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Visualizing unique vasculature in the human spleen with two- and three-dimensional imaging

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Visualizing unique vasculature in the human spleen with two- and three-dimensional imaging

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Perna on ihmisen suurin toissijainen lymfaattinen elin. Sillä on merkittäviä tehtäviä elimistön homeostaasin ja systeemisen immuniteetin ylläpitämisessä. (Alexandre & Mueller, 2023) Perna siivilöi jatkuvasti verta hävittäen taudinaiheuttajia sekä vanhoja punasoluja verenkierrosta (Lewis, Williams ja Eisenbarth, 2019). Tutkimusten mukaan potilaat, joilta puuttuu perna, ovat suuremmassa vaarassa saada bakteeri-, virus- ja parasiitti-infektioita (Alexandre ja Mueller, 2023).

Yllättäen imusuonten olemassaoloa ei ole pystytty osoittamaan ihmispernassa. Nykyisen oletuksen mukaan imusolut kulkevat elimeen veren mukana (Cesta, 2006). Kalucka ym. huomasivat tutkimuksessaan, että imusuoniston merkkiaineet puuttuvat täysin hiiren pernasta. On kuitenkin tiedossa, että hiiren ja ihmisen pernassa on paljon rakenteellisia eroja, joiden takia käsitystä imusuonten puuttumisesta ei voida suoraan soveltaa ihmispernaan (Medetgul-Ernar & Davis, 2022). Täten suoniston tarkempi selvittely ihmisnäytteitä hyödyntäen on ensisijaisen tärkeää.

Tämän tutkimuksen tarkoituksena oli visualisoida pernan ainutlaatuista suonistoa kaksi- ja kolmiulotteisilla kuvantamismenetelmillä. Turun yliopistollisesta keskussairaalasta saatujen ihmispernanäytteiden avulla onnistuttiin havainnollistamaan veri- ja imusuoniston rakenteita immunohistokemiallisia menetelmiä hyödyntäen. Lisäksi pernakudos tehtiin läpinäkyväksi, mikä mahdollisti normaalia paksumpien näytteiden kuvantamisen. Paksummat näytteet antoivat entistä luotettavampaa tietoa suoniston rakenteista.

Tutkimuksen tärkeimpänä löydöksenä oli imusuonten kaltaisten suonirakenteiden visualisointi ihmispernassa. Tämän lisäksi MAdCAM-1-molekyylin havainnollistaminen pernan verisuonistossa ja sen yhteys LYVE-1 positiiviseen suonistoon oli merkittävä tulos. MAdCAM-1-molekyylillä tiedetään olevan tärkeä rooli imusolujen kuljettamisessa muissa kudoksissa, kuten suolistossa, mikä voisi viitata vastaavanlaiseen tehtävään myös pernassa. Kaiken kaikkiaan, tätä ainutlaatuista työtä voidaan hyödyntää osana ihmispernan rakenteen ja toiminnan laaja-alaista tutkimusta.

Avainsanat: perna, verisuonisto, imusuonisto, MAdCAM-1

Contents

1. Introduction

The spleen is the largest secondary lymphoid organ in the human body. It plays a crucial role in various immunological functions, such as initiating the adaptive immune system. The spleen is also essential for filtering old and damaged red blood cells from the circulation. (Lewis et al., 2019) According to recent research, patients lacking the spleen are at higher risk of infection due to encapsulated bacteria, parasites and viruses (Alexandre and Mueller, 2023).

Surprisingly, the function of different cells and regions of the spleen in textbooks of human anatomy are based nearly completely on research done with mouse or other rodent spleens. Nevertheless, striking anatomical differences between the mouse and human spleen have been discovered and therefore current information that relies on mouse data should be questioned and researched further. (Lewis et al., 2019)

Recent research reveals that the mouse spleen lacks lymphatic vessels, as visualized by single-cell RNA sequencing (Kalucka et al., 2020). The human spleen is known to have an absence of afferent lymphatics, which implies that lymphocytes enter the organ via blood circulation (Cesta, 2006). The existence of efferent lymphatics remains undefined in the human spleen. Lymphocytes are thought to exit the organ mainly through the splenic veins located in the red pulp. However, the role of splenic blood vasculature in the trafficking of lymphocytes is partially unknown. (Bronte and Pittet, 2013)

The aim of this study was to visualize unique structures of the vascular system in the human spleen with two- and three-dimensional imaging. Using methods of immunohistochemistry and tissue clearing, we were able to image lymphatic-like vessels utilizing lymphatic valves as proof. In addition, a novel molecule known as MAdCAM-1, was visualized in the blood vasculature of the spleen. MAdCAM-1 positive vessels connect to LYVE-1 positive vessels, which suggests that MAdCAM-1 could have a functional role in lymphocyte trafficking in the organ. All in all, this study provides critical information based entirely on human material and can be used in further research to discover the unknown functions of this enigmatic organ.

2. Review of the literature

2.1 Human spleen

The spleen is a dark red organ located in the left abdomen, directly beneath the diaphragm and adjacent to the greater curvature of the stomach (Cesta, 2006). It is covered by a capsule consisting of an outer layer of mesothelial cells as well as a dense fibrous matrix. The capsule is connected to the trabeculae, which support the structure of the spleen and are highly vascularized with blood vessels and nerves. (Alexandre & Mueller, 2023)

As the largest secondary lymphoid organ in the human body, the spleen plays a crucial role in various immunological functions, blood filtration, and hematopoiesis. Its physical structure facilitates interactions between antigen-presenting cells and lymphocytes, initiating the adaptive immune system. (Lewis et al., 2019) The spleen filters blood, clearing pathogens and damaged erythrocytes from the circulation. It also serves as a storage for oxygenated red blood cells in hypoxic condition and is able to perform extramedullary formation of blood cells. According to research, individuals without a spleen are at increased risk of infection from encapsulated bacteria, parasites and viruses (Alexandre and Mueller, 2023).

The structure of the spleen is divided into two anatomical compartments: red and white pulp. The red pulp (RP) makes up the majority of the splenic tissue (Lewis et al., 2019). It consists of venous sinuses and cords with an abundance of macrophages. Consequently, the red pulp functions in the clearance of pathogens as well as dead blood cells from the bloodstream. (Nolte et al., 2000) It is commonly recognized for removing old erythrocytes and recycling iron (Mebius & Kraal, 2005). Other important functions include extramedullary hematopoiesis and storage of cellular reserves of blood cells (Lewis, Williams and Eisenbarth, 2019).

On the contrary, the white pulp (WP) serves as the primary immunological region of the organ (Lewis et al., 2019). It is laden with leukocytes consisting of various subsets of B and T cells, dendritic cells as well as macrophages (Bronte & Pittet, 2013). The structure is formed of discrete areas of B and T cells. B lymphocytes form follicles, while T cells are situated around the central arteriole forming the periarteriolar lymphocyte sheath (PALS) (Nolte et al., 2000).

In between the red and white pulp is the perifollicular zone in humans and the marginal zone in mice (Lewis et al., 2019).

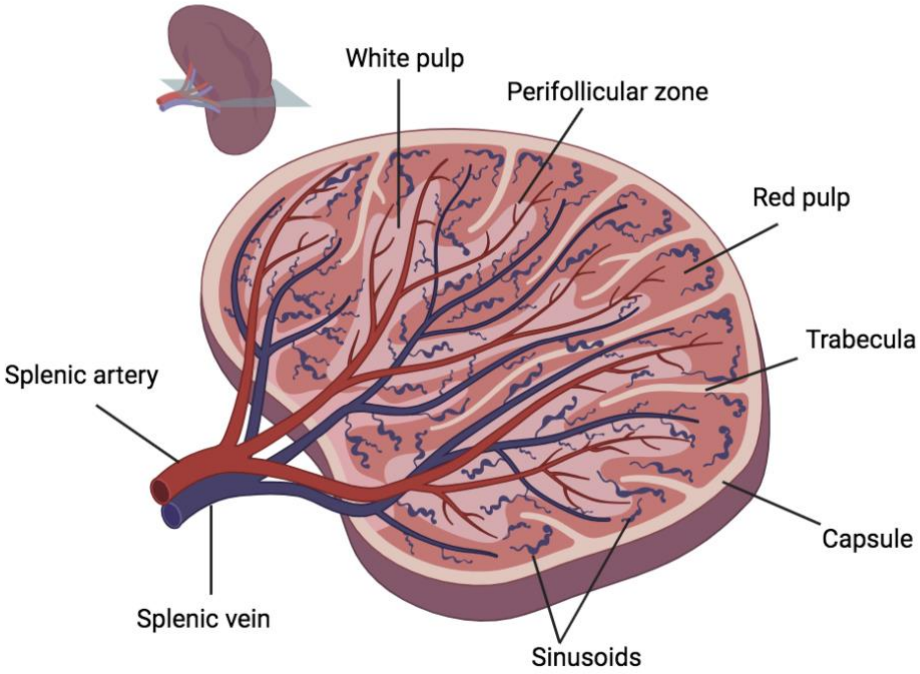

Figure 1. Cross section of the human spleen
Source: Created with BioRender

2.2 Blood vasculature

The blood and lymphatic vessel networks form the two coupled branches of the cardiovascular system in vertebrates (Petrova & Koh, 2020). The blood vasculature forms a highly organized network that comprises of arteries, veins and capillaries. This network functions by carrying oxygen, nutrients, circulating cells and soluble factors, such as hormones, to and from each tissue. In addition, capillaries function by removing metabolic waste products from the bloodstream (Marziano, Genet and Hirschi, 2021).

The splenic artery branches most commonly from the celiac trunk. The size of the artery is relatively large compared to the spleen as it supplies also a portion of the stomach and pancreas.

Close to the splenic hilum, the artery often divides into inferior and superior terminal branches. The superior branch provides the majority of the splenic arterial supply. Eventually, each of the branches further divide into four to six segmental intrasplenic branches. (Vanhoenacker et al., 2007)

The spleen consists of an open blood circulation. This means that afferent arterioles deliver oxygen-rich blood into sinusoids located in the perifollicular zone of the organ. (Bronte & Pittet, 2013) The PFZ is also where lymphocytes are released from blood circulation to reach the white pulp (Lewis et al., 2019). From there, blood flows through cords in the parenchyma into venous sinuses in the red pulp (Bronte & Pittet, 2013). Eventually, the venous sinuses collect into trabecular veins that unite at the hilum forming the splenic vein (Vanhoenacker et al., 2007). In addition to blood, the exit of lymphocytes occurs mainly through the same route (Bronte & Pittet, 2013).

2.3 Lymphatic vasculature

The lymphatic system is comprised of lymphatic vasculature, lymph nodes and associated lymphoid organs such as the spleen (Potente & Mäkinen, 2017). Lymphatic vasculature is defined as a unidirectional conduit system that returns interstitial arterial fluid and macromolecules back to the blood circulation. (Oliver et al., 2020)

Lymphatic vessels have conventionally been considered to be a passive conduit for fluid and cells. However, their significance is based on their crucial role in disease regulation as well as their contribution to the spread of pathogens and cancer cells. They also transport antigens and various immune cells into the lymph nodes (LNs), triggering immune activation or tolerance. (Takeda et al., 2019) In addition, they play an active role in the absorption of dietary fat in the gastrointestinal tract. (Oliver et al., 2020)

In normal physiological conditions, interstitial fluid continuously leaks out from blood capillaries and is taken up by initial lymphatic vasculature. These lymphatic vessels contain only one layer of loosely connected lymphatic endothelial cells (LECs). Furthermore, LECs are connected to each other through discontinuous button-like junctions that enable the efficient uptake of interstitial fluid and tissue metabolites. The contents of initial lymphatics are drained

into larger collecting lymphatic vessels that are specialized in the transport of lymph. (Oliver et al., 2020) Unlike initial lymphatics, collecting lymphatics comprise of LECs that are inter-connected through tighter, zipper-like junctions. Collecting lymphatic vessels are also covered by specialized smooth muscle cells that enable contractions to assist the flow of lymph. In addition, collecting lymphatics contain valves to ensure the unidirectional flow of lymph. (Hu et al., 2024)

Lymph is then transported to LNs by collecting afferent vessels (Von Andrian and Mempel, 2003). The immunological functions of LNs are based on efficient antigen display to large numbers of lymphocytes (Takeda, Salmi and Jalkanen, 2023). Lymph is eventually returned to blood circulation through an efferent lymphatic vessel and major lymphatic ducts, such as the thoracic duct, which is connected to the left subclavian vein (Von Andrian and Mempel, 2003). Moreover, afferent and efferent lymphatics have a significant functional difference: most leukocytes can enter the lymph node through afferent lymphatic vessels, however only lymphocytes are able to exit the LN via the efferent vessel under steady-state conditions. Hence, access to efferent lymphatics seems to function as an important control point in the trafficking of leukocytes and tumor spread. (Iftakhar-E-Khuda et al., 2016)

Surprisingly, current research suggests that the mouse spleen lacks lymphatic vessels, as visualized by single-cell RNA sequencing (Kalucka et al., 2020). In addition, it is believed that the human spleen lacks afferent lymphatic vessels, implying that lymphocytes enter the organ via blood circulation (Cesta, 2006). The exit of lymphocytes occurs mainly through the splenic veins (Bronte & Pittet, 2013).

Previously research has proposed that a small fraction of lymphocytes exit the mouse spleen through efferent lymphatics originating from the white pulp. This, however, does not apply to the human spleen, as the work cited in the mentioned review was performed on mice and conducted in 1990. (Pellas & Weiss, 1990) Lymphatic markers (PROX1, LYVE-1, and PDPN) were discovered only in 1999 (Hu et al., 2024), which does not allow us to claim the finding of efferent lymphatic vessels even in mice. Similar studies have not been conducted in humans.

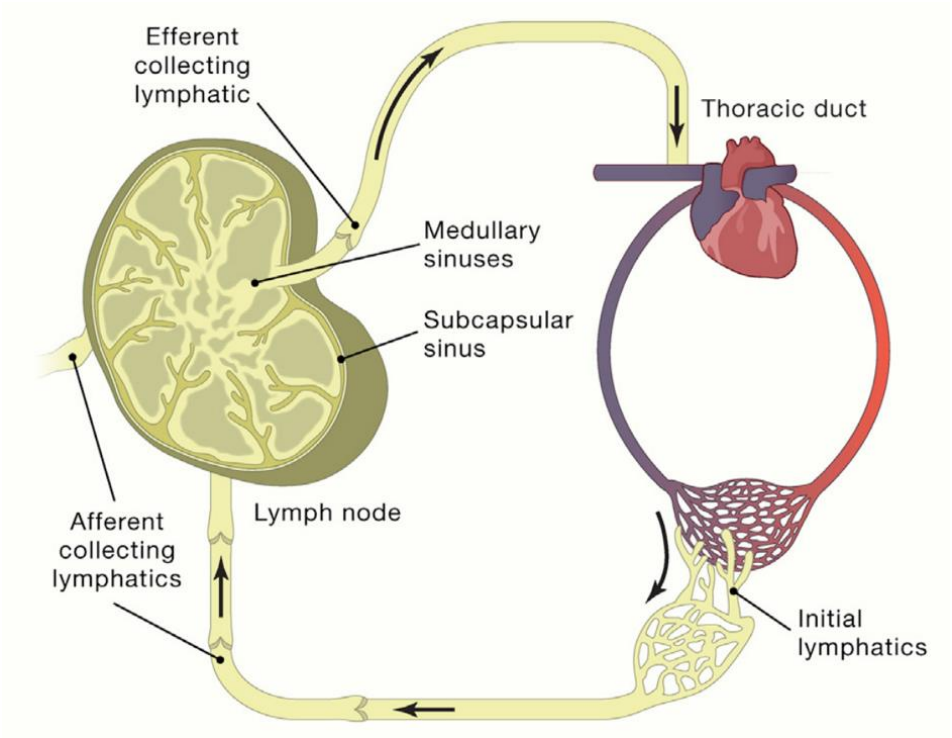

Figure 2. An overview of the main components forming the lymphatic system in humans
Source: *Cell* 2020: 182(2), 270–296

2.4 Endothelial cell markers

Endothelial cells (ECs) form the inner lining of all lymphatic and blood vessels. These cells are in immediate contact with the circulating fluid and function as first responders to circulating factors. (Marziano et al., 2021) Despite having a common mesodermal origin and sharing certain functions, ECs are not all alike. (Castro et al., 2018; Potente & Mäkinen, 2017) In fact, they are a heterogeneous population of specialized cells that are characterized by the physiological needs of the vessels they constitute (Marziano et al., 2021). Depending on the vessel type they reside, EC's can be divided into lymphatic endothelial cells (LECs) and blood endothelial cells (BECs) (Montenegro-Navarro, García-Báez and García-Caballero, 2023).

Lymphatic endothelial cells (LECs) form the inner lining of lymphatic vasculature and are specialized according to the needs of the tissue that the vessels supply (Potente & Mäkinen, 2017). Certain markers are expressed by all LECs, such as the prospero-related homeodomain transcription factor 1 (PROX1), vascular endothelial growth factor receptor-3 (VEGFR3),

membrane glykoprotein podoplanin (PDPN) and lymphatic vessel hyaluronan receptor-1 (LYVE-1) (Alitalo, 2011). LYVE-1 is also known to be a common marker for endothelial cells that line venous sinuses in the splenic red pulp (Martinez-Pomares et al., 2005). In addition, Claudin-11 (CLDN-11) has been identified as a marker for lymphatic valve endothelial cells. According to accumulating evidence, LECs play a crucial role in the immune system providing functions such as recruitment of dendritic cells and lymphocytes, maintenance of T cell survival, antigen presentation and immune tolerance. (Takeda et al., 2019)

Blood endothelial cells (BECs) form the inner lining of blood vasculature and therefore are in direct contact with the components of blood (Krüger-Genge et al., 2019). They have many functions including the control of coagulation, thrombolysis, vascular tone, permeability, angiogenesis and inflammation (Pusztaszeri et al., 2006). In addition, a recent study has shown that the BECs in the spleen are transcriptionally diverse and contain genes involved with scavenging and lipid metabolism. This finding suggests potential significance of the cells in maintaining splenic homeostasis. (Alexandre & Mueller, 2023)

Mucosal addressin cell adhesion molecule 1 (MAdCAM-1) is a novel BEC marker that is known for lymphocyte-endothelial interactions in the process of leukocyte recirculation. Numerous studies have indicated that continuous recirculation of lymphocytes between the blood and lymphoid organs is required to distribute the entire range of antigens throughout the body to enable efficient immune responses. MAdCAM-1 is expressed in some high endothelial venules (HEVs) as well as flat-walled venules of the lamina propria in the gut. (Salmi et al., 2001) In the spleen, stromal cells that surround the B-cell follicles and periarteriolar lymphoid sheath are positive for MAdCAM-1 (Alexandre and Mueller, 2023). In addition, the expression of MAdCAM-1 has been discovered in the sinus-lining cells of the perifollicular zone. Therefore, lymphocytes entering the white pulp have to pass through the rim of cells, which could indicate a further functional role for this molecule in the spleen. (Kraal et al., 1995)

In addition, to specific markers for LECs and BECs, there are also markers that are commonly used for ECs in both blood and lymphatic vessels. For example, CD31 and CD34 are used as common endothelial cell marker expressed in both vessel types. According to research done with the spleen, sinusoidal ECs are diffusely positive for CD31 and in red pulp CD34 marks capillaries. (Pusztaszeri et al., 2006)

3. Materials and methods

3.1 Tissue samples

The spleen samples were collected in collaboration with Turku University Hospital from patients undergoing surgery for pancreatic cancer. In order to reach the caput of the pancreas a part of the spleen had to be removed. All the patients gave their consent for the use of surgical resection material in research purposes. In addition, the samples were confirmed to be histologically normal according to pathological examinations. The tissue samples were preserved in 4% PFA at +4°C and some were frozen in OCT blocks with O.C.T.™ compound (Tissue-Tek®, 4583).

6-µm-thick spleen sections were cut from frozen OCT blocks using a cryostat. Also, 50 and 500-µm-thick sections were cut with a vibratome. For vibratome cutting spleen samples were cut into 1x1cm pieces and embedded in 4% low melting temperature agarose (Lonza, Cat. No. 50080) using a plastic mold. The vibratome was set to 0.36 mm/s speed, 1.0 mm amplitude and 50µm or 500µm auto feed (slice thickness). Serial sections were cut each time changing from 50µm to 500µm in between.

3.2 Immunofluorescence staining on 6-µm-thick human spleen sections

Immunofluorescence stainings were performed on 6-µm-thick tissue sections. Fixation was done by immersing the slides with spleen sections in cold acetone for 5 minutes. Next, the slides were left to dry at RT for 10 minutes. Then they were washed in 1xPBS for 2x5minutes at RT. A blocking solution of 10% FBS (Sigma Aldrich, S160B-500) and 90% BSA (ICN Biomedicals, 810034) (0.5% in PBS) was used to cover the sections for 1 hour at RT. Blocking was performed to prevent nonspecific binding of antibodies.

After blocking the tissue sections were stained with an antibody solution. The solution contained the following primary unconjugated antibodies: LYVE-1 1/100 (r&D Systems AF2089), MAdCAM-1 1/3 (in-house) and CD34 1/100 (abcam, ab81289). The antibodies were diluted in blocking solution. LYVE-1 was used to locate the red pulp of the spleen and CD34

as a common endothelial cell marker. MAdCAM-1 was used to characterize specific venules. The staining was performed overnight in a dark moisture chamber at +4°C.

After the overnight incubation, the slides were washed in 1xPBS for 3x5 minutes at RT. Then a secondary antibody mix was prepared in blocking solution. The secondary antibodies included: donkey anti-mouse A488 1/400 (Invitrogen, A32766), donkey anti-rabbit A555 1/400 (Invitrogen, A32794) and donkey anti-goat A647 1/400 (Invitrogen, A32849). The samples were incubated for 1 hour in a dark moisture chamber at RT.

When the tissue sections had been stained, they were washed once more in 1xPBS for 3x5 minutes at RT. Then the slides were mounted to ensure preservation of the samples. The nuclei were stained using NucBlue Live Cell stain (Thermo Fisher Scientific, R37605). After this, ProLong™ Gold antifade reagent (Life Technologies Corporation, P36930) was added simultaneously with a coverslip. The slides were preserved at +4°C in the dark and imaged within a week with the Panoramic MIDI Scanner. The imaging was completed with a 20x objective. The laser excitation times were 30ms for nuclei staining, 800ms for MAdCAM-1, 200ms for LYVE-1 and 600ms for CD34.

3.3 Immunofluorescence staining on 50-µm-thick human spleen sections

Immunofluorescence staining on 50-µm-thick tissue sections was performed in order to stain blood and lymphatic-like vasculature as well as lymphatic valves. In contrast to 6-µm-thick tissue sections, these had to be stained in wells. Firstly, the samples were washed 3x5 minutes in 1xPBS on a nutator at RT. A blocking solution of 10% FBS and 90% BSA (0,5% in PBS) was used to cover the sections for 1 hour at RT.

After blocking the tissue sections were stained with an antibody mixture diluted with blocking solution. The first solution contained the following primary unconjugated antibodies: LYVE-1 1/100 (r&D Systems, AF2089) and MAdCAM-1 1/3 (in-house). The second solution contained primary conjugated Podoplanin A647 1/100 (BioLegend, 337007) and unconjugated Claudin-11 1/100 (Sigma Aldrich, HPA013166). The staining was performed overnight protected from light at +4°C. Once the incubation was complete, the tissue sections were washed for 3x10minutes in a solution containing 0.5% Triton™X-100 (Sigma-Aldrich,

SLCJ7494) and 99.5% BSA (0.5% in PBS). Triton was added to reduce the surface tension of the water-based solution. After washing a secondary antibody solution was prepared in the usual block of 10% FBS and 90% BSA (0,5% in PBS). The secondary antibodies included donkey anti-mouse A488 1/400 (Invitrogen, A32766) and donkey anti-goat A557 1/400 (r&D Systems, NL001). For Claudin-11 donkey anti-rabbit A488 1/400 (Invitrogen, A32790) was used as the secondary antibody. The samples were incubated in the secondary antibody solution overnight at +4°C protected from light.

After the incubation, the samples were washed for 2x10minutes in the washing solution containing BSA and Triton. For the third wash, a 1:5000 dilution of DAPI (Invitrogen, D1306) in the washing solution was prepared. The third wash containing DAPI lasted for 10 minutes. Lastly, the spleen sections were mounted onto glass slides by adding ProLong™ Gold antifade reagent simultaneously with two spacers and a coverslip. The slides were preserved at +4°C in the dark and imaged within a week with Leica Stellaris 8 Falcon FLIM. The name of the objective was HC PL APO CS2 20x/0.75 DRY and the pinhole was 56.6µm. Four laser lines were used: 405nm, 494nm, 553nm and 653nm. 24 Z-stacks with 1.49µm interval were taken with 13619 x 13572 tiles (0.11 x 0.11 pixel size) 1494.26µm x 1489.1µm tile area.

3.4 FLASH

3.4.1 Depigmentation and antigen retrieval

The spleen sections had been cut with a vibratome to a thickness of 500µm. They were stored in 1xPBS for approximately a week before starting the FLASH protocol. As the spleen is considered a high pigmented tissue, the first step of the protocol is depigmentation of the sample. To start the sections were washed in wells for 3x5minutes on a nutator (Gerhardt Bonn) with speed 30 at RT. A depigmentation solution was prepared from DMSO (Sigma-Aldrich, 67-68-5) and H_2O_2 (Fisher Scientific, 7722-84-1) in PBS with a ratio of 1:1:4. After washing, the samples were incubated in depigmentation solution overnight at RT covered from light.
Once the incubation had finished, the tissue sectiones were washed 3x20minutes with rotation at RT. An antigen-retrieval solution was prepared with 4% (wt/vol) SDS (Acros Organics, 151-21-3) in 200mM borate (Sigma-Aldrich, B6768-500G). The samples were incubated in the

antigen-retrieval solution for 1 hour at RT with rotation. Lastly, the samples were moved to a hybridizing oven (Hybaid) and incubated overnight (<16 hours) at +54°C.

3.4.2 Blocking and immunolabeling

The tissue sections were washed 3x1 hour at RT with rotation in a washing solution containing 0.2% Triton™X-100 (Sigma-Aldrich, SLCJ7494) diluted in PBS (PBT). Meanwhile, a blocking buffer was prepared with 10% (vol/vol) FBS, 1% (wt/vol) BSA, 5% (vol/vol) DMSO in PBT. The buffer was also filtered using a sterile 0.22-µm filter (Millex® GS, Ref No. SLGSR33SS) to purify the solution from BSA aggregates. After the washing was completed, the samples were incubated for 1 hour in blocking buffer at RT with rotation. A primary antibody solution was prepared in the blocking buffer. The antibodies included: LYVE-1 1/100 (r&D Systems, AF2089), MAdCAM-1 1/3 (in-house) CD34 1/100 (abcam, ab81289) and Podoplanin A647 1/100 (BioLegend, 337007). The tissue sections were covered from light and incubated in the staining solution for 2 nights with rotation at RT.

Once the incubation was done, the samples were washed 3x20 minutes in PBS. A secondary antibody solution was prepared in the previously described blocking buffer. The secondary antibodies included: donkey anti-rabbit A488 1/400 (Invitrogen, A32790), donkey anti-mouse A555 1/400 (Invitrogen, A32773) and donkey anti-goat A750 1/400 (abcam, ab175744). The tissue sections were covered from light and incubated in the staining solution for 2 nights with rotation at RT.

3.4.3 Dehydration, clearing and mounting

The tissue sections were washed in PBS 3x20 minutes at RT with rotation. After washing they were placed in 1.2% low melting agarose (Lonza, 50080). After the agarose had solidified, they were gradually dehydrated with 30%, 50%, 75% and 2x100% (vol/vol) MetOH (Fisher Scientific, 67-56-1) in mQH_2O. Each step lasted 30 minutes and was done at RT with rotation. Next, the samples were gradually cleared using 25%, 50%, 75% and 2x100% (vol/vol) MetSal (Sigma Aldrich, 119-36-8) in MetOH, 30 minutes per step at RT with rotation.

Once the samples were dehydrated and cleared, they were mounted onto glass slides (Epredia, Ref. J1800AMNZ) with two 500-µm-thick silicone isolators (Electron Microscopy Sciences, 70337-70) on top of each other. A few drops of MetSal were added to keep the sample immersed in liquid and a 24x50mm cover glass (VWR, 631-0146) was placed on top. The edges were sealed with nail polish to prevent any leakage of liquid onto the microscope.

3.4.4 Imaging and 3D rendering

A confocal microscope, Leica Stellaris 8 Falcon FLIM, was used to image the 500-µm-thick tissue sections. The name of the objective was HC PL APO CS2 10x/0.40 DRY and the pinhole was 52.8µm. Two laser lines were used: 555nm and 728nm. 184 Z-stacks with 2,4µm interval were taken with 2048 x 2048 tiles (0.757 x 0.757 pixel size) 1550µm x 1550µm tile area.
Three-dimensional rendering and image processing were completed in Leica Application Suite LAS X 3D Software version 4.7.0. Background noise reduction was adjusted to 0.80 and deconvolution to 3.

4. Results

4.1 500-µm-thick human spleen section after tissue clearing

The spleen is a heme-rich and highly pigmented organ. These qualities could be considered as reasons why conventional methods to visualize structures deep within the organ have been limited. Tissue clearing protocols, such as the FLASH, have made it possible to visualize splenic vasculature with good quality. They have also enabled thicker tissue sections to be exploited. With thicker tissue sections, we acquire more reliable results of how the structures are located in relation to each other.

In the representative images, the cleared splenic tissue has been stained with anti-MAdCAM-1 visualized in green and anti-LYVE-1 seen in grey (figure 3). The picture emphasizes the red pulp of the spleen and more precisely a MAdCAM-1 positive venule. The main focus is the connection between LYVE-1 positive red pulp sinusoids and a MAdCAM-1 positive vessel. Unfortunately, there is significant autofluorescence in green, which disguises some of the grey sinusoids in this particular area.

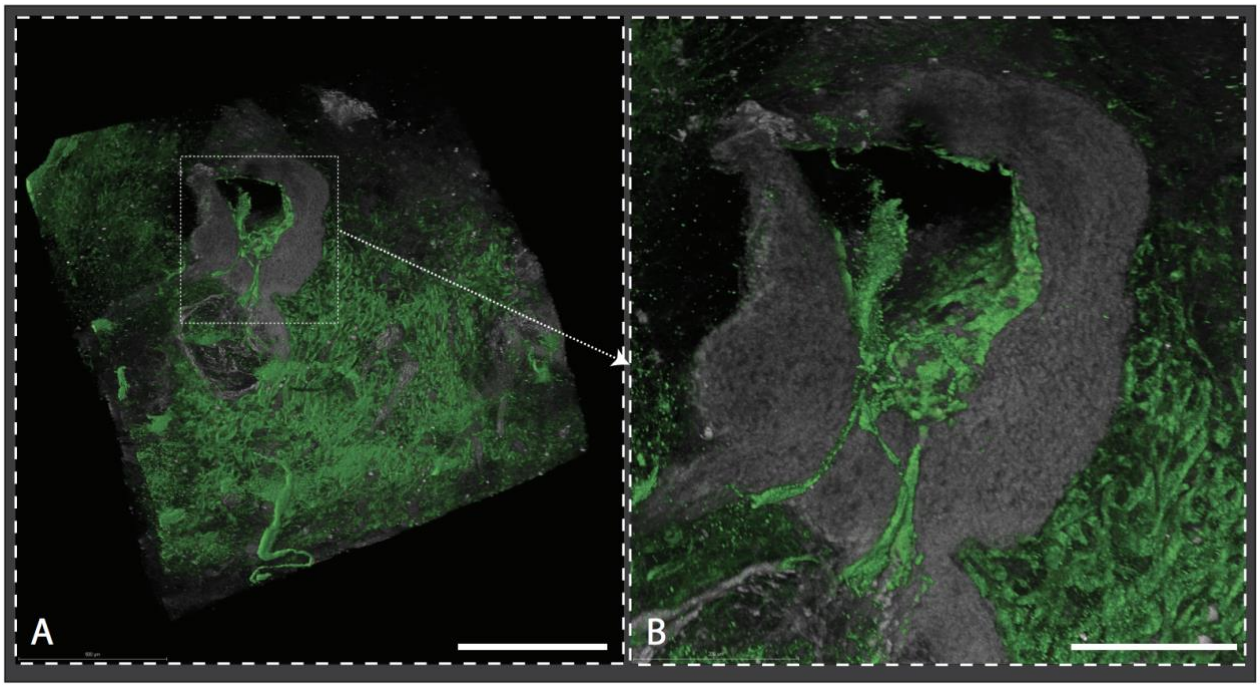

Figure 3. 500-µm-thick human spleen section after tissue clearing with FLASH. MAdCAM-1 is demonstrated in green and LYVE-1 in grey. Scale bars: A 500 µm and B 200 µm

4.2 Immunofluorescence of a 6-µm-thick human spleen section with MAdCAM-1 and LYVE-1 double positive vessels

As mentioned in chapter 2.1 the spleen is divided into two anatomical compartments: the red and white pulp. The red pulp consists of venous sinusoids and cords that form the open blood circulation. In figure 4, these sinusoids can be seen in orange as they are positive for LYVE-1. In addition, we can see multiple structures that are double positive for LYVE-1 and MAdCAM-1 (green), which are displayed in yellow. In figure 4A, it seems as though one side of the vessel is double positive while the other is only LYVE-1 positive.

MAdCAM-1 is also positive for stromal cells around B-cell follicles in the white pulp. However, it lies beside our interest as it has already been discovered. CD34 (red) is a common endothelial cell marker that demonstrates blood vasculature in the spleen and therefore, positive in veins. Our aim is to show the transition between red pulp sinusoids and veins that lie through novel MAdCAM-1 positive vessels.

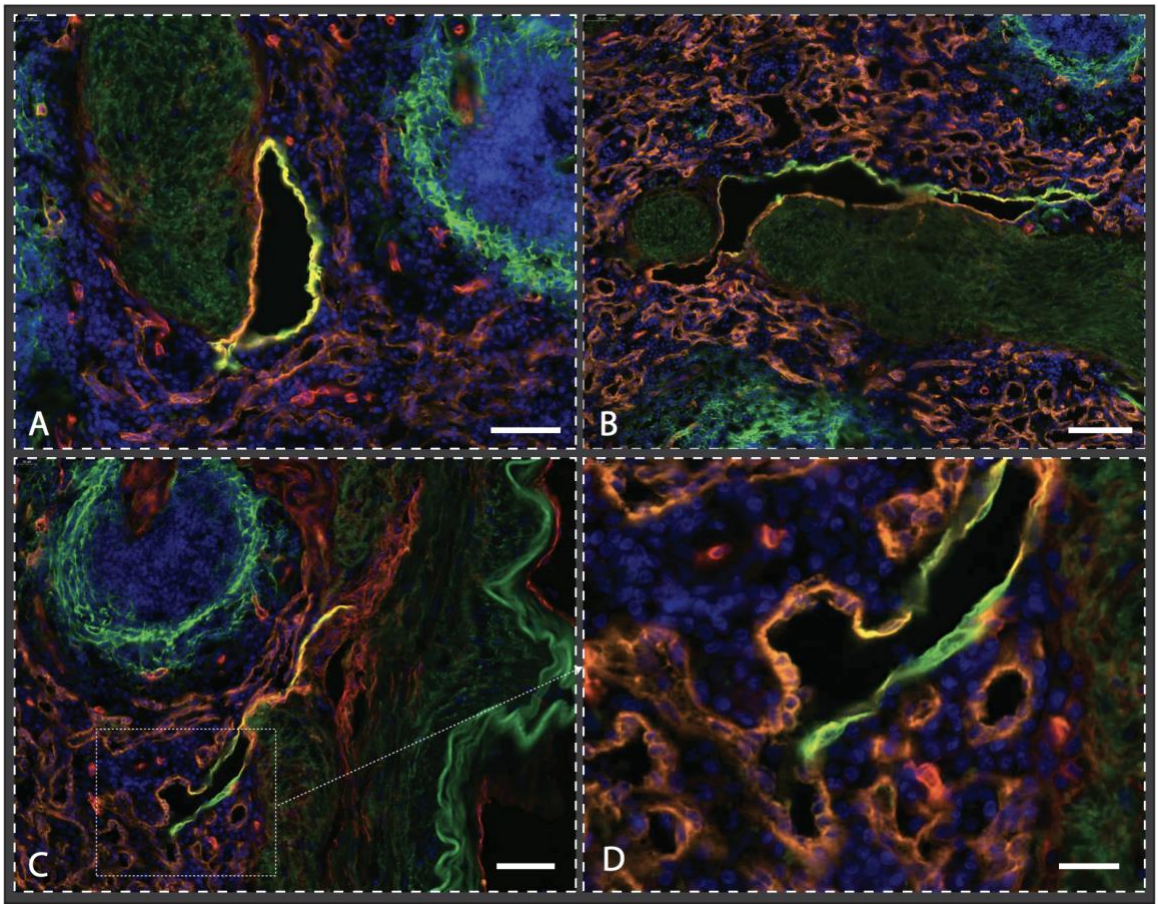

Figure 4. Immunofluorescence of a 6-µm-thick human spleen section with MAdCAM-1+ (green) vessel, LYVE-1+ (orange) red pulp sinusoids, CD34+ (red) blood vasculature and DAPI (blue) Scale bars: A and C 60 µm, B 100 µm, D 20 µm.

4.3 Immunofluorescence of 50- and 500-µm-thick human spleen sections with connection between MAdCAM-1+ and LYVE-1+ vessels

These three-dimensional images demonstrate the connection between MAdCAM-1 positive vessels and LYVE-1 positive red pulp sinusoids. In figure 5, a 50-µm-thick human spleen section was stained without clearing. The image demonstrates a larger MAdCAM-1 positive venule (green) which is merging into smaller LYVE-1 positive sinusoids shown in red. As mentioned before, in addition to red pulp structures, a white pulp B-cell follicle with MAdCAM-1 positive stromal cells around is noticeable.

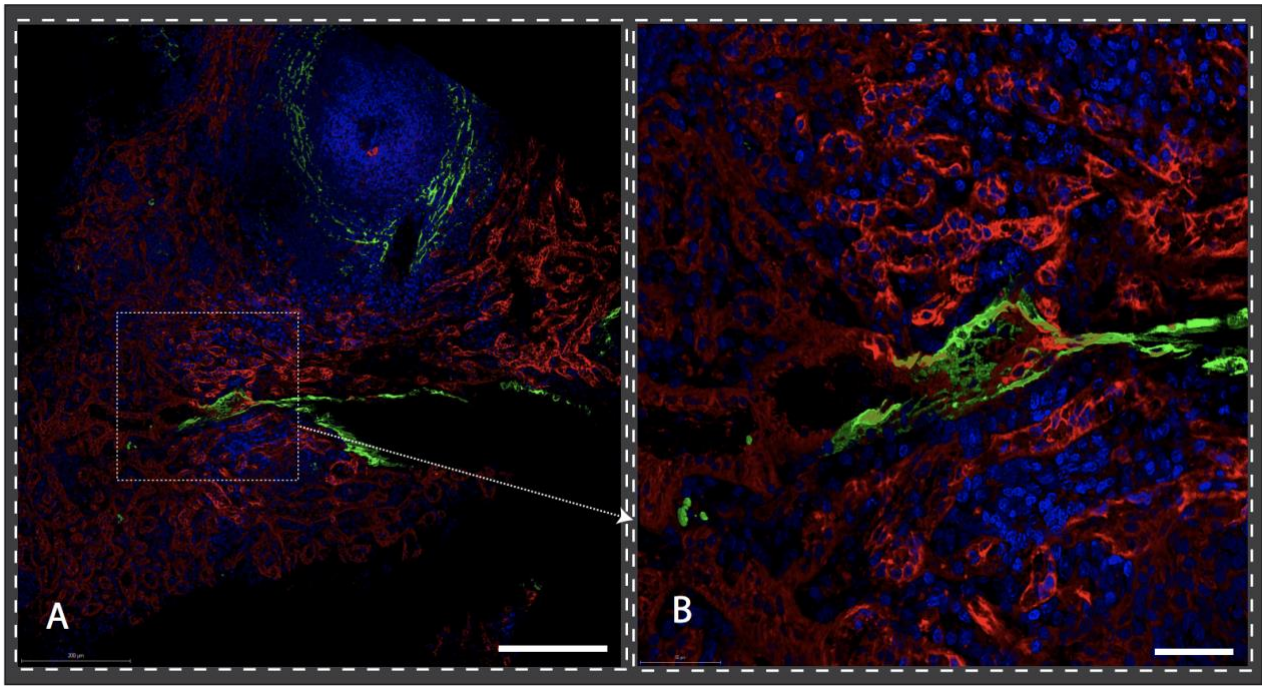

Figure 5. Immunofluorescence of a 50-µm-thick human spleen section with MAdCAM-1 (green), LYVE-1 (red) and DAPI (blue). Scale bars: A 200 µm and B 50 µm.

In figure 6, a 500-µm-thick human spleen section was stained using the FLASH protocol for clearing. This provided us with a three-dimensional image with more depth. The image demonstrates a connection between a larger LYVE-1+ vein and the red pulp sinusoids. The

pathway between these structures appears as a yellow MAdCAM-1 positive venule. In addition to real signal, autofluorescence is noticeable in yellow. It can be explained by sample thickness.

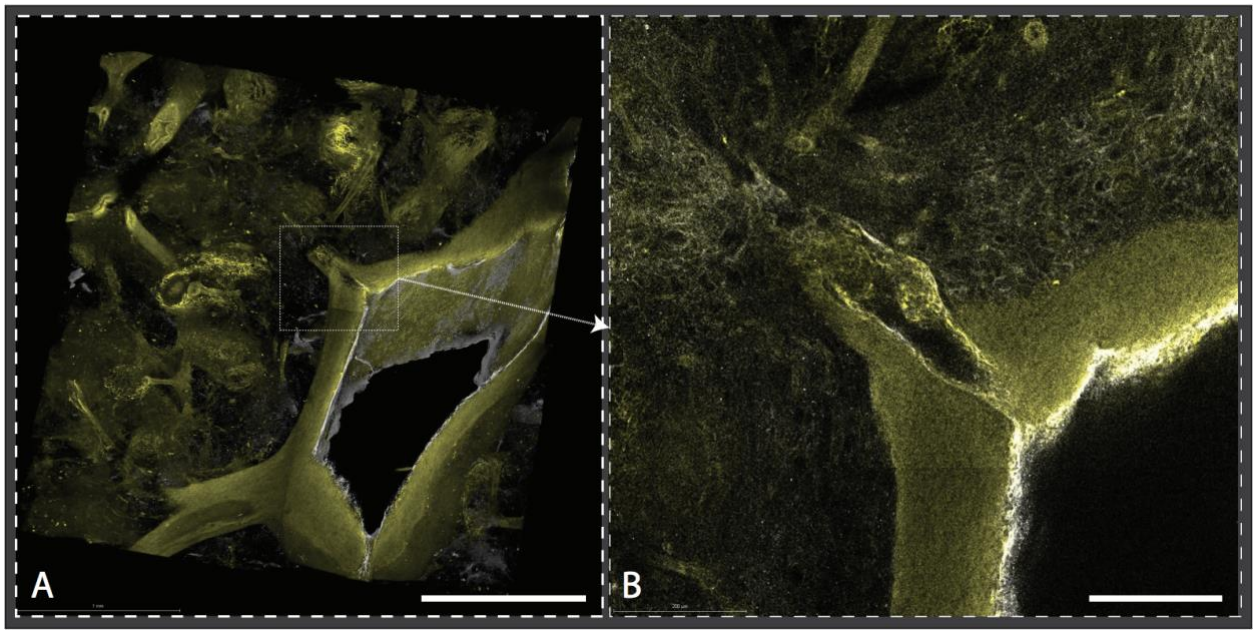

Figure 6. Immunofluorescence of 500-µm-thick cleared human spleen section with MAdCAM-1 (yellow) and LYVE-1 (white). Scale bars: A 1 mm and B 200 µm

4.4 Immunofluorescence of a 50-µm-thick human spleen section with PDPN+ lymphatic-like vessels and CLDN-11+ lymphatic valves

According to research, the human spleen lacks afferent lymphatic vessels, which suggests that lymphocytes enter the organ through blood circulation. On the other hand, it is still unclear how lymphocytes travel out of the organ and inside of it. Experiments on the human spleen have not been conducted to confirm the existence or absence of efferent lymphatic vessels. In figure 7, small vessels in the vascular sheath of an artery seem to express PDPN in red, which is a common lymphatic marker. To confirm that these vessels could be related to lymphatics, the lymphatic valves were stained with anti-CLDN-11. As expected, the valves appear to be present inside these vessels.

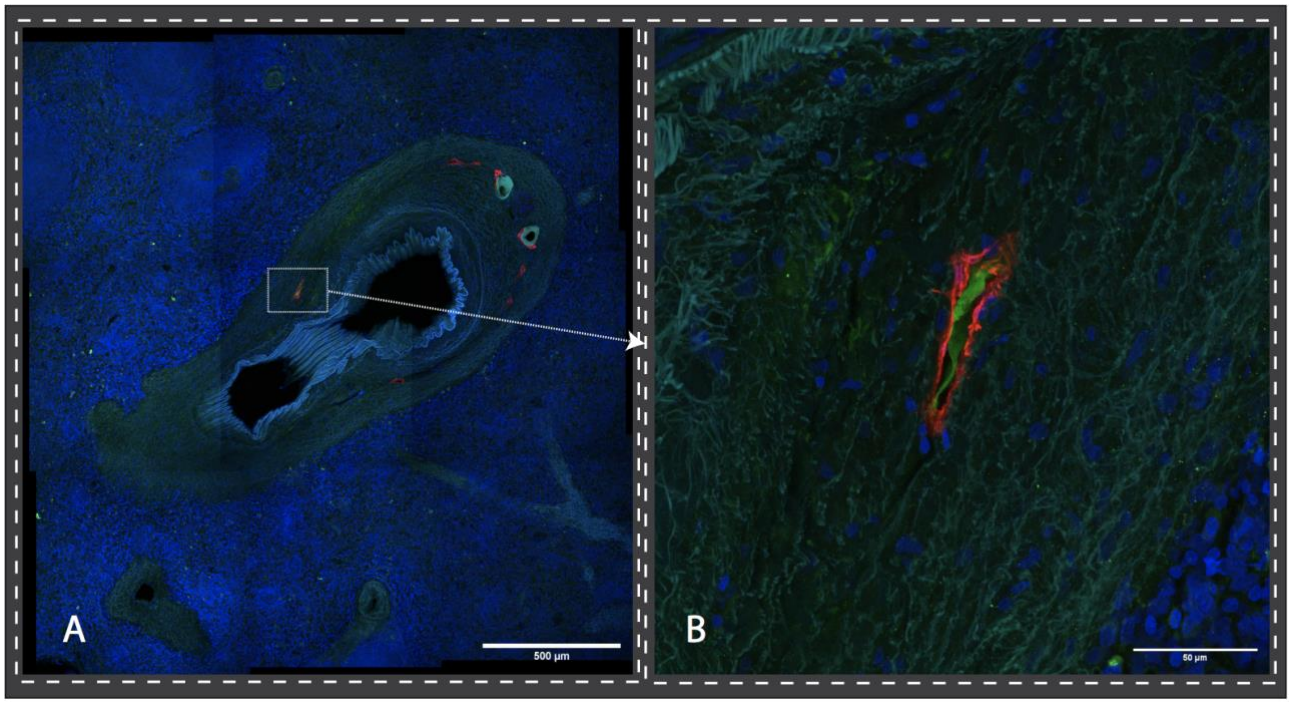

Figure 7. Immunofluorescence of a 50-µm-thick human spleen section with CLDN-11 (green), PDPN (red) and DAPI (blue). Scale bars: A 500 µm and B 50 µm.

5. Discussion

The spleen is an encapsulated and highly vascularized organ with significant roles in homeostasis and systemic immunity in the human body. Interestingly, it consists of an open blood system and is believed to lack afferent lymphatic vessels. (Alexandre & Mueller, 2023) In fact, recent research suggests that the mouse spleen lacks lymphatic vessels in general. This conclusion is based on single-cell RNA sequencing results that indicate the deficit of typical LEC markers in the mouse spleen. (Kalucka et al., 2020) Even though mice have been used as a common model to explain the mechanisms of the immune system, they have significant limitations when it comes to their ability to be informative regarding aspects such as genetic heterogeneity or microbial influences. Especially, striking differences in the structure of the spleen have emerged in studies comparing the organ between mice and humans. (Medetgul-Ernar & Davis, 2022)

One major difference in the structure of the spleen is the border between white pulp and red pulp, which is called the perifollicular zone in humans and the marginal zone in mice. This region differs especially in B-cell populations and layering. (Lewis et al., 2019) In addition,

the composition of the white pulp follicles is different. In the human spleen T cells are distributed in a diffuse manner in the germinal centers, whereas in mice they form discrete clusters. Whether these significant differences have an impact on immune responses is unknown for now. (Medetgul-Ernar & Davis, 2022) It has to be noted that the applications of advanced imaging techniques to the human spleen have been limited and therefore the extent of analogy between the organ in mice and humans is still not clear. Consequently, the current knowledge based on mouse data should be questioned and researched further.

The heme-rich and highly pigmented structure of the spleen could be considered as one of the reasons why advanced imaging techniques remain to be limited. In order to overcome this issue, we have used a relatively new tissue clearing protocol known as FLASH. As mentioned in the methods, it involves a depigmentation step in order to improve the transparency of the sample and the quality of images. This 7-day protocol had to be optimized in order to achieve desirable results. As a limitation, the protocol is suitable for only 500-µm-thick samples, which makes up a small fraction of the entire human spleen.

One of the main aims of the study was to prove that certain antibodies can be used with the FLASH tissue clearing protocol. As seen in figure 3 we were able to obtain three-dimensional images of 500-µm-thick human spleen sections that express MAdCAM-1 and LYVE-1. This proves that these markers are suitable for this specific clearing technique. We also attempted to image PDPN-positive vessels using this clearing method; however, the antibody did not work in these conditions, resulting in no signal being detected. In addition, a control sample without antibody staining was used to evaluate the amount of autofluorescence from the spleen. We concluded that channel 488 exhibited a significant level of autofluorescence and, as a result, decided to eliminate this channel from our images. Autofluorescence can also be seen in channel 555 with the MAdCAM-1 staining. The thickness of the sample also contributes to the amount of autofluorescence detected.

Another important aim of this project was to visualize vascular structures in the human spleen. Surprisingly, we found multiple connections between MAdCAM-1 and LYVE-1 positive vessels. In figure 4, several two-dimensional vessels with double positivity for these markers appear in yellow. It seems as though one side of the vessel expresses a single marker while the other side is double positive. This finding could suggest that MAdCAM-1 has a role in lymphocyte homing and trafficking in the vessel. In figure 4D, the merge of MAdCAM-1

positive vessel into the LYVE1+ red pulp sinusoid can be seen. The aim is to show the transition between red pulp sinusoids and veins that lie through novel MAdCAM-1 positive vessels.

The connection between MAdCAM-1+ vessels and LYVE-1+ red pulp sinusoids is visualized in more detail with three-dimensional imaging. While figure 4 shows only one plane of the sample, figure 5 shows multiple stacks of planes. This makes it more reliable to evaluate how the structure of the vessels is formed. Figure 5 shows the merging of MAdCAM1+ vessel into the red pulp sinusoid. However, in figure 6 we can detect how the MAdCAM-1+ vessel connects to the LYVE-1+ venule. This indicates a role for the MAdCAM-1+ vessel to function as a connection between the red pulp and the venule. As reviewed in chapter 2.4 MAdCAM-1 is also expressed by mucosal venules and has a functional role directing lymphocyte traffic into Peyer's patches and lamina propria in the intestines. Perhaps the molecule could have similar functions in the spleen.

Lastly, we decided to attempt to image some lymphatic-like vessels in the human spleen. Even though we did not obtain successful results with PDPN in cleared tissue, we managed to visualize it in 50-µm-thick spleen sections as shown in figure 7. Figure 7A suggests that the location of PDPN+ vessels is around arteries in the vascular sheath. Moreover, the anti-CLDN-11 staining seen in figure 7B demonstrates lymphatic valves inside the vessel. As reviewed in chapter 2.3, lymphatic valves are commonly recognized in efferent collecting lymphatic vessels. Lymphatic-like vessels could contribute to the trafficking of lymphocytes out of the organ. This finding is significant as lymphatic-like vessels have not been confirmed to exist in the human spleen. Further studies need to be conducted to figure out their precise architecture and function.

In conclusion, with the patient samples acquired from the Turku University Hospital, we were able to visualize novel vascular structures in the human spleen using two- and three-dimensional imaging techniques. Surprisingly, we managed to image lymphatic-like vessels in the spleen utilizing lymphatic valves as proof. This finding is important as these vessels have not been visualized in the human spleen before. In addition, we optimized the tissue clearing protocol for a highly pigmented organ and proved that antibodies such as LYVE-1 and MAdCAM-1 are compatible with the protocol. This study provides essential information on the connection between LYVE-1+ and MAdCAM-1+ vessels in the human spleen. Moreover,

the location of MAdCAM-1+ vessels in between venules and the red pulp sinusoids indicates a further functional role for MAdCAM-1 molecule in the spleen. In the future, more studies should be conducted in order to discover the specific function of these MAdCAM-1 positive vessels in lymphocyte trafficking. All in all, these results contribute to the understanding of the precise architecture of splenic vasculature, which is essential for studies on various functions of the human spleen.

6. References

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