in regulating immunity at the tumor site (7, 8). The presence of TLS in resected solid cancers has been correlated with prolonged patient outcome in some cancers such as breast cancer (9, 10), melanoma (11, 12) and lung cancer (13). In other cancers, TLS have been shown to either promote carcinogenesis such as virus-associated hepatocellular carcinoma (14), or to accumulate with disease progression such as in primary breast carcinoma (15). In colorectal cancer, TLS often containing germinal centres (Crohn's-like aggregates), develop in the surrounding peritumoural stroma as well as inside cancerous tissue. The number of TLS in colorectal cancer has been reported to correlate either with improved patient outcome or with disease progression depending on the stage of the disease (16-19).

In breast cancer and melanoma, the density of HEV correlated with improved patient outcome highlighting the important role that HEV play in orchestrating anti-cancer immunity (20, 21). HEV neogenesis correlates with regression of established tumours in preclinical mouse tumour immunotherapy models, such as depletion of $Foxp3^+$ regulatory T cells (22, 23) or combined checkpoint blockade inhibition and angiogenesis therapy (24). HEV neogenesis and tumour regression are also seen when the TNF superfamily member LIGHT (TNSF14) (which signals *via* LTβR, an important driver of lymphoid organ development) is expressed by tumour cells or targeted to tumours or tumour blood vessels (25-27). It is thought that the anti-tumour effects are due to the generation of cancer cell-destroying lymphocytes from naïve T cells which have been recruited from the bloodstream into the cancer by newly formed HEV.

PNAd-expressing blood vessels lined by flat endothelial cells, rather than the cuboidal or plump endothelial morphology typical of mature HEV, are found inside

cancer-induced ectopic lymphoid aggregates that are not organised into distinct T/B cell areas (28). These could represent immature HEV-containing structures in the process of forming TLS, or de-differentiating HEV as found in reactive LN or following disruption of the LN microenvironment (5, 29). Interestingly, PNAd-expressing blood vessels that form following depletion of $Foxp3^+$ regulatory T cells from methylcholanthrene induced tumour-bearing mice are not generally associated with histologically distinct, TLS (22) which suggests that HEV neogenesis may occur independently of lymphoid neo-organogenesis. The development of HEV in the absence of full-blown TLS correlates with T cell infiltration and cancer regression in this experimental model, highlighting the important role of HEV in controlling anti-tumoural immunity.

PNAd expressing blood vessels have been reported in preclinical mouse tumour models particularly following immunotherapy, In tumour cell transplant models, PNAd expression is induced on tumour blood vessels by infiltrating $CD8^+$ T cells and NK cells (30). In marked contrast to HEV development in LN, PNAd expression is not dependent on LTβR signaling but is stimulated instead by Lymphotoxin α3 (LTα3). Although comprising <10% of the tumour vascular network and lined by flat endothelial cells PNAd-expressing tumour blood vessels are functional in that they recruit naïve, L-selectin expressing T cells from the bloodstream into the tumour where they are activated to kill tumour tissue (30). In methylcholanthrene-induced fibrosarcomas in mice, HEV neogenesis is also not dependent on LTβR but is driven mainly by T cell-derived TNF-α and LTα3 (23). However, HEV neogenesis during anti-angiogenic/anti-PD-L1 therapy is dependent on LTβR signalling and in poorly infiltrated tumours where HEV neogenesis is low, such as glioblastomas, the combination of agonistic antibodies to LTβR alongside anti-angiogenic/anti-PD-L1

therapy boosts HEV neogenesis (22). Together, these findings suggest that TNFR and/or LTβR signaling in endothelial cells stimulates the development of PNAd expressing, HEV blood vessels that promote anti-tumour immunity by recruiting naïve T cells into cancerous tissues.

We are studying the role of homeostatic T cell trafficking *via* HEV and the mechanisms underpinning HEV neogenesis in controlling local immunity inside mouse tumours and human cancers. We use immunohistochemistry to identify HEV in clinical and murine tissues by detection of PNAd, a specific marker of HEV (**Figure 1**). We measure L-selectin-dependent recruitment of naïve T lymphocytes from the bloodstream into tissues, *via* PNAd-expressing HEV, in mice using short-term homing assays and analyse tissue-infiltrating T cells by flow cytometry (**Figure 2**) (31-33) and immunohistochemistry to co-locate infiltrating T cells and HEV (**Figure 3**) (31, 34).

2. Materials

All reagents which are not commercially available should be prepared with deionised, sterile water. All reagents stored at +4^{o}C unless otherwise specified by the manufacturer. Tissues should be collected into sterile phosphate buffered saline, lymphocytes isolated in growth media and resuspended in saline for injection into mice. All procedures apart from tissue collection and injections into mice should be performed in a biological safety cabinet. The following reagents are used in all methods: calcium- and magnesium-free phosphate-buffered saline (PBS), complete RPMI 1640 growth media supplemented with penicillin and streptomycin (RPMI), heat-inactivated (30 min at 56^{o}C) foetal calf serum (FCS), 70% alcohol for

sterilisation. The antibodies used for immunofluorescence staining of frozen or paraffin-embedded tissue sections and flow cytometry of T cells are listed in **Table 1**.

2.1 Immunolabelling HEV and TLS in murine tumour cryosections

1. Tissue-Tek plastic base molds (Thermo Fisher Scientific).
2. Optimum cutting temperature (OCT) compound.
3. Dry ice with or without ethanol for snap freezing murine tissue in OCT.
4. Cryostat capable of cutting cryosections.
5. Hydrophobic pen for creating a hydrophobic barrier around tissue section (e.g. Pap pen, Vector).
6. Humidified chamber containing wet paper towel to prevent evaporation of staining reagents.
7. PBS for washing steps.
8. Fixative of choice (see **Note 1**). For example:
    a. Acetone stored in glass Pyrex bottle at -20^{o}C.
    b. Fresh 4% formaldehyde. Dilute 16% formaldehyde in PBS and adjust pH to 7.4; 16% formaldehyde can either be bought in ampules (Thermo Fisher Scientific) or prepared from paraformaldehyde powder (Sigma) and stored at -20^{o}C. Limit exposure to paraformaldehyde by preparing in a fume hood. Store 4% formaldehyde at 4^{o}C and use within 1 week.
    c. Periodate-lysine-paraformaldehyde fixative (PLP; 0.075 M lysine, 0.37 M sodium phosphate (pH 7.2), 2% formaldehyde, and 0.01 M $NaIO_4$). Mix the following to create a buffered lysine solution: 0.36g Lysine (Sigma), 7.5 mL NaH_2PO_4 (0.1M, pH 7.2; Sigma), 2.5 mL Na_2HPO_4

(0.1M, pH 7.2; Sigma) and 10 mL H_2O. Immediately before use, add 15 mL of the buffered lysine solution to the following: 5 mL 8% formaldehyde and 50 mg Sodium meta-periodate ($NaIO_4$; Sigma). It is not necessary to pH the final solution.

9. If using formaldehyde or PLP to fix sections then prepare 0.3 M glycine (Thermo Fisher Scientific) in PBS, pH 7.4, to quench free aldehydes.
10. **Optional**: Avidin/Biotin blocking kit if a biotinylated primary or secondary antibody is used.
11. Serum blocking solution to block non-specific binding of antibodies. 2.5-5% serum of the species in which the secondary antibody is raised is often used. Other sera, for example horse or mouse serum,may also be used.
12. Primary antibodies diluted in 1% Bovine serum albumin (BSA, Sigma) in PBS. Rat anti-mouse PNAd antibody (clone MECA-79, rat IgM) is used at a working concentration of 2.5 □g/mL to detect HEV (see **Notes 2** and **3**). Alternatively, MECA-79 hybridoma supernatant can be prepared in-house and titrated on LN sections before staining HEV in tumour sections. Additional antibodies can be used simultaneously to detect other cells or structures such as TLS. Example 1: Biotinylated rat anti-mouse pan-EC antigen (clone MECA-32, rat IgG2a, used at a working concentration of 2 □g/mL, Biolegend) can be used to confirm that MECA-79 stains endothelial structures (**Figure 1**) (see **Note 4**). Example 2: Biotinylated rat anti-mouse MAdCAM-1 (clone MECA-367, rat IgG2a, used at a working concentration of 2 □g/mL, Biolegend) can be used to detect immature HEV in tumours (**Figure 1**). Example 3: The presence of TLS in tumour tissue, characterized by T/B cell segregation and follicular dendritic cell (FDC) networks may be revealed using antibodies to T cells (e.g.

rabbit anti-human CD3, which also detects mouse CD3, DAKO A0452, used at 2 □g/mL), B cells (biotinylated rat anti-mouse/human CD45R/B220, clone RA3-6B2, rat IgG2a, used at a working concentration of 2 □g/mL, Biolegend), and FDC (rat anti-mouse FDC, clone FDC-M1, rat IgG2c, used at a working concentration of 2 □g/mL, BD Biosciences).

13. For every antibody used, an isotype control antibody should be used at the same working concentration, in parallel on another section to check the staining is specific. A rat IgM isotype control antibody is used to check MECA-79 staining is specific. Biotinylated rat IgG2a is used to check pan-EC antigen (clone MECA-32), MAdCAM-1 (clone MECA-367) and B cell (clone RA3-6B2) staining is specific (**Figure 1**). Rat IgG2c is used to check FDC (clone FDC-M1) staining is specific and rabbit IgG polyclonal isotype control is used to check CD3 (DAKO A0452) staining is specific.
14. Fluorescently conjugated secondary reagents diluted in 1% BSA in PBS. To detect MECA-79, a fluorescently conjugated anti-rat IgM may be used. Other primary antibodies can be detected with fluorescently conjugated species-specific secondary antibodies or fluorescently conjugated streptavidin reagents. The fluorochromes chosen should be compatible with the specific capabilities of the microscope being used to analyse the tissues i.e. the laser systems and detectors. The Alexa Fluor dyes are excellent conjugates since they are very bright and resistant to photobleaching; Alexa Fluor 488, 594 and 647 emission spectrum are sufficiently separated to provide minimal overlap between fluorochromes and are compatible with most standard widefield and confocal fluorescent microscopes.

15. Optional: nuclear counterstain. Example Hoescht or DAPI (Sigma) diluted in PBS.
16. Mounting media. Example: Vectashield (Vector) or Prolong gold (Thermo Fisher Scientific).
17. Glass coverslips (thickness #1.5 coverslips should be used in fluorescence microscopy to obtain the brightest images with minimal spherical aberration; Thermo Fisher Scientific).

2. 2 Immunolabelling HEV and TLS in paraffin-embedded murine tumour sections

1. Tissue-Tek plastic base molds (Thermo Fisher Scientific).
2. Neutral buffered formal saline (NBFS).
3. Paraffin wax (Shandon Histoplast, Thermo Fisher Scientific).
4. Microtome capable of cutting paraffin sections (e.g.Thermo Fisher Scientific).
5. Blades (MX35, Thermo Fisher Scientific).
6. Hydrophobic pen for creating a hydrophobic barrier around tissue section (e.g.Pap pen, Vector).
7. Humidified chamber containing wet paper towel to prevent evaporation of staining reagents.
8. 100% Xylene.
9. Graded alcohols: 100%, 95%, 90%, 80% and 70% ethanol.
10. PBS for washing steps.
11. Peroxidase blocking solution: either 1% hydrogen peroxide/methanol or a commercial blocking reagent (e.g. Bloxall; Vector) (see **Note7**).

12. Serum blocking solution to block non-specific binding of antibodies. Often 2.5-5% serum of the species in which the secondary antibody is made is used. Other sera for example horse or mouse serum may also be used.
13. Primary antibody diluted in 1% BSA in PBS. Rat anti-mouse PNAd antibody (MECA-79, rat IgM, used at a working concentration of 2.5 □g/mL) can be used to detect HEV.
14. A rat IgM isotype control antibody should be used at the same working concentration in parallel on another section to check MECA-79 staining is specific.
15. Enzyme conjugated secondary antibody complexes to detect primary antibody. Example Immpress anti-rat horseradish peroxidase (HRP) kit (Vector).
16. Enzyme substrate to visualize immunolabelled target. Example: DAB, Impact VIP or Impact SG (Vector).
17. Optional: nuclear counterstain. Example: Haematoxylin (Sigma).
18. Mounting media. Example: distyrene, plastizer and xylene (DPX; Raymond Lamb).
19. Glass coverslips (Menzel; Thermo Fisher Scientific).

2.3 L-selectin dependent homing of naïve T lymphocytes in mice

1. The following equipment is required: 0.001g balance in animal unit for weighing tissues, humidified CO_2 incubator at 37^{o}C, refridgerated bench top centrifuge, trypan blue solution and Neubauer chamber or other equipment for cell counts and viability, 2 mL plastic syringes, 70 μm cell strainers, 50 mL

polypropylene tubes. T cell donor mice: 8-12-week-old, sex-matched and syngeneic to experimental mice.

2. Experimental mice with HEV-containing cancers as recipients of donor T cells.
3. Sterilised instruments (dissecting scissors, forceps and scalpel).
4. Labelled tubes containing 1 mL ice-cold PBS for collection of spleens from T cell donor mice.
5. Labelled tubes containing 1 mL ice-cold PBS for collection of cancerous tissues, lymph nodes and spleens from experimental mice after injection of donor T cells (labelled with mouse number (e.g.1-5) and organ (e.g. cancer, spleen, LN).
6. Red blood cell lysis buffer.
7. Naïve T cell isolation kits using negative selection.
8. Cell tracker dye: carboxyfluorescein diacetate-succinimidyl ester (CFSE, Molecular Probes).
9. Fluorescently conjugated antibodies to TCR (clone H57-597, Armenian hamster IgG), L-selectin/CD62L (clone MEL-14, rat IgG2a) and CD44 (clone IM7, rat IgG2b).
10. Rat-anti mouse PNAd (clone MECA 79, rat IgM), fluorescently conjugated or biotinylated anti-rat IgM to detect MECA79 in tissue sections.
11. Fixative: 2% formaldehyde/5% sucrose in PBS, 20% sucrose in PBS.
12. Freezing and storing tissues: liquid nitrogen.

3. Methods

3.1. Immunolabelling HEV and TLS in murine tumour cryosections

1. Collect tumour tissue and place immediately in optimal cutting temperature compound (OCT) in a plastic mould. Add sufficient OCT to completely cover the tissue.
2. Snap-freeze in dry ice and store blocks in small plastic zip-lock bags at -80^{o}C until needed.
3. Cut 5-10 μm sections on a cryostat and mount sections on glass slides. Typically, we mount 2 sections per slide.
4. Allow slides to dry for approximately 1 hour at room temperature (RT) and store in a slide box at -80^{o}C until needed.
5. Remove slides from freezer and allow them to warm up to RT and wipe away any residual moisture, taking care not to touch the tissue section.
6. Fix sections in ice-cold 100% acetone, 4% formaldehyde or PLP fixative for 10 minutes (see **Note 1**). Wash slides 3 times in PBS over 5 minutes at RT.
7. Drain slides and carefully wipe off excess liquid before next step. Be careful not to let the section dry out.
8. Draw a circle round each section with a hydrophobic marker.
9. **Optional**: if fixed in 4% formaldehyde or PLP quench free aldehydes with 0.3 M glycine in PBS for 10 min at RT and then wash slides 3 times in PBS over 5 minutes at RT.
10. **Optional**: if using a biotinylated antibody block endogenous biotin with avidin/biotin block and then wash slides 3 times in PBS over 5 minutes at RT.
11. Block non-specific protein binding with serum blocking solution for 30 min at RT.
12. Drain slides (no need to wash the slides).

Incubate with MECA-79 antibody or rat IgM isotype control in 1%BSA/PBS overnight at 4°C or for 1 hour at RT in a humidified chamber. **Optional**: include other antibodies such as MECA-367 to identify immature HEV, MECA-32 to confirm that MECA-79 is detected on EC (see **Figure 1**) or T cell, B cell and FDC antibodies (**Table 1**) to determine if TLS are present.

13. Wash slides 3 times in PBS over 5 minutes at RT.
14. Incubate with fluorescently conjugated secondary antibodies in 1% BSA/PBS for 30-60 min at RT (see **Note 5**). Counterstain with nuclear dye for 10 min at RT (e.g. Hoescht or DAPI).
15. Wash slides 3 times in PBS over 5 minutes at RT.
16. Mount slides in mounting media and coverslip.
17. Slides should be stored in the dark at +4°C until analysis and image acquisition by fluorescence microscopy. Staining may be preserved for at least 2 months if Alexa Fluor dyes are used and slides are stored correctly.

3.2 Immunolabelling HEV and TLS in paraffin-embedded murine tumour sections

1. Collect tumour tissue and fix in neutral buffered formal saline (NBFS) for 24-48 hours.
2. Process in tissue processor (Leica) as follows:
    - 70% Ethanol - 1 hour at 45°C
    - 80% Ethanol - 1 hour at 45°C
    - 95% Ethanol - 1 hour at 45°C
    - 100% Ethanol – 1 hour at 45°C

- 100% Ethanol – 1 hour at 45ºC
- 100% Ethanol – 1 hour at 45ºC
- 100% Xylene – 1.5 hours at 45ºC
- 100% Xylene – 2 hours at 45ºC
- 100% Xylene – 2 hours at 45ºC
- Wax - 1.5 hours at 65ºC
- Wax – 2 hours at 65ºC
- Wax – 2.5 hours at 65ºC
- Embed in wax and cool

3. Store tissue blocks in a cool dry place until ready to cut.
4. Pre-cool tissue blocks on ice or on a cold plate (Leica).
5. Cut 5-10 μm sections on a microtome and mount on glass slides.
6. Place slides in a slide rack at 60ºC for 1 hour to overnight to melt off wax.
7. Hydrate sections in a slide chamber with 3X 100% Xylene washes, 5 min each and then descending alcohols (100%, 100%, 90% and 70%) for 3 min each. Wash in running water for 5 min and then rinse in dH_20.
8. Use either Tris/EDTA (10 mM Tris base, 1 mM EDTA solution, 0.05% Tween 20, pH 9.0) or Sodium citrate (10 mM Sodium citrate, 0.05% Tween 20, pH 6.0) antigen retrieval buffer for heat induced epitope retrieval (see **Note 6**).
9. Wash slides 3 times in PBS over 5 minutes.
10. Drain slides and carefully wipe off excess water before next step. Be carefully not to let the section dry out.
11. Draw a circle round each section with a hydrophobic marker.

12. Neutralize endogenous peroxidase activity with 1% hydrogen peroxide/ methanol for 10 min at RT or alternatively use a commercial blocking reagent (e.g.Bloxall, Vector; see **Note 7**).
13. Wash slides 3 times in PBS over 5 minutes.
14. Block non-specific protein binding with blocking solution for 30 min at RT.
15. Drain slides (no need to wash).
16. Incubate with MECA-79 antibody or rat IgM isotype control in 1%BSA/PBS overnight at 4^{o}C or for 1hour at RT in a humidified chamber.
17. Wash slides 3 times in PBS over 5 minutes.
18. Incubate with anti-rat Immpress reagent (Vector) or other HRP or conjugated secondary antibody and incubate at RT for 30 min.
19. Wash slides 3 times in PBS over 5 minutes.
20. Incubate with freshly prepared DAB Chromogen solution and monitor under a microscope until colour develops.
21. Rinse with deionised H_2O and then wash in fresh deionised H_2O.
22. **Optional**: It is possible to sequentially stain the same tissue section with other antibodies in order to identify other cells and structures using different coloured chromogens (see **Note 8)**. For example, the presence of TLS containing organized B and T cell zones can be verified with rat anti-mouse CD45R/B220 (Biolegend) detected with anti-rat Immpress reagent (Vector) and Vector-VIP (purple; Vector) followed by rabbit anti-mouse CD3 (DAKO) detected with anti-rat Immpress reagent (Vector) and Vector-SG (grey; Vector).
23. Counterstain in Haematoxylin (10-20 secs) and Blue in Scotts tap water (10 sec) (see **Note 9**).

24. Dehydrate in ascending alcohols (70%, 90% 100% and 100%) for 3 min each and 3X 100% Xylene washes for 5 min each.
25. Mount in mounting media and coverslip.

3.3 L-selectin-dependent homing of naïve T lymphocytes in mice

1. Cull T cell donor mice by CO_2 inhalation and/or cervical dislocation and pin out on dissecting board ventral side up. Swab ventral surface with 70% alcohol to sterilise and wet fur, open abdominal cavity by midline incision using scalpel. Remove spleen carefully using forceps and scissors and avoid damaging the surface. Collect spleen into PBS in labelled tubes and transport on ice to biological safety cabinet (see **Notes 10** and **11**).
2. Place cell strainer in neck of a labelled 50 mL polypropylene tube in a tube rack and pour spleen and PBS through cell strainer.
3. Split open paper covering of 2 mL plastic syringe, remove plunger using paper covering to maintain sterility of flat end (not rubber seal end). Mash spleen using flat end of plunger. Wash strainer using 5 mL of PBS, repeat if necessary to completely disaggregate tissue; discard strainer.
4. Seal tubes and collect splenocytes by centrifugation at 250 g for 5 minutes at +4^{o}C. Aspirate or remove PBS by pouring.
5. Tap bottom of tube to dislodge cell pellet, carefully resuspend in 2 mL red cell lysis buffer using 10 mL strippette, make up to 5 mL with red cell lysis buffer and incubate for 5 minutes on ice. Add 10 mL PBS, collect cells by centrifugation and repeat once.

6. Isolate naïve T cells by negative selection using commercially available kits according to manufacturer's instructions (see **Note 12**)
7. Resuspend T cells to 5 x 10^7/mL in FCS-free PBS for labelling with CFSE (see **Note 13**) and remove 1 x 10^5 cells for staining to check naïve T cell purity (see **Note 14**).
8. Label T cells in 2 µM CFSE in FCS-free PBS in the dark for 15 min at 37ºC, wash twice in 5-10 mL PBS containing 1% FCS, resuspend to 2.5 x 10^7 cells/mL in saline and split into 2 equal aliquots. Collect cells by centrifugation and preincubate one aliquot with 100 µg/mL rat anti-mouse L-selectin (clone MEL-14, rat IgG2a) and the second aliquot with 100 µg/mL isotype control (clone MAC193, rat IgG2a) for 30 min at 4ºC.
9. Inject 0.2 mL of T cells plus antibody (5 x 10^6 cells) intravenously into experimental mice (see **Notes 10, 15** and **16**).
10. Cull mice after 1, 4 or 24 hours (see **Note 17**) by CO_2 inhalation and/or cervical dislocation Collect blood by cardiac puncture from the left ventricle using a 1 mL syringe with 27G needle into 1.5 mL heparinised tubes Eppendorf tubes containing 100 µl of 10 units/mL heparin or citrate-EDTA. Store carcasses on ice and prepare for dissection, as described in Step 1.
11. Collect tumour, spleen, peripheral (axillary, brachial and inguinal) LN and mucosal (mesenteric or mediastinal) LN, and process as follows: cut tumour, spleen and mucosal LN into two halves, one half to analyse infiltrating T cells by histology and the other half to isolate infiltrating T cells for flow cytometric analysis. Pool right peripheral LN and use to isolate infiltrating T cells. Collect left axillary, brachial and inguinal LN separately for immunolocalisation of infiltrating T cells. Fix LN, spleen and tumour in 2% formaldehyde/5% sucrose

in PBS for 2 hours, transfer to 20% sucrose in PBS for 2 hours, snap freeze in liquid nitrogen, and store at -80°C for up to 2 years. Collect lymphocytes from one half of a tumour, one half of spleen and pooled right peripheral LN, as described in Steps 2-5 above (see **Notes 18** and **19).**

12. Lyse red blood cells in spleen, tumour and peripheral blood, as described in Step 5.
13. Analyse CFSE-labeled lymphocytes on a flow cytometer analyzer using the fluorescein isothiocyanate filter. Data on 2 x 10^5 viable cells in spleen and blood, and 5-10 x 10^5 viable cells for LN is acquired and the data analyzed using FlowJo software. The percentage of CFSE-labeled cells recovered in the spleen, blood, and each group of LN is determined and compared between control and treated animals. If L-selectin deficient T cells are available label using CMTMR, mix them 1:1 with CFSE labelled L-selectin sufficient T cells and inject a total of 20 x 10^6 cells into a single group of experimental mice. In tissues with functional HEV, L-selectin sufficient T cells are highly enriched over L-selectin deficient T cells (**Figure 2**).
14. Cryostat sections of 8 μm are cut from fixed, frozen tissues (see Step 11), HEV stained using MECA-79 and red fluorescence–conjugated secondary antibody, as described in **Section 3.1**, Step 12 but without additional fixation. The presence of TLS in fixed tumour tissue may be revealed using antibodies that detect T and B cells in formalin-fixed paraffin embedded tissues (**Table 1**). The position of CFSE-labeled lymphocytes (green) in relation to HEVs (red) is determined by fluorescence microscopy using a confocal laser scanning microscope. For each tissue, 10 to 15 images containing complete cross-sections through 30 to 55 HEVs (average total HEV area 2500 □m^2) are

collected and analyzed using Image J-NIH software. The total cross-sectional area of HEVs and the remaining area within each image are calculated. CFSE-labeled cells “inside HEVs” are those attached to the luminal surface of the vessel wall and within the HEV wall (**Figure 3A**; arrows). The remaining CFSE-labeled cells are scored as “outside HEVs” (**Figure 3A**; arrowheads). Total numbers of lymphocytes counted ranged from 200-500 cells. The numbers of CFSE-labeled cells inside and outside HEVs are not significantly different in inguinal and brachial LN of individual mice or between animals in the same experimental group (31). Results are pooled from inguinal and brachial LN of mice within each experimental group and, where the results were similar, between experimental groups and are expressed as means $\pm$ SEM CFSE-labeled cells/mm^2 inside and outside HEVs (34).

4. Notes

1. Fixation is required to maintain tissue morphology. The choice of fixative depends on the antigen and antibody used to detect it. Formaldehyde is a cross-linking fixative and maintains good tissue architecture however, some antigens cannot easily be detected after formaldehyde fixation (e.g. mouse CD4). Acetone removes lipids, dehydrates cells and precipitates proteins so cell structure is poorly preserved but may allow better detection of some antigens (e.g. mouse CD4). PLP can be prepared with varying concentrations of formaldehyde and was designed to primarily cross-link carbohydrates to main antigenic sites on proteins. Since membranes are rich in glycoproteins and glycolipids, PLP provides good

preservation of membrane antigens of the immune system. HEV are detected well with all of these fixation methods, however if other cells of the immune system are also immunolabelled then different fixation protocols should be tested.

2. MECA-79 is a rat IgM and can precipitate over time resulting in speckly staining at which point the antibody needs to be discarded.
3. MECA-79 can detect HEV in both mouse and human cryosections or paraffin sections. However other antibodies e.g. clones MECA-32 and MECA-367 are mouse specific so alternative human specific antibodies would be needed.
4. When two rat primary antibodies are used simultaneously (e.g. clones MECA-79 and MECA-367 or MECA-32) fluorescently or biotin conjugated primary antibodies or isotype specific secondary antibodies should be used so that anti-rat secondaries are avoided.
5. Since MECA-79 is a rat IgM antibody, it can be detected with an anti-rat IgM secondary antibody. However this secondary antibody will cross-react to endogenous mouse IgM, which can be detected in plasma cells in mouse tissue. Plasma cells can be identified by co-staining with anti-mouse CD138 or alternatively MECA79 staining of HEV can be positively identified by morphology or co-staining with a pan-EC marker.
6. Either Tris/EDTA or Sodium citrate antigen retrieval methods are both suitable for detecting HEV in paraffin tissue sections. The choice of antigen retrieval is determined by other antibodies used to sequentially stain tissue sections.
7. Hydrogen peroxide/methanol will quench endogenous peroxidase that

would otherwise react with the HRP substrate (e.g. DAB) resulting in undesirable background. Some proteins may be sensitive to methanol or hydrogen peroxide treatment and may reduce staining of the antigen, in this case quenching with hydrogen peroxide/methanol may be performed after the primary antibody incubation step or alternatively a commercial blocking reagent such as Bloxall may used.

8. It is difficult to distinguish by eye two colocalized stains on a single structure or cell by immunohistochemistry e.g. MECA-79 and an EC marker. If this is required immunofluorescence is recommended. However, spatially separated antigens can be detected by immunohistochemistry.
9. Light staining with Haematoxylin for 10-20 sec is usually sufficient. However, if sections are stained sequentially with multiple antibodies, it may be unnecessary to counterstain with Haematoxylin since the antibody staining might be obscured.
10. Each mouse receives 5-10 x 10^6 labelled donor T cells and two groups of 5 mice are required per experiment, one group receiving naïve T cells pretreated with control antibody and the second pretreated with anti-L-selectin antibody to inhibit binding of naïve T cells to HEV. The total number of naïve T cells per experiment is therefore 50-100 x 10^6 T cells. The yield of T cells is 30-50 x 10^6 cells per spleen so 2-3 donor mice will provide sufficient T cells for each experiment. If donor mice are limiting, T cells can be isolated from peripheral (axillary, brachial and inguinal) and mesenteric LN and pooled with splenic T cells.
11. Dissecting instruments sterilized by autoclaving in sealed bags or by submerging in 70% alcohol.

12. We have used kits for isolating T cells and $CD8^+$ T cells from Miltenyi Biotec and Stem Cell technologies.
13. We have used carboxyfluorescein diacetate-succinimidyl ester (CFSE, Molecular Probes) 5-(and 6)-([{4-chloromethyl}benzoyl]amino) tetramethylrhodamine (CMTMR; Cell Tracker Orange; Molecular probes), PKH26 (Molecular Probes) or SNARFJ-1 dye (Molecular Probes) to label naïve T cells for short-term homing of wildtype L-selectin expressing T cells. Instead of blocking L-selectin on donor T cells, PNAd-dependent homing can be tested by injecting CFSE-labelled naïve T cells into groups of recipient mice injected either with 100 µg MECA-79 or 100 µg isotype control antibody immediately before donor T cells are injected.
14. Naïve T cells are CD62L positive and CD44 negative/low.
15. Where L-selectin deficient T cells are available we have used CMTMR to label L-selectin deficient T cells, mixed them 1:1 with CFSE labelled L-selectin sufficient T cells and injected a total of 20 x 10^6 cells into a single group of experimental mice. In tissues with functional HEV, L-selectin sufficient T cells are highly enriched over L-selectin deficient T cells (**Figure 2**). Cell tracker dyes need to be reversed to check for effects of cell tracker dyes on T cell recruitment from the bloodstream *via* HEV in wild-type mice (**Figure 3B**).
16. To avoid using cell tracker dyes, we have injected unlabeled T cells isolated from CD90.2 (Thy.1.2) mice and injected in CD90.1 (Thy.1.1) cancer bearing mice and used antibodies to CD90.2 to detect donor T cells by flow cytometry and immunohistochemistry.

17. To measure recruitment directly from the bloodstream into tissues time points of 1-3 hours are preferred since this is before T cells exit 3rd party organs such as the spleen or LN. However, the level of T cell recruitment into cancerous tissues may be low and longer time points of 24-48 hours are required (35) (30).
18. Collect peripheral LN as positive controls for L-selectin/CD62L dependent homing (axillary, brachial, inguinal LN). L-selectin/CD62L-independent organs and tissue compartments include mediastinal and mesenteric LN, spleen and peripheral blood (**Figure 2**).
19. Tissue disaggregation using enzymes may increase the yield of infiltrating T cells from fibrotic tissues and cancers over mechanical disaggregation; this needs to be determined empirically for each type of tumour.

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Figure Legends

Figure 1. HEV can be detected in mouse fibrosarcomas after regulatory T cell depletion in FoxP3^{DTR} mice using diptheria toxin. Frozen tumor sections, were stained with anti-PNAd (MECA-79, red) + anti-pan-EC (MECA-32, green) antibodies (A and B) and anti-PNAd (MECA-79, red) + anti-MAdCAM-1 (MECA- 367, green) antibodies (C and D). Cell nuclei were counterstained with DAPI (blue). Low power images are shown in Panels A and C, and high power images of boxed regions are shown in Panels B and D. Scale bars are shown in white.

Figure 2. L-selectin dependent homing of T cells in mice. (A) L-selectin sufficient (CD62L$^{+/+}$) and L-selectin deficient (CD62L$^{-/-}$) donor T cells were labelled with either CFSE or CMTMR and mixed 1:1 (Pre-transfer). 1-3 hours following intravenous administration to recipient (A) mice, organs were analysed for CD62L$^{+/+}$ and CD62L$^{-/-}$ T cells and identified as either dependent or independent of CD62L/L-selectin. (B) The percentages of CD62L$^{+/+}$ and CD62L$^{-/-}$ T cells pre-transfer and recruited into inguinal (Ing), axillary (Ax) and mediastinal (Med) LN, spleen and in peripheral blood (PB) were plotted. T cell homing to periphal LN such as inguinal and axillary is L-selectin dependent. In contrast, T cell homing to the mucosal-associated mediastinal LN is not dependent on L-selectin. HEV are not found in the spleen so not dependent on L-selectin for T cell entry. Peripheral blood is analysed to determine whether changes to the ratio of T cell populations inside tissues results from a change in the ratio in the bloodstream.

Figure 3. Localisation of homed T lymphocytes in HEV containing tissues . (A) CFSE-labelled naïve T lymphocytes were injected into naïve mice and after 60 minutes peripheral lymph nodes analysed for infiltrating T cells (green) and HEV stained using MECA-79 (red). Representative image showing CFSE labelled (green) T lymphocytes in the process of transmigration and inside HEV (arrows) and CFSE labelled T lymphocytes which have completed transmigration and outside HEV (white arrows). Bar, 50 μm. (B) Naïve T lymphocytes were split into two and labelled with either CFSE or CMTMR, mixed 1:1 and injected into naïve mice. After 60 minutes, peripheral lymph nodes were stained for HEV using MECA-79 and the ratio of CFSE (green) to CMTMR (orange) T cells inside HEV (white arrows) and outside HEV (arrowheads) determined.

Table 1. Antibodies used for immunofluorescence staining and flow cytometry.

Antigen	Conjugate	Clone	Isotype	Company	Working concentration (□g/mL)
Primary antibodies for immunolabeling frozen sections					
PNAd	Purified	MECA-79	Rat IgM, □	Biolegend	2.5
Pan endothelial cell (pan-EC)	Biotinylated	MECA-32	Rat IgG2a, □	Biolegend	2
MAdCAM-1	Biotinylated	MECA-367	Rat IgG2a, □	Biolegend	2
CD3	Purified	-	Rabbit polyclonal	DAKO	2
CD45R/B220	Biotinylated	RA3-6B2	Rat IgG2a, □	Biolegend	2
Follicular dendritic cell (FDC)	Purified	FDC-M1	Rat IgG2c, □	BD Biosciences	2

Secondary antibodies/reagents for immunolabeling frozen sections					
Anti-rat IgM	Alexa Fluor 594	-	Goat polyclonal	Invitrogen	1
Streptavidin	Alexa Fluor 488	-	-	Invitrogen	1
Streptavidin	Alexa Fluor 647	-	-	Invitrogen	1
Anti-rabbit	Alexa Fluor 594	-	Goat polyclonal	Invitrogen	1
Anti-rat IgG2c	Alexa Fluor FITC	MARG2c-5	Mouse IgG2a	Invitrogen	1
Isotype control antibodies for immunolabeling frozen sections					
Rat IgM □	Purified	RTK2118	Rat IgM □	Biolegend	2
Rat IgG2a, □	Biotinylated	RTK2758	Rat IgG2a, □	Biolegend	2
Rabbit polyclonal	Purified	-	Rabbit polyclonal	Abcam	2
Rat IgG2c, □	Purified	RTK4174	Rat IgG2c, □	Biolegend	2
Primary antibodies for immunolabeling paraffin sections					
PNAd	Purified	MECA-79	Rat IgM, □	Biolegend	2.5
CD45R/B220	Purified	RA3-6B2	Rat IgG2a, □	Biolegend	2
CD3	Purified	-	Rabbit polyclonal	DAKO	2
Secondary reagents for immunolabeling paraffin sections					
Immpress anti-rat	HRP	-	Goat	Vector	-
Immpress anti-rabbit	HRP	-	Horse	Vector	-

Isotype control antibodies for immunolabeling paraffin sections					
Rat IgM □	Purified	RTK2118	Rat IgM □	Biolegend	2
Rat IgG2a, □	Purified	RTK2758	Rat IgG2a, □	Biolegend	2
Rabbit polyclonal	Purified	-	Rabbit polyclonal	Abcam	2
Primary antibodies for flow cytometry of T lymphocytes					
CD62L/L-selectin	PE	MEL-14	Rat IgG2a	Biolegend	0.2
TCR	FITC	H57-597	Armenian Hamster IgG	BD Pharmingen	0.2
CD44	APC-Cy7	IM7	Rat IgG2b	Biolegend	0.2