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Liberation and Phenotyping of monocytic cells in human venous tissues

Freisetzung und Phänotypisierung von monocytären Zellen in humanen venösen Geweben

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I dedicate this thesis to my husband and my family.

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LIST OF ABBREVIATIONS

°C	degree Celsius
%	percent
µg	microgram
µl	microliter
Aqua dest.	Aqua destillata, distilled water
ASA classification	American Society of Anesthesiologists physical status classification system
BV	bypass vein
BVx	Brilliant Violet x
CA	California
CDx	cluster of differentiation
CD99L2	CD99 antigen-like protein 2
CSFx	colony stimulating factor x
CSFxR	colony stimulating factor x receptor
CVD	chronic venous disease
CEAP classification	Clinical-Etiology-Anatomy-Pathophysiology classification
CHIVA	Cure Conservatrice et Hémodynamique de l´Insuffisance Veineuse en Ambulatoire
CVI	chronic venous insufficiency
DAMP	damage-associated molecular pattern
DNase	deoxyribonuclease
EDTA	ethylenediaminetetraacetic acid
FACS	Fluorescence Activated Cell Sorting
FFPE	formalin-fixed paraffin-embedded
GB	Great Britain
GmbH	Gesellschaft mit beschränkter Haftung
GM-CSF	granulocyte-macrophage colony-stimulating factor
HBSS	Hank´s Balanced Salt Solution
HEPES	2-(4-(2-Hydroxyethyl)piperazin-1-yl)ethane-1-sulfonic acid
HFE	hemochromatosis gene
HLA	human leukocyte antigen
HSA	human serum albumin, Human Albumin 20%, salzarm

Ig	immunoglobulin
Inc.	Incorporation
JAM	junctional adhesion molecule
JAML	junctional adhesion molecule-like
KLF4	Kruppel-like factor 4
LFA-1	lymphocyte function-associated antigen 1
Ltd.	limited
MA	Massachusetts
mAb	monoclonal antibody
Mac-1	Macrophage-1 antigen
mg	milligram
MHC	major histocompatibility complex
min	minutes
mL	milliliter
mm	millimeter
mmHg	millimeter mercury column
MMP	matrix metalloproteinase
MN	Minnesota
mRNA	messenger ribonucleic acid
MTS-VES	Masson’s trichrome stain with Verhoeff’s elastica stain
MO	Missouri
NaCl	sodium chloride
NADHP	nicotinamide adenine dinucleotide phosphate
NaH_2PO_4 x·$2H_2O$	sodium phosphate monobasic dihydrate
nm	nanometer
NJ	New Jersey
NY	New York
ICAM-1	intercellular adhesion molecule 1
IL	Illinois
IL-x	interleukin-x
IRF8	interferon regulatory factor 8
PA	Pennsylvania
PAMP	pathogen-associated molecular pattern

PB	Pacific Blue
PBMC	peripheral blood mononuclear cell
PBS	phosphate-buffered saline
PECAM-1	platelet endothelial cell adhesion molecule
PRR	pattern recognition receptor
Prof.	professor
PSGL-1	P-selectin glycoprotein ligand-1
s	seconds
TIPP	transilluminated powered phlebectomy
TNF-α	tumor necrosis factor alpha
TLR4	toll-like receptor 4
USA	United States of America
WA	Washington
WI	Wisconsin
VCAM-1	vascular cell adhesion protein 1
VE-cadherin	vascular endothelial-cadherin

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1. INTRODUCTION

Chronic venous disease is a common disease affecting the general population. Varicose veins can be one of the many, visible symptoms of chronic venous disease.

In general, the venous system contains at least 60 % of the resting blood volume of which half is in the postcapillary venules in the lower extremity (Wittens et al., 2015). The venous system in the lower extremities consists of a superficial and deep system connected through perforator veins as shown in Figure 1.

Anatomically, among others, the femoral vein, deep femoral vein, popliteal vein and tibial veins form the deep venous system in the lower extremity (Schünke, 2014). The superficial vein system is formed mainly by great saphenous vein, small saphenous vein and femoral-popliteal vein (Schünke, 2014). Perforating veins are anatomically grouped by location. The Dodd's group is located in the middle third of the upper leg. The Boyd's group is located below the knee and the Cockett's group is located in the lower leg.

Valves are directing the venous flow from the superficial system into the deep system which drains the majority of the venous blood. Primary varicose veins are characterized by a retrograde circuit or a venous-venous shunt caused by valve dysfunctions (Goren 1996).

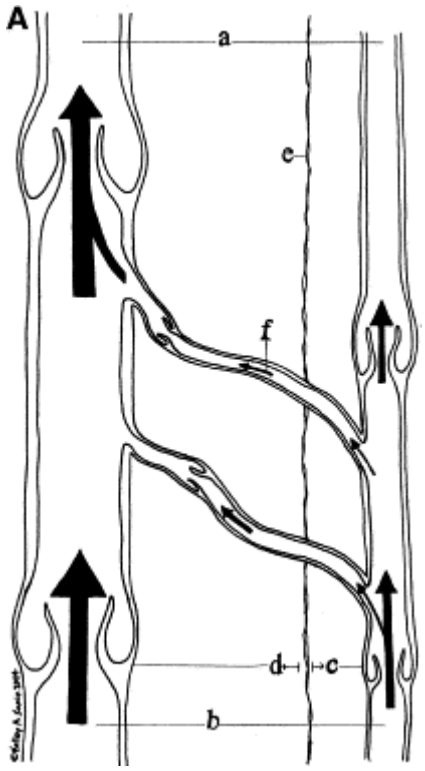

Figure 1: Overview of the venous system of the lower extremity with normal vein flow: (a) proximal, (b) distal, (c) superficial compartment and vein, (d) deep compartment and vein, (e) muscle fascia, (f) flow from superficial to deep veins. The Epidemiology of Chronic Venous Insufficiency and Varicose Veins (Annals of Epidemiology, 15) by Beebe-Dimmer J. et al. Copyright 2004 by Elsevier Inc. Reproduced with permission of Elsevier Inc. in the format of print and electronic via Copyright Clearance Center.

To accurately diagnose chronic venous disease and determine the severity of the disease a diagnostic work up including a detailed patient history, physical examination and duplex ultrasound as gold standard diagnostic is necessary (Krasinski et al., 2021, De Maeseneer et al., 2022). Duplex ultrasound is a noninvasive method allowing to examine the superficial venous system (Krasinski et al., 2021). Standardized duplex ultrasound examination should be performed in a physiological standing position with external rotation of the examined limb in a relaxed position (Wittens et al., 2015). Varicose veins are defined by superficial reflux lasting longer than 0.5 seconds and vessel diameter > 3.5 mm (De Maeseneer et al., 2022). In addition to a written duplex ultrasound report ‘venous mapping’ is recommended as accurate graphical representation of the findings (De Maeseneer et al., 2022). Other examples of diagnostic methods for special indications are magnetic resonance venography, computed tomography venography, intravascular ultrasound, photoplethysmography and venous occlusion plethysmography (De Maeseneer et al., 2022, Pannier, 2019).

There are a variety of treatment options available for chronic venous disease. Varicose veins are normally treated with phlebectomy or foam sclerotherapy (De Maeseneer et al., 2022).

Although chronic venous disease can be diagnosed and treated, the etiology of chronic venous disease is still not fully understood. It has been hypothesized that valvular incompetence is caused by inflammatory phenomena leading to changes in the venous valves causing reflux (De Maeseneer et al., 2022). So far mostly histological studies have been performed to gain a further insight into the immune cells present in the tissue (Buján et al., 2008, Cherian et al., 1999, Chu et al., 2013, Sayer and Smith, 2004). This study strives to provide further insight into the cell types using flow cytometry.

The human immune system consists of the innate and adaptive immune system (Austermann et al., 2022). The innate system can detect the general characteristics of pathogens and acts rapidly (Austermann et al., 2022). The innate immune system is comprised of different cell types including myeloid cells such as of monocytes, macrophages, dendritic cells and granulocytes (Austermann et al., 2022). The adaptive immune system, comprised of B cells and T cells, recognizes all kinds of antigens and provides immunological memory (Austermann et al., 2022). The immune system functions plays a causative or indirect role in homeostasis and in any kind of human disease (Austermann et al., 2022). As the innate system can detect pathogens through general characteristics via preserved receptors, it initiates the adaptive immune response (Austermann et al., 2022).

This study focuses on monocytic cells. Monocytes are bone-marrow-derived mononuclear cells in the blood which among other functions have been shown to orchestrate the immune response (Austermann et al., 2022). Macrophages reside in every tissue of the body and have diverse functions (Austermann et al., 2022). There have also been described tissue-resident macrophages with the ability to self-renew (Austermann et al., 2022, Davies et al., 2013). Some of these cells with self-renewal capacity have been shown to seed in the tissues during embryonic development (Ginhoux and Jung, 2014). Another possibility of replenishment of macrophages is the differentiation of monocytes when entering tissues (Austermann et al., 2022). It is organ-dependent to what degree macrophages are replenished by bone marrow-derived monocytes (Nobs and Kopf, 2021). Monocytes and macrophages can both change their phenotypes according to the microenvironment in the tissue (Austermann et al., 2022). In a basal state the cells execute homeostatic functions while they convert into a proinflammatory state in inflammation and an anti-inflammatory phenotype to resolve inflammation and restore homeostasis (Austermann et al., 2022). One organ can have multiple specialized macrophage

populations fulfilling specific functions e.g., the central nervous system where microglia as well as choroid plexus macrophages can be distinguished in humans (Nobs and Kopf, 2021).

1.1. Aim of this thesis

Previous histological studies showed the presence of immune cells in varicose veins and bypass vein grafts (Buján et al., 2008, Cherian et al., 1999, Chu et al., 2013, Sayer and Smith, 2004). Luther et al. (2016) further investigated immune cells in varicose veins focusing on T cells using flow cytometry.

This study intended to investigate the composition of inflammatory cell infiltrate in varicose veins and human bypass vein grafts. Single cell suspensions were generated adapting two different digestion protocols. Thereafter, inflammatory cell infiltrates were further phenotyped using flow cytometry.

2. DISSCUSION OF LITERATURE

2.1. Chronic venous insufficiency

The term *chronic venous insufficiency (CVI)* has to be separated from the term *chronic venous disease (CVD)*. Chronic venous disease is defined as signs and symptoms of long-term abnormalities of the venous system indicating the need for further investigation or care (Eklof et al., 2009). In contrast chronic venous insufficiency refers to disease of greater severity defined as classes C_3 to C_6 of the CEAP classification (Bergan et al., 2006, Eklöf et al., 2004, Eklof et al., 2009).

The CEAP classification system was established in 1994 (Lurie et al., 2020, Rabe and Pannier, 2012). As a descriptive classification it was established to unify clinical and scientific communication (Lurie et al., 2020) and is gold standard for classification of CVD (Rabe and Pannier, 2012). For evaluative classification other scores have been developed (Lurie et al., 2020, Vasquez and Munschauer, 2008). The revised Venous Clinical Severity Score is the most widely used clinical scoring tool designed as a dynamic assessment of patient status over time after treatment (De Maeseneer et al., 2022). Further, validated patient reported outcome measures which can be generic or disease specific are often used to evaluate success after treatment (De Maeseneer et al., 2022).

2.1.1. Epidemiology

Chronic venous disease is a common medical problem (Beebe-Dimmer et al., 2005, Bergan et al., 2006). Multiple studies regarding the prevalence of chronic venous insufficiency have been conducted (Robertson et al., 2008, Beebe-Dimmer et al., 2005, Salim et al., 2021). The prevalence of chronic venous insufficiency ranges from <1 % to 17 % in men and <1 % to 40 % in women (Robertson et al., 2008). This thesis focused on varicose veins. The prevalence of varicose veins has been reported to range from 7 % to 40 % in man and 25 % to 32 % in women, increasing with age (Robertson et al., 2008). The Framingham Study reported the two year incidence rate of varicose veins with 39.4 per 1000 for men and 51.9 per 1000 for women (Robertson et al., 2008). Salim et al. (2021) showed in a systematic review with pooled prevalence analysis a prevalence for varicose veins of 19 % showing highest prevalence in Europe and lowest in Africa. In addition they reported an annual incidence of varicose veins ranging from 0.22 % to 2.3 % (Salim et al., 2021).

2.1.2. Pathogenesis

The CEAP classification categorizes the etiology of CVD as primary, secondary, secondary – intravenous, secondary extravenous, congenital and no cause identified as shown in Table 1 (Lurie et al., 2020).

E class	Description
E_p	Primary
E_s	Secondary
E_{si}	Secondary – intravenous
E_{se}	Secondary – extravenous
E_c	Congenital
E_n	No cause identified

Table 1: The 2020 revision of CEAP: summary of etiologic (E) classification. The 2020 update of the CEAP classification system and reporting standards (Volume 8, Issue 3) by Lurie et al. Copyright 2020 by the Society for Vascular Surgery and published by Elsevier Inc. Reproduced with permission of Society for Vascular Surgery in the format of print and electronic via Copyright Clearance Center.

According to the CEAP classification pathophysiology of CVD is either reflux or obstruction or both (Table 2) (Lurie et al., 2020).

P class	Description
P_r	Reflux
P_o	Obstruction
$P_{r,o}$	Reflux and obstruction
P_n	No pathophysiology identified

Table 2: The 2020 revision of CEAP: summary of pathophysiologic (P) classification. The 2020 update of the CEAP classification system and reporting standards (Volume 8, Issue 3) by Lurie et al. Copyright 2020 by the Society for Vascular Surgery and published by Elsevier Inc. Reproduced with permission of Society for Vascular Surgery in the format of print and electronic via Copyright Clearance Center.

Often chronic venous disease has a primary etiology, meaning, it is not preceded by a known pathology (Segiet et al., 2015). An example for a common intravenous secondary etiology is deep vein thrombosis including post-thrombotic syndrome (De Maeseneer et al., 2022). The underlying pathophysiology in this case can be obstruction, if the thrombosis does not resolve, or reflux, if valves are damaged or destroyed by resolved thrombosis, or both (De Maeseneer et al., 2022). Examples for extravenous secondary etiology are extrinsic vein compression, raised venous pressure in case of right heart failure, an impaired muscle pump as well as obesity (Bergan et al., 2006, De Maeseneer et al., 2022).

In general, there are a variety of risk factors associated with chronic venous disease. Among them are female gender, pregnancy and multiparity, age, positive family history and prolonged standing (Chaitidis et al., 2022, De Maeseneer et al., 2022, Pannier, 2019).

Although the pathophysiologic mechanism for chronic venous disease is still uncertain generally impairment of larger superficial or deep veins occurs with subsequent impairment of the microcirculation and surrounding tissues of the skin (De Maeseneer et al., 2022). Multiple factors such as changes in the hydrostatic pressure, valvular incompetence, ineffective function of calf muscle, biochemical and structural alterations of the vessel wall, extracellular matrix abnormalities, impaired balance between growth factors or cytokines influence pathogenesis (Segiet et al., 2015). The return of venous blood can be then impaired by valvular incompetency and dysfunction of the calf muscle pump leading to increased venous pressure also called venous hypertension (Chaitidis et al., 2022). This venous hypertension can then lead to anatomic, physiologic and histologic changes causing chronic venous disease (Chaitidis et al., 2022). The valvular incompetence is caused by inflammatory phenomena leading to changes within the venous wall and valves in superficial veins and in the vasomotor tone as well as reflux (De Maeseneer et al., 2022). It remains unknown what initiates the inflammatory events in venous valves and walls (Bergan et al., 2006). One theory is sterile inflammation in which case altered shear stress might be important (Bergan et al., 2006). The abnormal venous blood flow as response to changes has been hypothesized to in itself also cause changes in the venous valves and vein walls (Bergan et al., 2006). The changes in venous valves includes stretching, splitting, tearing, thinning and adhesion of valve leaflets and infiltration of valve leaflets and venous wall by monocytes and macrophages (Bergan et al., 2006). The occurring chronic inflammation plays a key role in the skin changes associated with chronic venous disease (Bergan et al., 2006). Vascular remodeling and degenerative changes such as loss of elastin and collagen occur and in combination with fibrosis change the wall thickness and promote the development of varicose veins (De Maeseneer et al., 2022). Therefore, varicose veins contain an increased collagen content and decreased number and disorganization of smooth muscle cells and decreased amount of elastin with disruption of

elastic fibers (Wittens et al., 2015). The vein wall changes present heterogenous as hypertrophic segments alternate with thinner atrophic segments (Bergan et al., 2006). Physiologically valves prevent retrograde flow and modulate the venous flow (Lurie et al., 2003). Opened valve leaflets form a stenosis causing a jet and creating a vortex behind the valves possibly preventing thrombosis (Lurie et al., 2003). When dilatation occurs the valve leaflets lose contact leading to valvular incompetence (Wittens et al., 2015). Reflux can occur in the superficial or deep vein system or both (Bergan et al., 2006). If valvular incompetence occurs in perforating veins, high pressures generated in the deep vein system by calf-muscle contraction can be transmitted to the superficial vein system (Bergan et al., 2006). Overall, reflux and changing wall compliance result in faster refilling of the veins, less efficient venous emptying and venous hypertension especially while standing or walking (De Maeseneer et al., 2022).

Positive family history has been established as a risk factor for developing primary chronic venous disease (Baylis et al., 2021, Bergan et al., 2006). Chronic venous disease has also been described in specific genetic disorders suggesting a genetic component (Baylis et al., 2021, Segiet et al., 2015). One of the first genes identified was *FOXC2* (forkhead box protein C2) responsible for lymphedema distichiasis (Segiet et al., 2015). Patients affected by this rare genetic disease suffer from dysfunctional lymphatic vessels, extra eyelashes and varicose veins (Baylis et al., 2021, Segiet et al., 2015). In the Klippel-Trenaunay syndrome varicose veins develop in 76 % to 100 % of patients and even venous hypoplasia, atresia, agenesis are described due to a balanced de novo translocations t(8;14)(q22.3;q13) and t(5;11) (Segiet et al., 2015). There are several other rare genetic diseases which are accompanied by varicose veins among other symptoms. When deriving genetic insights from congenital diseases, it should be considered that these particular mutations only account for a small subset of patients affected by chronic venous disease (Baylis et al., 2021). As a result of technical advances the deciphering of the multifactorial genetics behind chronic venous disease in patients without rare genetic diseases has started (Baylis et al., 2021). Baylis et al. (2021) reviewed the current state of knowledge on genetics of chronic venous disease. Comparison of transcriptomic changes in varicose vein tissue and healthy vein tissue showed numerous differentially expressed genes to be enriched in pathways dictating extracellular matrix organization and vascular morphogenesis in patients with chronic venous disease (Baylis et al., 2021). With the genome-wide association study approach became more feasible as more data is available and risk loci for a varicose veins have been identified (Baylis et al., 2021).

In general, chronic venous disease itself is progressive (Chaitidis et al., 2022). Progression can occur in an ascending pattern when first tributaries are affected and then the saphenous trunk and junction are affected (De Maeseneer et al., 2022). Progression can also occur in a

descending pattern when first the saphenous junction is affected and then the saphenous trunk and tributaries are affected (De Maeseneer et al., 2022). Varicose veins has a progression rate of 22 % to developing a venous leg ulcer in six years (De Maeseneer et al., 2022). Regarding venous leg ulcer mutations in the hemochromatosis gene including *HFE C282Y* and *HFE H63D* causing a deficiency of iron metabolism were associated with a 5-fold increase in risk of development of venous ulcer in chronic venous disease patients (Baylis et al., 2021). Other genes that have been identified with increased risk for venous leg ulcer are *SLC40A1* (ferroportin-1), *MTHFR* (methylenetetrahydrofolatereductase), *MMP12* (matrix metalloproteinase-12), *FGFR2* (fibroblast growth factor receptor 2) and *FXIII* (coagulation factor XIII) (Baylis et al., 2021).

2.1.3. Symptoms

The CEAP classification describes clinical classes by various clinical signs as shown in Table 3 (Lurie et al., 2020),

C class	Description
C_0	No visible or palpable signs of venous disease
C_1	Telangiectasias or reticular veins
C_2	Varicose veins
C_{2r}	Recurrent varicose veins
C_3	Edema
C_4	Changes in skin and subcutaneous tissue secondary to CVD
C_{4a}	Pigmentation or eczema
C_{4b}	Lipodermatosclerosis or atrophie blanche
C_{4c}	Corona phlebectatica
C_5	Healed
C_6	Active venous ulcer
C_{6r}	Recurrent venous ulcer

Table 3: The 2020 revision of CEAP: summary of clinical (C) classification. Each clinical class is subcharacterized by a subscript indicating the presence (symptomatic, s) or absence (asymptomatic, a) of symptoms attributable to venous disease. The 2020 update of the CEAP classification system and reporting standards (Volume 8, Issue 3) by Lurie et al. Copyright 2020 by the Society for Vascular Surgery and published by Elsevier Inc. Reproduced with

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Symptoms reported by patients suffering from chronic venous disease are variable (Wittens et al., 2015). Symptoms include heaviness, tired legs, feeling of swelling, pruritus, nocturnal cramps, throbbing, burning pain, restless legs, aching of legs exacerbated by prolonged standing or sitting as well as venous claudication during exercise (De Maeseneer et al., 2022, Bergan et al., 2006, Chaitidis et al., 2022). Symptoms like cramps and restless legs are less specific for chronic venous disease (De Maeseneer et al., 2022). While heaviness, sensation of swelling, burning, itching, pain/aching are associated with a higher clinical classification in the CEAP classification (De Maeseneer et al., 2022). In general, symptoms do not necessarily correlate with presence or severity of venous hypertension (De Maeseneer et al., 2022). Symptoms increase with age and are more commonly reported by women (De Maeseneer et al., 2022). The described symptoms have a negative impact on the patients' quality of life especially when the patients are suffering from chronic venous insufficiency (van Korlaar et al., 2003). Surprisingly, quality of life has been studied less in patients with chronic venous disease in comparison to other chronic diseases (van Korlaar et al., 2003).

2.1.4. Treatment

The European clinical practice guidelines on the management of chronic venous disease of the lower limbs from 2022 provides a detailed overview of the treatment options available for chronic venous disease. Conservative management is part of the multimodal treatment at all stages of chronic venous disease (De Maeseneer et al., 2022). It includes physical exercise to strengthen the lower limb muscle and increase ankle mobility as well as physiotherapy and leg elevation (De Maeseneer et al., 2022). Other methods with less evidence are massage, balneotherapy and cooling therapy (De Maeseneer et al., 2022). As social and psychological factors contribute to chronic venous disease the guideline recommends an holistic approach (De Maeseneer et al., 2022). Another column of conservative treatment is compression therapy commonly using elastic compression stockings exerting a pressure of at least 15 mmHg at the ankle or adjustable compression garments (De Maeseneer et al., 2022). Elastic and inelastic bandages and intermittent pneumatic compression also present options for compression therapy but are used less commonly (De Maeseneer et al., 2022). Compression therapy should be combined with lifestyle changes such as weight reduction, exercise and leg elevation (Chaitidis et al., 2022). Pharmacological treatment can be used to relieve symptoms in patients

with symptomatic chronic venous disease who are not undergoing interventional treatment, awaiting intervention or have persisting symptoms after interventions (De Maeseneer et al., 2022). Natural as well as synthetic drugs provide symptomatic relieve by decreasing capillary permeability, reducing inflammatory mediator and improving venous tone (De Maeseneer et al., 2022). Available drugs are ruscus extract, micronized purified flavonoid fraction, calcium dobesilate, horse chestnut extract, hydroxyethylrutosides, red vine leaf extract and sulodexide (De Maeseneer et al., 2022). When choosing a pharmacological treatment, the efficacy profile based on the available evidence for each individual drug as well as the patients symptoms should be considered in order to develop the best treatment strategy (De Maeseneer et al., 2022). In addition to conservative treatment, interventional treatment is available and recommended for patients with superficial venous incompetence presenting with symptomatic varicose veins (De Maeseneer et al., 2022). The established standard for superficial venous incompetence is endovenous treatment with or without ambulatory phlebectomy (De Maeseneer et al., 2022). Postprocedural compression therapy is recommend (De Maeseneer et al., 2022). Endovenous treatment options are ultrasound guided foam sclerotherapy, endovenous thermal ablation, endovenous laser ablation, endovenous radiofrequency ablation, endovenous steam ablation as well as mechano-chemical endovenous therapy (Wittens et al., 2015, De Maeseneer et al., 2022). The European clinical practice guidelines from 2022 recommends consideration of concomitant tributary treatment for patients with an incompetent saphenous trunk treated with endovenous thermal or non-thermal ablation – especially as part of a shared decision process (De Maeseneer et al., 2022). For patients with uncomplicated varicose veins equivalent of CEAP clinical class C2 phlebectomies with preservation of saphenous trunk can be considered (De Maeseneer et al., 2022). Phlebectomy has been performed in the ancient times and was described as ambulatory surgery by Muller in 1966 (Wittens et al., 2015). A phlebectomy is performed via multiple small incisions over the preoperatively marked varicose veins (De Maeseneer et al., 2022). The varicose veins are removed using phlebectomy hooks and fine tipped mosquito clamps (De Maeseneer et al., 2022). In general complications associated with this surgery are infrequent and mild (De Maeseneer et al., 2022). Possible complications include blisters (5.4 %), hyperpigmentation (4.6 %), matting (3.6 %), superficial venous thrombosis (2.8 %), deep vein thrombosis (0.02 %), dysesthesia (0.4 %), lymphocele (0.2 %), postoperative hemorrhage (0.1 %), large hematoma (0.1 %) and infection (0.07 %) (De Maeseneer et al., 2022). Another ambulatory treatment option for tributaries is foam sclerotherapy using maximal 10 to 20 mL foam per treatment session (De Maeseneer et al., 2022). The treatment can be repeated and can also be performed ultrasound-guided (De Maeseneer et al., 2022). In contrast to phlebectomy anesthesia is not needed when injecting foam in varicose veins (De Maeseneer et al., 2022). Ambulatory phlebectomy and ultrasound guided foam sclerotherapy can also be combined to

treat varicose veins (De Maeseneer et al., 2022). Both treatment options should also be considered for patients with symptomatic recurrent varicose veins without truncal incompetence (De Maeseneer et al., 2022). A new treatment option for varicose veins is transilluminated powered phlebectomy (TIPP) (Chaitidis et al., 2022). In contrast to conventional phlebectomy this novel modified phlebectomy technique can be performed using fewer incisions, has a larger varicose vein clearance in less time and has shown better cosmetic score outcomes (Chaitidis et al., 2022). Unfortunately, early postoperative pain seems to be significantly increased (Chaitidis et al., 2022). Further investigation is needed to determine whether TIPP has a clear benefit over conventional phlebectomy (Chaitidis et al., 2022).

The current German guideline for treatment of varicosis from 2019 emphasizes that the patient's wish after detailed information about the methods as well as their success rates and financial implications has to be considered within the shared decision for multimodal treatment (Pannier, 2019). Also, the origin of reflux using duplex ultrasound should be identified and considered in the therapy concept (Pannier, 2019). Conservative treatment recommended in the German guideline also consists of physiotherapy, leg elevation, compression therapy and pharmacological therapy (Pannier, 2019). The pharmaceuticals approved in Germany are standardized red vine leave extract, standardized horse chestnut extract and oxerutin (Pannier, 2019). It is emphasized that symptomatic relief is delayed and medication has to be taken for two to four weeks before showing effects (Pannier, 2019). The German guideline differentiates between vein ablative and vein preservative invasive treatment methods (Pannier, 2019). The vein ablative invasive treatment methods are crossectomie, vein stripping and phlebectomy and should be performed according to stage of disease removing only diseased vein parts (Pannier, 2019). The German guideline also lists relevant intraoperative and postoperative complications of phlebectomy which are both scarce if the procedure is performed correctly (Pannier, 2019). Intraoperative complications include hemorrhage (0.01 – 0.1 %), nerve injury (0.01 – 6.6 %) and injury to major vessels (0.01 – 0.1 %) (Pannier, 2019). Relevant postoperative complications include postoperative hemorrhage/hematoma (0.06 – 2.0 %), wound infection (0.1 – 2.8 %), wound healing disturbance (0.05 – 1.38 %), lymphatic drainage disturbance (0.02 – 1.82 %), superficial vein thrombosis (0.2 -0.3 %) and deep vein thrombosis and/or pulmonary embolism (0.01 – 0.24 %) (Pannier, 2019). Patients report better quality of life after surgery (Pannier, 2019). In general, absolute contraindications for vein ablative invasive treatment methods are acute deep vein thrombosis in leg or pelvic veins, peripheral arterial disease (stage III or higher), known pregnancy and the patient being terminal ill (ASA classification 5) (Pannier, 2019). Vein preservative treatment methods are Cure Conservatrice et Hémodynamique de l`Insuffisance Veineuse en Ambulatoire (CHIVA) and

extraluminal valvuloplasty which is scarcely used in Germany (Pannier, 2019). Indication for vein preservative treatment methods is foremost a high probability that a vein will be used as a graft due to arteriosclerosis (Pannier, 2019). Endovenous methods for treatment are also available using either thermal, laser, radiofrequency or steam ablation as well as mechano-chemical therapy (Pannier, 2019). The German guideline for treatment of varicosis will expire on the 30th March 2024 and will be updated.

2.1.5. Complications

In general acute complications are uncommon with chronic venous disease (De Maeseneer et al., 2022). Although among the possible complications superficial vein thrombosis is the most common, deep vein thrombosis may occur and recur (De Maeseneer et al., 2022). Müller-Bühl et al. (2012) showed a 5.6 % occurrence of deep vein thrombosis in patients with varicose veins compared to 0.9 % in patients without varicose veins in a family practice population. In the general population varicose veins are considered a minor risk factor in developing a deep vein thrombosis (De Maeseneer et al., 2022). Superficial vein thrombosis can occur in patients with varicose veins spontaneous or after minor local trauma and can extent into the deep venous system (De Maeseneer et al., 2022). Hemorrhage from varicose veins can occur when the overlying skin becomes thinner (De Maeseneer et al., 2022). If the skin stays intact bruising occurs in case of injury (De Maeseneer et al., 2022). If an injury breaks the skin, excessive variceal hemorrhage which has the potential to be fatal in exceptional cases can occur (De Maeseneer et al., 2022). Immediate treatment of hemorrhage is elevation and external compression (De Maeseneer et al., 2022). The European clinical practice guidelines on the management of chronic venous disease of the lower limbs from 2022 recommends a referral for urgent assessment and treatment for patients who have had acute spontaneous bleeding from superficial veins (De Maeseneer et al., 2022). In addition to the aforementioned complications the German guideline for treatment of varicosis additionally lists guide vein insufficiency and arthrogenic stasis syndrome as possible complications (Pannier, 2019). In the Gutenberg Health Study it was shown that chronic venous insufficiency was independently associated with an increased prevalence of arterial hypertension, obesity, active smoking and cardiovascular disease (Prochaska et al., 2021). It was shown that cardiovascular disease and chronic venous insufficiency are interdependent and share risk factors (Prochaska et al., 2021). Advanced chronic venous disease was specifically associated with peripheral artery disease and venous thrombo-embolism (Prochaska et al., 2021). The Gutenberg Health Study also showed that the all-cause mortality was significantly higher in participants with chronic

venous insufficiency in comparison to participants not suffering from chronic venous insufficiency (Prochaska et al., 2021). Therefore, chronic venous insufficiency represents a strong predictor for all-cause mortality in the general population (Prochaska et al., 2021). As mentioned before chronic venous disease is a progressive disease (Chaitidis et al., 2022). This presented a new complication during the SARS-CoV2-pandemic as surgical treatment was no longer available and symptoms worsened with a more sedentary lifestyle during lockdown (Krasinski et al., 2021). As restrictions are now lifted future studies have to be performed to understand the full impact of the limitations during the pandemic on progression of chronic venous disease.

2.2. Monocytic cells (monocytes/macrophages)

Monocytes and macrophages are some of the myeloid cells comprising part of the innate immune system (Austermann et al., 2022). The innate immune system is important in the initiation of the adaptive immune response (Austermann et al., 2022). Additionally, it plays a key role in surveillance and homeostasis as well as restoration of homeostasis (Austermann et al., 2022).

Monocytes and macrophages are heterogenous cell populations with high plasticity (Das et al., 2015). Both cell types are able to undergo phenotypic and functional changes in response to stimuli from the local microenvironment (Das et al., 2015, Austermann et al., 2022). Under physiological conditions monocytes and macrophages have homeostatic functions but are able switch to a proinflammatory phenotype in case of inflammation and an anti-inflammatory phenotype after resolution of inflammation and reestablishment of homeostasis (Austermann et al., 2022).

Monocytes compose approximately 10% of nucleated cells in circulation in humans (Austermann et al., 2022, Das et al., 2015, Martinez, 2009). Different monocyte populations have different life spans (Austermann et al., 2022). Classical monocytes remain in circulation for approximately one day before differentiating into a different phenotype or entering tissues (Guilliams et al., 2018). Non-classical monocytes on the other hand have a certain flexibility regarding their life span (Guilliams et al., 2018).

In addition to orchestrating the immune response monocytes and macrophages fulfill various functions in development, homeostasis and inflammation by e.g., phagocyting microbes and producing cytokine (Austermann et al., 2022, Coillard and Segura, 2019, Cormican and Griffin, 2020, Das et al., 2015).

Macrophages are found in every tissue in the human body (Austermann et al., 2022). Some macrophage populations arise from embryonic precursors seeded in tissues before birth with the ability to proliferate and maintain through self-renewal (Ginhoux and Jung, 2014, Austermann et al., 2022). Even though monocytes can differentiate into macrophages in certain settings, their contribution to the population of tissue-resident macrophages during homeostasis depends on the organ (Nobs and Kopf, 2021). In inflammation replenishment of the tissue-resident macrophages depends not only on the tissue but also on the type of inflammation (Austermann et al., 2022, Nobs and Kopf, 2021).

Monocytes and macrophages both arise from the myeloid cell lineage (Coillard and Segura, 2019).

Monocytes and macrophages are activated using the same mechanism. In inflammation monocytes and macrophages are prone to a proinflammatory phenotype by microbial products and proinflammatory cytokines such as interferon-γ, TNF-α (Austermann et al., 2022).

To initiate an adequate immune response during infection or tissue damage monocytes and macrophages must recognize pathogens (Austermann et al., 2022). Both cell types rely on pattern recognition receptors (PRRs) to activate them by identifying pathogen- and tissue-damage associated molecular patterns (PAMPs, DAMPs) (Austermann et al., 2022).

Pathogen-associated molecular patterns are evolutionarily conserved structures associated with pathogens such as viruses, bacteria, fungi, parasites while damage-associated molecular patterns are exposed by damage in host tissues (Austermann et al., 2022). The pattern recognition receptors are classified into four different families called toll-like receptors (TLRs), C-type lectin receptors (CLRs), cytoplasmic proteins like retinoic acid-inducible gene-I-like receptors (RLRs) and NOD-like receptors (NLRs) (Austermann et al., 2022, Fujiwara and Kobayashi, 2005). One abundant pattern recognition receptor on monocytes and macrophages is TLR4 (Austermann et al., 2022). After recognition of PAMPs/DAMPs, pattern recognition receptors with exception of NOD-like receptors upregulate via different signaling pathways the transcription of genes involved in inflammation response enhancing e.g., phagocytosis and cytokine production (Austermann et al., 2022).

Proinflammatory monocytes and macrophages are one of the first defense mechanism against invading pathogens (Austermann et al., 2022). Both cell types produce cytokines such as IL-1β, IL-6, IL-12, IL-23, TNF-α, nitrogen oxide, radical oxygen species and facilitate complement-activated phagocytosis (Austermann et al., 2022). The efficiency of monocytes and macrophages to eliminate the pathogens determines the duration of the activation phase as it ends with the termination of the stimuli (Austermann et al., 2022). After removal of the pathogen and control of the inflammation the infection has to be resolved in order to return to

homeostasis (Austermann et al., 2022). To effectively terminate the inflammation and reestablish homeostasis macrophages have to acquire an anti-inflammatory and pro-resolving phenotype (Austermann et al., 2022). Anti-inflammatory mediators such as IL-4, IL-13, glucocorticoids, IL-10, tumor growth factor-β and lipid mediators e.g., lipoxins, resolvins, protectins promote differentiation into an anti-inflammatory phenotype (Austermann et al., 2022).

2.2.1. Monocyte origin, peripheral subtypes

Monocytes originate in the bone marrow from hematopoietic stem cells with the ability to self-renew (Guilliams et al., 2018). The hematopoietic stem cells give rise to either common lymphoid progenitors or common myeloid progenitors (Guilliams et al., 2018). From the common myeloid progenitor a megakaryocyte and erythrocyte progenitor and a granulocyte and macrophage progenitor rise (Guilliams et al., 2018). The granulocyte and macrophage progenitor gives rise to the common monocyte progenitor by a monocyte/dendritic cell progenitor (Guilliams et al., 2018). The common monocyte progenitor differentiates into monoblasts and promonocytes before becoming mature monocyte (Das et al., 2015, Guilliams et al., 2018). For the differentiation into monocytes hematopoietic growth and transcriptions factors such as IRF8 and KLF4 are important (Guilliams et al., 2018). For IRF8 an autosomal recessive deficiency has been described resulting in less circulating monocytes in humans (Guilliams et al., 2018). Further research is needed to determine if monocytes solely arise in the bone marrow or if peripheral reservoirs are present in the human body (Guilliams et al., 2018).

For the circulating monocytes a nomenclature was proposed by Ziegler-Heitbrock et al. (2010) and has been established. Blood monocytes are distinguished phenotypically into three subsets using CD14 and CD16 (Ziegler-Heitbrock et al., 2010). Subsets are classical monocytes expressing $CD14^{++}$ $CD16^{-}$, intermediate monocytes expressing $CD14^{++}$ $CD16^{+}$ and non-classical monocytes expressing $CD14^{+}$ $CD16^{++}$ (Ziegler-Heitbrock et al., 2010).

Classical monocytes comprise 80 to 90% of the monocyte population under physiological conditions (Guilliams et al., 2018). When exiting the bone marrow monocytes are $CD14^{++}$ $CD16^{-}$ (Cormican and Griffin, 2020). Hence, classical monocytes are also the first population to reappear after transient monocytopenia induced by lipopolysaccharide injection in humans followed by intermediate monocytes and non-classical monocytes (Guilliams et al., 2018). Classical monocytes remain in the blood stream for approximately one day after egress from the bone marrow (Guilliams et al., 2018). Their high differentiation potential enables

classical monocytes to become either non-classical monocytes or macrophages by entering tissues (Martinez, 2009, Guilliams et al., 2018). The transition of classical monocytes to non-classical monocytes seems to be based on an evolutionary conserved program and has been observed after ablation of circulating monocytes using clodronate-loaded liposomes, cell transfer and pulse labelling experiments in different species including humans (Guilliams et al., 2018).

Non-classical monocytes and intermediate monocytes each comprise approximately 5 to 10% of the monocyte population under physiological conditions (Coillard and Segura, 2019, Cormican and Griffin, 2020). Non-classical monocytes remain in the blood stream approximately seven days in homeostasis in humans (Guilliams et al., 2018). Further investigation is needed to determine whether non-classical monocytes can also develop omitting classical monocytes (Guilliams et al., 2018). Non-classical monocytes seem to have a certain flexibility to increase their life-span ensuring constant numbers when monocyte recruitment occurs under pathological conditions (Guilliams et al., 2018). The ratio of classical and non-classical monocytes in the blood is determined by tissue infiltration, release from the bone marrow or maybe also peripheral reservoirs (Guilliams et al., 2018). Under pathological conditions recruitment to the site of injury or inflammation in a rapid manner requires large numbers of monocytes (Guilliams et al., 2018). To ensure a successful recruitment monocytes are constantly generated and mobilized from the bone marrow (Guilliams et al., 2018).

Regarding their function, non-classical monocytes have been considered to locally surveil tissues including the endothelium of the vasculature (Cormican and Griffin, 2020, Ginhoux and Jung, 2014, Martinez, 2009). This idea is supported through experiments using adoptive transfer into immune-compromised mice. Hereafter, non-classical monocytes exhibit patrolling behavior (Guilliams et al., 2018).

Intermediate monocytes are a heterogenous cell population (Guilliams et al., 2018). It has been demonstrated that both subsets, intermediate and non-classical monocytes, which both express CD16 produce proinflammatory cytokines like TNFα, IL-6, IL-10 more efficiently than classical monocytes (Martinez, 2009).

With gene expression analysis further insight into the heterogenous cell populations of monocytes was gained. Overall, three genetically distinct monocytes subsets have been identified using gene expression analysis (Cormican and Griffin, 2020).

Monocyte expressing CD16 show an increased expression of pro-inflammatory genes and co-stimulatory molecules (Martinez, 2009). In addition to overexpression of CD16, genes important for Fc mediated phagocytosis are increased fitting with the increased ability of Fc mediated phagocytosis in intermediate and non-classical monocytes (Martinez, 2009). Other

genes that are highly expressed in CD16^{+} monocytes are genes involved in cytoskeletal dynamics e.g. *CKB* (creatinine kinase B), *EML4* (echinoderm microtubule associated protein like 4) as well as genes important for tissue invasion in inflammation like *CTSL1* (cathepsin 1) and genes suggesting terminal differentiation and cellular maturity like e.g. *CDKNIC* (cyclin-dependent kinase inhibitor 1C) and *HES4* (hairy and enhancer of split 4) (Cormican and Griffin, 2020).

While monocytes expressing CD16 share certain expressed genes, both populations show differences at gene level. Intermediate monocytes show a high expression of genes related to MHC-II-restricted antigen processing and presentation (Cormican and Griffin, 2020). Additionally, genes involved in superoxide regulation are highly expressed in intermediated monocytes in conformity with their high basal production of reactive oxygen species (Cormican and Griffin, 2020). Non-classical monocytes show a high expression of genes involved in cell movement (Cormican and Griffin, 2020). Further differences between the populations were identified using de novo motif detection in regions of subset specific histone modifications and cap analysis of gene expression (CAGE) cluster expression (Cormican and Griffin, 2020). The motif signature of intermediate monocytes is dominated by NF-κB (nuclear factor-κB), E-box and MEF2 (myocyte enhancer factor 2) (Cormican and Griffin, 2020). The motif signature of non-classical monocytes is dominated by E2F, NRF1 (nuclear respiratory factor 1) and OCT (octamer transcription factor) (Cormican and Griffin, 2020).

Regarding transcripts CD16 expressing monocytes show higher expression of e.g., TNF (tumor necrosis factor), CX3CR1 (fractalkine receptor), IFNG (interferon γ) while non-classical monocytes in addition highly expressed transcripts related to cytoskeletal organization (Cormican and Griffin, 2020).

On mRNA level CD16 expressing monocytes show a higher expression of e.g. CSF1R (colony stimulating factor 1 receptor), IL12RB1 (receptor for macrophage colony-stimulating factor and complement component factors C1QA, C1QB, C3, IL-12 receptor), HMOX1 (heme oxygenase 1, VIL2 (villin 2), HCK (hematopoietic cell kinase) and LYN (tyrosine protein kinase Lyn) (Martinez, 2009).

At protein level higher expression has been shown for actin-related protein 1/3 complex (ARP2, ARP3), hematopoietic cell kinase (HCK), tyrosine protein kinase Lyn (LYN) and colony stimulating factor 1 receptor (CSFR1) among others (Martinez, 2009).

Classical monocytes are more effective in producing reactive oxygen species in response to bacteria in comparison to CD16 expressing monocytes (Martinez, 2009). In accordance with this finding, classical monocytes show a higher expression of genes involved in responses to bacterial infection and inflammation like *TLR4* (toll-like receptor 4), *TREM1* (triggering receptor

on myeloid cells-1), *CCR2* (C-C chemokine receptor type 2) as well as genes involved in inflammasome signaling such as e.g. *NLRP3* (NLR family pyrin domain containing 3) and *NLPR12* (NACHT, LRR and PYD domains-containing proteins 3 and 12) (Cormican and Griffin, 2020). Classical monocytes also show a high expression of genes involved in low density lipoprotein (LDL) uptake, *ITGAM* (CD11b) and genes for S100 proteins (Cormican and Griffin, 2020).

The motif signature of classical monocytes identified using de novo motif detection in regions of subset specific histone modifications and cap analysis of gene expression (CAGE) cluster expression are dominated by AP-1 (activator protein 1) and CEBP (CCAAT enhancer binder protein) (Cormican and Griffin, 2020). As FOS transcription factor is a component of AP-1 it is also higher expressed in classical monocytes (Cormican and Griffin, 2020). Other highly expressed transcripts include SELL2 (L-Selectin), CCR1/2 (C-C chemokine receptors 1 and 2) as well as TRL2/4/5/6/8 (toll like receptors 2/4/5/6/8) (Cormican and Griffin, 2020).

At mRNA level a higher expression of IL-13 receptor (IL13RA1), myeloperoxidase (MPO), lysozyme C (LYZ), protein S100-A9 (S100A9), eosinophil cationic protein (RNASE3), phospholipase B domain containing 1 (PLBD1, also known as FLJ22662) and colony stimulating factor 3 receptor (CSF3R) has been demonstrated in classical monocytes (Martinez, 2009). At protein level higher expression has been shown for cathepsin G (CTSG), myeloperoxidase (MPO), lysozyme C (LYZ), protein S100-A9 (S100A9), receptor for complement component C1q1 (CD93) and colony stimulating factor 3 receptor (CSF3R) (Martinez, 2009).

Regarding cell surface marker expression levels correlate with expression levels of the related genes across the monocyte subsets (Cormican and Griffin, 2020).

In 2017 unbiased single-cell RNA sequencing was performed to identify subpopulation of blood monocytes and dendritic cells in healthy human subjects (Villani et al., 2017). Four transcriptionally distinct clusters regarding monocytes were identified (Villani et al., 2017). The two largest clusters were named Mono1 and Mono2 (Villani et al., 2017). These clusters contained classical and non-classical monocytes as well as the majority of intermediate monocytes (Villani et al., 2017). Additionally, two smaller clusters termed Mono3 and Mono4 were identified (Villani et al., 2017). The distinct transcriptional signatures of Mono3 were linked to cell cycle, differentiation and trafficking while the Mono4 cluster expressed a cytotoxic gene signature (Cormican and Griffin, 2020, Villani et al., 2017). In another single-cell RNA sequencing study blood of patients with primary non-small cell lung cancer was used (Zilionis et al., 2019). In this study three monocyte clusters were identified matching the previously identified Mono1, Mono2 and Mono3 (Cormican and Griffin, 2020, Zilionis et al., 2019).

2.2.2. Macrophage origin and functions

While monocytes develop in the bone marrow, tissue-resident macrophages can have different origins in different tissues (Das et al., 2015, Davies et al., 2013, Ginhoux and Jung, 2014, Nobs and Kopf, 2021). Tissue-resident macrophages can derive from embryonic progenitors that seed in the tissues during embryonic period (Ginhoux and Jung, 2014). Human hematopoiesis starts in the blood islands in the yolk sac around day 18 to 19 of estimated gestational age lasting for approximately three to six weeks (Ginhoux and Jung, 2014). Some tissue-resident macrophages directly derive from primitive precursors in the yolk sac such as microglia, tissue-resident macrophages in the central nervous system (Nobs and Kopf, 2021). The majority of tissue-resident macrophages, such as alveolar macrophages in the lung and Kupffer cells in the liver, derive from a monocyte intermediate stage in the fetal liver (Nobs and Kopf, 2021). The fetal liver harbors the hematopoiesis during development around four to five weeks of estimated gestational age (Ginhoux and Jung, 2014). When seeded into fetal tissues, progenitor cells develop into fetal tissue macrophages with the ability to self-renew (Ginhoux and Jung, 2014).

In adulthood embryonic derived macrophages and monocyte-derived macrophages contribute to the tissue macrophage population in steady state to varying degrees in an organ-dependent manner (Ginhoux and Jung, 2014, Guilliams et al., 2018, Nobs and Kopf, 2021). For example, there are several macrophage populations found in the human central nervous system (Nobs and Kopf, 2021). In mice, microglia, perivascular and subdural meningeal macrophages have been demonstrated to exclusively arise from embryonic precursors, while the population of choroid macrophages is maintained by bone marrow derived monocytes (Nobs and Kopf, 2021). In the heart two major populations of macrophages have been found in humans as well as mice (Nobs and Kopf, 2021). In mice, embryonic-derived cardiac macrophages are important for tissue remodeling, coronary development and cardiac repair but decrease with age while monocyte-derived cardiac macrophages increase in atherosclerosis and accumulate in atherosclerotic plaques (Nobs and Kopf, 2021). In contrast to humans, tissue-resident macrophage localized in the aortic intima have been demonstrated in mice (Nobs and Kopf, 2021). These cells seem to play a pivotal role in the initiation of atherosclerosis by participating in early accumulation of cholesterol and can be replaced by inflammatory monocytes acquiring the gene signatures of these macrophages during advanced stages of cardiovascular disease (Nobs and Kopf, 2021). In humans further research is needed to shed light on the origin of tissue-resident macrophages in different organs (Nobs and Kopf, 2021).

Macrophages are heterogenous regarding their surface markers and their functions as they have a high plasticity and flexibility (Austermann et al., 2022, Das et al., 2015, Tarique et al., 2015). Hence, a variety of factors can change their phenotype and function (Austermann et al., 2022, Das et al., 2015, Tarique et al., 2015).

Macrophages can be separated based on their functions, defense, wound healing and immune regulation, or based on activation stimuli (Das et al., 2015, Tarique et al., 2015). Macrophages polarized by cytokines typically produced by type 1 helper T cells e.g., interferon-γ and tumor necrosis factor-α, were defined as M1, also known as classically activated, macrophages (Das et al., 2015). In vitro stimulation of in vitro monocyte-derived uncommitted macrophages in a basal state, named M0, with lipopolysaccharides also results in a M1 phenotype (Tarique et al., 2015). When deriving M1 macrophages in vitro in presence of granulocyte-macrophage colony-stimulating factor (GM-CSF) their phenotype is defined by CD64 and CD80, resulting in a $CD64^{high}CD80^{-}$ or $CD64^{+}CD80^{+}$ phenotypes (Tarique et al., 2015). Functional in vitro experiments showed that these M1 macrophages secret interferon-γ induced protein 10, interferon-γ, IL-8, TNF-α, IL-1β and chemokine ligand 5 (Tarique et al., 2015). Functions attributed to M1 macrophages are rather inflammatory nature such as pathogen killing (Das et al., 2015). M1 macrophages have an enhanced production of reactive oxygen species requiring NADPH provided by the pentose phosphate pathway induced by increased glucose uptake for aerobic glycolysis, the metabolic pathway in M1 macrophages (Das et al., 2015).

Macrophages polarized by cytokines typically produced by type 2 helper T cells e.g., IL-4 and IL-10, were defined as M2, also known as alternative activated, macrophages (Das et al., 2015). In vitro stimulation of M0 macrophages with IL-13 also results in a M2 phenotype (Tarique et al., 2015). When deriving M2 macrophages in vitro in presence of granulocyte-macrophage colony-stimulating factor their phenotype is $CD11b^{+}CD209^{+}$ (Tarique et al., 2015). The functions attributed to M2 macrophages were related to tissue repair and remodeling (Das et al., 2015). It was also proposed to further separate M2 macrophages into three subtypes, M2a, b and c (Das et al., 2015). M2a macrophages, induced by IL-4 or IL-13, have been attributed profibrotic functions (Das et al., 2015). Interestingly, it has been demonstrated that IL-4 and IL-13 also increase pinocytosis and endocytosis capacity in macrophages (Martinez, 2009). The combined occurrence of immune complexed and toll-like receptor or IL-1 receptor agonists can produce M2b macrophages (Das et al., 2015). Lastly, M2c macrophages, induced by IL-10, transforming growth factor-β as well as glucocorticoids, have been attributed neovascularizing functions (Das et al., 2015). Glucocorticoids are a pharmacological treatment option in humans used to substitute a lack of endogenous synthesis as well as to treat inflammatory diseases e.g., acute exacerbation of autoimmune diseases such as multiple sclerosis, chronic inflammatory diseases such as bronchial asthma. Glucocorticoids have been

suspected to inhibit proinflammatory macrophages and promote the differentiation of anti-inflammatory phenotype in monocytes and macrophages with e.g., increased abilities to fight bacterial infections, to modulate the immune response preventing overwhelming inflammation (Austermann et al., 2022). After prolonged glucocorticoid treatment both cell types show a strong expression of CD163 enabling macrophages to bind and scavenge hemoglobin-haptoglobin complexes leading to release of IL-10 and carbon monoxide as strong anti-inflammatory effects to downregulate inflammatory response in the resolution phase (Austermann et al., 2022). While M1 macrophages rely on glycolysis for their metabolism, M2 macrophage metabolism is associated with oxidative phosphorylation and fatty acid oxidation (Das et al., 2015, Nobs and Kopf, 2021).

In vitro stimulation of M2 macrophages with different cytokines result in expression of different surface markers e.g., stimulation with GM-CSF induced CD206, but within 12 days activated macrophages derived in presence of GM-CSF can also revert to the so-called M0 basal state (Tarique et al., 2015).

Tarique et al. (2015) analyzed transcriptional profiles of in vitro induced M1 and M2 macrophages using M0 macrophages as controls demonstrated an upregulation of Cox-2 (cyclooxygenase-2), APOL3 (apolipoprotein L3), CXCL11 (C-X-C motif chemokine 11), IRF5 (interferon regulatory factor 5), TNF-α in M1 macrophages and an upregulation of IFR4 (interferon regulatory factor 4), FN1 (fibronectin) and CCL18 (C-C motif chemokine ligand 18) in M2 macrophages (Tarique et al., 2015). The in M1 upregulated transcriptional profiles were suppressed in M2 macrophages and vice versa (Tarique et al., 2015).

Molecular network analysis of in vitro monocyte-derive M1 and M2 macrophages have shown oxidized low-density lipoproteins partly regulate their gene expression profiles, for instance Maresin derived from docosahexaenoid acid influences the transition of M1 to M2 macrophages (Das et al., 2015).

As macrophages have a high plasticity and depend on environmental cues for their phenotype classifying macrophages into M1 and M2 macrophages, even with subclassification of M2 macrophages, is oversimplified and depicts macrophages only inadequately (Austermann et al., 2022, Nobs and Kopf, 2021).

Instead, macrophages can be distinguished more broadly into proinflammatory and anti-inflammatory regarding phenotype and functions (Austermann et al., 2022). In inflammation microbial product and proinflammatory cytokines such as interferon-γ, TNF-α, induce a proinflammatory phenotype in macrophages which produce cytokines such as IL-1β, IL-6, IL-12, IL-23, TNF-α, nitrogen oxide critical for immunomodulation in inflammation (Austermann et al., 2022, Fujiwara and Kobayashi, 2005). Proinflammatory macrophages present antigens

and are effective in recognition, phagocytosis and killing of pathogens in phagolysosomes by producing reactive nitrogen species and reactive oxygen species mainly mediated by NOX2 (NADPH oxidase 2) (Austermann et al., 2022, Fujiwara and Kobayashi, 2005). After removal of the pathogen and control of the inflammation anti-inflammatory macrophages induced by anti-inflammatory mediators such as IL-4, IL-13, glucocorticoids, IL-10, tumor growth factor-β and lipid mediators e.g., lipoxins, resolvins, are needed to resolve the acute inflammation preventing transformation into low-grade chronic infection (Austermann et al., 2022). Anti-inflammatory macrophages expressing surface markers such as CD204, CD206 and arginase 1, have many abilities to promote resolution of infection, wound healing, tissue repair and return to homeostasis (Austermann et al., 2022). Among those are the negative regulation of proinflammatory cytokines, the release of anti-inflammatory mediators e.g., IL-10, tumor growth factor-β, phagocytosis of apoptotic cells, coordination of tissue integrity (Austermann et al., 2022).

The ability of macrophages to transdifferentiate into endothelial-like cells, endothelial progenitor cells and endothelial cells in vitro and in vivo is important for regenerative processes (Das et al., 2015). Pleiotrophin promotes this transdifferentiation of macrophages and is expressed by macrophages in ischemia and regulated by GATA-2 and GATA-3 on transcription level (Das et al., 2015).

2.2.3. Monocyte tissue entry and local differentiation into macrophages

Monocytes are recruited to the side of injury or inflammation through transmigration and differentiate into macrophages after entering tissues in response to environmental cues or inflammation (Gerhardt and Ley, 2015, Guilliams et al., 2018, Martinez, 2009).

After activation by inflammatory cytokines, mainly TNF-α, IL-1β produced by tissue-resident macrophages, endothelium cells express adhesion molecules like E- and P-selectin, intercellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1) and tethered chemokines on their luminal surface (Gerhardt and Ley, 2015). Selectins can interact with O-glycosylated carbohydrate provided by P-selectin glycoprotein ligand-1 (PSGL-1) expressed on monocytes which subsequently begin to roll on the endothelium and conquer the blood flow's high shear stress (Gerhardt and Ley, 2015). In inflammation, monocytes rolling additionally depends on CD44 and very late antigen-4 (integrin α4β1) which mediates slow rolling by interaction with VCAM-1 and facilitates the transition to firm arrest of monocytes (Gerhardt and Ley, 2015). C-C and C-X-C chemokines such as chemokine (C-C motif) ligand 2 (CCL2) and IL-8 ensure the firm adhesion of monocytes to the endothelium (Gerhardt and

Ley, 2015). Monocyte arrest on endothelium is followed by a directional chemotactic and mechanotactic step consisting of monocytes spreading, polarizing and locomoting laterally to preferred sites of extravasation (Fujiwara and Kobayashi, 2005, Gerhardt and Ley, 2015). Intraluminal crawling of monocytes differs from their strictly intravascular patrolling and depends on leucocyte integrins lymphocyte function-associated antigen 1 (LFA-1), macrophages-1 antigen (Mac-1) and the endothelial ligands ICAM-1 and ICAM-2 and probing of the apical surface with membrane protrusions called lamellipodia (Gerhardt and Ley, 2015).

Transmigration is hardly ever reversed in tissue entry of monocytes and depends on the negotiation of not only the endothelium but also the lamina basalis and embedded pericytes (Gerhardt and Ley, 2015). The endothelium is a thin monolayer of endothelial cells posing the primary physical barrier between blood and tissue without true tight junctions (Gerhardt and Ley, 2015). A network formed by adherens junctions between cells, integrin and cadherin anchoring on the basement membrane restrains leucocyte transendothelial migration (Gerhardt and Ley, 2015). Monocytes predominately transmigrate through junctions between endothelial cells, the paracellular route, requiring junctional remodeling and control of endothelial junctions determining time and location of leucocyte extravasation (Gerhardt and Ley, 2015). To activated endothelial cells adherent monocytes can disrupt the association between endothelial-specific vascular endothelial tyrosine phosphatase and vascular endothelial-cadherin (VE-cadherin), an essential stabilizing component of adherens junctions linked to actin cytoskeleton through α-catenin, through phosphorylation of VE-cadherin by non-receptor protein tyrosine kinase Src as well as in Pyk2-dependent manner (Gerhardt and Ley, 2015). Another molecule crucial in monocyte transmigration is junctional adhesion molecule (JAM) expressed on endothelial cells as well as sometimes leucocytes (Gerhardt and Ley, 2015). JAM-C is required to prevent monocytes to reverse back into the vascular lumen (Gerhardt and Ley, 2015). A closely to JAM family related protein is JAM-like protein (JAML) which is upregulated on monocytes in inflammation and important for monocyte migration across the endothelium in vitro (Gerhardt and Ley, 2015). Platelet endothelial cell adhesion molecule (PECAM-1) is along with CD99 and CD99 antigen-like protein 2 (CD99L2) highly expressed at endothelial junctions and diffusely expressed on monocytes and facilitates transmigration through homophilic binding between molecules on monocytes and endothelial cells (Gerhardt and Ley, 2015). Furthermore, PECAM-1, CD99, CD155, JAM-A and other molecules form a transmigration complex residing in a subjunctional, intracellular endothelial membrane reticulum called lateral border recycling compartment (LBRC) (Gerhardt and Ley, 2015). Kinesin-microtubule motors transport the lateral border recycling compartment actively to the site of diapedesis where it surrounds the transmigrating cell (Gerhardt and Ley, 2015). The lateral border recycling compartment also participates in the transcellular migration of

monocytes (Gerhardt and Ley, 2015). Transcellular migration is transmigration of monocytes through fusing vesicles in endothelial cell cytoplasm and constitutes only up to 30% to monocyte trafficking across the endothelium but can be increased by strong activating stimuli (Gerhardt and Ley, 2015). Even though some steps of monocyte tissue entry are seemingly understood in vitro there is further research needed to understand monocyte transmigration in vivo. One example for the need of research are metalloproteinases as their inhibition significantly prolongs the duration of the diapedesis step in monocytes which under this conditions express more Mac-1 (Gerhardt and Ley, 2015).

As for the differentiation of monocytes into macrophages adhesion to the endothelial already provides the first trigger of regulation of extravasation-specific genes and initiates changes towards a more differentiated phenotype (Gerhardt and Ley, 2015). Further differentiation depends on cues of the microenvironment depending on tissue and homeostatic or inflammatory context (Guilliams et al., 2018). When differentiating into macrophages monocytes can either replenish the pool of macrophages or in context of inflammation contribute to the inflammatory response (Coillard and Segura, 2019, Das et al., 2015, Martinez, 2009). Monocyte-derived macrophages can functionally overlap with resident macrophages regarding pro-inflammatory and anti-inflammatory activities, antigen-presentation, tissue remodeling but they can also gain functions not fulfilled by resident macrophages or conventional dendritic cells (Guilliams et al., 2018).

For human monocytes many in vitro studies have been performed to identify possible differentiation signals without proofing the applicability in vivo. In general macrophage colony-stimulating factor is a potent maturation signal for monocytes and required for the development of many tissue-resident macrophages (Martinez, 2009). For antigen-presentation, in vitro experiments have shown that human monocytes stimulated with GM-CSF or a combination of GM-CSF with IL-4 differentiated into $CD11c^{+}MHCII^{+}CD11b^{+}$ cells that are able of soluble antigen-presentation (Guilliams et al., 2018). Tarique et al. (2015) demonstrated that in vitro monocytes can differentiate into uncommitted macrophages and be polarized into M1 phenotypes using LPS and IFN-γ and into M2 phenotypes using IL-4 and IL-13.

On gene level, it has been demonstrated that monocyte-derived macrophages have significant gene modifications compared to circulating monocytes (Guilliams et al., 2018). During adaptation to the local tissue environment monocyte-derived macrophages acquire transcriptomic signatures similar to tissue-resident macrophages of embryonic origin retaining epigenetic and transcriptional as well as functional differences (Guilliams et al., 2018).

3. MATERIALS AND METHODS

3.1. Materials

3.1.1. Laboratory equipment

Equipment	Manufacturer
Autoclave (Varioklav Steam Sterilizer)	HP Medizintechnik GmbH, Oberschleißheim, Germany
Centrifuges (Heraeus, Biofuge primo R; Heraeus, Multifuge 3L-R)	Thermo Fisher Scientific Inc., Waltham, MA, USA
CO_2 Incubator (Heracell 150i)	Thermo Fisher Scientific Inc., Waltham, MA, USA
Flow Cytometer (BD LSR II)	BD Biosciences, Becton, Dickinson and Company, Franklin Lakes, NJ, USA
-20°C freezer (economic Froster)	Robert Bosch GmbH, Stuttgart, Germany
-80°C freezer (Hera freeze HFU B series)	Therma Fisher Scientific Inc., Waltham, MA, USA
Fridge (FKS 1800-20, profi line)	Liebherr-International AG, Bulle, Switzerland
Ice Flaker (Scotsmann AF80)	Hubbard Systems, Ipswich, GB
Micro scale (MC1 Analytic AC 210 S)	Sartorius, Data Weighing Systems, Elk Grove Village, IL, USA
Microscopes (Eclipse TS100 and E100, Olympus BX51 microscope)	Nikon, Chiyoda, Tokio, Japan Olympus K.K., Shinjuku, Tokio, Japan
Neubauer Counting Chamber, improved, depth 0.100 mm (0.0025 mm^2)	LO-Laboroptik, Lancing, GB
Pipets (2, 10, 20, 100, 200, 1000 µl Pipetman)	Gilson Inc., Middleton, WI, USA
Vortex Mixer (Vortex Genie 2)	Bender & Hobein AG, Zürich, Switzerland
Work bench (Herasafe KS)	Thermo Fisher Scientific Inc., Waltham, MA, USA

Table 4: Laboratory equipment.

3.1.2. Plasticware

Consumables	**Manufacturer**
96-well round bottom cell culture plate	Greiner Bio-One GmbH, Frickenhausen, Germany
Disposable gloves	VWR International, Radnor, PA, USA
FACS-Tubes, 5 ml	Corning Inc., Corning, NY, USA
Falcon (15 ml; 50 ml)	Greiner Bio-One GmbH, Frickenhausen, Germany
Feather disposable scalpel	Feather Safety Razor Co., Ltd., Osaka, Japan
Glass bottle, 100 ml	VWR International, Radnor, PA, USA
Glass pipets, 10 ml	Greiner Bio-One GmbH, Frickenhausen, Germany
Nylon Cell strainer 70 µm	Thermo Fisher Scientific Inc., Waltham, MA, USA
Petri dish, 94 x 16 mm	Greiner Bio-One GmbH, Frickenhausen, Germany
Pipet tips (2, 10, 100, 200, 1000 µl)	Greiner Bio-One GmbH, Frickenhausen, Germany
Sage seal reaction tubes, 2 ml	Eppendorf AG, Hamburg, Germany

Table 5: Plasticware.

3.1.3. Reagents, kits and chemical solutions

Reagents and solutions	**Manufacturer**
Albumin Fraction V, free from biotine (BSA) EC-No.: 292-322-5	Roth GmbH & Co. KG, Karlsruhe, Germany
Aqua dest. (ddH_2O)	B.Braun Melsungen AG, Melsungen, Germany
BD Cytofix/Cytoperm kit	BD Biosciences, Becton, Dickinson and Company, Franklin Lakes, NJ, USA

BD FACS Flow™	BD Biosciences, Becton, Dickinson and Company, Franklin Lakes, NJ, USA
BioColl density 1,077 g/ml, isotonic	Merck, Darmstadt, Germany
Collagenase Type II EC-No.: 232-582-9	Thermo Fisher Scientific Inc., Waltham, MA, USA
Collagenase Type IV EC-No.: 232-582-9	Worthington Biochemical Cooperation, Lakewood, NJ, USA
Deoxyribonuclease I (DNase I) from bovine pancreas EC-No. 232-667-0	F. Hoffmann-La Roche AG, Basel, Switzerland
Dispase II (neutral protease, grade II) from bacillus polymyxa EC-No.: 232-642-4	F. Hoffmann-La Roche AG, Basel, Switzerland
Ethanol 70% denatured EC-No.: 200-578-6	Roth GmbH & Co. KG, Karlsruhe, Germany
Ethanol 100% EC-No.: 200-578-6	Roth GmbH & Co. KG, Karlsruhe, Germany
Ethylenediaminetetraacetic Acid (EDTA) Disodium Salt Dihydrate ($C_{10}H_{14}N_2O_8Na_2 \times 2H_2O$) EC-No.: 205-358-3	AppliChem GmbH, Darmstadt, Germany
Hank's Balanced Salt Solution (1x) (HBSS)	Thermo Fisher Scientific Inc., Waltham, MA, USA
2-(4-(2-Hydroxyethyl)piperazin-1-yl)ethane-1-sulfonic acid (HEPES, $C_8H_{18}N_2O_4S$) EC-No.: 230-907-9	Sigma-Aldrich, St. Louis, MO, USA
Human Albumin 20%, salzarm (HSA)	CSL Behring, King of Prussia, PA, USA
MMP Sense TM 645 FAST	Pekrin Elmer, Boston, USA
Penicillin-Streptomycin (10.000 Units Penicillin, 10 mg Streptomycin/ml in 0,9% NaCl)	Sigma-Aldrich, St. Louis, MO, USA
Privigen (normal Immunoglobulin for humans IVIg 100 mg/ML)	CSL Behring LLC, King of Prussia, PA, USA
Sodium Chloride (NaCl) EC-No.: 617-042-6	Roth GmbH & Co. KG, Karlsruhe, Germany
Trypan Blue Solution (0,4%)	Sigma-Aldrich, St. Louis, MO, USA

Trypsin Inhibitor EC-No.: 232-987-0	Worthington Biochemical Cooperation, Lakewood, NJ, USA
X-Vivo 15	Lonza Group AG, Basel, Switzerland
Zinc formalin fixative	Sigma-Aldrich, St. Louis, MO, USA

Table 6: Reagents, kits and chemical solutions.

3.1.4. Antibodies

Antibody	**Clone**	**Isotype**	**Conjugated Fluorochrome**	**Manufacturer**
CD11b	HI11b	Mouse IgG1	PerCP	ImmunoTools, Friesoythe, Germany
CD11b	ICRF44	Mouse IgG1, kappa	PE	BD Pharmingen, BD Biosciences, Becton, Dickinson and Company, Franklin Lakes, NJ, USA
CD11c	3.9	Mouse IgG1, kappa	BV421	BioLegend, San Diego, CA, USA
CD14	HCD14	Mouse IgG1, kappa	PE-Cy7	BioLegend, San Diego, CA, USA
CD14	61D3	Mouse IgG1, kappa	PE-Cy7	ThermoFisher, Scientific Inc., Waltham, MA, USA
CD16	3G8	Mouse IgG1, kappa	eFluor 450	BioLegend, San Diego, CA, USA
CD28	CD28.2	Mouse IgG1, kappa	BV650	BioLegend, San Diego, CA, USA
CD36	5-271	Mouse IgG2a, kappa	APC-Cy7	BioLegend, San Diego, CA, USA

CD45	H130	Mouse IgG1, kappa	FITC	BioLegend, San Diego, CA, USA
CD56	CMSSB	Mouse IgG1, kappa	PE-Cy5	eBioscience, ThermoFisher Scientific Inc., Waltham, MA, USA
CD62L	HI62L	Mouse IgG2a	PerCP	AAT Bioquest, Pleasanton, CA, USA
CD68	Y1/82/7	Mouse IgG2b, kappa	BV711	BD Horizon, BD Biosciences, Becton, Dickinson and Company, Franklin Lakes, NJ, USA
CD69	FN50	Mouse IgG1, kappa	PE	BioLegend, San Diego, CA, USA
CD101	BB27	Mouse IgG1	PE	Miltenyi Biotec, Bergisch Gladbach, Germany
CD163	GHI/61	Mouse IgG1, kappa	BV421	BioLegend, San Diego, CA, USA
CD163	GHI/61	Mouse IgG1, kappa	PE	ThermoFisher Scientific Inc., Waltham, MA, USA
CD206	15-2	Mouse IgG1, kappa	APC-Cy7	BioLegend, San Diego, CA, USA
CD206	DCN228	Mouse IgG1	PE-Vio770	Miltenyi Biotec, Bergisch Gladbach, Germany
CD209	9E9A8	Mouse IgG2a, kappa	PE	BioLegend, San Diego, CA, USA
Fixable Viability Dye			eFluor 506	eBioscience, ThermoFisher Scientific Inc., Waltham, MA, USA
HLA-DR	LN3	Mouse IgG2b, kappa	APC	eBioscience, ThermoFisher Scientific Inc., Waltham, MA, USA

KLF2	665333	Mouse IgG2b	APC	R&D Systems, Minneapolis, MN, USA

Table 7: Antibodies and fluorochromes used in flow cytometry.

3.1.5. Buffers

Buffer	**Composition**
70% ethanol	35 ml > 99,9% ethanol, 15 ml Aqua dest.
FACS-buffer	1 L 1xPBS, 25 ml HSA, 2 ml 0.5 M EDTA, 200 µl Privigen Infusion Solution
Fc-blocking buffer (unlabeled mAb to block unspecific binding of Fc receptor)	10 ml 1xPBS, 10 µl Privigen
1x PBS	0.1 L 10x PBS, 1 L Aqua dest.
PBS-EDTA-buffer	1 L 1x PBS, 2 ml 0.5 M EDTA

Table 8: Buffers.

3.1.6. Software

Software	**Manufacturer**
FlowJo version 10.0	Tree Star, Inc., Ashland, OR, USA
GraphPad Prism 8.0.1	Graphpad Software, Inc., La Jolla, CA, USA
Image J version 1.51m9	Wayne Rasband, National Institutes of Health, USA
Microsoft Office 2019	Microsoft Corporation, Redmond, WA, USA

Table 9: Software.

3.2. Methods

Tissue preparations were performed at a sterile work bench at room temperature (22-26°C) if not stated otherwise.

3.2.1. Tissue samples

Initially ethical approval was obtained from the Ethics Committee of the Landesärztekammer Rheinland-Pfalz, Mainz, Germany. In the course of the research for this thesis the University Medical Center Mainz established a general consent for the research use of surplus material obtained during procedures which obviated the need for an ethical approval. All individuals provided written informed consent.

Human varicose veins were obtained through phlebectomy (Figure 2) performed as an elective ambulatory surgery in the department of dermatology at the University Medical Center Mainz. Samples were used completely anonymized.

Additionally excess human vein grafts used for coronary bypass surgery performed at the department for heart and vascular surgery at the University Medical Center Mainz were obtained.

3.2.2. Mechanical processing and tissue digestion

3.2.2.1. Established digestion

Varicose veins were obtained through phlebectomy as shown in Figure 2. Phlebectomies were performed as an elective ambulatory surgery at the Department of Dermatology at the University Medical Center Mainz.

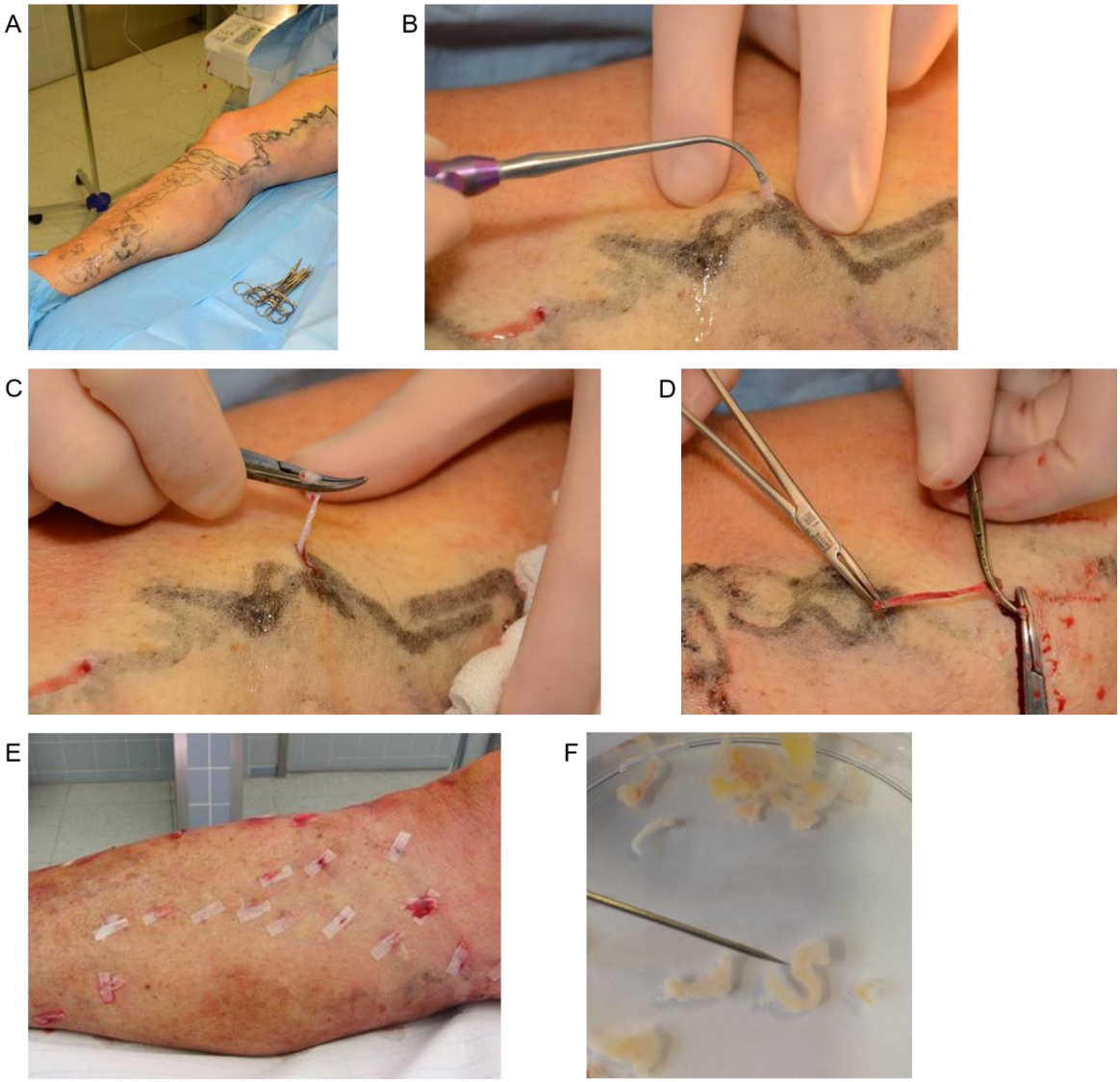

Figure 2: Phlebectomy. A) Preoperatively varicose veins are marked while patient is standing. B) After injection of local anesthetic varicose veins are pulled through a stab incision using a hook. C) The varicose vein is clamped with a hemostat. D) Extracted vein. E) Stab incisions are closed using butterfly bandages. F) Representative varicose vein after removal of adherent blood. Tortuosity of vein marked by needle.

All tissue samples were stored in 20 ml X-Vivo15 with 1% penicillin-streptomycin in a 50 ml falcon at 4 °C up to 24 h until further analysis.

Samples were transferred into petri dishes and covered with sterile 1 x PBS as shown in figure 3.

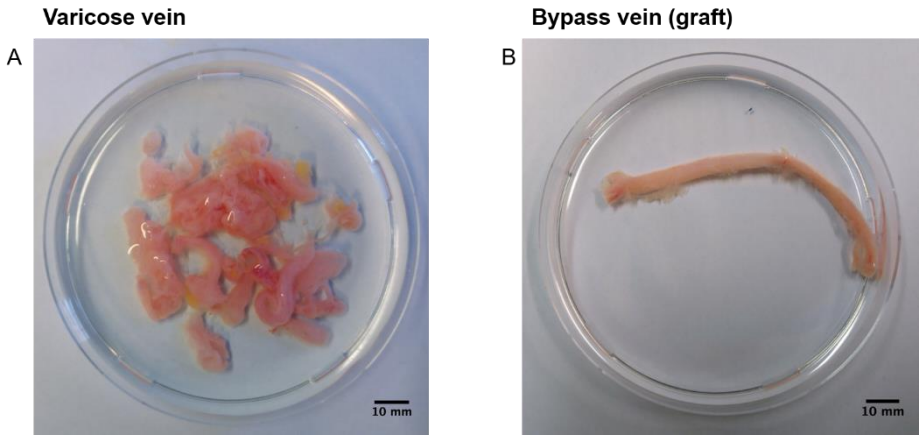

Figure 3: Tissue samples examples.

Adherent blood was removed by cutting the vessel open and slightly scratching the inner surface with a scalpel followed by repeated shaking of the tissue in the wash medium (Figure 4). After the first cleaning step were cut to small pieces using a disposable scalpel and shaken up again in the medium with the aid of a vortexer. All washing media up to this point were kept for flow cytometric analysis.

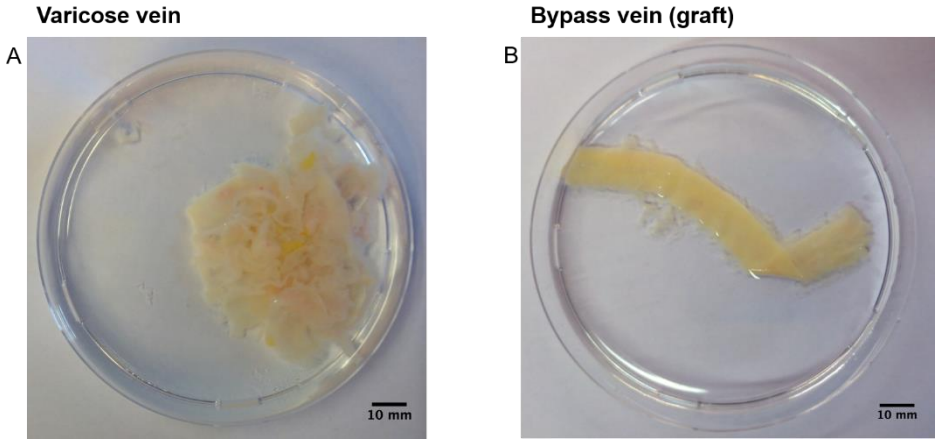

Figure 4: Tissue samples after opening and removal of adherent blood.

Fragmented samples were lysed for 20 to 30 min at 37°C and 5% CO_2 using a mixture of collagenase type II (1 mg/ml) and DNase I (0.1 µg/ml) in 5 ml HBSS (Luther et al., 2016). Every 5 min samples were mixed for 30 to 60 s using a vortex mixer. The resulting cell suspension was passed through a 70 µm nylon cell strainer moistened with 1 x PBS containing 1% HSA into 50 ml falcon tubes and filled up with 1 x PBS, then centrifuged for 10 min at 4 °C and 350 g. Pellets were resuspended in 5 ml transferred to 15 ml falcon tubes with FACS - buffer.

After a second wash step cell pellets were resuspended in 100-200 µl left medium for further analysis.

3.2.2.2. Alternative digestion

Alternative to the established digestion, tissue fragments were lysed for 60 min at 37 °C and 5% CO_2 using a mixture of collagenase IV (450 U/ml), DNase I (500 U/ml), trypsin inhibitor (1 mg/ml) and HEPES (4.8 mg/ml) in 5 ml HBSS (Patino et al., 2006).

3.2.3. Isolation of PBMC from human blood

Buffy coats were obtained from the transfusion center at the department of transfusion medicine at the University Medical Center Mainz. After surface disinfection, the blood bag was opened and transferred to a 100 ml glass bottle. Four 50 ml falcons were filled with 15 ml Bicoll Separating Solution and the blood of one buffy coat layered on top without intermixture of both layers using a 10 ml glass pipette. The falcons were centrifuged for 30 min at 25 °C and 350 g. Subsequently to centrifugation three distinct layers were definable. The layer with PBMCs was carefully transferred to 50 ml falcons using a 10 ml glass pipette filled up with PBS–EDTA-buffer and centrifuged for 6 min at 4 °C and 350 g. Pellets were resuspended in 25 ml PBS–EDTA-buffer and centrifuged again for 6 min at 4 °C and 350 g. Thereafter, the samples were combined. Samples were additionally washed until the supernatant was clear. A cell sample was counted upon Tryptan blue staining using a Neubauer Improved Counting Chamber and the cell number was calculated.

3.2.4. Flow cytometry

Flow cytometry was used for rapid multi-parametric analysis of single cell suspensions based on light scattering and surface marker staining with fluorescent dye coupled specific antibodies.

3.2.4.1. Fluorochromes

Fluorochrome	Abbreviation	Excitation peak [nm]	Emission peak [nm]
Allophycocyanin	APC	650	660
Allophycocyanin-Cyanine7	APC-Cy7	650	774
Brilliant Violet 421	BV421	405	421
Brilliant Violet 650	BV650	405	645
Brilliant Violet 711	BV711	405	711
-	eFluor 450	405	445
-	eFluor 506	419	508
Fluorescein isothiocyanate	FITC	493	525
Pacific Blue	PB	410	455
R-Phycoerythrin	PE	565	575
R-Phycoerythrin-Cyanine5	PE-Cy5	565	670
R-Phycoerythrin-Cyanine7	PE-Cy7	565	774
Peridinin-chlorophyll protein complex	PerCP	482	675
Pycoerythrin-Vio770	PE-Vio770	565	775

Table 10: Fluorochromes used on flow cytometry antibodies.

3.2.4.2. Cell surface staining

Isolated single cell suspensions were transferred to 96 well round bottom cell culture plates, washed with FACS-buffer and pelleted by centrifugation at 350 g for 10 min at 4 °C. All isolated cells of the veins were used. To block nonspecific Fc receptor-mediated binding of staining antibodies all samples were mixed well with 20 µl Fc-blocking buffer and incubated for 30 min at 4 °C. Cells were subsequently stained with fluorochrome-conjugated antibodies for 30 min at 4 °C protected from light. After two washing steps with 100 µl FACS-buffer to remove unbound antibodies, cells were re-pelleted by centrifugation for 10 min at 4 °C and 350 g and were fixed using 100 µl BD Cytofix/Cytoperm fixation solution per well. Stained cells were stored in at 4 °C overnight, washed the next day using 1 x Permeabilisation buffer (mixing 1 part of 10 x concentrate with 9 parts distilled water). After two additional washing steps with FACS buffer and pelleting cells were resuspended in 200 µl FACS buffer and transferred to FACS tubes for flow cytometric data acquisition.

3.2.4.3. Staining intracellular (nuclear) targets

Cells which were stored at 4 °C overnight were washed two times using 1 x Permeabilisation buffer and pelleted by centrifugation for 10 min at 4 °C and 500 g. The cells were then incubated for 30 min at 4 °C protected from light with the desired antibody. Two washing steps using 1 x Permeabilisation buffer, then using FACS buffer were performed to remove unbound antibodies. Samples were resuspended in 200 µl FACS buffer and transferred to FACS tubes for flow cytometric data acquisition.

3.2.4.4. Flow cytometric analysis

Flow data were acquired on a BD LSR II cytometer. Data were subsequently analyzed using Flow Jo Version 10.0.

3.2.5. Statistical Analysis

Statistical analysis was performed with GraphPad Prism 8.0.1. Normal distribution was tested using D'Agostino–Pearson test. If data were normally distributed, an unpaired t-test was performed for two unmatched groups. Multiple groups were analyzed by one–way ANOVA. If data were not normally distributed, a Mann – Whitney test was performed. Statistical significance was assumed if the p value < 0.05.

3.2.6. Histology

Regions with tortuosities and abnormalities were macroscopically identified in the tissue samples before samples were cut open. The regions were harvested and fixed in 2-3 ml 4% zinc formalin in a 15 ml falcon. After 24 hours zinc formalin was removed and the falcon filled with 70% ethanol. Marina Thielen (group Prof. Schäfer, Cardiology, University Medical Center Mainz) kindly embedded, cut and stained samples using Masson's trichrome staining with Verhoeff's elastica staining and performed immunohistochemistry for CD68 according to the standard operating procedures (Drosos et al., 2019).

Immunohistochemistry is using an antigen-antibody-reaction where an antibody coupled with a detection system binds specifically and strongly to the desired antigen making it visible in the tissue (Lang, 2012).

All pictures of the completed object slides were taken using an Olympus BX51 microscope.

4. RESULTS

4.1. Human bypass vein graft

4.1.1. Histology

4.1.1.1. Vein structure

In general, a healthy vein consists of three layers: at the luminal side the *tunica intima* consists of a single endothelial cell layer, followed by the *tunica media* formed by several layers of smooth muscle cells (Rehfeld et al., 2017). The *tunica adventitia* or *tunica externa* forms the outer layer and consists of connective tissue with vasa and nervi vasorum (Ovalle and Nahirney, 2013). Masson's trichrome stain with Verhoeff's elastica stain (MTC-VES) allowed to distinguish muscle cells (red), the extracellular matrix (green/blue) and elastic fibers (black) (O'Connor and Valle, 1982). A representative MTC-VES staining of a human bypass vein graft shows the tissue structure including connective tissue (blue) and the internal elastic membrane (Figure 5).

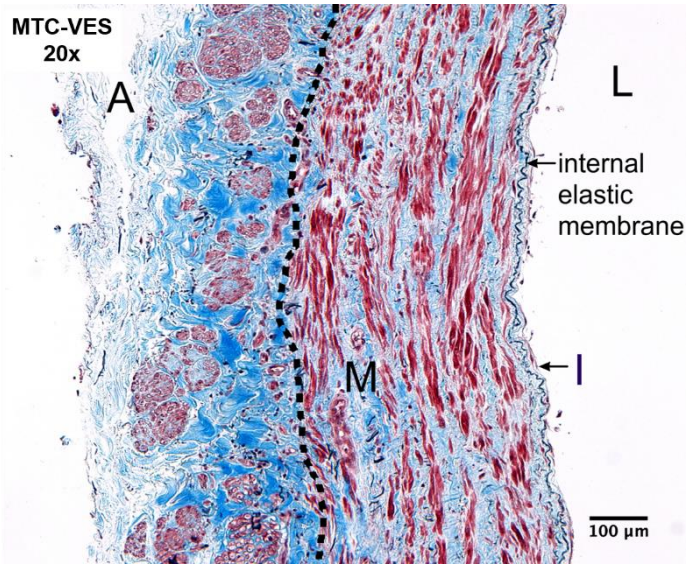

Figure 5: Bypass vein structure. Representative Masson's trichrome and Verhoeff's elastica staining (MTC-VES) of a saphenous vein bypass sample FFPE section. (A) tunica adventitia, (I) tunica intima, (M) tunica media, (L) lumen, internal elastic membrane marked by arrow. The

border between the tunica media and tunica adventitia was marked up by a dotted line. Magnification 20x on Olympus BX51 microscope.

4.1.1.2. CD68 - expressing cells

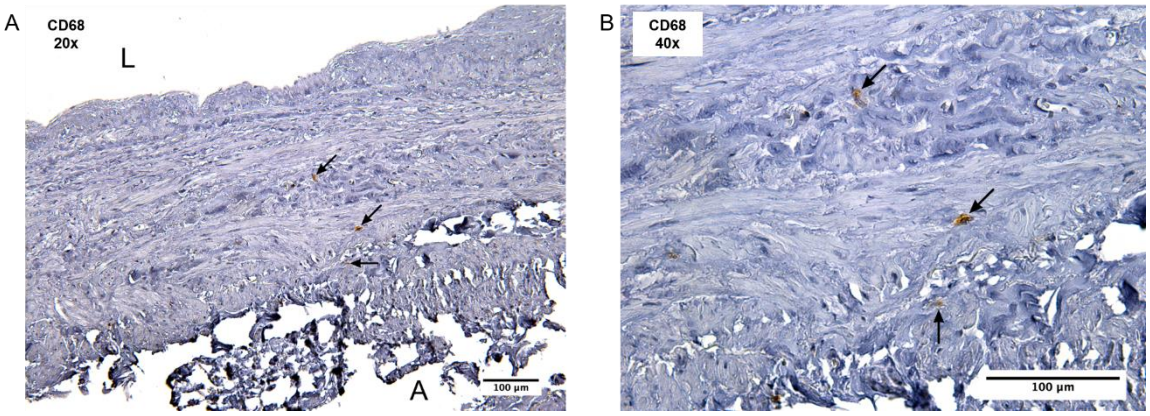

Figure 6: CD68$^+$ cells in saphenous vein bypass samples. Representative CD68 staining of a saphenous vein bypass sample FFPE section. A) Overview, 20x magnification. B) 40x magnification. (A) tunica adventitia, (L) lumen, CD68$^+$ cells marked by arrows. All pictures were taken on Olympus BX51 microscope.

CD68 staining showed isolated cells in the tunica media, but not in the intima or adventitia. This suggests that the respective cells are tissue macrophages. As the staining was spiky, it probably marked cell spurs.

4.1.2. Flow cytometry

4.1.2.1. Frequency of CD45$^+$immune cells

As part of the vascular system and due to the surgical procedure vein samples contained peripheral blood. To minimize blood-borne immune cells and detect immune cells from the tissue, washing steps were performed before tissue digestion and the amount of detached cells analyzed by flow cytometry (Figure 7). Corresponding statistical analysis is shown in Figure 8.

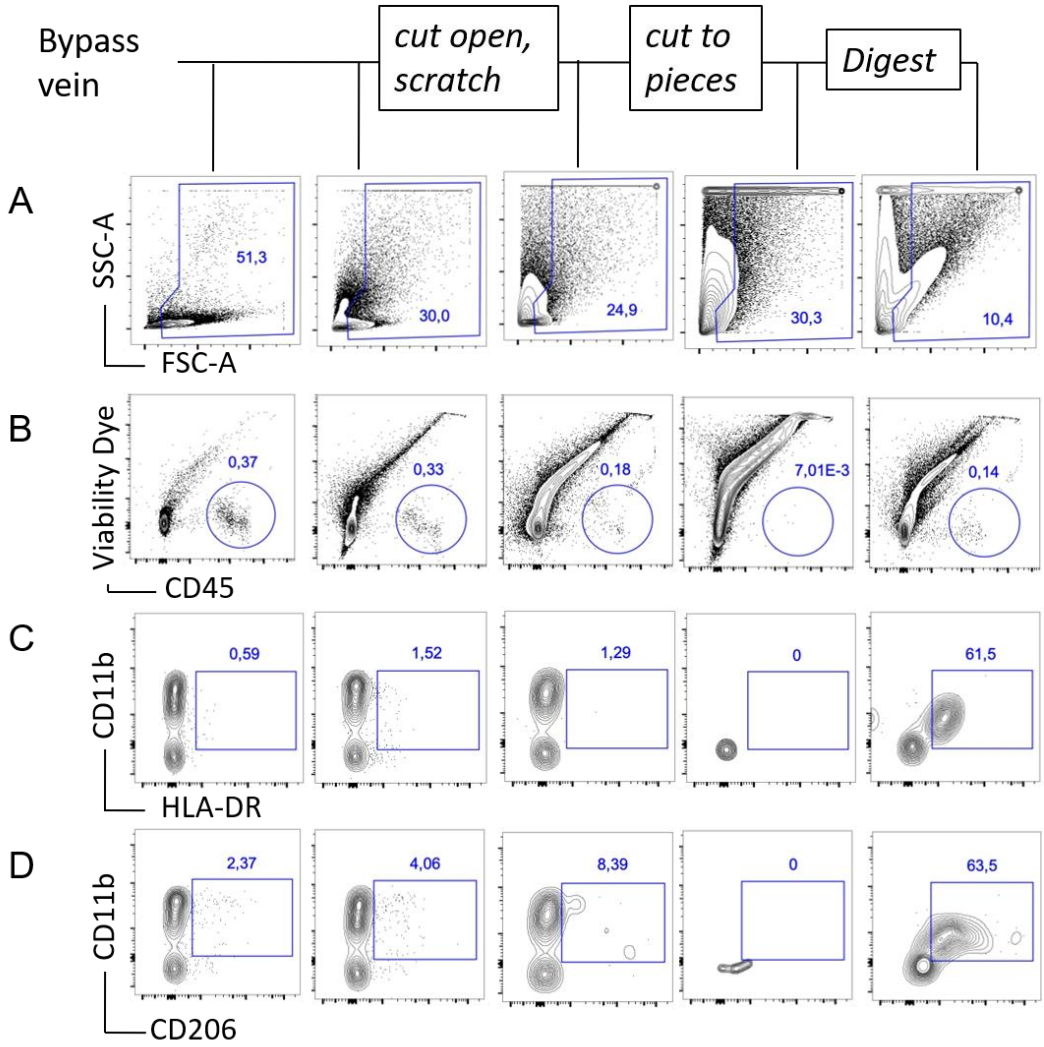

Figure 7: Flow cytometry of cells detached by washing steps in bypass vein tissue. A) Flow cytometric plot of scatter parameters of examined single cells suspensions during washing steps. B) Representative flow cytometric representation of percentage of detached live $CD45^+$ immune cells. C) Representative flow cytometric representation of percentage of detached $CD11b^+$ $HLA\ DR^+$ cells pregated on viable $CD45^+$ singlets (gating strategy shown in Figure 9). D) Representative flow cytometric representation of percentage of detached $CD11^+$ $CD206^+$ cells pregated on viable $CD45^+$ singlets.

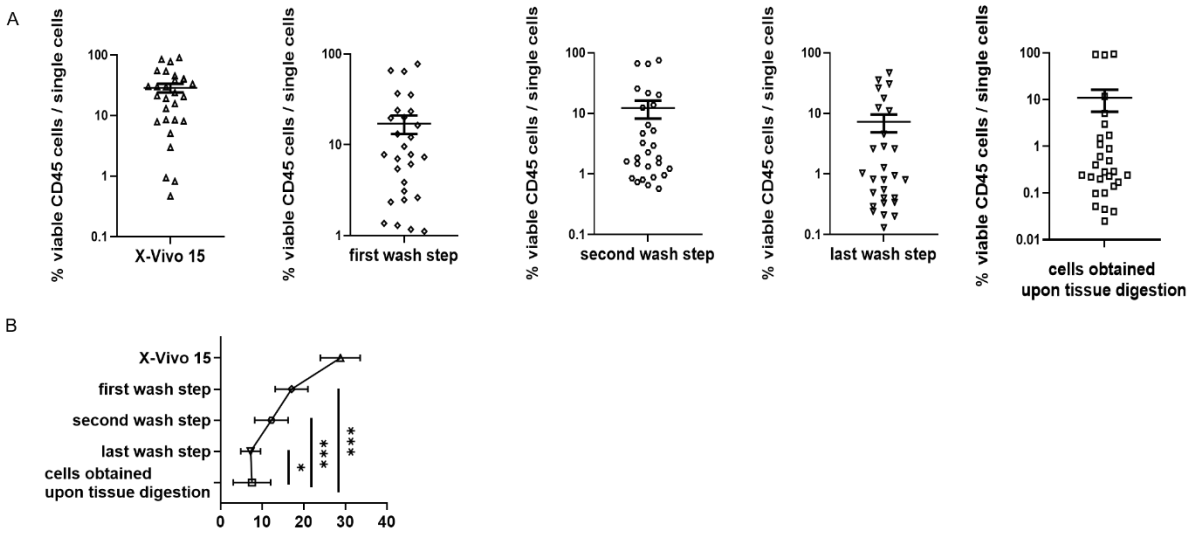

Figure 8: Statistical analysis of the percentage of CD45$^+$ immune cells detached by washing steps in bypass vein tissue. A) Percentage of viable CD45$^+$ single cells detached in each washing step. B) Statistically significant decrease of CD45$^+$ immune cells detached by each wash step in comparison to CD45$^+$ immune cells isolated by digestion. Data show mean ± SEM, ***p < 0.0001, *p 0.0151. Data representative of at least twenty independent experiments.

In order to free cells from tissue for flow cytometric analysis, the tissue had to be digested. The digestion process for varicose veins was previously established in the Becker lab and published in Luther et al. (2016). Compared to varices, the tissue of saphenous veins is more resistant. To obtain immune cells from the tissue without damaging their surface markers by the digestive enzymes, an alternative digestion previously published by Patino et al. (2006) for the isolation of macrophages from carotid artery plaques was tested. A comparative flow cytometric analysis of cells isolated from bypass vein tissue using the established and the alternative digestion is shown in Figure 9. Comparison of the two digestion methods showed that the approach of Patino et al. isolated a lower number of CD45$^+$ immune cells from the tissue.

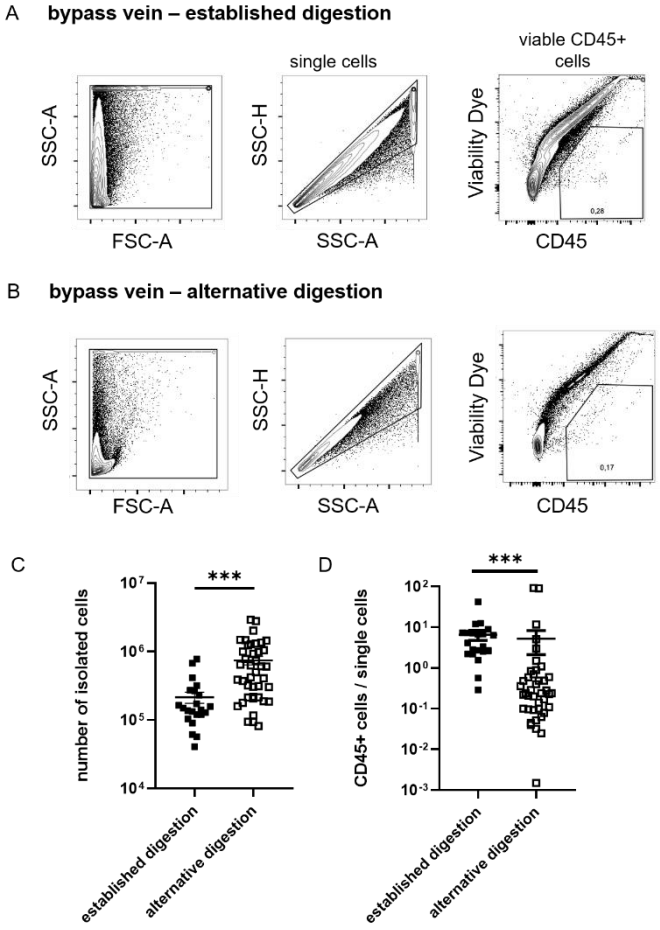

Figure 9: Comparison of immune cell yield from saphenous bypass vein by two digestions methods. A) and B) Gating strategy for $CD45^+$ cells with exclusion of duplicates and dead cells. A) Digestion according to Luther et al. B) Digestion according to Patino et al. C) Statistical analysis of total number of isolated cells from bypass vein tissue according to established (Luther) and alternative (Patino) digestion. Data show mean ± SEM. ***p 0.0008. D) Statistical analysis of frequency of isolated cells from bypass vein tissue according to established (Luther) and alternative (Patino) digestion. Data show mean ± SEM. ***p < 0.0001. Data are representative of at least ten independent experiments.

PBMC were similarly digested in parallel to evaluate the influence of tissue digestion on cell surface marker expression (Figure 10).

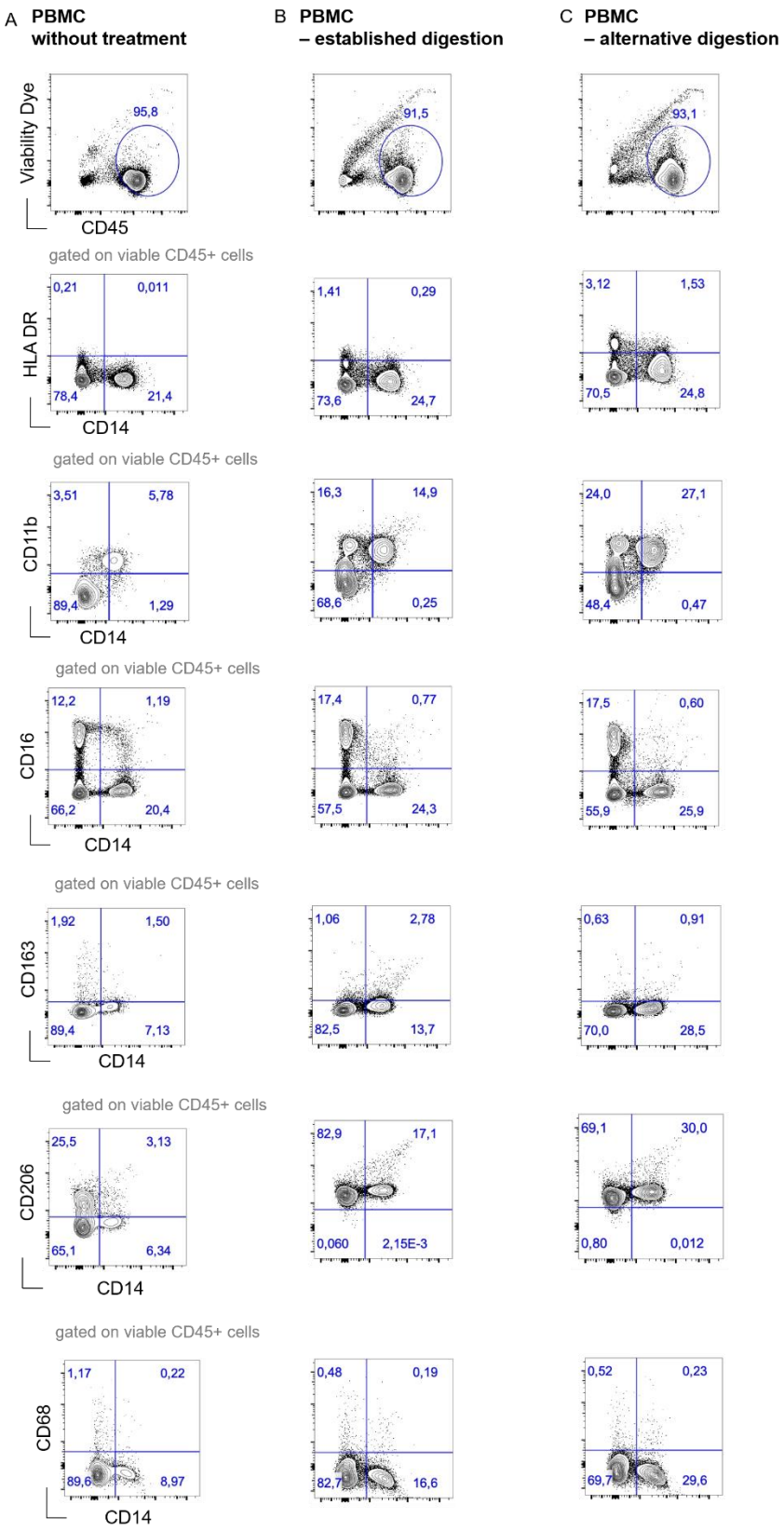

Figure 10: Both digestion methods did not affect the detection of cell surface markers. A) undigested PBMC. B) established digestion (Luther et al.). C) alternative digestion (Patino et al.).

The majority of bypass vein samples contained very low numbers of immune cells (less than 1%). In a few samples, these cells could be analyzed by flow cytometry using additional markers. This showed that the immune compartment in the bypass veins consisted predominantly of monocytic cells ($CD11b^+$ $CD14^+$ HLA DR^+) (Figure 11).

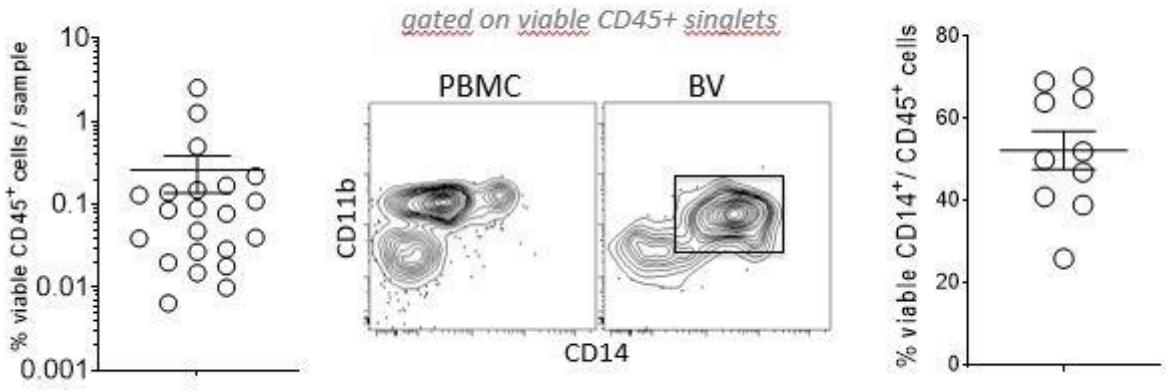

Figure 11: Saphenous bypass veins contain few immune cells mainly consisting of monocytic $CD45^+CD11b^+CD14^+$ cells (varicose veins: mean 3%, SD 2.2; bypass vein: mean 0.26%, SD 0.57, p-value bypass vs varicose < 0.0001). Data show mean ± SEM. Data are representative of at least eight independent experiments.

4.2. Varicose veins

4.2.1. Histology

4.2.1.1. Varicose vein structure

We used MTC-VES to show the structure of obtained varicose veins as shown in Figure 12.

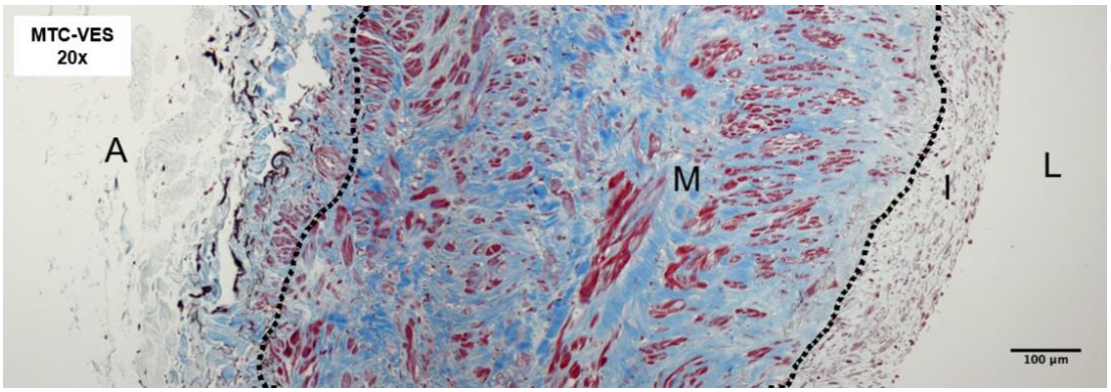

Figure 12: Varicose vein structure. Representative Masson's trichrome and Verhoeff's elastica staining (MTC-VES) of a varicose vein sample FFPE section. (A) tunica adventitia, (I) tunica intima, (M) tunica media. The borders between the tunica media, tunica adventitia and tunica intima were marked up by a dotted line. Magnification 20x on Olympus BX51 microscope.

4.2.1.2. CD68 – expressing cells

In contrast to saphenous veins used for bypass surgery, $CD68^+$ cells in varicose veins were found predominantly in the adventitia, as well as in the tunica media (Figure 13). In contradiction to the assumption of an immigration of immune cells from the blood via the luminal side, no $CD68^+$ cells were found in or near the tunica intima.

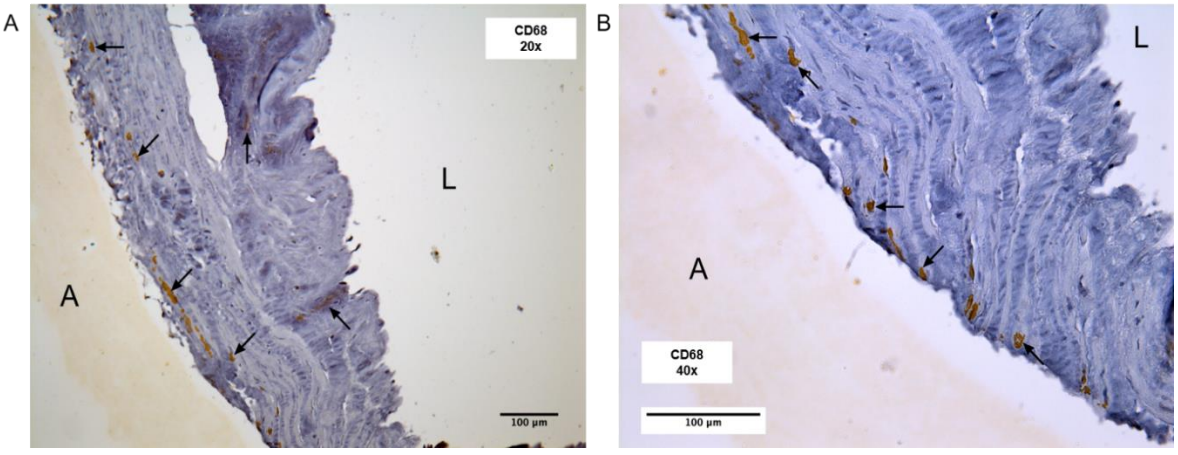

Figure 13: $CD68^+$ cells in varicose vein samples. Representative CD68 staining of a varicose vein sample FFPE section. A) Overview, 20x magnification. B) 40x magnification. C) 40x magnification. (A) tunica adventitia, (L) lumen, $CD68^+$ cells marked by arrows. All pictures were taken on Olympus BX51 microscope.

CD68 staining shows grouped cells in the tunica media and the tunica intima. This suggests that the cells are not only tissue macrophages but may include newly recruited monocytic cells.

4.2.2. Flow cytometry

4.2.2.1. Frequency of $CD45^+$ immune cells

Like veins from bypass surgery before, varices were first cleared of adherent blood cells by washing. The tissue was then cut into small pieces and shaken in medium to further remove adherent cells. In the final washing step, hardly any immune cells could be found (Figure 8). After subsequent digestion, however, immune cells were again retrieved, demonstrating that the latter were freed from the tissue by digestion.

To minimize contamination with blood and increase likelihood of analyzing $CD45^+$ cells isolated from tissue same washing steps as with bypass vein were performed and washing media analyzed as shown in Figure 14.

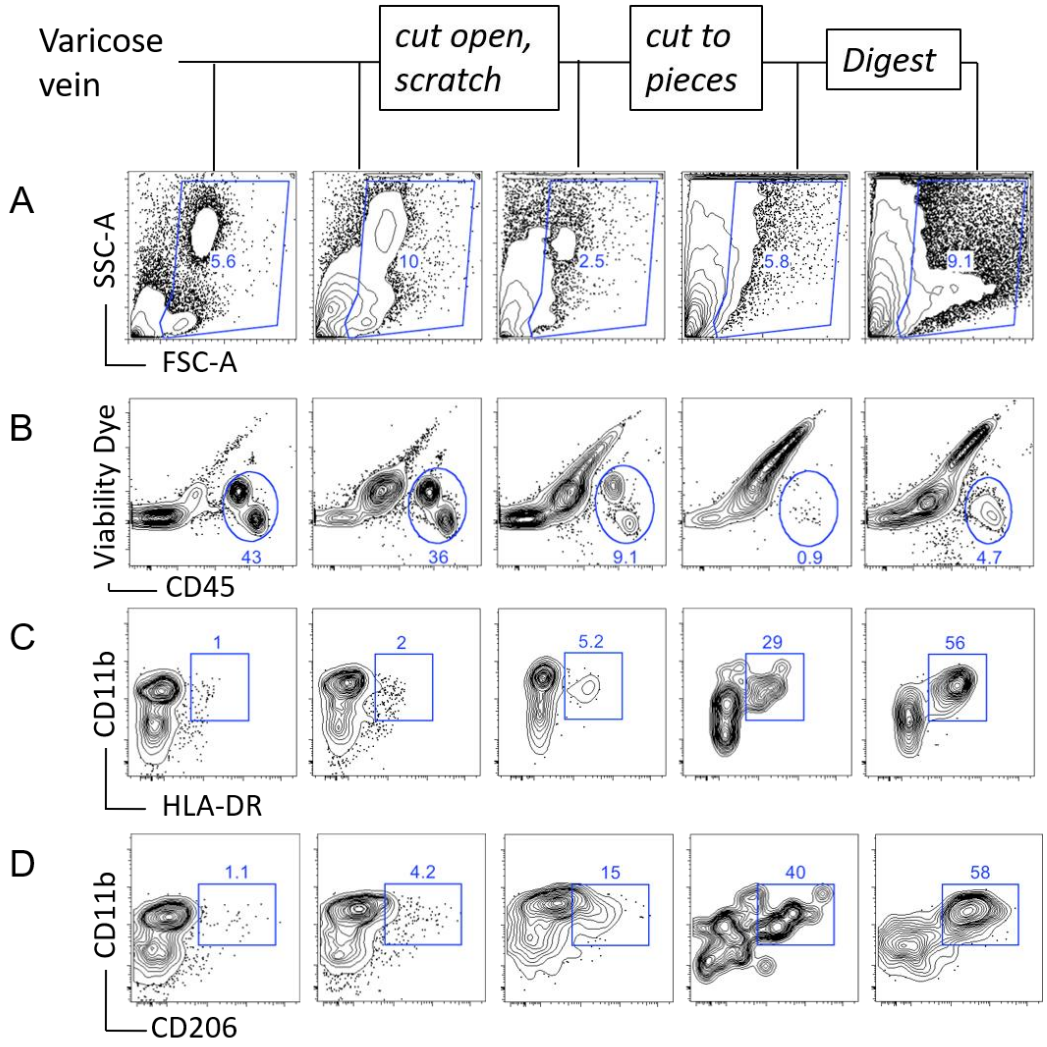

Figure 14: Immune cells frequencies detached by tissue dissection/washing and subsequent tissue digestion from varicose vein samples. A) Flow cytometric plot of scatter parameters of examined single cell suspensions during preparation steps. Ungated representative dot plots are shown. B) Representative flow cytometric representation of percentage of detached live $CD45^+$ immune cells. C) Representative flow cytometric representation of percentage of detached $CD11b^+$ $HLA\ DR^+$ immune cells gated on viable $CD45^+$ immune cells (gating strategy shown in Figure 9). D) Representative flow cytometric representation of percentages of detached $CD11b^+$ $CD206^+$ immune cells gated on viable $CD45^+$ immune cells (gating strategy shown in Figure 9).

The alternative digestion tested on bypass veins was also tested on varicose veins. Compared to the previously established digestion regimen, the alternative digestion resulted in an increased yield in number of isolated cells but a decreased frequency of $CD45^+$ cells

(Figure 15). Based on this observation data sets were pooled only if based on the same isolation method.

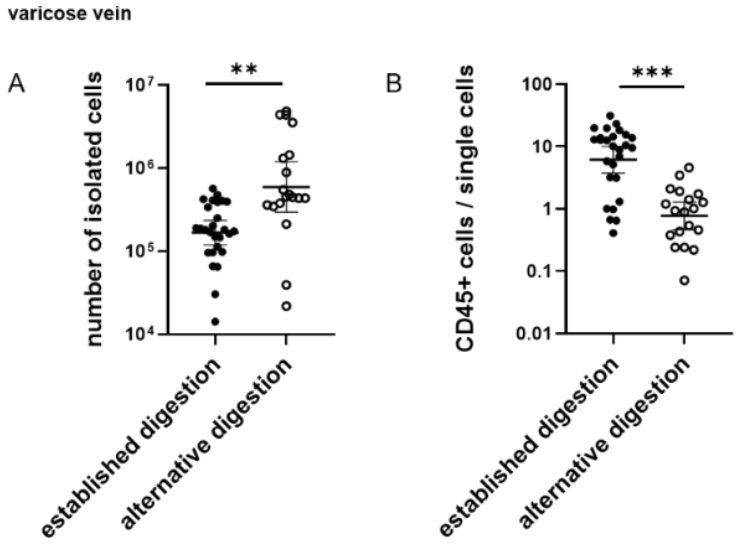

Figure 15: Isolated CD45$^+$ immune cells from varicose vein tissue using established and alternative digestion. A) Statistical analysis of total number of isolated cells from varicose vein tissue using established and alternative digestion. Data show mean ± SEM, **p 0.0001. B) Statistical analysis of frequency of CD45$^+$ cells isolated from varicose vein tissue using established and alternative digestion. Data shown mean ± SEM, ***p < 0.0001. Data are representative of at least ten independent experiments.

As observed with bypass vein samples before, the digestion method of Patino et al. appeared to result in a higher cell number per sample, but fewer CD45$^+$ immune cells from the tissue. The digestion method of Patino et al. apparently breaks down the tissue more into single cells but does not release more immune cells from tissue.

4.2.2.2. Definition of lineage markers for monocytic cells

4.2.2.2.1. Immune cells in varicose vein infiltrate

To analyze the composition of CD45$^+$ immune cell infiltrate we looked at T cells (Figure 16), monocytic cells, dendritic cells and natural killer cells (Figure 17).

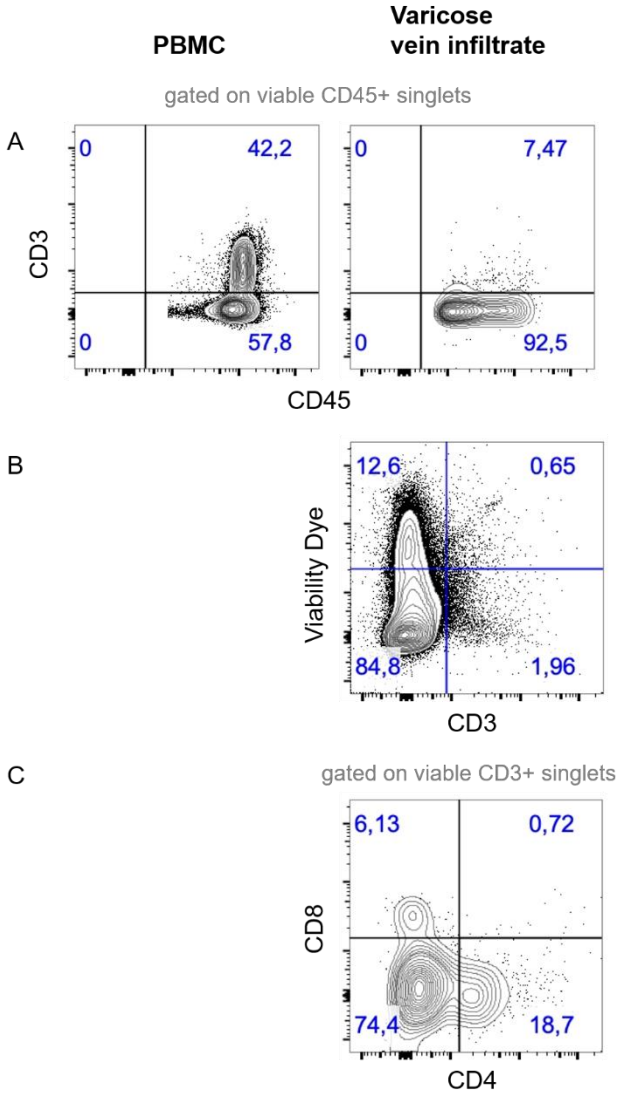

Figure 16: Flow cytometric analysis of T cells in PBMC and varicose vein infiltrates. A) Representative flow cytometric plots of $CD3^-$ and $CD3^+$ cells of $CD45^+$ immune cells. The majority of $CD45^+$ immune cells in varicose veins are $CD3^-$. B) Representative flow cytometric plots of single cells. Ungated representative dot plots are shown. C) Representative flow cytometric plots of $CD4^+$ and $CD8^+$ T cells pregated on viable $CD3^+$ singlets.

The majority of $CD45^+$ immune cells in varicose veins are $CD3^-$.

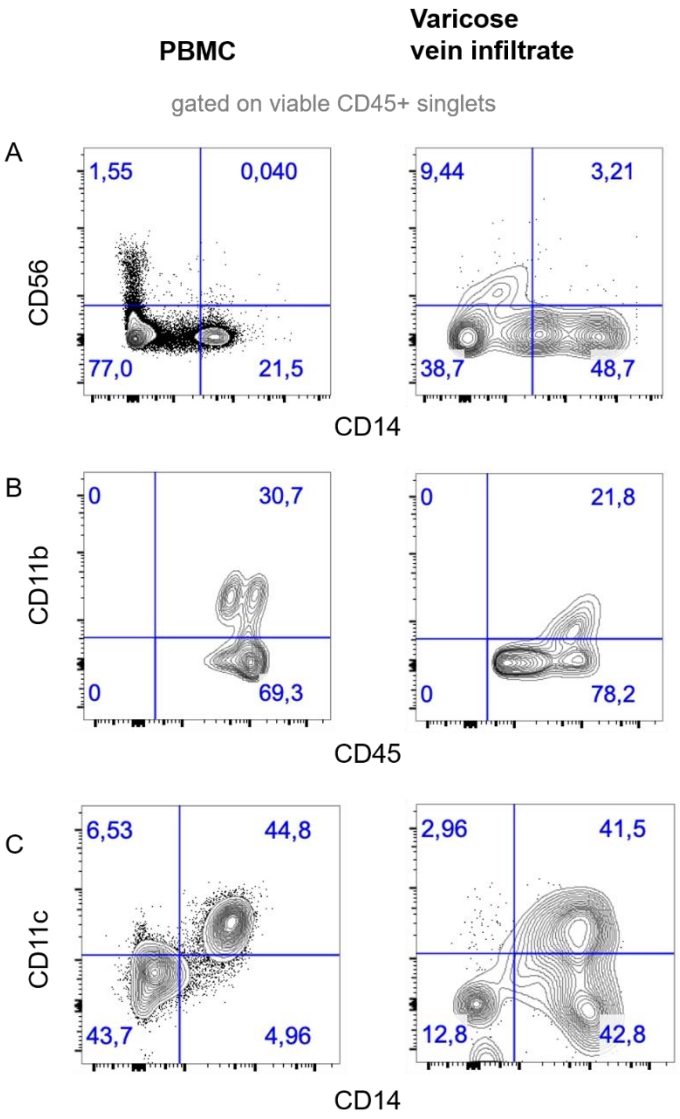

Figure 17: Flow cytometric analysis of dendritic cells and natural killer cells in PBMC and varicose vein infiltrate. A) Representative flow cytometric plot of natural killer cells defined as $CD14^-$ $CD56^+$ gated on viable $CD45^+$ singlets (gating strategy shown in Figure 9). The varicose veins contain more natural killer cells than PBMC. B) Representative flow cytometric plots of $CD45^+$ $CD11b^+$ cells gated on viable $CD45^+$ singlets (gating strategy shown in Figure 9). C) Representative flowcytometry of percentages of dendritic cells defined as $CD14^-$ $CD11c^+$. The varicose vein infiltrate only contains few dendritic cells.

Varicose vein infiltrate contains only few dendritic cells defined as $CD14^-$ $CD11c^+$ as shown in Figure 17. In the varicose vein infiltrate only few natural killer cells were found as shown in Figure 17. Therefore, the majority of $CD45^+$ $CD3^-$ cells are monocytic cells.

4.2.2.2.2. CD11b

Human myeloid cells express CD11b (Todd 3rd et al., 1981, Lambert et al., 2015). HLA DR and CD14 are established markers for flow cytometric identification of monocytes (Cossarizza et al., 2019). We combined CD11b with HLA DR and CD14 to identify monocytic cells. The corresponding gating strategy is shown in Figure 18. The corresponding representative flow cytometric plots and statistical analysis are shown in Figure 19.

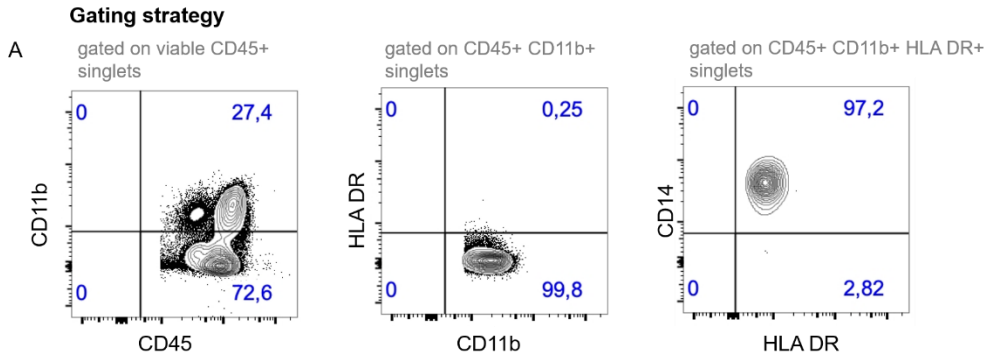

Figure 18: Gating strategy using CD11b, HLA DR and CD14 to identify monocytic cells. CD11b, HLA DR and CD14 were gated on single viable $CD45^+$ cells as shown in Figure 9. If CD11b was not stained, gating strategy was adapted by gating on $CD45^+$ $HLA\text{-}DR^+$ immune cells before gating on CD14.

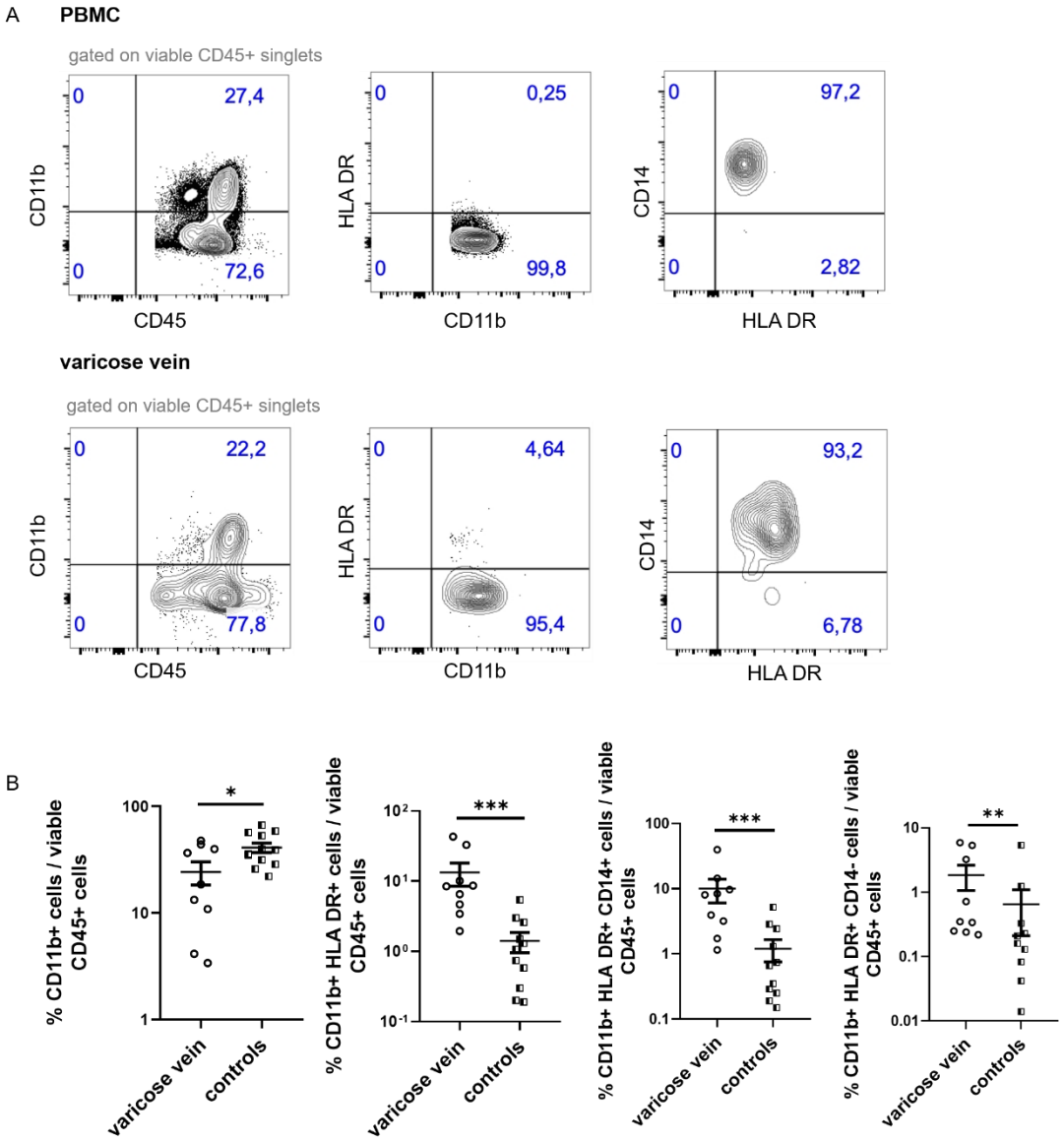

Figure 19: Frequency of CD11b^{+}, HLA DR^{+} and CD14$^{+/-}$ cells in varicose veins compared to controls (data pooled from PBMC and X – Vivo 15). A) Representative flow cytometric plots of CD11b^{+}, HLA DR^{+} and CD14$^{+/-}$ cells in controls and in a varicose vein sample. Gating strategy for single viable CD45^{+} cells shown in Figure 9. B) Statistical analysis. Data show mean ± SEM, *p 0.0001, **p 0.0091, ***p 0.0007. Data representative of at least seven independent experiments.

4.2.2.3. Additional markers

4.2.2.3.1. CD16

The expression of CD16 on human monocytes has been previously investigated and has been used to classify human monocytes (Cossarizza et al., 2019, Ziegler-Heitbrock et al., 2010). We adapted this strategy and looked for expression of CD16 on CD45$^+$ cells (figure 20).

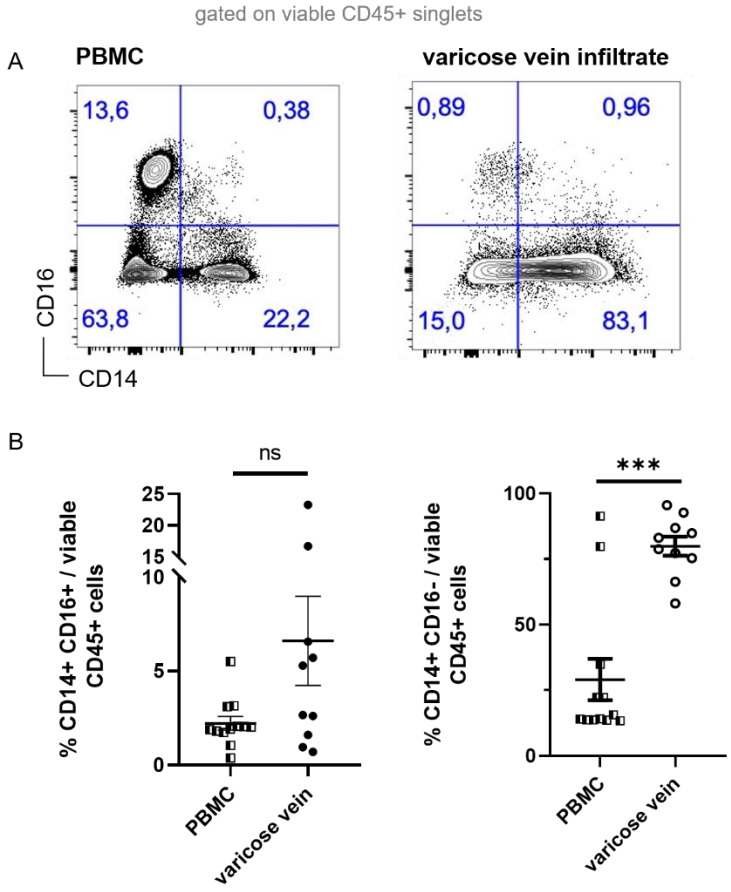

Figure 20: CD14 and CD16 expression on CD45$^+$ immune cells in varicose vein infiltrate compared to PBMC. A) Representative flow cytometric plot of CD45$^+$-pregated viable cells (gating strategy shown in Figure 9). B) Statistical analysis. Data show mean ± SEM, ns non-significant, ***p <0.0001. Data representative of at least eight independent experiments.

4.2.2.3.2. HLA DR, CD68, CD206 and CD163

CD68 is a protein mainly localized within the endosomal compartment and expressed in monocytes and macrophages among other non-myeloid cell populations (Gottfried et al., 2008).

CD206, also known as macrophage mannose receptor, is expressed on human macrophages (Gordon, 2003) and differentiated monocytes (Austermann et al., 2022). Enhanced expression of CD206 on macrophages has been shown after alternative activation (Porcheray et al., 2005, Goerdt and Orfanos, 1999).

CD163, also known as haemoglobin scavenger receptor, is exclusively expressed in monocytes and macrophages (Kristiansen et al., 2001, Pulford et al., 1992).

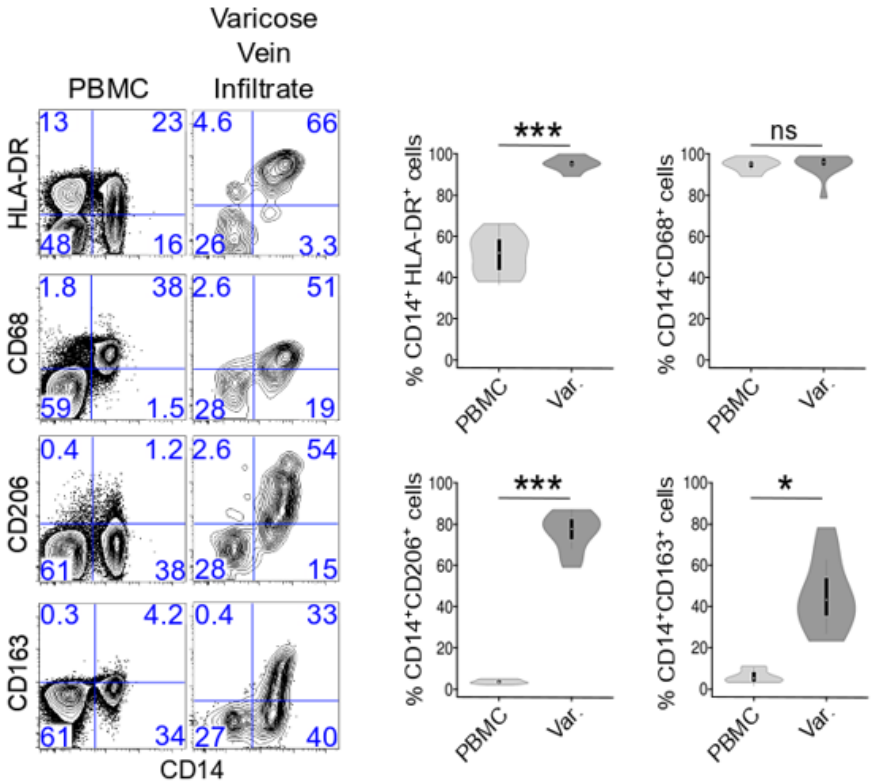

Figure 21: Analysis of CD68, CD206 and CD163 on $CD14^+$ cells in varicose veins compared to PBMC. Representative flow cytometric plots with corresponding statistical analysis. Data show mean ± SEM. Data representative of at least fifteen independent experiments.

CD14+ cells in varicose vein infiltrates express uniformly HLA DR+ and CD68+ and partially CD206+ and CD163+.

4.2.2.3.3. CD101

CD101 has been shown to be expressed on human CD14$^+$ monocytes and GM-CSF differentiated macrophages (Ohradanova-Repic et al., 2016, Damasceno et al., 2016).

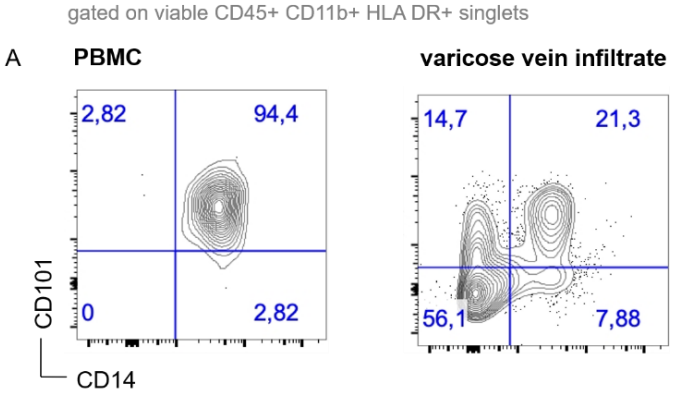

Figure 22: Analysis of CD101 on CD14$^+$ cells in varicose veins compared to PBMC. Representative flow cytometric plots of viable CD45$^+$ CD11b$^+$ HLA DR$^+$ cells (gating strategy shown in Figure 18).

5. DISCUSSION

This study established a method to isolate single cells from bypass veins without damaging cell surface markers for flow cytometric analysis. It also showed that varicose veins contain more $CD45^+$ immune cells in comparison with bypass veins. The majority of these cells were monocytic cells.

For this work, surplus material was obtained from patients. The study population was not further defined as no information regarding preoperative investigations was obtained. This approach guaranteed more samples to analyze by accepting an additionally higher biological variance. As for further investigation on varicose veins it would be useful to at least obtain the CEAP classification for the patient as additional information. For further investigations on bypass veins which are intended as healthy controls preoperatively excluding venous reflux and chronic venous disease in patients undergoing bypass surgery seems to be necessary for a more uniform population.

The differences between bypass veins and varicose vein were shown histologically. Varicose veins showed a thickening of the intima and media as well as disorganization of the media much as previously described by Cherian et al. (1999) and Sayer and Smith (2004). In contrast to bypass veins the elastic internal membrane was not detectable in varicose veins which might be because the tissues are from different anatomical locations of the venous system. This study showed that in both tissues CD68 – expressing cells were present in accordance with previous results (Buján et al., 2008, Cherian et al., 1999, Sayer and Smith, 2004). Although immunohistochemistry using CD68 is an established method, it has been shown to not be specific for the identification of macrophages (Kunisch et al., 2004). In addition to Buján et al. (2008), Cherian et al. (1999) and Sayer and Smith (2004) Chu et al. (2013) also demonstrated the presence of inflammatory cells. Further investigations are needed to histologically analyze immune cells in both tissues.

Single cells were isolated from the tissues through enzymatic digestion. Interestingly the alternative digestion by Patino et al. increased the total number of isolated cells but did not increase the frequency of $CD45^+$ immune cells. Speculatively, the alternative digestion method by Patino et al. isolated more tissue cells. $CD45^+$ immune cells seem to be rather loosely in tissues and easier to extract if the tissue is not completely dissolved as accomplished with the established digestion by Luther et al.

Both digestion protocols did not damage cell surface markers. Expression of CD45, CD163, CD16 and CD68 were unaffected by both digestion methods. Luther et al. (2016) demonstrated that their digestion method did not damage CD62L which is a molecule susceptible to damage

by enzymatic tissue disintegration in mice (Autengruber et al., 2012). Cells isolated by either enzymatic digestion method showed a higher expression of CD11b and CD206.

Buján et al. (2008) and Sayer and Smith (2004) showed histologically that bypass veins contain few $CD45^+$ immune cells. In this study bypass vein grafts also contained few $CD45^+$ cells in flowcytometry. Further investigation is needed to characterize $CD45^+$ immune cells found in bypass vein grafts more efficiently, but the low cell number made further flow cytometric analysis impracticable. However, immune cell infiltrates in vena saphena samples used as bypass grafts might be clinically relevant as inflammation contributes to saphenous vein graft disease influencing the longevity of the grafts (Guida et al., 2020). Specific testing could therefore identify low value grafts prior to transplantation. This could open up immunomodulating treatment options prior to implantation of the grafts as well as immunomodulating therapy options throughout the course of saphenous vein graft disease.

Varicose veins contained more immune cells the great saphenous vein bypass samples enabling further flow cytometric analysis. Previous histological studies showed the presence of T cells and B cells (Buján et al., 2008, Chu et al., 2013, Sayer and Smith, 2004). In flow cytometric analysis only few $CD4^+$ and $CD8^+$ T cells were present. Luther et al. (2016) showed by flow cytometric analysis that T cells isolated from varicose veins were mainly effector-memory or resident memory T cells of which many also expressed the activation marker CD69. It must be assumed that T cells are probably locally activated and contributing to inflammation. T cells can be activated by antigens. As varicose veins are not associated with any infections, T cells would have to be activated by a self-antigen. This scenario is unlikely as the inflammation is chronic but localized. As T cells need multiple signals to be activated it is highly likely that cytokines are contributing to activation of T cells (Curtsinger and Mescher, 2010). The majority of the $CD45^+$ $CD3^-$ immune cells from the varicose vein infiltrate are monocytic cells which could be creating the microenvironment using cytokines to activate T cells.

This study showed that the varicose vein infiltrate contained statistically significant more $CD11b^+$ HLA DR^+ $CD14^+$ monocytic cells compared to PBMC. As CD11b is expressed on phagocytic cells (Arnaout, 1990) including dendritic cells and macrophages (Ling et al., 2014) HLA DR and CD14 (Cossarizza et al., 2019) were additionally used to identify monocytic cells. As human monocytes have been identified using CD14 and CD16 (Passlick et al., 1989, Ziegler-Heitbrock et al., 2010) this study also investigated the expression of those markers. The varicose vein infiltrate had higher frequencies of $CD14^{++}CD16^-$ and $CD14^{++}CD16^+$ cells in comparison to PBMC. Whether these cells represent $CD14^{++}CD16^-$ and $CD14^{++}CD16^+$ monocytes needs to be further investigated by gene expression analysis.

Monocytes are in general very heterogenous and can be precursors of macrophages and are subject to ongoing further definition (Merah-Mourah et al., 2020, Hamers et al., 2019). Hence, in this study additional markers were examined showing a statistically significant higher frequency of $CD14^+CD163^+$ cells and $CD14^+CD206^+$ cells in comparison to PBMC. CD163 and CD206 are scavenger receptors (Nielsen et al., 2020). CD163 is also called hemoglobin-haptoglobin scavenger receptor and expressed on monocytes and macrophages (Kristiansen et al., 2001). CD163 is also present as a soluble form in normal plasma and increased in inflammation (Etzerodt and Moestrup, 2013). It can be used as biomarker for macrophage activation in various diseases (Møller, 2012).

CD206 is also called mannose receptor. In this study $CD206^+$ immune cells were only detectable in high frequencies after enzymatic digestion. This suggests that $CD206^+$ immune cells had to be freed from the tissue. PBMC and washing steps only contained none to very few $CD206^+$ immune cells. These observations suggest that $CD206^-$ monocytic cells might migrate through the connective tissue and differentiate into $CD206^+$ monocytic cells in the vein wall.

As CD206 is exclusively expressed on macrophages (Gordon, 2003), there seem to be macrophages within the immune cell infiltrate in varicose vein walls. As CD163 is expressed on both monocytes and macrophages, it seems likely that also monocytes are present. Histologically CD68 – expressing cells were observed mainly in the tunica media. This makes an infiltration of immune cells from the blood into the tissue seem unlikely. Speculatively, the immune cells infiltrate the tissue from connective tissue of the tunica adventitia. Further investigation is needed to define the monocytic cells further and gain insights into the tissue entry of immune cells in varicose veins.

Genetic and epigenetic investigation into chronic venous disease has already started (Baylis et al., 2021). For further investigation a prior genetic analysis of isolated monocytic cells would allow a more unbiased investigation instead of studying single cell surface markers.

Overall, this study provided further insight into the inflammation in varicose veins. This study is a further step towards new therapeutic approaches targeting the inflammation in chronic venous disease in general and varicose veins in specific.

6. ABSTRACT

Varicose veins (varices) are a common, progressive degenerative disease of the superficial venous system. Previous histological studies revealed infiltrating immune cells including dendritic cells, macrophages and T cells (Buján et al., 2008, Cherian et al., 1999, Chu et al., 2013, Sayer and Smith, 2004). In the present study, the composition of monocytic cells in varicose veins was analyzed using flow cytometry. For this purpose, a step-by-step mechanical cleansing/tissue digestion process was developed to generate undamaged single cells. Large saphenous veins, removed for bypass surgery, served as non-inflamed control tissue. Flow cytometric analysis of the immune cell infiltrate in varicose veins and bypass veins showed that varicose veins contained up to ten times more $CD45^{+}$ immune cells than bypass veins and that the immune infiltrate consisted predominantly of monocytic cells. While the outer connective tissue mainly contained $CD206^{neg}$ myeloid cells, deeper layers of the vein, which became only accessible through digestion, harbored $CD206^{pos}$ myeloid cells. These observations suggest that $CD206^{neg}$ monocytes migrate via the connective tissue and differentiate into $CD206^{pos}$ macrophages in the vein wall.

6. ZUSAMMENFASSUNG

Krampfadern (Varizen) sind eine häufige, fortschreitende degenerative Erkrankung des oberflächlichen Venensystems. Frühere histologische Studien zeigten infiltrierende Immunzellen, darunter dendritische Zellen, Makrophagen und T-Zellen (Buján et al., 2008, Cherian et al., 1999, Chu et al., 2013, Sayer and Smith, 2004). In der vorliegenden Studie wurde die Zusammensetzung monozytischer Zellen in Krampfadern mittels Durchflusszytometrie analysiert. Zu diesem Zweck wurde ein schrittweiser mechanischer Reinigungs-/Gewebeaufschlussprozess entwickelt, um unbeschädigte Einzelzellen zu erzeugen. Als nicht entzündetes Kontrollgewebe dienten große Stammvenen, die im Rahmen von Bypass-Operationen entfernt wurden. Die durchflusszytometrische Analyse des Immunzellinfiltrats in Krampfadern und Bypassvenen zeigte, dass Krampfadern bis zu zehnmal mehr $CD45^{+}$-Immunzellen enthielten als Bypassvenen und dass das Immuninfiltrat überwiegend aus monozytischen Zellen bestand. Während das äußere Bindegewebe hauptsächlich myeloische $CD206^{neg}$-Zellen enthielt, befanden sich in tieferen Schichten der Vene, die erst durch die Verdauung zugänglich wurden, myeloische $CD206^{pos}$-Zellen. Diese Beobachtungen legen nahe, dass $CD206^{neg}$-Monozyten über das Bindegewebe wandern und sich in der Venenwand zu $CD206^{pos}$-Makrophagen differenzieren.

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8. ACKNOWLEDGEMENT

9. CURRICULUM VITAE