Elucidating the interconnection of GvHD and Western diet-induced atherosclerosis

Aufklärung des Zusammenhangs von GvHD und durch westliche Ernährung induzierter Atherosklerose

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Chapter 1

Summary

Allogeneic hematopoietic cell transplantation (Allo-HCT) is the main and only treatment for many malignant and non-malignant haematological disorders. Even though the treatment has improved through the years and patient life expectancy has increased, graft versus host disease (GvHD) is still considered the main obstacle and one of the main reasons for increased mortality. Furthermore, improved patient's survival and life expectancy brought into question the late post-HCT complications. The leading cause of late death after allo-HCT is the relapse of primary disease as well as chronic GvHD (cGvHD) [1]. However, a clear relationship was also described with pulmonary complications, endocrine dysfunction and infertility, and cataracts in post-HCT patients [2, 3, 4, 5, 6]. In the last years big concern regarding a cumulative cardiovascular incidence in long-term survivors has been raised [7, 8, 9]. Severe cardiovascular disease (CVD) is caused by atherosclerosis which is considered a chronic inflammatory disease of blood vessels. As such, it takes a long time from endothelial damage, as the onset event, and followed plaque formation to a manifestation of severe consequences, such as stroke, coronary heart disease, or peripheral arterial disease [10, 11, 12]. Endothelial damage is well documented in patients post-HCT [13, 14]. In the context of allo-HCT, the endothelial damage is induced by the conditioning regimen with or without total body irradiation (TBI) [15, 16]. Furthermore, endothelial cells (ECs) have been documented as a target of GvHD [14] and increased concentration of circulating endothelial cells (CEC) coinciding with an increase in the number of circulating alloreactive T cells [17]. According to 2021 ESC Guidelines on CVD prevention [18], the main atherosclerotic CVD (ASCVD) risk factors are blood apolipoprotein B (ApoB)-containing lipoproteins (of which low-density lipoprotein (LDL) is the most abundant), high blood pressure, cigarette smoking and diabetes mellitus (DM). GvHD is considered a high-risk factor for the onset of dyslipidaemia, hypertension, and DM [19, 20, 21, 22, 23]. Overall, the risk of premature cardiovascular death is 2.7 fold increased in comparison to the general population [1], while the cumulative incidence of cardiovascular complications was shown to be up to 47% at ten years after reduced intensity conditioning (RIC), post-HCT [8]. However, up to date, there are no available studies elucidating the interconnection between GvHD and atherosclerosis. The goal of this study was, therefore, to investigate the involvement of GvHD in the progression of atherosclerosis as well as to elucidate whether cytotoxic, CD8^{+} T cells that were shown to play a significant role in endothelial damage during the course of skin GvHD [14] on one hand, and inducers of formation of unstable plaque on the other, are involved in this interconnection. For that purpose we established a novel minor histocompatibility antigens (miHAg) allo-HCT Western diet (WD)-induced atherosclerosis mouse model. We were able to show that GvHD has a significant impact on atherosclerosis development in B6.Ldlr$^{-/-}$ recipient mice even in the absence of overt clinical disease activity. It seems that the impact is at least partly induced by CD8^{+} T cells, that showed significantly increased infiltration of aortic lesions in mice facing subclinical GvHD. As studies have

shown in regular atherosclerotic models as well as in humans [24, 25, 26, 27, 28], these $CD8^+$ T cells exhibited not only increased expression of genes involved in activation, survival and differentiation to cytotoxic phenotype, but also some genes pointing out their exhaustion, that were absent in $CD4^+$ T cell cluster. When anti-CD8β antibody was applied once per week along with WD feeding for eight weeks, the plaque formation was significantly reduced in aorta and aortic root pointing out the importance of these cells in an alloreactivity induced lesion formation. Furthermore, anti-CD8β treatment led to significantly decreased necrotic core formation followed by overall increase in plaque stability. Strikingly, bone marrow plus T cells (BMT) recipients fed WD showed significantly increased serum cholesterol levels in comparison to bone marrow (BM) (a group lacking alloreactive T cells that induce GvHD). This effect was reversed when anti-CD8β treatment was applied, suggesting, at least partly, an impact of alloreactive $CD8^+$ T cells on cholesterol levels. Expression of genes responsible for lipid metabolism pointed out the tendency of the liver to regulate the increased cholesterol levels, however, the mechanism behind this phenotype still remains to be revealed. On the other hand, the impact of obesity, induced by chronic high-fat diet (HFD) feeding, has been shown to be an independent risk factor for gastrointestinal GvHD [29]. Similarly, in major histocompatibility complex (MHC) disparate allo-HCT mouse model, we have noticed that even short-term WD intake leads to a significant decrease in survival of mice post-HCT. When the concentration of transplanted alloreactive T cells was reduced, the survival was improved, pointing out the involvement of these cells in the pathogenesis. Additionally, bioluminescence imaging (BLI) during initiation and effector phase of acute GvHD (aGvHD) revealed increased infiltration of alloreactive T cells in mice fed WD. As Khuat et al. [29] suggested in an obesity model, we could confirm the involvement of specifically $CD4^+$ T cells in WD induced impact, as the relative number of these cells was significantly increased in small intestine on day six post-HCT in mice fed WD. This increased intestinal infiltration was preceded by increase in the number of alloreactive T cells expressing intestine homing receptor ($\alpha4\beta7$ integrin) in peripheral lymph nodes (LNs). Even though the number of T cells was not changed in the spleen of WD fed mice, the subset of $CD4^+$ and $CD8^+$ T cells that were highly secreting TNFα was increased as well as the expression of genes regulating pro-inflammatory cytokines such as IL-6 and interferon (IFN)γ pointing out significant WD-induced inflammation. Moreover, slight tendency towards increased intestinal permeability and load of translocated luminal bacteria, that we observed, could induce severe endotoxemia and dysregulated systemic immune response that could lead to detrimental induction of cell death. Justifying our speculations, we noted increased levels of transaminases and an increase in lactate dehydrogenase (LDH) levels (pointing out significant tissue damages). However, the exact mechanism behind this detrimental WD impact still remains to be elucidated.

Zusammenfassung

Die allogene hämatopoetische Zelltransplantation (engl.: allogeneic hematopoietic cell transplantation; allo-HCT) ist die wichtigste und einzige Behandlung für viele bösartige und nicht bösartige hämatologische Erkrankungen. Auch wenn sich die Behandlung im Laufe der Jahre verbessert hat und die Lebenserwartung der Patienten gestiegen ist, gilt die Transplantat-gegen-Wirt-Krankheit (engl.: graft versus host disease; GvHD) nach wie vor als Haupthindernis und ist einer der Hauptgründe für die erhöhte Sterblichkeit. Darüber hinaus hat die Verbesserung der Überlebensrate und der Lebenserwartung der Patienten dazu geführt, dass die Spätkomplikationen nach der HCT in Frage gestellt werden. Die Hauptursache für den späten Tod nach einer allo-HCT ist das Wiederauftreten der Primärerkrankung und die chronische GvHD (cGvHD). Es wurde jedoch auch ein eindeutiger Zusammenhang mit pulmonalen Komplikationen, endokriner Dysfunktion und Unfruchtbarkeit sowie Katarakten bei Patienten nach einer HCT beschrieben [2, 3, 4, 5, 6]. In den letzten Jahren wurde große Besorgnis hinsichtlich einer kumulativen kardiovaskulären Inzidenz bei Langzeitüberlebenden geäußert [7, 8, 9]. Schwere Herz-Kreislauf-Erkrankungen werden durch Atherosklerose verursacht, die als chronische Entzündungserkrankung der Blutgefäße gilt. Von der Endothelschädigung als Beginn und der anschließenden Plaquebildung bis zur Manifestation schwerwiegender Folgen wie Schlaganfall, koronare Herzkrankheit oder periphere arterielle Verschlusskrankheit vergeht eine lange Zeit [10, 11, 12]. Endothelschäden sind bei Patienten nach HCT gut dokumentiert [13, 14]. Im Zusammenhang mit der allo-HCT wird die Endothelschädigung durch das Konditionierungsschema mit oder ohne TBI induziert [15, 16]. Darüber hinaus wurde dokumentiert, dass Endothelzellen ein Ziel der GvHD sind [14] und dass eine erhöhte Konzentration zirkulierender Endothelzellen (engl: circulating endothelial cells; CEC) mit einem Anstieg der Anzahl zirkulierender alloreaktiver T-Zellen korreliert [17]. Nach den ESC-Leitlinien 2021 zur Prävention von Herz-Kreislauf-Erkrankungen sind die wichtigsten Risikofaktoren für atherosklerotische Herz-Kreislauf-Erkrankungen (engl.: atherosclerotic cardiovascular disease; ASCVD) Apolipoprotein B (ApoB)-haltige Lipoproteine im Blut (von denen das Low-Density-Lipoprotein (LDL) am häufigsten vorkommt), Bluthochdruck, Zigarettenrauchen und Diabetes mellitus (DM). GvHD gilt als Hochrisikofaktor für das Auftreten von Dyslipidämie, Bluthochdruck und DM [19, 20, 21, 22, 23]. Insgesamt ist das Risiko eines vorzeitigen kardiovaskulären Todes im Vergleich zur Allgemeinbevölkerung um das 2,7-fache erhöht [1], während die kumulative Inzidenz kardiovaskulärer Komplikationen zehn Jahre nach einer Konditionierung mit reduzierter Intensität (RIC) nach einer HCT [8] bei bis zu 47% lag. Bislang gibt es jedoch keine Studien, die den Zusammenhang zwischen GvHD und Atherosklerose aufklären. Ziel dieser Studie war es daher, die Beteiligung der GvHD am Fortschreiten der Atherosklerose zu untersuchen und zu klären, ob zytotoxische $CD8^+$ T-Zellen, die einerseits eine bedeutende Rolle bei der Endothelschädigung im Verlauf der Haut-GvHD spielen und andererseits die Bildung instabiler Plaques induzieren, an diesem Zusammenhang beteiligt sind. Zu diesem Zweck haben wir ein neuartiges miHAg-allo-HCT Atherosklerose-Mausmodell etabliert. Wir konnten zeigen, dass GvHD einen signifikanten Einfluss auf die Entwicklung von Atherosklerose in B6.Ldlr$^{-/-}$-Empfängermäusen hat, selbst wenn keine klinische Krankheitsaktivität vor-

liegt. Es scheint, dass dieser Einfluss zumindest teilweise durch $CD8^+$ T-Zellen induziert wird, die bei Mäusen mit subklinischer GvHD eine signifikant erhöhte Infiltration von Aortenläsionen zeigten. Dies wurde auch in Studien in regulären Atherosklerose-Modellen sowie beim Menschen gezeigt. [24, 25, 26, 27, 28]. Diese $CD8^+$-T-Zellen wiesen nicht nur eine erhöhte Expression von Genen auf, die an der Aktivierung, dem Überleben und der Differenzierung zum zytotoxischen Phänotyp beteiligt sind, sondern auch einige Gene, die auf zelluläre Erschöpfung hinweisen, die im $CD4^+$-T-Zell-Cluster fehlten. Wurde ein Anti-CD8β-Antikörper einmal wöchentlich zusammen mit der Fütterung von WD acht Wochen lang verabreicht, so wurde die Plaquebildung in der Aorta und der Aortenwurzel signifikant reduziert, was auf die Bedeutung dieser Zellen bei der durch Alloreaktivität induzierten Läsionsbildung hinweist. Darüber hinaus führte eine Anti-CD8β-Behandlung zu einer signifikant verringerten Bildung eines nekrotischen Kerns, gefolgt von einer allgemeinen Zunahme der Plaquestabilität. Auffallend ist, dass BMT-Empfänger, die mit WD gefüttert wurden, im Vergleich zu BM (einer Gruppe ohne alloreaktive T-Zellen, die GvHD induzieren) signifikant erhöhte Serumcholesterinwerte aufwiesen. Dieser Effekt kehrte sich um, wenn eine Anti-CD8β-Behandlung durchgeführt wurde, was zumindest teilweise auf einen Einfluss alloreaktiver $CD8^+$-T-Zellen auf den Cholesterinspiegel schließen lässt. Die Expression von Genen, die für den Lipidstoffwechsel verantwortlich sind, wies auf die Tendenz der Leber hin, den erhöhten Cholesterinspiegel zu regulieren; der Mechanismus, der diesem Phänotyp zugrunde liegt, muss jedoch noch aufgeklärt werden. Andererseits hat sich gezeigt, dass die durch chronische Fütterung induzierte Fettleibigkeit ein unabhängiger Risikofaktor für gastrointestinale GvHD ist. In ähnlicher Weise haben wir in dem MHC disparaten allo-HCT-Mausmodell festgestellt, dass selbst eine kurzfristige WD-Zufuhr zu einer signifikanten Verringerung des Überlebens der Mäuse nach der HCT führte. Wenn die Konzentration der transplantierten alloreaktiven T-Zellen reduziert wurde, verbesserte sich die Überlebensrate, was auf die Beteiligung dieser Zellen an der Pathogenese hinweist. Darüber hinaus zeigte die Biolumineszenz-Bildgebung (engl.: bioluminiscence imaging; BLI) während der Initiations- und Effektorphase der aGvHD eine erhöhte Infiltration alloreaktiver T-Zellen bei Mäusen, die mit WD gefüttert wurden. Wie von Khuat et al. [29] in einem Adipositasmodell vorgeschlagen, konnten wir die Beteiligung von spezifisch $CD4^+$ T-Zellen an der WD-induzierten Wirkung bestätigen, da die relative Anzahl dieser Zellen im Dünndarm am sechsten Tag nach der HCT bei Mäusen, die mit WD gefüttert wurden, signifikant erhöht war. Dieser erhöhten Darminfiltration ging ein Anstieg der Zahl alloreaktiver T-Zellen voraus, die den Darm-Homing-Rezeptor ($\alpha4\beta7$-Integrin) in den peripheren LNs exprimieren. Obwohl sich die Anzahl der T-Zellen in der Milz von mit WD gefütterten Mäusen nicht veränderte, war die Untergruppe der $CD4^+$- und $CD8^+$-T-Zellen, die in hohem Maße TNFα sezernierten, ebenso erhöht wie die Expression von Genen, die pro-inflammatorische Zytokine wie IL-6 und IFNγ regulieren, was auf eine signifikante WD-induzierte Entzündung hinweist. Darüber hinaus könnte die von uns beobachtete leichte Tendenz zu einer erhöhten intestinalen Permeabilität und Belastung mit translozierten luminalen Bakterien eine schwere Endotoxämie und eine dysregulierte systemische Immunantwort auslösen, die zu einer schädlichen Induktion des Zelltods führen könnte. Zur Untermauerung unserer Spekulationen stellten wir erhöhte Transaminasenwerte und einen Anstieg der LDH-Werte fest (was auf erhebliche Gewebeschäden hinweist).Jedoch verbleibt der genaue Mechanismus, der zu den verheerenden Auswirkungen von WD führt, ungeklärt.

Glossary

ABC ATP-binding cassette

ABCA1 ABC subfamily A member 1

ABCG ABC subfamily G

ACAT acyl coenzyme A-cholesterol acyltransferase

aGvHD acute GvHD

Allo-HCT Allogeneic hematopoietic cell transplantation

ApoA-I apolipoprotein A-I

ALT alanine aminotransferase

AML acute myeloid leukaemia

APCs antigen presenting cells

ASCVD atherosclerotic CVD

AST aspartate aminotransferase

ATP adenosine triphosphate

ApoB apolipoprotein B

BAFF B cell activating factor

BCL-6 B cell lymphoma 6

BCR B cell receptors

BLI bioluminescence imaging

BM bone marrow

BMT bone marrow plus T cells

Bregs regulatory B cells

CAD coronary artery disease

CA cholic acid

CB cord blood

CCR CC-chemokine receptor

CCL2 chemokine (C-C motif) ligand 2

CD137L CD137 ligand

CD40L CD40ligand

CDCA chenodeoxycholic acid

CEC circulating endothelial cells

cGvHD chronic GvHD

CLS capillary leak syndrome

ConA concanavalin A

CRP C-reactive protein

CSF-1R colony stimulating factor 1 receptor

CTLA-4 cytotoxic T lymphocyte antigen 4

CTL cytotoxic T lymphocyte

CVD cardiovascular disease

CXCR3 CXC-chemokine receptor 3

CYP27A1 cytochrome P450 family 27 subfamily A Member 1

CYP7A1 cytochrome P450 family 7 subfamily A Member 1

CYP7B1 cytochrome P450 family 7 subfamily B Member 1

CYP8B1 cytochrome P450 family 8 subfamily B polypeptide 1

CyTOF cytometry by time of flight

CsA Cyclosporine

CSF-1 colony stimulating factor 1

CXCL C-X-C Motif Chemokine Ligand

DAH diffuse alveolar haemorrhage

DAMP danger-associated molecular pattern

DCA deoxycholic acid

DCs dendritic cells

DLI donor lymphocyte infusion

DM diabetes mellitus

DNA deoxyribonucleic acid

DSS Dextran sulfate sodium

ECs endothelial cells

ED endothelial dysfunction

EPC endothelial progenitor cell

ERAD ER-associated degradation

ER endoplasmic reticulum

FO follicular

FasL Fas-ligand, CD95L

FoxP3 forkhead box protein P3

FOXO1 forkhead box protein O1

FXR farnesoid X receptor

GAPDH glyceraldehyde-3-phosphate dehydrogenase

G-CSF granulocyte colony-stimulating factor

GC germinal centre

G-PBSC G-CSF-mobilized PBSC

GIT gastrointestinal tract

GM-CSF granulocyte-macrophage colony-stimulating factor

Gmzb Granzyme B

GvHD graft versus host disease

GvL graft versus leukemia effect

GvT graft versus tumor effect

HCT hematopoietic cell transplantation

HDLs high-density lipoproteins

HFD high-fat diet

HF heart failure

HIF hypoxia-inducible factor

HNF Hepatocyte nuclear factor

HLA Human Leukocyte Antigen

HMGCR 3-hydroxy-3-methylglutaryl coenzyme A reductase

HMGB1 high mobility group box 1

HR high resolution

HSP60/65 heat shock protein 60/65

HSPs heat shock proteins

HUVECs human umbilical vein ECs

ICOS inducible T cell co-stimulator

ICAM-1 intercellular adhesion molecule 1

IDO indoleamine-2,3-dioxygenase

IDOL Inducible degrader of the LDLr

IL interleukin

IL-1R IL-1 receptor

ILC1s innate lymphoid cells 1

ILC2 innate lymphoid cells 2

INSIGs insulin-induced genes

iNOS inducible NO synthase

IRA innate response activator

IRF IFN regulatory factor

IFN interferon

LAG-3 lymphocyte activity gene 3

LCAT lecithin:cholesterol acyltransferase

LCA lithocholic acid

LDLr LDL receptor

LDL low-density lipoprotein

LDH lactate dehydrogenase

LNs lymph nodes

LPS lipopolysaccharide

LpX lipoprotein X

LRH1 liver receptor homolog-1

LVEF left ventricular ejection fraction

LXR liver X receptor

Lp-PLA2 lipoprotein-associated phospholipase A2

M-CSF macrophage colony-stimulating factor

MCP-1 monocyte chemoattractant protein-1

MHC major histocompatibility complex

MI myocardial infarction

MIC MHC class I Chain

miHAg minor histocompatibility antigens

MRD matched related donor

mTOR mammalian target of rapamycin

MZ marginal zone

NETs neutrophil extracellular traps

NF nuclear factor

NK natural killer cell

NLR nucleotide-binding oligomerization domain-like receptor

NMDP National Marrow Donor Program Committee

NO nitric oxide

NPC1L1 Niemann–Pick type C1-like 1

NRF1 nuclear factor erythroid 2-related factor 1

NRM non-relapse mortality

nSREBP2 nuclear SREBP2

oxLDL oxidized LDL

PAI-1 plasminogen activator inhibitor-1

PAMP pathogen-associated molecular pattern

PBSCs peripheral blood stem cells

PD-1 programmed cell death 1

PD-L1 programmed cell death ligand 1

PDCD1 gene encoding PD-1 protein

PCSK9 proprotein convertase subtilisin/kexin type 9

PDGF platelet-derived growth factor

PLT platelets

Prf perforin

RBC red blood cell

RIC reduced intensity conditioning

RLRs RIG-I-like receptors

ROS reactive oxygen species

RXR retinoid X receptor

SD standard diet

SCAP SREBP-cleavage activating protein

SCFAs short-chain fatty acids

SLOs secondary lymphoid organs

SMC smooth muscle cell

SREBP2 sterol regulatory element-binding protein 2

SREBF2 sterol regulatory element-binding transcription factor 2

scRNA-seq single-cell RNA sequencing

T-bet T-box transcription factor TBX21

TAM transplant-associated microangiopathy

TBI total body irradiation

TCR T cell receptor

Tc cytotoxic, $CD8^+$ type T cell

TF tissue factor

Tfh T folicular cell

TIM-3 T cell immunoglobulin and mucin domain-3

TLRs Toll-like receptors

TM thrombomodulin

TNF tumor necrosis factor

TNFRSF9 TNF receptor superfamily member 9

TNFRs TNF receptors

Tox thymocyte selection-associated high mobility group box

Traf-1 TNF receptor-associated factor 1

TRAIL TNF-related apoptosis-inducing ligand

Tcon T conventional cells

Th T helper

Tr1 type 1 regulatory T cells

Treg regulatory T cells

URD unrelated donor

VCAM-1 vascular cell adhesion molecule 1

VLDL very-low-density lipoproteins

VOD veno-occlusive disease

VSMC vascular SMC

vWF von Willebrand factor

WD Western diet

Part I

Introduction

This part covers the theoretical background of the two projects that will be discussed in this thesis.

Chapter 2

Hematopoietic cell transplantation

HCT is considered a procedure where hematopoietic stem and progenitor cells of any donor type and any source are given to a recipient with the intention of repopulating and replacing completely or partly the hematopoietic system of a recipient. Before HCT recipients are treated with high-dose chemotherapy and/or fractional radiation to eradicate malignant hematopoietic cells and to allow donor cells to repopulate the BM niche [30, 31]. Similar to the solid organ transplantation, depending on the origin of the graft, HCT can be divided into three common types: *autologous*, where hematopoietic cells are derived from the recipient; *syngeneic*, where the cells originate from the twin sibling, and *allogeneic*, where the cells and the immunological repertoire are derived from a non-related donor. In all cases, hematopoietic cells are infused into a patient in order to establish donor-derived hematopoiesis and immunity [31, 32]. Over the last 50 years, HCT has evolved from a highly experimental technique to the standard, well-established procedure used for the treatment of many congenital or acquired hematological disorders, benign or neoplastic, including those of the immune system and metabolic disorders (Figure.2.1) [32, 33, 34, 35, 36, 31].

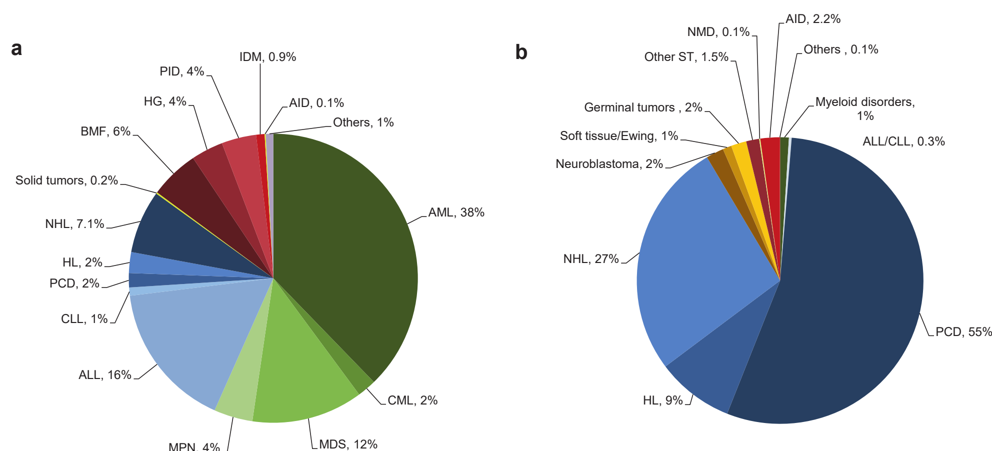

Figure 2.1: Relative proportion of disease indications for allogeneic (a) and autologous (b) HCT in Europe 2019. BMF:bone marrow failure; PID:primary immunodeficiencies; IDM:inherited diseases of metabolism; AID:autoimmune diseases; AML:acute myeloid leukaemia; ALL:acute lymphocytic leukaemia, CML:chronic myeloid leukaemia; MDS:myelodysplastic syndrome; MPN:myeloproliferative neoplasm; CLL:chronic lymphocytic leukaemia; PCD:plasma cell disorders; HD:Hodgkin disease; NHL:non-Hodgkin lymphoma. Reproduced from open access article (CC BY) [36].

The outcome of HCT treatment (in the sense of the success of engraftment and induction

of undesirable immune reactions, such as GvHD) is primarily dependent on the origin of the graft and established MHC compatibility of donor and recipient.

2.1 MHC histocompatibility and transplantation immunology

MHC molecules were initially defined as antigens that stimulate an immunologic response in the organism to transplanted organs and tissues. In the 1950s, skin graft experiments done in mice showed that graft rejection was an immune reaction mounted by the host organism against foreign tissue. The host recognized the MHC molecules on cells of the graft tissue as foreign antigens and attacked them [37]. Today we know that the strongest transplant antigens are coded by these MHC genes. When tissues containing nucleated cells are transplanted, T cell reaction to the highly polymorphic MHC molecules almost always triggers a response against the grafted organ.

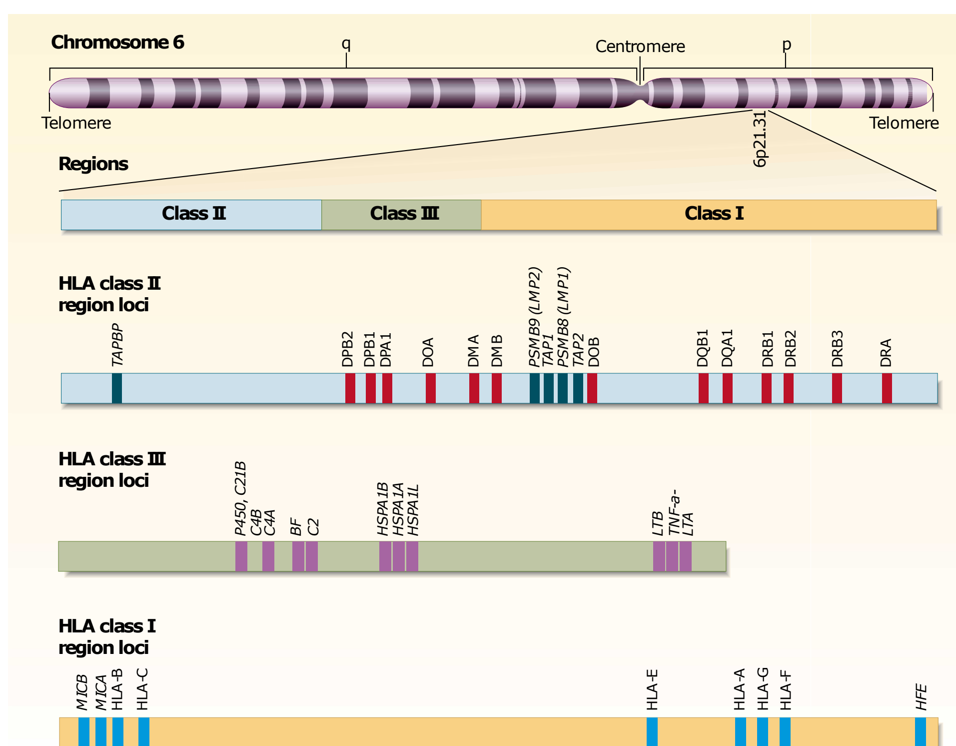

Figure 2.2: Location and organization of the HLA Complex on Chromosome 6. Reproduced with permission from [38], Copyright Massachusetts Medical Society. HLA: human leukocyte antigen, MIC: MHC class I Chain.

The MHC is a locus on the vertebrate deoxyribonucleic acid (DNA), which is well known as an inevitable part involved in the regulation of the immune system. Cell surface proteins crucial for the adaptive immune system are encoded by the set of closely linked polymorphic genes located in this locus. In humans, this locus is located on chromosome 6 (Figure.2.2) and is called Human Leukocyte Antigen (HLA) system. The name of this complex comes from the notion that these antigens were first identified and characterized using alloantibodies against leukocytes, where graft rejection was associated with the development of antibodies against allogeneic leukocytes [39, 40]. HLA complex contains

over 200 genes and more than 40 of which encode leukocyte antigens [39, 41, 38]. The complex is arranged in three genomic regions which encode three different types of MHC molecules.
The more distal region corresponds to MHC class I, which carries the genes that code for the class I α heavy chains, HLA- A,-B, and -C. An extraordinary degree of polymorphism characterizes these genes (2,735 alleles at HLA-A, 3,455 alleles at HLA-B and 2,259 alleles at HLA-C loci). This region also contains so-called non-classic HLA loci coding for the expressed, though less variable, genes HLA- E, -F, -G, HFE, and the MHC class I Chain (MIC)- related MICA and MICB. Nearly all class I HLA (HLA I) molecules (with the exception of MIC genes) form heterodimers with the invariant β2-microglobulin chain, coded by a gene on chromosome 15 [42]. The α chain has five domains: two peptide-binding domains (α1 and α2), one immunoglobulin-like domain (α3), the transmembrane region, and the cytoplasmic tail. α1 and α2 domains are folded and form walls of a cleft on the surface of the molecule. This part of the molecule is the area where peptide binds; therefore, it is known as peptide-binding cleft or peptide-binding groove. The major differences between the different allelic forms of MHC genes are located in this peptide-binding cleft. By contrast, β2 microglobulin, which does not contribute directly to peptide binding, is not polymorphic [43]. MHC class I is expressed on the surface of nearly all somatic cells (except red blood cell (RBC)) and is able to specifically bind to CD8 T cells, by which it plays its role in immune regulation and pathology (Figure.2.3) [38].

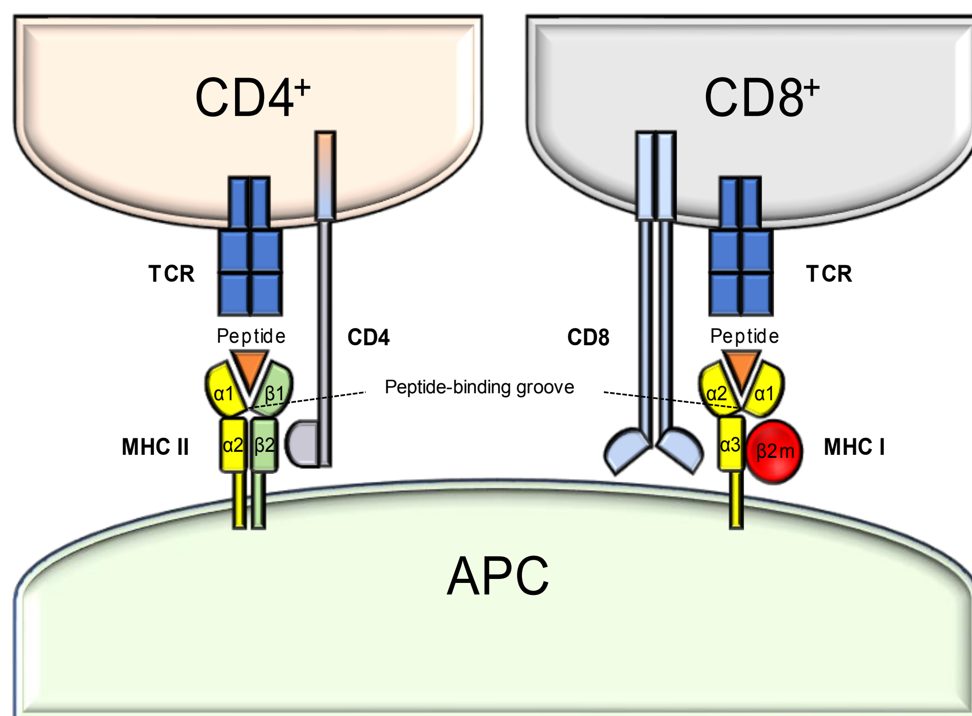

Figure 2.3: Structure of MHCI, MHCII molecules and MHC-peptide-TCR complexes. The figure is made according to [44, 38]. TCR:T cell receptor, APC:antigen presenting cell, MHC:major histocompatibility complex, β2m:beta-2 microglobulin.

On the other hand, class II MHC molecule consist of non-covalent complex of two chains, α and β (Figure.2.3) [43]. Genes encoding MHC II are located in the more centromeric part (Figure.2.2) of the MHC region and they are coding for both chains that will form the functional heterodimer, HLA-DR, HLA-DQ, HLA-DP, HLA-DM, and HLA-DO (2,649 known alleles) [42]. These genes exhibit restricted expression, being predominantly ex-

pressed in antigen presenting cells (APCs), such as macrophages, dendritic cells (DCs), Langerhans and Kupffer cells, as well as B lymphocytes. However, inflammatory processes (anomalous conditions) can induce the expression of these genes in some cells (for example, intestinal epithelial cells [45] and thyrocytes [46]). As surface molecules expressed specifically on APCs, MHC II molecules are binding to $CD4^+$ T cells by which they play their role in the immune response (Figure.2.3).
Because of such polymorphism and polygenicity of MHC molecules, during transplantation, matching the recipient and donor MHC type increases the success rate of the graft survival and the perfect matching is only possible between related donor and recipient. However, even in those cases, genetic differences at other loci (known as miHAg) can trigger rejection, although less severely [43]. Based on the outcome of many studies, the gold standard of unrelated donor (URD) matching, according to the guidelines of the European Society for Blood and Marrow Transplantation, is the identification of 10 alleles in 5 HLA loci, namely, HLA-A, -B, -C, -DRB1, and -DQB1 using high resolution (HR) typing (so-called 10/10 matching) [47, 48, 49]. Alternatively, another matching algorithm, which is recommended by the National Marrow Donor Program Committee (NMDP), is to look for an HLA-A, -B, -C, and -DRB1-compatible donor (8/8 allelic identity) [50, 49], as HLA-DQB1 differences are rare if the donor is matched for HLA-A, HLA-B, HLA-C, and particularly HLA-DRB1 [51, 52, 53].
The type of transplantation in use for a patient, autologous or allogeneic, depends on the type of malignancy, availability of a suitable donor, age of the recipient, the ability to collect a tumor-free autograft, the stage and status of disease (BM involvement, chemosensitivity to conventional chemotherapy, bulk of disease), and the malignancy's susceptibility to graft versus tumor effect (GvT) effects.

2.1.1 Allogeneic hematopoietic cell transplantation

Barnes and colleagues published in 1956 the experiment on two groups of mice with acute leukemia: both groups were irradiated for an anti-leukemic-effect therapy, and both were rescued, by BM transplantation, from marrow aplasia. The first group was transplanted with syngeneic marrow (from mice of the same strain), and most of the mice died from leukemia relapse. Allogeneic marrow (from a different mouse strain) was transplanted in the second group, resulting in the absence of the disease relapse, but all the animals died from a "wasting syndrome" [54]. This experiment demonstrated three *major principles of allo-HCT*:

1) the preparative anti-leukemic regimen role in HCT;

2) the capability of the new engrafted immune system to prevent leukemia relapse (later known as graft versus leukemia effect (GvL)); and

3) the activity of the engrafted immune system against the recipient (today known as GvHD) [32].

Allogeneic transplant was based for years on the premise of using maximally intensive myeloablative radiation with or without chemotherapy or chemotherapy-only regimens (such as busulfan and cyclophosphamide) to perform three functions: (1) eradicate cancer cells, (2) suppress the recipient's immune system to prevent rejection of the graft, and (3) create a "space" in the BM to facilitate donor cell engraftment. [55]. These maximally intense regimens carry significant acute and long-term toxicities and could not be used in the elderly, where, ironically, the greatest incidence of hematologic malignancies is

seen [31]. An increasing understanding of the GvL effect and its potency in eradicating malignant disease led to the development of RIC regimens in canine models, where they demonstrated reliable engraftment, reduced toxicity, and potent antitumor effects [56, 31]. Nowadays, there are specific regimens for each indication. The regimens are categorized by their intensity level as full myeloablative, reduced-toxicity, or reduced-intensity and non-myeloablative ones [32]. The source of progenitor and stem cells has also changed over the years. BM harvested from the posterior iliac crests under general anaesthesia had been used as the source of hematopoietic cells for transplantation for many years. However, in the 1990s, two new source options, namely, granulocyte colony-stimulating factor (G-CSF)-mobilized peripheral blood stem cells (PBSCs) and cord blood (CB), became available for clinical use. The choice of the cell source depends on the age of the donor and recipient, clinical comorbidities as well as disease stage, and it varies depending on the preferences of different centers and donors [48]. Even though the care of patients has improved, taking into account immunosuppressive therapy, usage of antibiotics, and RIC regimen, HCT is still frequently followed by life-threatening complications such as tumour relapse, infections, organ failure, and GvHD. After day 100, the relapsed disease remained the most common cause of death (57% with matched related donor (MRD), 46% with URD), followed by infection (7% in MRD and 10% in URD) and GvHD (7% in MRD and 9% in URD) [57].

2.2 GvT effect

Immune reaction noticed after allo-HCT that disabled the occurrence of leukemia relapse is nowadays known as GvL. The remaining malignant cells post-conditioning are eradicated due to the alloreactivity of donor T cell, which is a major mechanism for the curative effect of allo-HCT [58]. Although different subsets of donor immune cells are likely to contribute to GvL response, current clinical evidence suggests that T cells exert the most potent and clinically relevant anti-leukemic effect [59]. Both T cell subsets ($CD4^+$ and $CD8^+$) are thought to be important for this response. Acute myeloid leukaemia (AML) cells are recognised as they express MHC molecules on their surface, enabling interaction with corresponding T cell receptor (TCR) on T cells. Interestingly, high levels of MHC II expression have been seen in most cases of AML [60, 61, 62], which is recognized by $CD4^+$ T cells, whereas $CD8^+$ T cells recognise peptides in the complex with MHC I. However, this immune reaction needs to be balanced with alloreactivity against normal, healthy tissues that manifest as GvHD and can be detrimental to the host. GvHD is considered the major allo-HCT complication.

2.3 Graft versus host disease

The “wasting syndrome”, whose onset was observed and documented in 1956 in mice exposed to a lethal dose of TBI and administered allogeneic splenocytes, was characterized by decreased survival, the onset of diarrhea, weight loss, and presence of skin lesions [54, 63, 64]. Initially, this syndrome was named a “secondary disease”, which was later changed to GvHD. The three crucial requirements for the development of GvHD were then postulated:

1) immunologically competent cells have to be contained inside the transplanted graft;

2) the rejection and elimination of the transplanted cells by the recipient must be disabled; and

3) tissue antigens that are not present in the graft must be expressed by the recipient; thus, donor cells recognise the recipient antigens as foreign [64].

Today, it is known that T lymphocytes are the immunocompetent cells that are present in the hematopoietic cells inoculum and are essential to mount an effective immune response [65]. The usage of immune-ablative agents (chemotherapy and/or radiotherapy) compromises the recipient's immune system, thereby disabling the rejection of the transplanted cells. Furthermore, the tissue antigens which differ between the recipient and the donor are major and minor HLA, whose expression is crucial for the activation of allogeneic T cells and initiation of GvHD [66, 67]. The first definitions of GvHD in patients counted that aGvHD occurs in the first 100 days after the transplantation, and cGvHD was considered as the one occurring after day 100. Nowadays, it is clear that aGvHD can occur after day 100, or it can appear as an overlap syndrome of both aGvHD and cGvHD. The current consensus is that clinical manifestations, and not the time to symptomatic onset after transplantation, determine whether the clinical syndrome of GvHD is considered acute or chronic [68].
aGVHD is a cytolytic, tissue-destructive process that involves skin, gut, liver, and, recently demonstrated, lung and central nervous system, and it usually occurs in the first 3–12 weeks after HCT [69]. This process is initiated by innate immune cells activated during conditioning-regimen-induced inflammation and by tissue injury and amplified by adaptive immune responses. **cGVHD**, on the other hand, is immunologically distinct from aGVHD and is characterized by T helper (Th)17- rather than Th1-skewed responses, immune dysregulation, autoimmunity, and/or fibrosis [70]. It usually occurs more than six months after HCT and it can affect most of the tissues, typically skin with lichenoid plaques, eyes with sicca symptoms, joints, skin and lung fibrosis [71], as detailed below.

2.3.1 Acute GvHD

The pathophysiology of aGvHD is explained in three different stages. Initially, pre-conditioning induces tissue damage that, in turn, activates host APCs. The second phase, known as the afferent phase, or phase of immune priming, is considered a stage of donor T cell activation by APCs, whereas the third, efferent phase or effector phase, is the stage of target tissue damage induced by activated donor T cells [67] (Figure.2.4).
Phase I - Activation of APCs
As mentioned above, before the transplantation, patients are subjected to a pre-conditioning regimen of different intensities accompanied by broad-spectrum antibiotics that disrupt the microbiota ecosystem of gastrointestinal tract (GIT), especially the ileum, where the constant interaction of microbiome and innate immune cells occurs [73, 74]. Such treatments invoke inflammation, translocation of intestinal microbiota (pathogen-associated molecular pattern (PAMP)), and release of danger-associated molecular pattern (DAMP) molecules from dying host cells [69]. Innate immune cells, such as monocytes/macrophages and DCs, are being activated after stimulation of their Toll-like receptors (TLRs) by PAMPs (such as lipopolysaccharide (LPS) or peptidoglycans derived from bacteria) that finally leads to initiation of an inflammatory cytokine cascade of interleukin (IL)-12, IL-23, and IL-6 [73, 74]. The inflammatory cytokine secretion, then, becomes amplified by released DAMPs (high mobility group box 1 (HMGB1), adenosine triphosphate (ATP), and uric acid), culminating in high local levels of IL-12 in GIT [74]. Such a pro-inflammatory

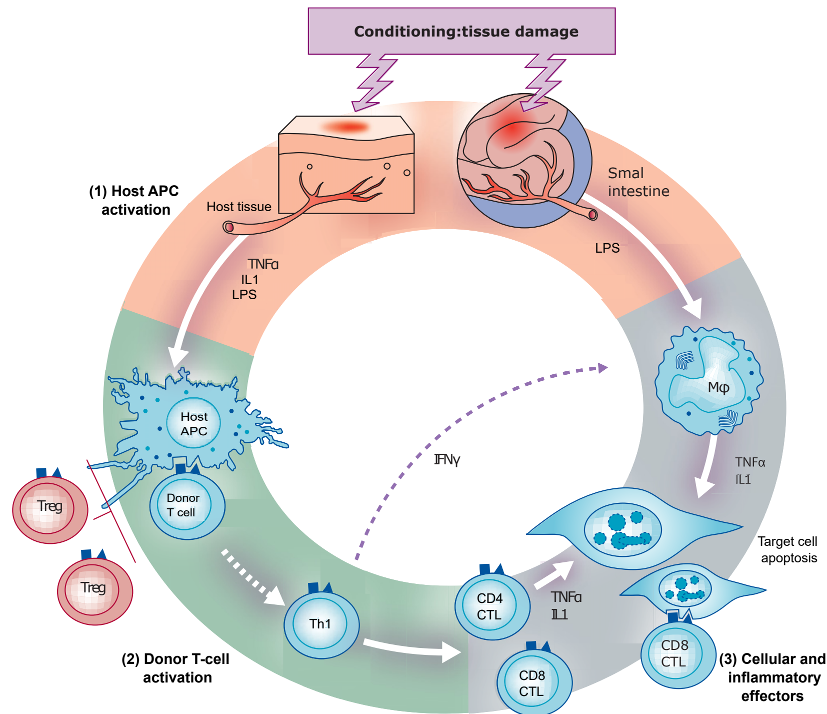

Figure 2.4: Schematic overview of aGvHD pathophysiology. Reprinted by permission from Elsevier, The Lancet [72]. APC:antigen presenting cell, Treg:T regulatory cell, Th1:T helper 1, CTL:cytotoxic T lymphocyte, TNF:tumor necrosis factor, IL:interleukin, Mϕ:macrophage, LPS:lipopolysaccharides, IFN:interferon.

environment leads to increased expression of MHC II by intestinal epithelial cells, as IL-12 promotes IFNγ secretion by innate lymphoid cells 1 (ILC1s) [75, 76] and recipient T conventional cells (Tcon) cells, enabling APC function and final donor T cells priming to initiate lethal gut aGvHD [73].

Phase II - Activation of donor T cells

Interaction between recipient APC and donor T cell is considered the key to aGvHD mechanism [71, 74]. Differentiation of circulating naive donor T cells is initiated by activated recipient APCs in lymphoid structures [77]. Depending on the cell type that mainly induces the disease, GvHD has been conceptualized as MHC class I and MHC class II-dependent (i.e. $CD8^+$ and $CD4^+$ T cells, respectively) [78]. HLA class I and II mismatches are significant risk factors for severe GvHD and transplant-related mortality [79], where both, $CD8^+$ and $CD4^+$ T cells, are involved. Indeed, when either subset was depleted, it was insufficient to prevent GvHD [80, 81]. Noteworthy, the pathogenesis of GvHD after ***MHC-matched*** transplantation involves both T cell subsets and miHAg are presented and recognized within MHC class I and II [78]. As the origin of presented antigens and the process of presentation differ between these two pathways (MHC I and MHC II presentation) as well as between the different MHC compatibility models, it is essential to emphasise and comprehend these differences in order to understand the process behind the transplantation.

During MHC-matched hematopoietic cell transplantation (HCT), alloantigens presented within MHC class I are predominantly of endogenous origin [82], and their peptides are processed within the proteasome that is transported into the endoplasmic reticulum (ER), where aminopeptidases shorten peptides for loading into MHC class I before transferring

to the surface [83] (Figure.2.5). Conversely, MHC class II presents mainly alloantigens of exogenous origin, and presentation within MHC class II occurs by both recipient and donor APCs. The protein processing, in this case, takes place inside the lysosome and peptides are transported to the endosome for loading into MHCs. However, exogenous antigens can also be presented within MHC class I and this process is suggested to occur in sub-specialized DC subsets (CD8^{+} and/or CD103^{+} in mouse, CD141^{+} in humans) and is termed cross-presentation [78]. Furthermore, during periods of cellular stress, in a process known as autophagy, endogenous proteins of nuclear, mitochondrial and cytoplasmic origin can also be presented within MHC class II (Figure.2.5). In this case, proteins are incorporated into autophagosomes that are fusing with a lysosome to allow antigen delivery into the MHC class II pathway. In both cases, donor T cells recognize polymorphic recipient peptides presented within MHC that are shared by both the donor and recipient [78]. Recently, the third "semi-direct" pathway of antigen presentation has been described where MHC molecules loaded with alloantigen are transferred from neighbouring cells in a cell contact or an exosome-secretion–dependent manner that might subsequently activate the donor T cell [84].

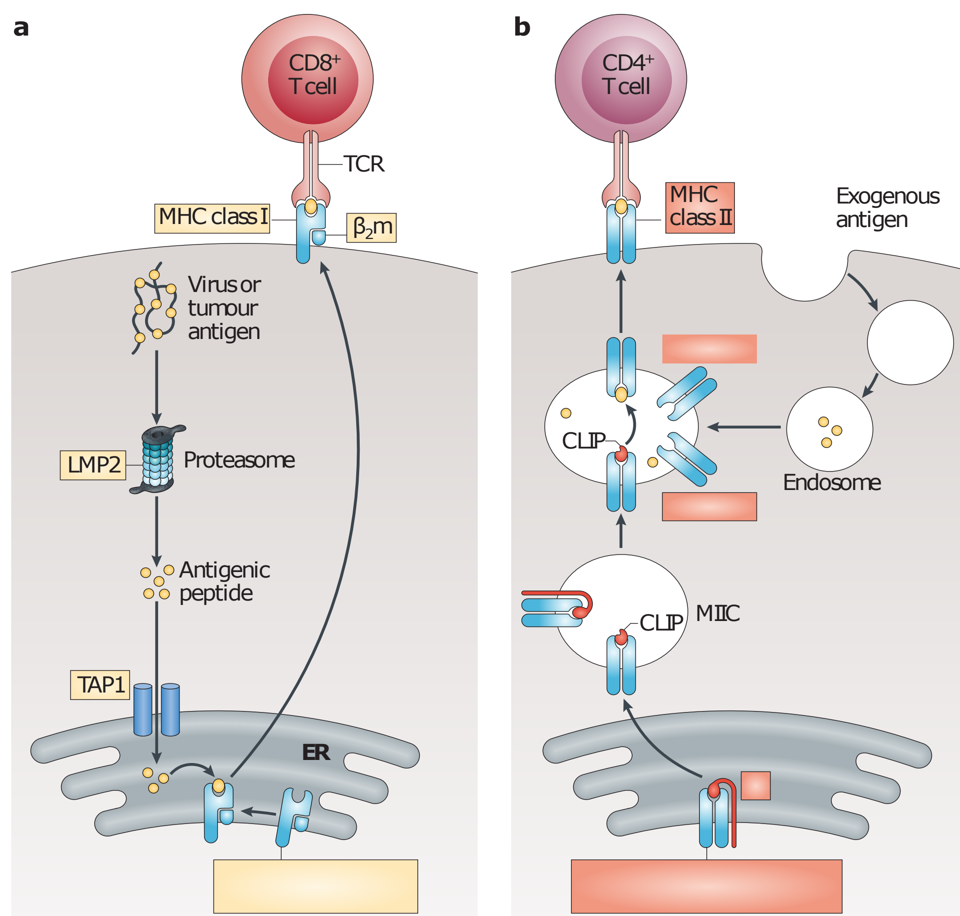

Figure 2.5: The MHC class I and MHC class II antigen-presentation pathways. Reprinted by permission from Springer Nature, Springer, Nature Reviews Immunology [85]. MHC:major histocompatibility complex, ER:endoplasmic reticulum, TAP1:transporter associated with antigen processing 1, β2m:beta-2 microglobulin, CLIP:class II-associated invariant chain peptide.

On the other hand, when it comes to the transplant settings, when ***MHC-mismatches*** are present, donor T cells react to recipient APCs at a very high frequency [86]. Nowadays,

it is known that in this setting, donor T cells can cross-react to non-self (host)-MHCs loaded with an antigenic peptide through the process known as molecular mimicry [86, 87, 88, 89]. MHC-disparate T cells have been shown to have an extensive capacity for recognition of conformational changes in the MHC-peptide complex and their TCRs to be capable of undergoing conformational "fine-tuning" in order to accommodate minor conformational alterations in MHC-peptide complexes [90]. Therefore, it is likely that these mechanisms are responsible for the severe GvHD risk when transplanting across multiple MHC mismatches relative to a single-locus mismatch [91, 92]. During MHC class I-dependent aGvHD, $CD8^+$ derived response, hematopoietic recipient APCs were shown to be of critical importance [82], whereas, so far, no single professional APC type has been identified as critical for MHC class II-dependant aGvHD, or $CD4^+$ derived response [74]. Noteworthy, non-professional APCs, such as epithelial and mesenchymal cells, were shown to be able to induce MHC II-dependant aGvHD following conditioning and microbiota disruption [74, 93, 94]. Overall, although immunological pathways involved in initiating MHC I- versus MHC II-dependant aGvHD differ, PAMPs and DAMPs signals are central to both [71]. Finally, upon DCs activation, they expand and migrate to mesenteric LNs, promoting T cell priming, differentiation, and expression of gut-homing integrin receptor ($\alpha4\beta7$) in a feed-forward cascade for aGVHD that is driven by donor T cell granulocyte-macrophage colony-stimulating factor (GM-CSF) secretion [95, 94].

Phase III - Effector phase with tissue damage

Following priming, naive T cells differentiate and become licenced for tissue destruction. The pro-inflammatory environment induced by PAMPs and DAMPs [96] and strong TCR stimulation by alloantigens favours Th1 over Th17 skewing [97]. High levels of IL-12 produced by macrophages and DCs as a reaction towards DAMPs and PAMPs; increased secretion of IFNγ by T, NK cells and APCs and IL-18 by macrophages, mononuclear cells and DCs lead to Th1 polarization and IFNγ, IL-2, and tumor necrosis factor (TNF)α secretion by Th1 cells [98]. IFNγ produced by donor T cells exhibits a direct cytotoxic effect on the intestinal stem cells during gastrointestinal murine aGvHD [99], but also it induces a host tissue-protective program by upregulating indoleamine-2,3-dioxygenase (IDO) [100] and programmed cell death ligand 1 (PD-L1) [101]. Excessive production of Th1 cytokines in mice gave an initial concept that aGvHD is primarily driven by Th1/Tc1- rather than Th2/Tc2-associated immunopathology [99]. Th1/Tc1 cells, as shown in mouse aGvHD models, utilize multiple overlapping and redundant cytotoxicity pathways from Fas/Fas-ligand, CD95L (FasL), perforin (Prf)/granzymes, to TNF-related apoptosis-inducing ligand (TRAIL) [102, 103]. As post-transplantation donor T cell activation is augmented by the conditioning regimen, excessive amounts of soluble cytokines are released that can cause the lethal syndrome, particularly in MHC-mismatched transplants where the precursor frequency of alloreactive donor T cells is very high. Subsequently, expanded alloreactive T cells are able to infiltrate and damage target tissues [104]. In MHC-mismatched transplants, it was observed that $CD4^+$ T cells (and to a lesser extent $CD8^+$ T cells) are able to induce GvHD without a direct contact with GvHD-target tissue and it was suggested that the development of disease (including lethality) is, at least partly, caused by the action of cytokines, such as TNFα and/or IL-1, as a consequence of the activation of high frequencies of alloreactive T cells [105]. Interestingly, a similar trend was observed in MHC-matched models, where $CD4^+$ T cells also did not require direct contact with recipient parenchymal tissues. However, in this, MHC-matched model, GvHD was non-lethal [106] and parenchyma needed to be allogeneic, as $CD4^+$ T-cell-mediated GvHD does not develop if only the hematopoietic system is allogeneic [107]. Donor $CD4^+$ T cells are, furthermore, interacting with donor-derived MHC class II positive cells, such as tissue

DCs and macrophages that indirectly present recipient antigens. In this way, $CD4^+$ T cells can become stimulated by tissue DCs to produce inflammatory mediators, whereas, at the same time, they are able to activate macrophages to induce tissue damage [104]. For $CD8^+$ T cells, conversely, cognate interaction with the target tissue was indispensable for GvHD induction [106]. As it was shown that a part of T cell-mediated MHC-mismatched GvHD can be induced without direct contact with the target tissue, the mechanism seems to be CD95L and/or Prf-independent. Consistently, T cells from CD95L-mutant mice, in MHC-matched model, induced lethal GvHD [102], while cutaneous and hepatic GvHD was reduced. Similarly, CD95-deficient recipient mice developed severe GvHD, but consistent with previous results, hepatic GvHD was reduced [108]. By contrast, Prf-deficient T cells induced GvHD with delayed kinetics, but without a change in histological features of cutaneous and hepatic GvHD in comparison to wild-type T cells. Furthermore, CD95L and Prf-deficient T cells managed to induce GvHD, confirming, at least partly, the role of indirect alloreactive $CD4^+$ T cell effect [109]. Such phenotype suggests that other killing mediators, such as TNFα and/or TRAIL, might be pathogenic. TNFα is involved in all stages of GvHD pathogenesis, and its inhibition in experimental allo-HCT models ameliorated the consequent apoptosis of GvHD-related damage to the GIT [110, 111]. By contrast, blockade of TRAIL cytotoxicity exhibited no difference in GvHD occurrence [112]. Th17 and cytotoxic, $CD8^+$ type T cell (Tc)17 are other subsets playing a significant role in the onset of GvHD. Recently has been shown that IL-6 mediated Th17/Tc17 expansion, early after allo-HCT, induces neutrophil recruitment to the inflammatory site by C-X-C Motif Chemokine Ligand (CXCL)8 secretion. Confirming the importance of this pathway, Th17/Tc17 specific ablation early after HCT protected mice from aGvHD [113, 114, 115], while the transfer of in vitro differentiated Th17 cells caused lethal aGvHD with skin and lung manifestations [116, 117] and neutralizing IL-17A [116] partially reversed aGvHD. Even though Tc17 cells post-transplantation are poorly cytotoxic, they produce pro-inflammatory cytokines IL-17A, IL-22, GM-CSF, and IFNγ, contributing to aGvHD [114]. GM-CSF not only stimulates donor-derived macrophages to produce inflammatory mediators but directly expands donor DCs to increase indirect alloantigen presentation and secrete co-stimulatory cytokines such as IL-23 that provides amplification of aGvHD [118, 119, 94].

2.3.2 Chronic GvHD

cGvHD is considered a major factor responsible for the non-relapse mortality (NRM) in patients surviving $\geq$2 years post allo-HCT [70]. The main risk factor for its development is the previous history of aGvHD, older recipient age, G-CSF-mobilized PBSC (G-PBSC), mismatched or URD grafts, and female-to-male transplantation. The treatment of cGvHD faces many obstacles because of the poor therapeutic responses induced by diverse pathologies and involvement of virtually every organ, the absence of specific diagnostic criteria, and valid biomarkers [71]. However, preclinical mouse models have helped to make significant progress in the last decades in the sense of understanding its pathogenesis, which was divided into three phases: (1) early inflammation induced by tissue injury; (2) chronic inflammation followed by thymic injury and dysregulation of T- and B-cell immunity and (3) tissue repair and fibrosis [120, 70]. Similar to aGvHD, tissue damage, during defined **phase 1**, leads to a release of PAMPs induced by translocation of bacteria, fungi, and their product through damaged epithelial barriers as well as DAMPs, induced by cytotoxic conditioning initiated cell death. PAMPs and DAMPs trigger the activation of TLRs, purinergic receptors, nucleotide-binding oligomerization domain-like receptor (NLR)P3

inflammasome, etc. During this phase, loss of microvessels, intimal arteritis, and increased plasma levels of von Willebrand factor (vWF) were observed. As T cells are already in contact with pre-activated DCs (as a consequence of pre-conditioning-induced tissue damage), T cells are activated during this phase. In **phase 2**, APCs prime effector T- and B-cell populations leading to their expansion and polarization. Conditioning and alloreactive T cells induced thymic injury leads to the loss of epithelial cells, therefore dysregulation in the thymic selection process, thymopoiesis, and finally, loss of regulatory cell subsets, including regulatory T cells (Treg), regulatory B cells (Bregs), regulatory natural killer cell (NK) cells, and invariant NK T cells, and type 1 regulatory T cells (Tr1), that are impaired or reduced in frequency or numbers. Auto- and alloreactive $CD4^+$ T cells, that have escaped immune regulation by thymic selection and peripheral mechanisms, produce IL-17A, which maintains the inflammation. The germinal centre (GC) formation is also stimulated by IL-21-producing T folicular cell (Tfh) cells and this is not counterbalanced because of the lack of follicular (FO) regulatory T cells [70]. During the last phase, **phase 3**, as a response to profibrotic cytokines, such as transforming growth factor β (TGFβ) and platelet-derived growth factor (PDGF) secreted by colony stimulating factor 1 receptor (CSF-1R) dependant macrophages, activated fibroblasts secrete collagen fibres that are accumulating and induce tissue fibrosis, which is the main manifestation of cGvHD [121, 122, 123, 124, 125, 126, 127, 70]. Differentiated B cells (plasma cells), stimulated by B cell activating factor (BAFF), produce isotype-switched immunoglobulins whereby pathogenic immunoglobulins are accumulated in various organs, contributing to the organ damage and fibrosis [70].

During allo-HCT, it has been shown that ECs are playing an important role in the induction of GvHD, but also in the other late post-HCT complications. From the beginning of the treatment, they are consecutively challenged by toxicities of the conditioning regimen, the drugs used for immunosuppressive prophylaxis, inflammatory molecules released by damaged cells and tissues, endotoxins due to damaged mucosal barriers, donor leukocyte engraftment, and alloreactive immune responses [128, 129]. Thus, possible consequences are EC activation and injury that may progress to an irreversible state of endothelial dysfunction (ED) leading to severe post-HCT complications.

2.3.3 Endothelial dysfunction and allo-HCT

The endothelium is a semipermeable, thin monolayer of ECs that is in direct contact with the blood/lymph and the circulating cells as it constitutes the inner cellular lining of the blood vessels (arteries, veins, and capillaries) and the lymphatic system [130]. As a highly active organ, endothelium is involved in the regulation of vascular tone, cellular adhesion and migration, coagulation, vessel wall permeability, and various inflammatory processes and plays a pivotal role in vascular homeostasis [131, 132]. During allo-HCT, many factors have a significant impact on functionality of endothelium, such as *pre-conditioning regimen*, *usage of immunosuppressive drugs*, *inflammatory molecules and endotoxins* released as the result of tissue injury and damage of mucosal barriers, *alloreactive immune responses*, etc. [128]. As a consequence, ECs may become activated and damaged, which might lead to an irreversible state of ED. ECs activation is characterized by increased endothelial permeability followed by enhanced expression of pro-coagulant and adhesive molecules, whereas ED is considered as the inability of ECs to exhibit vasodilatation of the vessel wall (Figure.2.6). ED is associated with decreased levels of nitric oxide (NO), but also increased permeability of endothelial layer and adhesiveness of leukocytes and platelets (PLT), and apoptosis of ECs [133].

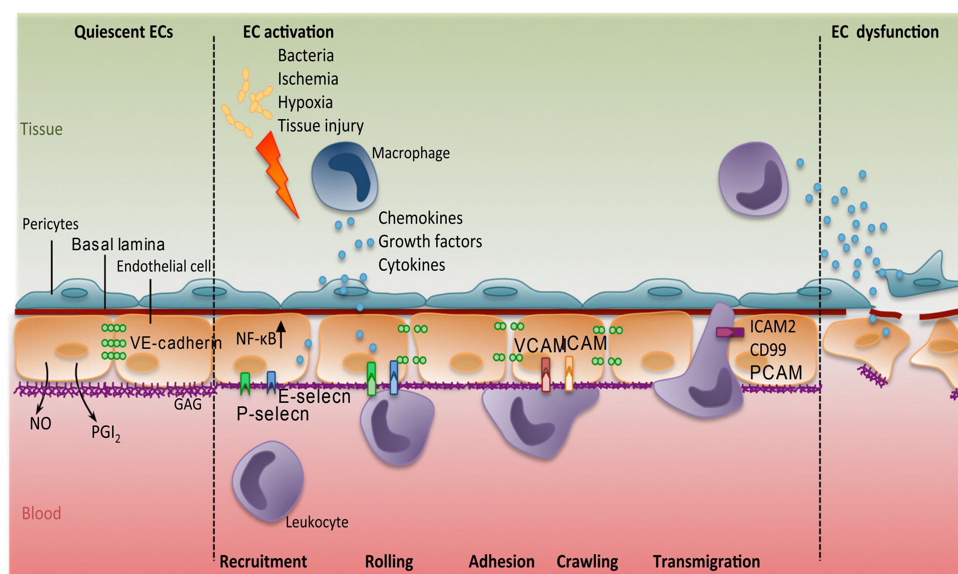

Figure 2.6: ECs through activation and dysfunction. Reprinted by permission from Springer Nature, Springer, Pflügers Archiv European Journal of Physiology [134]. EC:endothelial cell, NO:nitric oxide, VCAM:vascular cell adhesion molecule, ICAM:intercellular adhesion molecule, PECAM:platelet endothelial cell adhesion molecule, VE-cadherin:vascular endothelial cadherin, PGI2:Prostaglandin I2, GAG:glycosaminoglycan.

Radiotherapy as a part of the pre-conditioning regimen, has been demonstrated to induce EC damage. In vitro models suggested that 4 Gy irradiation, as a clinically relevant dose, induces apoptosis in micro-and macrovascular ECs model [135]. Irradiation of cultured human umbilical vein ECs (HUVECs) increased inducible NO synthase (iNOS) and nitrotyrosine expression, stimulating NO toxicity and peroxynitrite-induced cellular damage [136]. On the other hand, in vivo models revealed that irradiation-induced damage leads to recruitment of endothelial progenitor cell (EPC) to injured tissues, and irradiation induces leukocyte adhesion to ECs in a CD11/CD18 and intercellular adhesion molecule 1 (ICAM-1)-dependant manner and enhances vascular permeability [137].

Chemotherapeutic drugs are commonly used during conditioning regimens and they directly affect EC survival. Cyclophosphamide or methotrexate administration in mice directly induced EC damage observed through the increased levels of CEC shortly after their administration, noticed before the suppression of the immune system [138]. Likewise, busulfan and cyclophosphamide treatment leads to ultrastructural features of EC injury and death, as well as increased levels of EPCs and CECs [139]. Treatment with fludarabine induces enhanced EC lysis by cytotoxic T lymphocyte (CTL) by making ECs more allogeneic [140].

Immunosuppressive drugs are a common treatment used post-HCT in order to prevent GvHD. Cyclosporine (CsA) was suggested to induce ECs injury in vitro, characterized by an early detachment and cell lysis when used in a similar concentration to the present in patients [141]. Enhanced expression of adhesion molecules, such as ICAM-1, was observed in human microvascular ECs after the combination treatment of CsA or tacrolimus with sirolimus, where CsA showed an additional prothrombotic effect, with increased PLT adhesion and clot formation [142].

G-CSF is commonly used post-HCT in order to accelerate recovery from neutropenia after chemotherapy. Expression of adhesion molecules by ECs, such as ICAM-1, vascular

cell adhesion molecule 1 (VCAM-1), and E-selectin, was increased after their exposure to G-CSF, suggesting the impact of this factor on activation of ECs [143].
Inflammatory molecules and endotoxins. As mentioned before, all the treatments used pre- and post-HCT induce injuries in various organs that elicit the release of a wide range of pro-inflammatory molecules, PAMPs (LPS, proteoglycans) and DAMPs (HMGB1, ATP, and uric acid), followed by cytokines such as TNFα and IL-1. All of these factors are known to have an impact on endothelial function through stimulation of increased expression of adhesion molecules (ICAM-1, VCAM-1) and different prothrombotic factors, including plasminogen activator inhibitor-1 (PAI-1), vWF, tissue factor (TF), and decreased endothelial expression of thrombomodulin (TM) [144, 145]. Serum collected from patients undergoing even allo-transplantation with stem cells promotes ICAM-1 and VCAM-1 expression, leukocyte adhesion, and p38 activation [146] and is able to induce apoptosis of cultured ECs [147], while plasma of these patients is enriched in vWF and increases PLT adhesion to ECs underflow condition in vitro [148].
Alloreactivity has been shown as one of the important factors in the pathogenesis of post-HCT endothelial complications [149, 150]. Studies have shown that endothelial complications such as transplant-associated microangiopathy (TAM), veno-occlusive disease (VOD), capillary leak syndrome (CLS), diffuse alveolar haemorrhage (DAH) are more frequent in allogeneic than auto-HCT, despite the use of identical conditionings. Furthermore, T cell depletion, by which the allogenicity of the graft is reduced, leads to a decrease in the incidence of such complications [151]. Alloreactivity and chemotherapy-induced in vitro models of HUVECs caused ECs apoptosis [146].
EC damage was shown to correlate with the pathogenesis of steroid-resistant GvHD and increased non-relapse mortality in patients [152]. Sildenafil, a phosphodiesterase type 5 inhibitor known to protect ECs by improving metabolic activity and reducing apoptosis, decreased the severity of GvHD when used in combination with steroids in experimental models. It reduced EC damage in the liver and fibrinogen deposits in the colon while improving GvHD survival [153]. Clinical studies, as well as mouse models, have shown that markers of neovascularization and EC damage correlate with the occurrence of GvHD [154, 155]. GvHD mouse models have revealed that inhibition of neovascularization leads to amelioration of GvHD symptoms and reduced mortality [156, 154].

2.4 CVD as the late post-HCT complication

Even though most studies have focused on the role of ECs on the GvHD pathogenesis, endothelium is also considered a target organ, being located between the alloreactive donor T cells and the host tissue, exposed to direct and indirect injury by CTLs and cytokine storm [157]. Vascular ECs were proposed in several studies as targets of alloreactive and allospecific T-cell response during acute and chronic GvHD [128], and allogeneic reactions against ECs are associated with CD8$^+$ T cell infiltration in the skin and loss of dermal microvessels [14, 128].
Patients subjected to allo-HCT have an increased prevalence of CV risk factors such as hypertension, dyslipidaemia and DM [158, 159, 160, 161, 158, 162, 163], and the risk of CV death is 1.7-2.3 times that of the general population [164, 1, 165]. Dyslipidaemia appears as a factor that significantly changes during HCT. A 25 years long study including 1196 adult patients observed a significant change in the incidence of dyslipidaemia, rising from 36 and 28% before autologous and allogenic procedures, respectively, to 62 and 74% within only three months after HCT [166]. Hypertension was shown to be dependent on the immunosuppressive drug usage, as the treatment with CsA increased its incidence

within four weeks from the beginning of the therapy, and multivariate analysis showed that exposure to CsA at any time post-HCT was the sole factor predictive for development of new-onset hypertension within the first 2-years post-HCT [21].
CV complications in HCT are divided into early and late [9, 167]. Early post-HCT complications occur within 100 days to six months after the treatment and include atrio/ventricular arrhythmias, coronary artery disease (CAD), ischaemic event [168, 169], thromboembolism, and heart failure (HF). Late CV complications occur later in the form of CV events and stroke [158, 170]. Reduction in left ventricular ejection fraction (LVEF) from 62 to 55% occurred within six weeks post-HCT [171], while acute cardiotoxicity (where LVEF drop is 10% or more, or to less than 50%) can develop in 2-4 weeks after HCT with the majority of patients recovering by six weeks [9]. One hundred days post-HCT follow-up emphasised 50% higher CV-related events and mortality incidence in patients facing CV complications [172]. Noteworthy, the development of CV risk factors, such as hypertension, DM, and dyslipidaemia, has been associated with the presence of GvHD (grade II-IV) [158]. aGvHD was shown to be an independent risk factor for hypercholesterolemia and hypertriglyceridemia after allogeneic transplant [173]. Moreover, its association with conduction abnormalities, arrhythmias, myocarditis, and pericardial effusions was also postulated [174]. As a hyper-inflammatory condition, GvHD is associated with thrombosis and may increase the risk of epicardial and microvascular disease in the form of myocardial injury/infarction [175].
Taking into account that the majority of patients subjected to HCT are of older age, with already present CV risk factors, therefore established onset of atherosclerosis, high CVD incidence is expected. Therefore, the gaps in understanding of underlying mechanisms between atherosclerosis and GvHD is needed to be filled. An urgent need for systematic examination of large cohorts to identify risk factors for early CV events emerges as well as the role of blood and imaging biomarkers for risk stratification in HCT requires prospective validation [176].

2.4.1 HCT induced dyslipidaemia

As mentioned in Section 2.4, HCT patients are at high risk for dyslipidaemia and its pathogenesis appears multifactorial. Firstly, the usage of immunosuppressive drugs such as sirolimus (mTOR inhibitor) for a prolonged period of time showed a prominent impact on lipid homeostasis, resulting in a high incidence of hypercholesterolemia and hypertriglyceridemia [177]. When used in the treatment of steroid refractory GvHD, it was shown to be associated with 77% incidence of hypertriglyceridemia and 34% of hypercholesterolemia [178]. The occurrence of lipid abnormalities is quick to arise as they occur within one month of initiation of the therapy. The mechanism behind the sirolimus impact was explained as the expansion of free fatty acid pool and an increase in hepatic very-low-density lipoproteins (VLDL) synthesis [179]. mTOR inhibition results in an increase in lipolysis via augmentation of hormone-sensitive lipase (increasing circulating free fatty acids), interference with triglyceride metabolism, decreased triglyceride storage, and a disruption of the insulin-signalling pathway [179, 180, 181, 182]. Similarly, CsA, a calcineurin inhibitor, exhibited a significant impact on the levels of total cholesterol and LDL-cholesterol levels. A randomized, double-blinded, placebo-controlled trial of 36 non-transplant patients treated with this drug for only two months yielded 21%, and 31% mean increases in total cholesterol and LDL, respectively [183]. It is suggested that CsA impairs the conversion of cholesterol into bile acids via inhibition of steroid 26-hydroxylase and, in that way, leads to an increase in the total cholesterol levels. As a drug transported

via lipoproteins, it has been suggested that it blocks LDL receptor (LDLr) resulting in elevated serum LDL levels [184]. CsA has also been shown to affect VLDL and LDL clearance via alterations in lipase activities [184]. By contrast, tacrolimus, a more potent calcineurin inhibitor, was suggested to have a less prominent effect on lipid metabolism than CsA. Switching from CsA- to tacrolimus-based immunosuppression has resulted in improvements in lipid values, suggesting this significant difference in their impact on lipid metabolism [184]. However, larger studies in allo-HCT patients have not reported significant differences in rates of hyperlipidaemia between treatments with these two drugs [185, 186, 187]. Finally, glucocorticoids, which are known for their hyperglycaemic and appetite-stimulating effects, were also found to be associated with dyslipidaemia. It is suggested that they have an impact on lipase activity, stimulation of de novo lipogenesis and VLDL export [188], as well as in downregulation of LDLr, further perturbing lipid levels [184]. However, not only are immunosuppressive drugs appointed to contribute to dyslipidaemia in allo-HCT, but post-transplant complications can also affect lipid homeostasis. For instance, cGvHD of the liver results in severe elevations of total serum cholesterol and triglycerides and a specific appearance of lipoprotein X (LpX), as a serum cholesterol transporter, induced by the inability of cholesterol and bile acids to be cleared through the bile duct [189]. In a similar way, nephrotic syndrome, a rare but serious complication associated with cGvHD, was shown to cause dyslipidaemia [190]. Lastly, post-transplant endocrine complications, such as hypogonadism and hypothyroidism, are shown to be also associated with dyslipidaemia [182, 191]. Studies have pointed out that these abnormalities are specifically more frequent in patients subjected to irradiation as a pre-conditioning regimen [192, 190].

Chapter 3

Atherosclerosis

Atherosclerosis is a chronic inflammatory disease and the most common form of CVD. CVD is considered a leading cause of global death and one of the most serious problems worldwide. In 2016, it was shown that nearly one-third of all global deaths were caused by CVD [193], and it was projected that CVD will be accounted for more than 23 million deaths in 2030 around the world [194]. The importance and understanding of this disease were not recognized until the 1980s, even though the first connections were revealed far earlier. The first ideas about its origin as an inflammatory disease [195] and the disease elicited by cholesterol intake [196] were given during the 19th-20th century. The connection with inflammation was recognized through the studies of myocardial infarction (MI), observing high C-reactive protein (CRP) levels in these patients in 1982 [197], and nowadays, CRP is routinely used worldwide as a prognostic marker. It is well known that the main feature of the disease is lipid accumulation and inflammation of the large arteries that, in the long run, may lead to clinical complications, such as MI and stroke [198].

3.1 Pathogenesis of atherosclerosis

The wall of blood vessels is composed of different layers (Figure.3.1): (1) Monolayer of ECs - that borders the luminal blood flow; (2) Intima - mainly acellular layer consisting of glycosaminoglycans and collagen; (3) Media - the layer of smooth muscle cell (SMC); and (4) Adventitia - fibrous layer [198].
As shown in the Figure.3.1, ECs form a compact layer by making tight junction connections between each other and, in this way, they separate blood from the vessel wall and keep the layer resistant to attachment of blood leukocytes. During conditions of disturbed, turbulent blood flow, cellular alignment of ECs changes what leads to increased permeability of the EC layer and promotes the uptake of plasma LDL and triglyceride-rich lipoproteins either by trans-endothelial transport or diffusion at the cell-cell junctions [199]. Atherosclerosis specifically tends to occur in the arterial regions dominated by the turbulent blood flow, such as bifurcations, and it is mainly initiated by the accumulation of these plasma lipoproteins in the intimal region of the vessel. Lipoprotein accumulation is partly induced by interaction with the intimal glycosaminoglycans [200], which leads to their aggregation and chemical modification [198]. Myeloperoxidases, lipoxygenases, and reactive oxygen species (ROS), through the process of oxidative modifications, induce the formation of oxidized LDL (oxLDL), which can elicit the innate immune response [201]. As a subsequent response to lipid oxidation, activation of ECs occurs, resulting in the expression of adhesion molecules such as P-selectin, E-selectin, VCAM1, and ICAM1. Circulating monocytes are, therefore, enabled to bind to the ECs, enter the intima and differentiate into macrophages under the impact of macrophage colony-stimulating factor (M-CSF) and GM-CSF, which are produced by ECs and several other cell types.

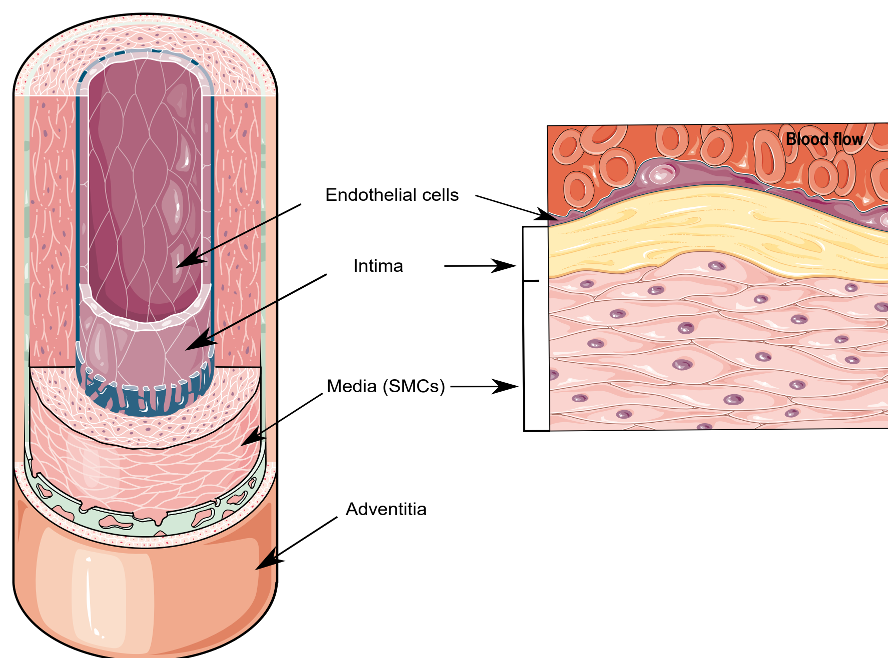

Figure 3.1: Anatomy of a healthy blood vessel. The figure is made by using smart.servier.com. SMC:smooth muscle cell.

These lesional macrophages engulf the modified lipoproteins by their scavenger receptors or phagocytosis of aggregated lipoproteins which leads to their modification into cholesterol-enriched "foam cells" [202, 198]. The migratory capacity of foam cells is then compromised, and these cells become trapped within the arterial intima [203]. Following these initial processes, lipids and foam cells continue to accumulate, which further leads to the infiltration of other leukocytes, particularly T cells, which enter the lesion and interact with macrophages. Over time, the foam cells undergo apoptosis or necrosis, giving rise to a growing "necrotic core" composed of apoptotic and necrotic cells, cholesterol crystals and other extracellular material. These processes also have an impact on SMCs in the media, which transform from the contractile to a proliferative state and migrate into the intima. The intimal SMCs, over time, secrete an extracellular matrix largely consisting of collagen that gives rise to plaque-protective fibrous cap [202, 198]. On the other hand, few migration-competent SMCs that enter intima undergo clonal expansion and redifferentiation into contractile SMCs able to transdifferentiate into macrophage-like and osteochondrogenic descendants [204, 205]. It was also shown that SMCs are able to acquire cholesterol from nearby macrophage foam cells [206], and they are able to produce M-CSF that drives the proliferation of macrophages in lesions [198]. In animal models, it was estimated that SMC-derived foam cells could account for as many as 50% of lesional foam cells [207]. An overview of atherosclerotic inflammatory processes is shown in Figure.3.2.

3.1.1 Innate immune response

As explained above, macrophages play the most important role in the pathogenesis of atherosclerosis. Mouse studies suggest that the abundance of macrophages during early lesions is dependent on monocyte recruitment, while more advanced lesion infiltration is mainly determined by macrophage proliferation [209]. OxLDL stimulates macrophage

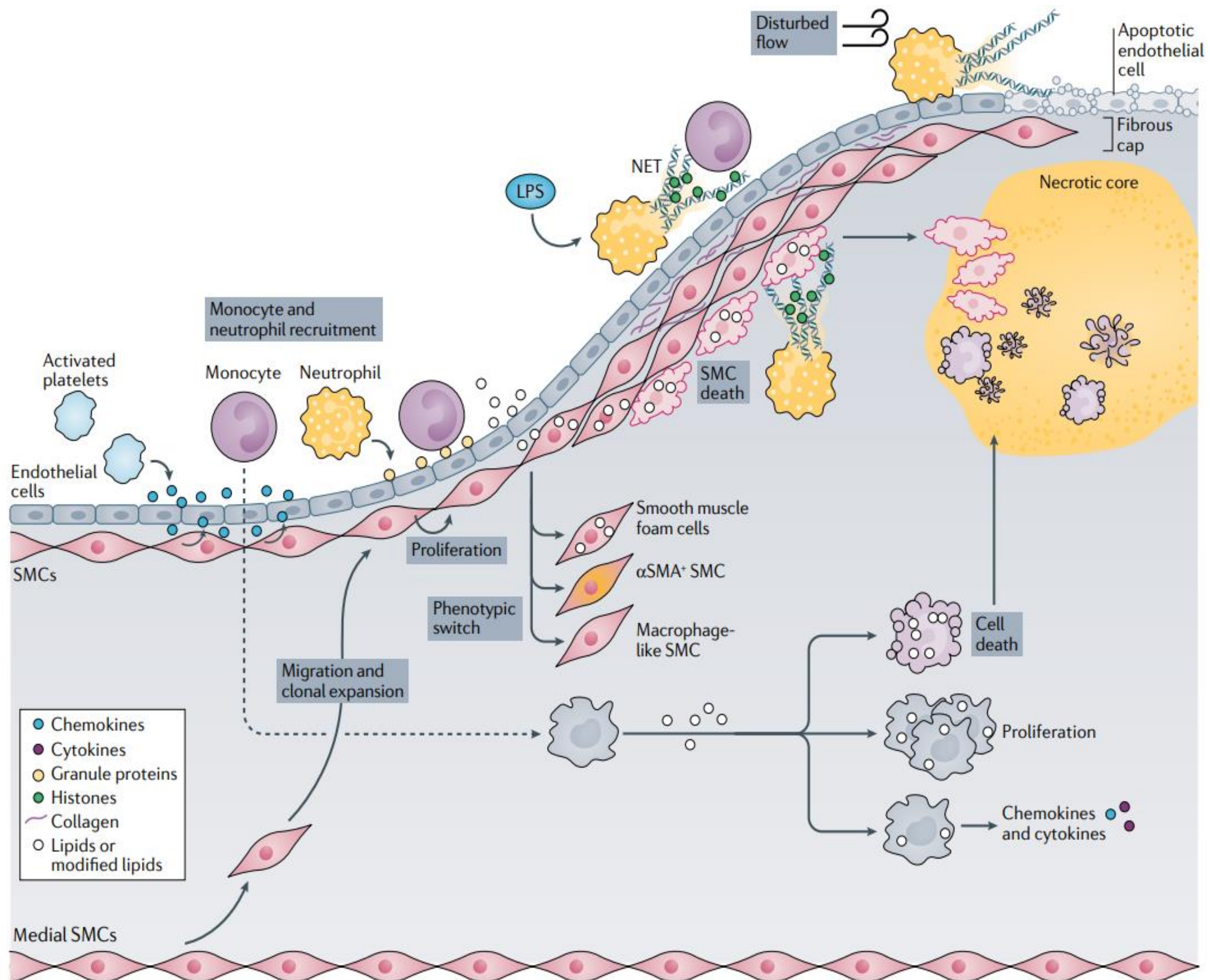

Figure 3.2: Overview of inflammatory processes during atherosclerosis. Reprinted by permission from Springer Nature, Springer, Nature Reviews Drug Discovery [208]. NET:neutrophil extracellular trap, SMC:smooth muscle cell, SMA:smooth muscle actin.

activation by binding TLRs, such as TLR2 and TLR4, expressed on the surface of macrophages [210, 211]. Apart from oxLDL, other endogenous and exogenous ligands for TLRs might also be present in plaques, such as heat shock proteins (HSPs), bacterial toxins and viral glycoproteins [212] that can induce their activation. On the other hand, LDL oxidation by lipoprotein-associated phospholipase A2 (Lp-PLA2) generates lysophosphatidylcholine and oxidized non-esterified fatty acids that were also shown to be capable of activating the innate immune system [213]. Although all of these factors seem to be important in the initiation of inflammation and to contribute to its maintenance during lesion formation, strategies to reduce the incidence of CV events by preventing innate immune activation using antioxidants [214, 215] or selective Lp-PLA2 inhibitors [216, 217] have failed in various patient populations. Apart from membrane bound receptors that are activated during the initial inflammation, oxLDL, as well as intracellularly formed cholesterol crystals in foam cells, can activate intracellular danger signal sensing NLRP3 inflammasome, which leads to release of IL-1β and IL-18 [218, 219, 208]. Briefly, activation of inflammasome leads to recruitment of caspase-1, which cleaves the pro-form of IL-1β and IL-18 to their functional, mature form. The released IL-1β stimulates SMCs to produce IL-6 [220, 221], which in turn signals to the liver to induce the acute phase re-

sponse, including CRP production [222, 10]. In comparison to macrophages, other innate immune cells, such as neutrophils, mast cells, NK and NK T cells, are considered minor plaque infiltrating populations. In hypercholesterolemic mice, neutrophil recruitment was noticed during the initiation of atherosclerosis, but they were not found in the later stages [223]. However, neutrophils were shown to have an important role in the control of macrophage migration through the secretion of several proteins with monocyte chemotactic activity, such as chemokine (C-C motif) ligand 2 (CCL2), cathelicidin, cathepsin G and α-defensins [224, 225, 226, 227]. These secretory products were shown not only to attract rather also to activate macrophages. In atherosclerotic mice, neutrophil extracellular traps (NETs) by stimulating NLRP3 inflammasome prime macrophages to produce IL-1β [228]. On the other hand, mast cells are suggested to have a role in plaque instability through the secretion of matrix-degrading enzymes [229], while NK and NK T cells aggravate atherosclerosis probably through their IFNγ release [230, 231]. Although many studies have shown the involvement of various populations of the innate immune response at different stages of disease development, the main innate immune effector cells in the plaque are macrophages.

3.1.2 Adaptive immune response

Myeloid cells, including macrophages, are considered the first line of defence against a broad spectrum of identified threats that are able to respond rapidly and uniformly. Conversely, adaptive immune response gives delayed but highly specific, 'target matched' immune response [232]. Adaptive immunity is mediated by T and B cells that recognize specific antigens through their surface receptors, TCR and B cell receptors (BCR), respectively [233]. Accordingly, different study approaches have shown that CD3$^+$ T cells account for 25–38% of all leukocytes in mouse aortic and human atherosclerotic plaques [234, 235, 236, 237, 238]. Plaque infiltrating DCs, as well as DCs residing in the adjacent adventitia [239], take up the antigens derived from plaque, including oxLDL and HSP60 [240], migrate to LNs and display the engulfed antigens to naive T cells [241]. Presentation of processed antigenic peptides to CD8$^+$ T cells and CD4$^+$ T cells by MHC I on all nucleated cells and MHC II on APCs, respectively, leads to activation and clonal proliferation of these T cells. However, their activation process is strictly controlled and able to occur only when a specific TCR is concomitantly bound with co-stimulatory molecules provided by APCs [242]. Upon activation, naive CD4$^+$ T cells are able to differentiate into various cell subsets such as effectors Th1, Th2, and Th17 and Treg [243] depending on the type of encountered antigen, the TCR signal intensity as well as on the local cytokine milieu [244, 245, 246]. On the other hand, upon activation, CD8$^+$ T cells differentiate into CTL [247]. Interestingly, both T cell subsets (CD4$^+$ and CD8$^+$) have been shown to accumulate in the shoulder region, the fibrous cap, adventitial tissue and the intima of human atherosclerotic plaque. They were suggested to account for $\approx$54% of all shoulder region infiltrating leukocytes and $\approx$14% of the necrotic core [248, 235], while dying macrophages are necrotic core dominating cells and B cells were only found in adventitial tissue [249, 236].

3.1.2.1 CD4$^+$ T cells

Lesional CD4$^+$ T cells, as mentioned above, have the capacity to differentiate into distinct Th cell (Th1, Th2, Th9, Th17, Th22, Tfh, and CD28null T cells) or Treg subtypes (FoxP3$^+$ Tregs and Tr1) [250, 251]. Each of the subtypes has a specific transcriptional and, therefore, cytokine profile that can modulate immune response - either activate or

dampen the response of other T cells; have direct pro-inflammatory or anti-inflammatory impact on tissue-resident cells; help B cells to produce high-affinity IgG antibodies, or have cytolytic activity [251, 252]. Therefore, $CD4^+$ T cells are considered multifunctional and very important cells in the pathogenesis of atherosclerosis. The polarization of their response (immunogenic or tolerogenic) is initiated and modulated by co-stimulatory signals and cytokines provided by antigen-loaded APCs migrating to the LNs [253, 252]. Several known types of cells can act as APCs, including plaque infiltrating macrophages, B cells from adventitia and several DC subsets, such as conventional, plasmacytoid [254], or IFN regulatory factor (IRF)8-dependent [255] DCs, activating antigen-experienced effector memory T cells [256]. Th1 polarization is, for example, induced by co-stimulatory interaction between CD40 on APCs and CD40ligand (CD40L) on T cells [257]. CD80 and CD86 on APCs are also co-stimulatory molecules that interact with T cells through cytotoxic T lymphocyte antigen 4 (CTLA-4) expressed on activated T cells or Tregs or with CD28 expressed on T cells. Disabling these interactions (CD40, CD80/CD86) or overexpressing of CTLA-4 was shown to improve atherosclerosis in mouse models [258, 259, 260, 261]. On the other hand, CD80 and programmed cell death 1 (PD-1) on T cells can interact with PD-L1 on APCs. PD1 was shown to have an important limiting effect on early T cell activation and exhaustion in cancer and chronic inflammation [262], which might be the case with atherosclerosis too. It was shown that T cells from human atherosclerotic plaque express high levels of PD-1 [263], as well as that pro-atherogenic T cell response, is limited by the PD1–PDL1 pathway [264]. CD137 (4-1BB or TNF receptor superfamily member 9 (TNFRSF9)) is expressed on activated T cells and its interaction with CD137 ligand (CD137L) on APCs increases T cell proliferation and survival, which can stimulate atherosclerosis development and progression [24]. Ox40 L-Ox40 interaction stimulates Th2 response, and its blockade seems to induce plaque regression in $LdLr^{-/-}$ mice [265].

Th1 cell subset (Figure.3.3) is defined according to their expression of T-box transcription factor TBX21 (T-bet) and chemokine receptors such as CXC-chemokine receptor 3 (CXCR3) and CC-chemokine receptor (CCR)5, and they are known to secrete IFNγ. Studies have shown that these cells are plaque-promoting and the most prominent Th cell subset in atherosclerotic plaque [266, 267]. They were found in increased numbers in patients facing recent stroke in comparison to patients with asymptomatic atherosclerosis [263]. Th1 cells from LNs of $ApoE^{-/-}$ mice express CCR5 which is considered a plaque-homing chemokine receptor. CCR5 expression was also defined in plaque-derived T cells in humans [263]. Apart from IFNγ, many $CD4^+$ T cells were shown to secrete other Th1-associated pro-inflammatory cytokines, such as IL-2, IL-3, TNFα and lymphotoxin that can accelerate the inflammatory response by activating macrophages, T cells, or other plaque infiltrating cells [250]. However, in the context of Th1 response, IFNγ is extensively studied. It was shown that IFNγ directly reduces plaque stability by inhibiting vascular SMC (VSMC) proliferation [268]. Other studies have shown its impact on macrophage polarization [269] and modulation of CV risk factors [270]. Overall, many studies have shown the importance of IFNγ during atherogenesis: deficiency of IFNγ, its receptor or T-bet, decreases atherosclerosis in mice [266, 271, 272], while IFNγ administration leads to increased lesion formation [273].

Th2 cells (Figure.3.3) are well known for their role in immune defence against parasites and allergic diseases, such as asthma. They are defined as cells expressing GATA3 transcription factor, which is the master regulator of their differentiation. During atherosclerosis, these cells were shown to secrete four main Th2-associated cytokines, IL-4, IL-5, IL-10 and IL-13 [236]. The exact role of these cells is still not clear. Different studies

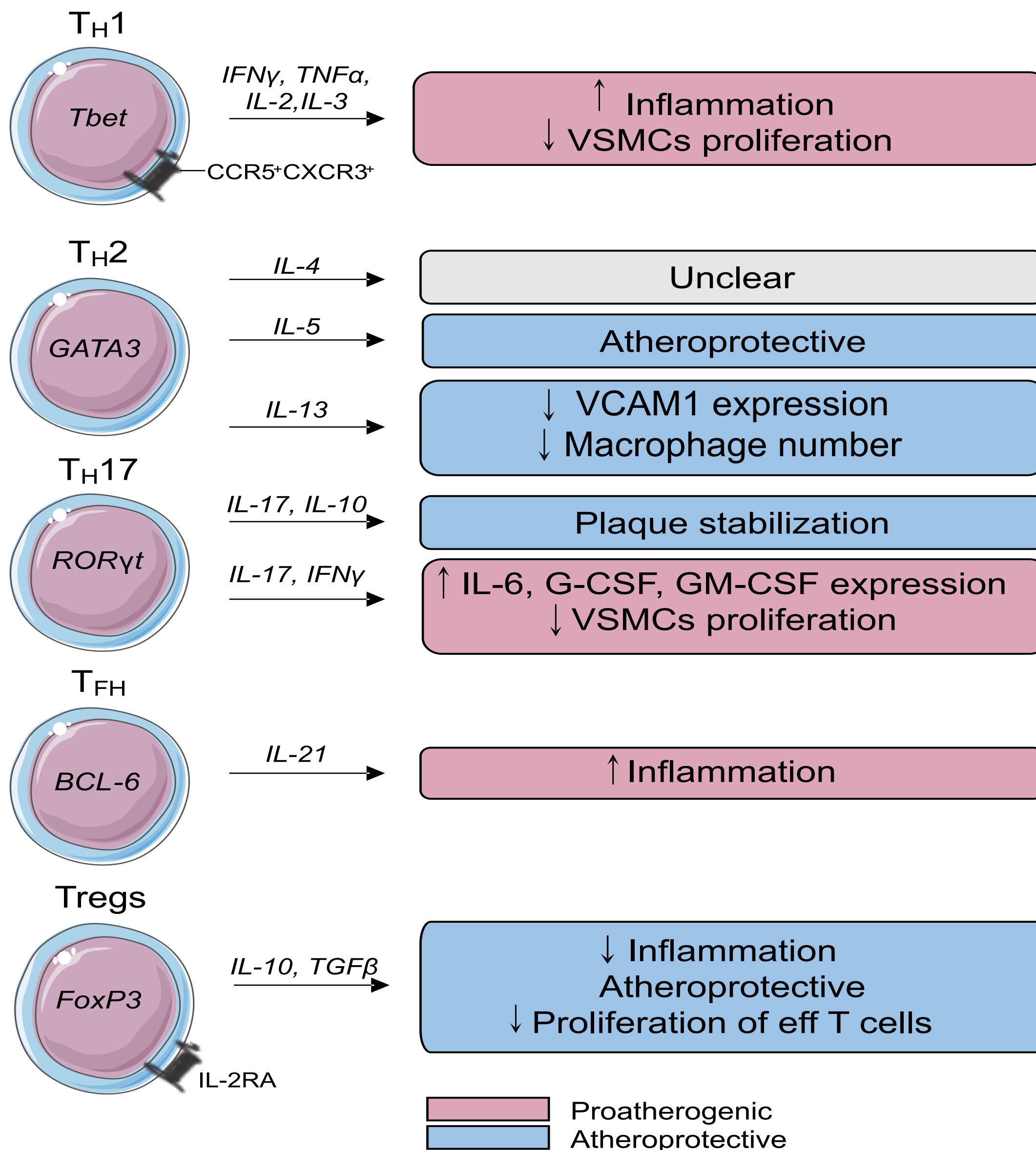

Figure 3.3: T helper cells during atherogenesis Scheme made by using smart.servier.com, according to [252]. VSMC:vascular SMC, G-CSF:granulocyte colony-stimulating factor, GM-CSF:granulocyte-macrophage colony-stimulating factor, eff T cells:effector T cells.

have shown opposite conclusions in regard to the role of these cells and the effect of their cytokines. IL-4 released from human activated mononuclear leukocytes has shown a negative correlation with clinical atherosclerosis in vitro [274]. The other study [275] suggested that IL-4 antagonizes Th1 responses and decreases atherosclerotic lesion formation in ApoE$^{-/-}$ mice, while others observed an atheroprotective effect when IL-4 is depleted in Ldlr$^{-/-}$ mice HFD [276]. Furthermore, IL-4 administration in ApoE$^{-/-}$ mice with angiotensin II-induced atherosclerosis did not reduce lesion formation [277]. As opposed to IL-4, the other cytokines, such as IL-5 and IL-13, were shown, more agreeably, to be atheroprotective. IL-5 plasma levels inversely correlate with carotid intima media thickness [278, 279], but increased plasma levels of IL-5 were associated with MI and the presence of unstable angina [280]. Mice immunization with modified LDL induces

a Th2 cell-skewed immune response characterized by antigen-specific production of IL-5 and IL-13 and small amounts of IL-4 and IFNγ compared with non-immunized mice and is shown to be atheroprotective [281]. IL-13 administration in Ldlr$^{-/-}$ mice fed HFD modulates already established atherosclerotic lesions by decreasing VCAM-1 expression, resulting in decreased macrophage infiltration in plaque, and by increasing lesional collagen content [282]. Interestingly, treatment with IL-33, as a cytokine that induces the production of Th2 cytokines, IL-4, IL-5 and IL-13, reduces atherosclerosis development in HFD-fed ApoE$^{-/-}$ mice and decreases the level of IFNγ in serum and LNs compared with PBS treated animals [283]. However, deficiency in IL-33 or its receptor ST2 in high cholesterol diet fed ApoE$^{-/-}$ mice showed no effect on atherosclerosis development [284]. Further studies are needed to clarify the role of these cells in the context of atherosclerosis, specifically with a focus on T cells, as innate lymphoid cells 2 (ILC2) produce the same cytokines (IL-1, IL5, IL-13) [285] making the interpretation of results controversial.

Th17 cells (Figure.3.3) are defined by their expression of transcription factor nuclear receptor RORγt. They are activated by IL-23, and the main cytokine that they secrete is IL-17 [286]. Depending on the inflammatory setting, these cells have distinct plasticity [287, 288, 289, 290, 291]. IL-17 induces the secretion of pro-inflammatory cytokines such as IL-6, G-CSF, GM-CSF and chemokines by immune, ECs and stromal cells [286], which are considered pro-atherogenic. However, IL-17 response studies have yielded discrepant results. The reason is probably that apart from Th17, other cell types ($\gamma\delta$T cells and ILC3 [292]) also secrete the same cytokine, IL-17, giving the opposite effect on atherogenesis. Furthermore, a specific subtype of Th17 cells is induced by IL-6 and TGF-β, which produce IL-10 concomitantly with IL-17 [293, 294], where IL-10 is atheroprotective [295].

Tfh cells (Figure.3.3) are cells defined by the expression of transcription factor B cell lymphoma 6 (BCL-6). They are found in B cell follicles and have a role in GC formation together with B cells and are required for antibody isotype switching [296]. Differentiation and maintenance of Tfh cells are dependent on co-stimulatory molecule inducible T cell co-stimulator (ICOS) and its ligand ICOSL as blocking of ICOS-ICOSL signalling pathway reduced atherosclerosis burden and led to lowering the number of Tfh cells in secondary lymphoid organs (SLOs) of ApoE$^{-/-}$ mice [297]. The pro-atherogenic environment stimulates autoimmune responses of CXCR3^{+} Tfh cells in atherosclerosis-prone mice [298]. However, marginal zone (MZ) B cells inhibited the response of Tfh cells resulting in limited atherosclerosis development and progression [299]. Noteworthy, Tfh cells can derive from “switched” Tregs, and these cells are considered pro-atherogenic as their depletion reduced atherosclerosis in ApoE$^{-/-}$ mice [297].

Tregs (Figure.3.3) are a subset of CD4^{+} T cells characterized by expression of the transcription factor forkhead box protein P3 (FoxP3), the IL-2 receptor subunit-α (IL-2RA; also known as CD25, which is part of the trimeric high-affinity IL-2 receptor), CTLA4 and by lack of CD127 expression [252]. Tregs were shown to be atheroprotective in mice [300, 301]. Their role was confirmed in a few clinical studies showing lower levels of IL-10, as the main cytokine that these cells secrete, in patients with MI in comparison to patients with stable angina or individuals without coronary disease [302, 303]. Furthermore, a large cohort study showed an increased rate of CV events in patients with a low Tregs to CD4^{+} T cell ratio [304]. Upon binding the cognate antigenic peptides in the complex with MHC II by their TCR, Tregs become activated and produce high levels of IL-10 and TGFβ. The deficiency of IL-10, as an anti-inflammatory cytokine, was shown to increase atherosclerosis in mice [295]. On the other hand, TGFβ has shown plaque-stabilizing effects in ApoE$^{-/-}$ mice [305]. Atheroprotective properties of Tregs are, apart from their secretion of anti-inflammatory cytokines (IL-10 and TGFβ),

also attributed to their capability of suppressing the proliferation of pro-inflammatory effector T cells [306]. Treg effector function is controlled by a splice variant of FoxP3 that was associated with human atherosclerotic plaque stability [307]. MHC II deficiency was shown to aggravate atherosclerosis via decreased Treg cell number attributed to the lack of antigen presentation via MHC II molecules in mice [308]. Hypercholesterolemia initially stimulates differentiation of Tregs, followed by increased TCR downstream signalling events in atherosclerosis-prone mice [309]. This effect was suggested to occur as a response to increased inflammation [310], intracellular lipid accumulation in Tregs [311], or to a specific antigen. In patients with subclinical atherosclerosis, plasma LDL levels were shown to correlate with the number of Tregs in the blood [312]. These findings suggest that Tregs respond to components of LDL particles or at all, with antigens responsible for increased LDL plasma levels. These Tregs, having specific TCR that respond to atherosclerosis-related antigens, have been demonstrated by using human and mouse MHC class II tetramers loaded with a sequence-identical human and mouse ApoB peptide [313]. However, different studies have shown that the immunosuppressive phenotype of Treg cells disappears as atherosclerosis progresses [314, 315, 310]. This change in the phenotype was named as a conversion of Tregs into pathological Th subtypes, as they lose their FoxP3 expression followed by a loss in the capacity to regulate and protect against atherosclerosis but upregulate BCL-6 [297], T bet, or RORγt [313] expression. Increased aortic lesion size is followed by a reduction in the number of circulating and atherosclerotic plaque-residing Tregs, whereas total effector CD4$^+$ T cells and splenic Tregs increase [297].

3.1.2.2 CD8$^+$ T cells

In contrast to CD4$^+$ T cells, CD8$^+$ recognizes peptide antigens in the complex with MHC class I. Among them, they can be differentiated into a few subsets, including short-lived effectors, Teff (with the high migratory ability and high capacity to produce cytokines and cytotoxins), effector memory cells, Tem (which accumulate in peripheral organs and become effectors upon re-encounter with antigens), central memory cells, Tcm (which rapidly proliferate and produce abundant cytokines, but few cytotoxic molecules upon antigen encounter), tissue-resident memory cells, Trm (that have the very limited migratory capacity, hence permanently reside in peripheral tissue, producing cytokines and cytotoxic molecules upon antigen encounter) [316, 317, 318, 319, 320] and regulatory cells, CD8$^+$ Tregs [316, 321]. In contrast to CD4$^+$ T cells, naive circulating CD8$^+$ T cells are activated upon cross-presentation by encountering peptide-loaded MHC I presenting DCs [322]. Furthermore, activation of these cells can be enhanced by cytokines such as IL-1β [323], IL-2, IL-12, IL-15 and IL-21 [324, 325]. CD8$^+$ T cells express many surface molecules that are important during the regulation of their activity, including PD-1, CTLA-4, T cell immunoglobulin and mucin domain-3 (TIM-3) and lymphocyte activity gene 3 (LAG-3) [326]. They can also express various selectins, integrins and chemokine receptors, such as PSGL-1 and CD44, CCR4, CCR5, CCR7, CCR9, CCR10, CXCR3, VLA-1 (integrin, α 1 subunit) and LFA-1 (integrin $\alpha L\beta 2$), that enable them to migrate and localize in different regions of the body [327, 328]. Effector CD8$^+$ T cells secrete many pro-inflammatory cytokines, including IFNγ and TNFα, but also IL-17A, IL-17F, IL-21 and IL-22 [329] and IL-14, IL-5 and IL-10. As the other killer cells, CD8$^+$ T cells express Prf and granzyme, which induce lysis of target cells [330, 331], FasL, inducing apoptosis through binding to its receptor Fas on the target cells [332, 333, 334] and TRAIL, as the other apoptosis-inducing cytokine [335]. Terminal differentiation, as well as strong or chronic antigen stimulation, can lead to the change in T cells phenotype to an exhausted,

hypofunctional phenotype [336]. In the case of $CD8^+$ T cells, the exhaustion phenotype is characterized by a decrease in the antigen-driven secretion of effector cytokines and elevated expression of inhibitory cell surface receptors, such as PD-1 [337, 338]. In regard to atherosclerosis, levels of cytotoxin-producing $CD8^+$ T cells have been shown to be increased in the blood of patients with CAD in comparison to healthy individuals [339, 340], and they were abundant in atherosclerotic plaque of humans and mice [341, 342, 343]. Advanced atherosclerotic lesions in humans are predominantly infiltrated by $CD8^+$ T cells, in comparison to $CD4^+$ [341, 344, 263], and these cells are mostly located in the area of fibrous cap [345]. $CD8^+$ T cell depletion models in atherosclerosis-prone mice demonstrated reduced plaque formation [346, 347, 348], pointing out the pro-atherogenic role of these cells. These pathogenic $CD8^+$ T cells were shown to have higher IFNγ and Granzyme B (Gmzb) production than $CD8^+$ T cells from non-atherosclerotic mice [348]. One of the studies even suggested that $CD8^+$ T cells regulate monopoiesis and the levels of peripheral monocytes via IFNγ production and, in this way, promote atherogenesis [346]. The antibody-mediated $CD8^+$ T cell depletion treatment also revealed a reduction in the area of plaque necrotic core [346, 347], implying a $CD8^+$ T cell contribution to cell death within the lesions. Another study using adaptive transfer in $ApoE^{-/-}Rag2^{-/-}$ delineated TNFα, Prf and Gmzb as the main drivers of pro-atherogenic effects of $CD8^+$ T cells [347]. The same study pointed out the involvement of $CD8^+$ T cells in the formation of plaque necrotic core by inducing the lesional cell death of macrophages, ECs and VSMCs by secretion of cytotoxic granules (Prf or Gmzb), leading to the formation of an unstable plaque phenotype during lesion development [347]. Recent single-cell RNA sequencing (scRNA-seq) and cytometry by time of flight (CyTOF) data have confirmed that $CD8^+$ constitutes a variable but a substantial proportion of the inflamed cellular plaque infiltrates in murine and human atherosclerosis [349, 237, 236, 238, 350, 263, 351]. CyTOF analysis of $CD8^+$ T cell compartment defined two different terminally differentiated effector memory subsets, including $CD103^+$ $CD8^+$ T cells that correspond to classical tissue-resident memory T cell subset [263]. Interestingly, an activated $CD8^+$ phenotype has been observed in asymptomatic patients, whereas in symptomatic patients, both clusters of lesional effector memory cells exhibited signs of T cell exhaustion, as suggested by increased expression of PD-1 and lower levels of Prf [263]. Furthermore, the activation status of plaque infiltrating $CD8^+$ T cells seems to be higher than its blood counterparts, suggesting quiescent $CD8^+$ T cell phenotype in blood and a distinct degree of their activation within the lesions. However, the answer to the mechanism behind the migration of these cells into the lesion sites is still unclear. In vitro invasion assay has suggested that migration into early lesion formations requires additional T cell activation [341], pointing out the potential role of TCR activation and cytokine milieu as the drivers of cell recruitment. Recently, an endothelium-homing receptor CX3CR1 expressing $CD8^+$ T cell population was described in humans [352, 353], suggesting a potential role of this chemokine in the recruitment of these cells into the inflamed vessel wall [353]. On the other hand, the question regarding the antigen specificity of these cells, their possible priming, or activation locally within the lesion also remains unclear. Several studies have suggested local antigen-specific T-cell responses within lesions [354, 355]. TCR mRNA analysis of atherosclerotic lesions during different stages of the disease development revealed skewing towards a highly restricted TCR repertoire in both fatty streaks and fibro-fatty plaques of $ApoE^{-/-}$ mice, suggesting the oligoclonal expansion of T cells [355]. During atherosclerosis, different lesion-related antigens can be detected, including oxLDL, ApoB-100 and HSPs, in humans and mice [356, 357, 358]. In regard to $CD4^+$ T cells, the presentation of these peptides within MHC II complex was already demonstrated [359, 356, 357, 358],

while the exact mechanism relevant to $CD8^+$ is not clear. In vitro data suggests that exclusively oxLDL, not the native form, can activate $CD8^+$ T cells in the presence of DCs and might thus serve as a self-antigen in atherosclerosis [360, 361]. Studies in mice using MHCI-specific pentamer, including a peptide fragment of ApoB-100, revealed the existence of antigen-dependent T cells [362]. As an antigen-specific activation requires MHCI-dependant antigen presentation, several studies have focused on MHCI-dependent $CD8^+$ T cell responses. However, so far, obtained data shows contradictory atherosclerotic effects of decreased $CD8^+$ response in the absence of functional MHCI [363, 364]. The other point of view is the communication between APCs and $CD8^+$ T cells. Antigen-specific response of T cells is controlled by the interaction between specific receptors on APCs and $CD8^+$ T cells, transducers of co-stimulatory or inhibitory signals [264, 365]. For example, PD-1 (CD279), a member of the extended CD28/CTLA-4 family of T cell regulators, is considered a co-inhibitory cell surface receptor that is essential for T cell tolerance [351]. In the atherosclerosis model, deficiency in PD-1 or its ligand PD-L1 triggered a massive lesion growth followed by abundant $CD4^+$ and $CD8^+$ infiltration despite the expansion of atheroprotective Tregs [264, 366, 367]. CD137 is another T cell regulatory molecule that was found to act in a TCR-independent manner and is expressed on activated T cells in human atherosclerotic plaque [25, 26]. As mentioned before, this receptor belongs to necrosis factor superfamily members and it stimulated T cell proliferation and cytokine production [25, 368]. Treatment with CD137 agonists in $ApoE^{-/-}$ mice caused increased infiltration predominantly of $CD8^+$ T cells and elevated levels of pro-inflammatory cytokines [25]. $CD137^+$ effector $CD8^+$ T cells seem to promote plaque infiltration of endogenous IFNγ-producing $CD8^+$ T cells, whereas CD137-deficient $CD8^+$ T cells showed impaired and, therefore, decreased vessel wall infiltration with minimal IFNγ production [351]. CD137 was shown to signal through the recruitment of two TNFR-associated factors, TRAF1 and TRAF2 [369, 370, 371]. TRAFs were found to be associated with many other receptors, such as TNF receptors (TNFRs), IL-1 receptor (IL-1R), TLRs, RIG-I-like receptors (RLRs), NLR, and receptors for IL-2, IL-17, IFN, and TGF-β [372, 373, 374, 375, 376]. The main biological role of TRAFs is in the regulation of cell survival, immunity, and inflammation. TRAF1 expression is mainly regulated by nuclear factor (NF)-κB activation and shows reduced expression in resting cells [377, 378]. Studies have shown that deficiency of TRAF1 impairs NF-κB signalling and favours the accumulation of pro-apoptotic signals in the cell [379, 380], as well as it impairs the survival of activated and memory $CD8^+$ T cells [380, 381, 382]. During HIV and influenza virus infections, TRAF1 was shown to be instrumental for antigen-specific $CD8^+$ T cell response, partially by promoting T cell survival and memory [378, 383]. During atherosclerosis, TRAF1 seems to be overexpressed in murine lesions and in neointima formation after arterial injury [27, 28]. Atherosclerosis-prone mice deficient in TRAF1 develop significantly smaller atherosclerotic lesions after 8 or 18 weeks of high cholesterol diet, suggesting its pro-atherogenic role [384]. Decreased de novo atherosclerosis was followed by the reduced content of lipids and macrophages in plaque, an effect likely caused by reduced VCAM-1 and ICAM-1 expression on ECs and reduced β1-integrin expression on macrophages [376]. TRAF1 blood mRNA expression was shown to be increased in patients with acute coronary syndrome [384], and its high expression was also noticed in fibrous atheromatous and aneurysmal atherosclerotic lesions of carotid arteries [27]. Together, these observations demonstrate that many co-stimulatory factors modulate the function of $CD8^+$ T cells in atherosclerosis by controlling their accumulation and activation. A schematic overview of $CD8^+$-induced effects during atherogenesis is shown in Figure.3.4.

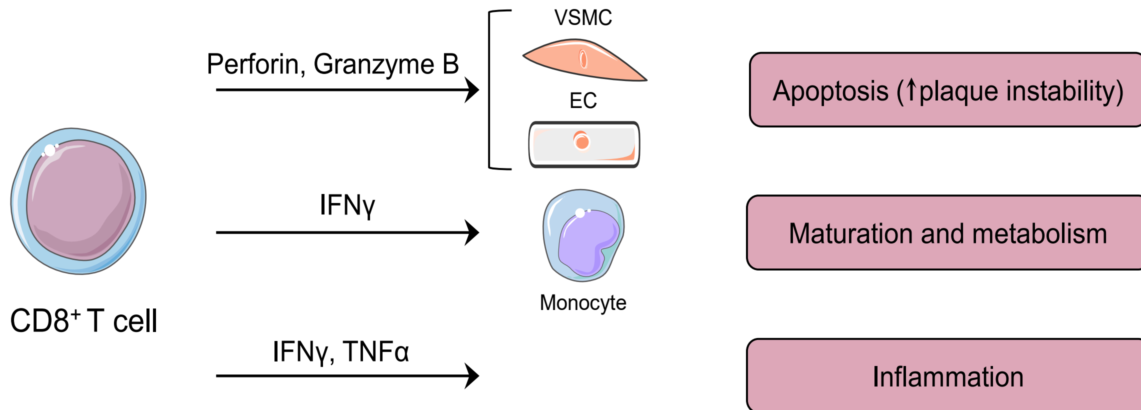

Figure 3.4: CD8$^+$ T cell effects during atherogenesis. Scheme made by using smart.servier.com, according to [252]). EC:endothelial cell, VSMC:vascular SMC.

CD8$^+$ Tregs are the subset of CD8$^+$ T cells that were shown to have immunosuppressive features. In atherosclerotic lesions of ApoE$^{-/-}$ mice, the presence of CD8$^+$ and CD25$^+$ T cells was noted, and their adaptive transfer decreased plaque size, reduced macrophage content, and inhibited CD4$^+$ T cells proliferation in comparison to CD8$^+$CD25$^-$ when there was no effect on the atherosclerotic burden [385]. Furthermore, CD8$^+$ T cell antibody-mediated depletion during advanced atherosclerosis resulted in less stable plaque formation with significantly reduced collagen, increased macrophage content and necrotic core in the aortic valve area compared to the controls [386] implying a protective role of CD8$^+$ subsets in the late stages of the disease.

3.1.2.3 B cells

Similarly to T cells, B cells are activated by antigens present in lesions, and they contribute to the chronic inflammation present in atherosclerosis [387]. These cells derive from BM progenitors, whereas their maturation takes place in the spleen. Each B cell possesses a unique BCR that recognizes a specific antigen leading to their transformation into antibody-producing plasma cells [198]. Among mature B cells, few subsets can be differentiated, including B1, FO, and MZ B cells, as well as Bregs and innate response activator (IRA) B cells [387]. B cells have been observed within healthy and atherosclerotic aortas surrounding the adventitia and are present within tertiary lymphoid structures of the aortic wall [388, 349]. Some B cell subsets are considered atheroprotective as they produce ‘natural’ antibodies that bind necrotic debris and oxidized epitopes of modified lipoproteins, thereby inhibiting inflammation. On the other hand, elevated levels of IgE immunoglobulins, which can stimulate pro-inflammatory responses in macrophages, are shown to be associated with atherosclerosis [198]. Prevalence of anti-oxLDL antibodies was observed in patients with CAD when compared with healthy individuals [389]. IgM and IgG anti-oxLDL antibodies have been detected during atherogenesis, indicating T cell involvement in antigen processing and immune response [390]. T cells reactive to heat shock protein 60/65 (HSP60/65) have also been isolated from atherosclerotic plaques [358, 391], while autoantibodies against HSP60/65 were shown to mediate cytotoxicity to ECs and induce fatty streak formation [392, 393].
Atherosclerosis is a silent but progressive disease. Its symptoms typically arise years after the onset of the disease, when the cap fails to withstand the pulsatile force from the blood

pressure that forms, near the edges of the plaque, superficial fissures [394]. Rupture-prone plaque is characterized by a thin fibrous cap, a large lipid-filled necrotic core, and ongoing inflammation [395, 396]. Even around 70% of coronary thrombosis events [397] are thought to be caused by plaque ruptures, while the remaining 30% are induced by endothelial erosion and possibly also by other forms of pathology that can trigger the clinical disease [202].

3.2 Cholesterol metabolism and hypercholesterolemia regulation by immune cells

Hypercholesterolemia is associated with the onset of atherosclerosis and the immune response that contribute to that onset and development is activated by ligands and antigens generated by hypercholesterolemia [342]. Cholesterol homeostasis in the organism is maintained mainly by de novo synthesis, intestinal absorption, and biliary and faecal excretion. Cholesterol is synthesized from acetyl-CoA through the enzymatic activity of many metabolic steps, and these enzymes are largely found in the membrane of the ER (Figure.3.5).
However, the source of cholesterol is also a diet. The absorption of dietary cholesterol takes place in the intestine where Niemann–Pick type C1-like 1 (NPC1L1) protein, expressed on the apical surface of enterocytes, takes up the cholesterol and enables its release as chylomicrons, which are taken up by the liver. The liver is the main site of cholesterol biosynthesis that provide both endogenously synthesized and exogenously acquired cholesterol to the bloodstream asVLDL. VLDLs are further metabolized to LDL, which can be taken up by peripheral cells via receptor-mediated endocytosis [398, 399]. The excess of cholesterol can be exported to lipid-free or lipid-poor apolipoprotein A-I (ApoA-I) produced by the liver, intestine and pancreas via passive or active mechanisms generating high-density lipoproteins (HDLs) [400]. Surplus cholesterol is esterified by acyl coenzyme A-cholesterol acyltransferase (ACAT) to cholesteryl esters [401] and can be stored as a cholesterol reservoir in cytosolic lipid droplets or released as a major constituent of plasma lipoproteins, such as chylomicrons, VLDLs, LDLs and HDLs. Finally, HDLs are transported from peripheral tissues back to the liver and intestine (where cholesterol is recycled or eliminated) or to the steroidogenic organs (where cholesterol is used to generate steroid hormones). These processes are tightly controlled by multiple transcriptional and post-translational regulatory mechanisms that function as an integrative system capable of responding to varying intracellular and physiological stimuli to ensure cholesterol homeostasis. The crucial players of the cholesterol biosynthetic pathway are: sterol regulatory element-binding protein 2 (SREBP2), which functions as a master transcriptional regulator of cholesterol biosynthesis, and two rate-limiting enzymes, namely, 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMGCR) and squalene monooxygenase. The expression of cholesterol biosynthetic enzymes is transcriptionally regulated by SREBP2. SREBP2 resides in ER as a part of a protein complex with two other proteins: SREBP-cleavage activating protein (SCAP) and insulin-induced genes (INSIGs) [402, 398]. The complex is sensitive to ER sterol levels and controls SREBP2 function. During high-sterol conditions, SREBP2 is held in the ER that effectively decreases cholesterol synthesis, whereas during decreased cholesterol levels, SREBP2 is cleaved, now named nuclear SREBP2 (nSREBP2), and moved to the nucleus where it transactivates the genes encoding enzymes involved in the cholesterol synthesis program, including HMGCR and SQLE (encoding squalene monooxygenase).

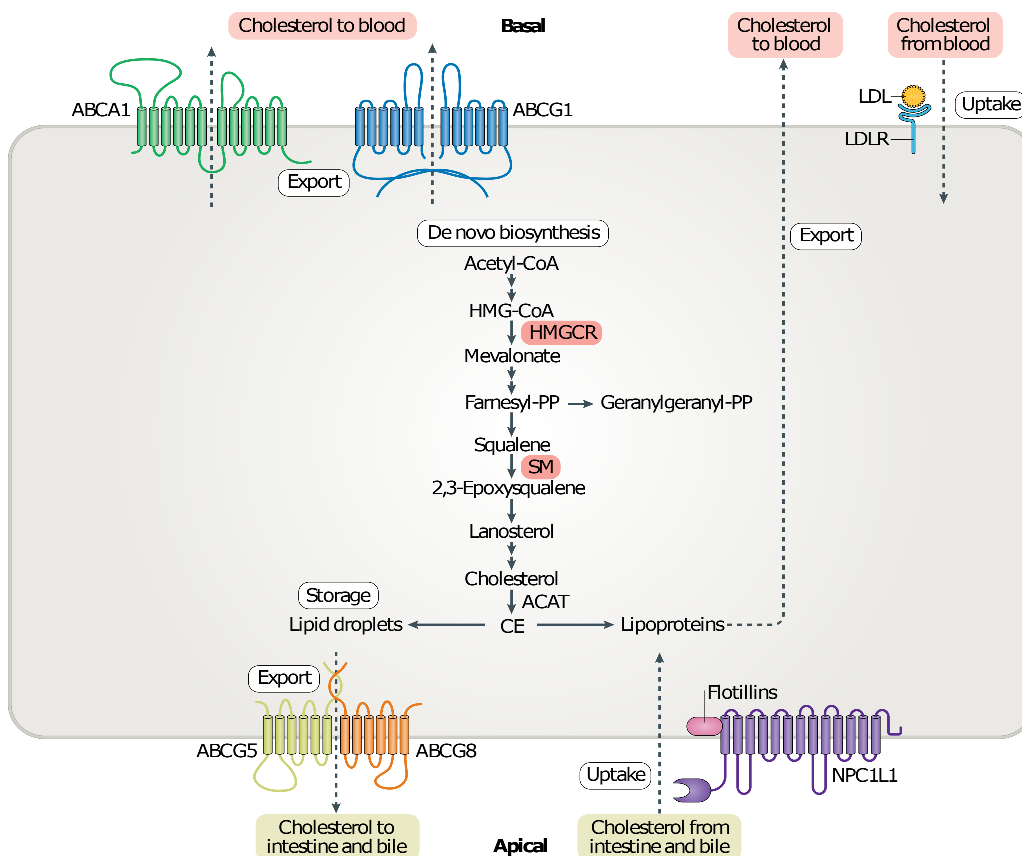

Figure 3.5: Major pathways of cholesterol metabolism in a polarized cell. Reprinted by permission from Springer Nature, Springer, Nature Reviews Molecular Cell Biology [398]. ABCA1:ABC subfamily A member 1, ABCG1/5/8:ABC subfamily G 1/5/8, NPC1L1:Niemann–Pick type C1-like 1, ACAT:acyl coenzyme A-cholesterol acyltransferase, CE:cholesterol ester, SM:squalene mono-oxygenase, HMGCR:3-hydroxy-3-methylglutaryl coenzyme A reductase, LDL:low-density lipoprotein, LDLR:LDL receptor.

HMGCR is an ER-residential protein that is as a rate-limiting enzyme highly regulated at transcriptional, translational and post-translational levels [403]. HMGCR gene is activated by nSREBP2 when sterol concentrations are low. Its degradation is induced mostly by oxysterols such as 25-hydroxycholesterol and 27-hydroxycholesterol, and methylated sterols such as lanosterol and 24,25-dihydrolanosterol [404, 405]. By contrast, cholesterol is a relatively weak signal for HMGCR degradation [404]. When lanosterol and oxysterols accumulate in the cell, INSIG1 is induced to bind the membrane domain of HMGCR [404, 405, 406], triggering proteasomal ER-associated degradation (ERAD). Interestingly, INSIG2 gene was recently proposed to be a direct target of hypoxia-inducible factor (HIF)-1α in the liver, which is a major transcription regulator of hypoxia [407]. Accordingly, upregulation of INSIG2 and accumulation of lanosterol and 24,25-dihydrolanosterol were observed in mice exposed to hypoxia, contributing to the reduced levels of hepatic HMGCR [407, 408]. Squalene monooxygenase is another rate-limiting enzyme in the cholesterol biosynthetic pathway [409] that also responds to sterol via SREBP2. Although HMGCR and SQLE are transcriptionally co-regulated by nSREBP2, different metabolic stimuli trigger their degradation, and it relies on different molecular machineries. Besides biosynthesis, diet-derived cholesterol and its subsequent uptake from the blood have key roles

in maintaining cholesterol homeostasis. As mentioned before, NPC1L1 is a key mediator of cholesterol absorption, responsible for cholesterol uptake in enterocytes via clathrin-mediated endocytosis. NPC1L1 is also activated by SREBP2 [410, 411]. Animals fed with a high-cholesterol diet have markedly reduced expression of NPC1L1 in the intestine [412, 413], suggesting a negative feedback loop between the cholesterol abundance and the pathway of its absorption. In addition to SREPB2, it was shown that Hepatocyte nuclear factor (HNF)4α mediates NPC1L1 activation in the liver and intestine in humans [414] while liver X receptor (LXR) activation or ablation of sortilin, sorting receptor closely implicated in CVD, reduce NPC1L1 expression [415, 416]. Circulating cholesterol, as a component of LDLs, is cleared by peripheral cells expressing LDLr. LDLr was shown to be a transcriptional target of SREBP2. Inducible degrader of the LDLr (IDOL) and proprotein convertase subtilisin/kexin type 9 (PCSK9) are considered important regulators that act in an independent but complementary manner and regulate LDLr stability. LDLr expression is also induced by thyroid hormones that were proposed to bind directly to its promoter [417]. In contrast to NPC1L1, in polarized cells, such as hepatocytes and enterocytes, LDLr is localized to the basolateral membranes. LDLr is very important in the regulation of cholesterol homeostasis, as disruption of its life cycle at any step may affect LDLr numbers or activities and, consequently, LDL clearance [398]. Although all mammalian cells can produce cholesterol, except for hepatocytes, adrenal cells and gonadal cells, a majority cannot catabolize it and therefore need to dispose of the excess out of the cell or store it as cholesteryl esters in lipid droplets. Four members of the ATP-binding cassette (ABC) transporter superfamily, including ABC subfamily A member 1 (ABCA1) and ABC subfamily G (ABCG) members 1, 5 and 8, are responsible for cholesterol efflux in a cell type-specific manner. ABCA1 was shown to have a particularly important role in macrophages where it promotes the removal of excess cholesterol (as a result of their prominent activity in scavenging lipoproteins from circulation), thereby preventing their transformation into foam cells and protecting against atherosclerosis [418, 419]. The primary acceptor for cholesterol efflux by ABCA1 is lipid-free ApoA-I [420], and this leads to the generation of nascent HDL particles. By the action of lecithin:cholesterol acyltransferase (LCAT), HDL matures and becomes competent for acquiring cholesterol from ABCG1 [421]. Consistent with its role, ABCA1 is upregulated by the activation of LXRs and retinoid X receptor (RXR) [422]. Accordingly, MeXis, an LXR-responsive long non-coding RNA, was shown to enhance the transcription of ABCA1 in mice [423]. Besides ABCA1, the other ABC transporter responsible for cholesterol efflux is ABCG1. This transporter was found to be abundantly expressed in macrophages and many other cell types except hepatocytes, where their expression was shown to be low, and it was absent from enterocytes [424]. Various acceptors of ABCG1 mediated cholesterol efflux are recognized, such as HDL, LDL, albumin, methyl-β-cyclodextrin and liposomes, but not lipid free ApoA-I unless ABCA1 is active [421, 424, 425, 426]. ABCA1 and ABCG1 were shown to be directly involved in the prevention of atherosclerosis, as combined deletion of ABCA1 and ABCG1 induced massive lipid accumulation in macrophage-rich tissues [427] and macrophage deficiency of ABCA1 and ABCG1 was sufficient to accelerate atherosclerosis in Ldlr$^{-/-}$ mice fed standard, chow diet standard diet (SD) [428]. Similarly to ABCA1, ABCG1 is also regulated by LXR and RXR [429]. Finally, ABCG5 and ABCG8 are transporters that mediate the excretion of neutral sterols, including plant sterols and cholesterol, into the bile and intestinal lumen, respectively and therefore, they are nearly exclusively expressed in the apical surface of hepatocytes and enterocytes [430]. When expressed alone, ABCG5 or ABCG8, serves only as a non-functional half-transporter as they must form the heterodimer to obtain sterol transport functionality [430]. As shown

in mice, hepatic ABCG5 and ABCG8 directly promote the efflux of liver sterol into the bile [431, 432, 433], whereas the intestinal counterparts are responsible for the disposal of plasma-derived cholesterol into the gut lumen [434, 435]. Several transcription factor have been recognized to be involved in the regulation of these transporters, such as the liver receptor homolog-1 (LRH1) [436], HNF4α [437], forkhead box protein O1 (FOXO1) [438], GATA-binding proteins [439, 437], LXR [440, 441, 442] and NF-κB [443]. Furthermore, agonists of FXR and bile acid are found to be able to induce ABCG5 and ABCG8 expression in human and mouse primary hepatocytes [444]. Bile acid were shown to act as signalling molecules that activate nuclear receptors and regulate bile acid and cholesterol metabolism [445].

Bile acids are synthesized in the liver and excreted into the bile, whereas in the ileum, they are reabsorbed and transported back to the liver via enterohepatic circulation, where they inhibit bile acid synthesis by suppressing the gene encoding the rate-limiting enzyme, cytochrome P450 family 7 subfamily A Member 1 (CYP7A1) [446]. Bile acid synthesis in the liver is accomplished through two distinct pathways [446] (Figure.3.6). The classical (or neutral) pathway is initiated by CYP7A1, the rate-limiting enzyme, and results in the formation of the primary bile acid, cholic acid (CA) and chenodeoxycholic acid (CDCA). CA and CDCA differential formation in the classical pathway is determined by cytochrome P450 family 8 subfamily B polypeptide 1 (CYP8B1), with CDCA being formed in the absence of CYP8B1 activity [447]. Cytochrome P450 family 27 subfamily A Member 1 (CYP27A1) initiates an alternative (or acidic) pathway by oxidation of the cholesterol side chain that is followed by cytochrome P450 family 7 subfamily B Member 1 (CYP7B1). Before undergoing biliary excretion, primary bile acid conjugated in the liver are metabolized by luminal bacteria to form hydrophobic and cytotoxic secondary bile acid, such as lithocholic acid (LCA) and deoxycholic acid (DCA) [448, 449]. The relative contribution of the acidic pathway to bile acid synthesis is not clear. As hydrophobic bile acids are toxic if accumulated in large quantities in hepatocytes, their synthesis and transport must be tightly regulated. The most important physiological mechanism for controlling the overall rate of bile acid biosynthesis is enterohepatic circulation [450]. An increase in bile acid synthesis and decreased levels of LDL cholesterol are noticed when an interruption in the enterohepatic circulation occurs [451, 447]. In humans, hypercholesterolemia, atherosclerosis, cholestasis, and cholelithiasis might be induced in the condition of increased cholesterol input and decreased output of bile acid [450, 452, 453]. Free cholesterol accumulation in the cell is additionally prevented by the formation of cholesteryl esters, mediated by ACATs, as it directs cholesterol for storage or secretion. Esterification is also an important part of maintaining the balance between free cholesterol and cholesteryl esters, and it is required for the absorption of cholesterol in the intestine. In mammals are described two ACAT isoenzymes, ACAT1 and ACAT2 [454]. ACAT1 is present throughout the body, most abundantly in macrophages, epithelial cells, and steroid hormone-producing cells [455, 456], whereas ACAT2 is predominately expressed in enterocytes and, to some extent, also in hepatocytes [456, 457, 458]. As a high level of ACAT1 was found in macrophages of human atherosclerotic plaque, ACAT1 was suggested to be involved in the pathology of atherosclerosis [459]. Inhibition of ACAT1 in $CD8^+$ T cells increases plasma membrane cholesterol levels and promotes T-cell receptor clustering and immunological synapse formation that eventually enhance the antitumor activity of these cells [460]. The human ACAT1 gene contains two promoters, P1 and P7, located in chromosomes 1 and 7, respectively [461]. It is thought that its expression is not regulated by SREBPs or LXRs, but rather by IFNγ, TNF [462], all-trans-retinoic acid [463] and the synthetic glucocorticoid dexamethasone [464]. To achieve homeosta-

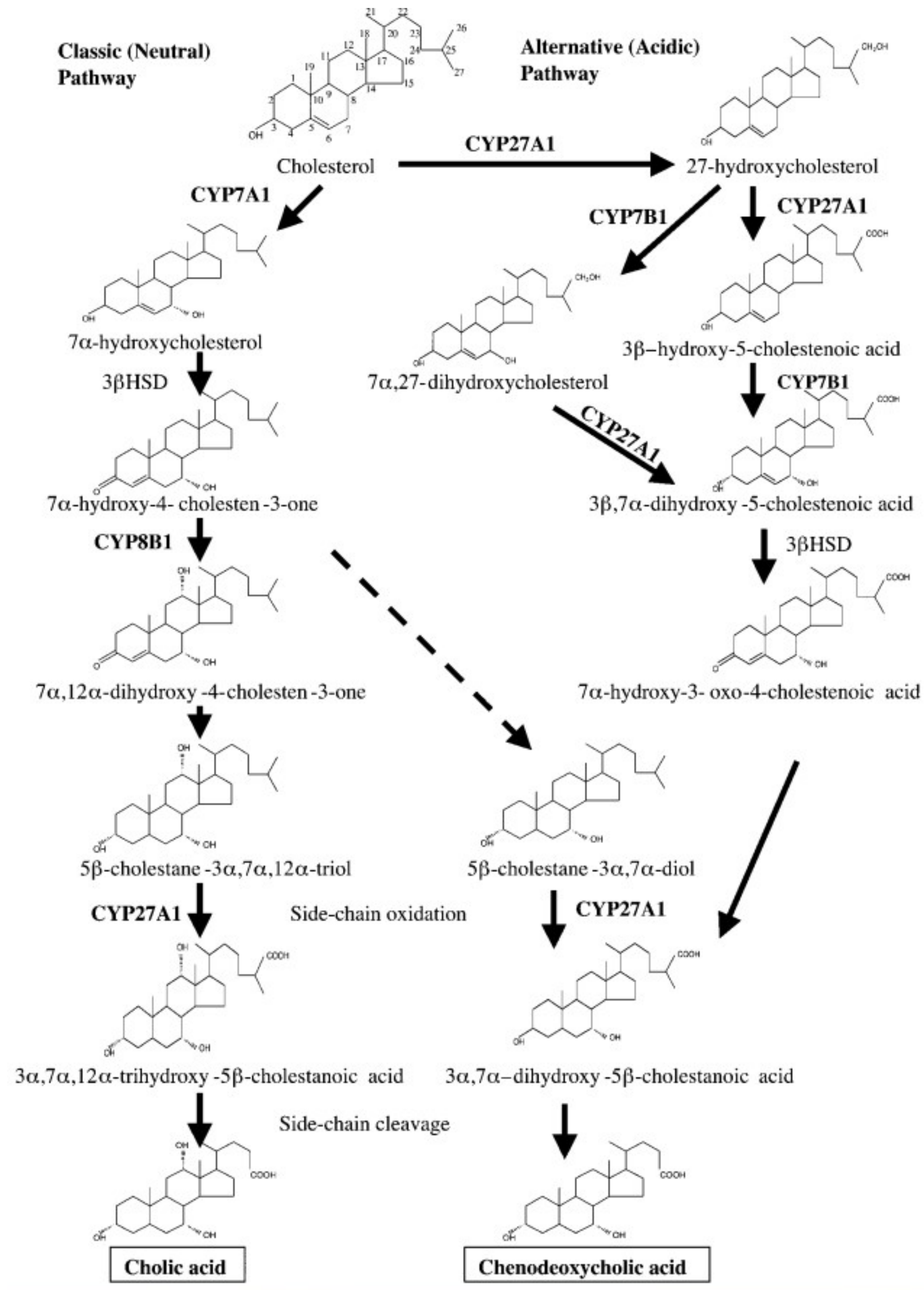

Figure 3.6: Bile acid biosynthetic pathways in the liver. Reprinted by permission from Oxford University Press, Endocrine Reviews [445]. CYP27A1:cytochrome P450 family 27 subfamily A Member 1, CYP7A1:cytochrome P450 family 7 subfamily A Member 1, CYP7B1:cytochrome P450 family 7 subfamily B Member 1, CYP8B1:cytochrome P450 family 8 subfamily B polypeptide 1, 3βHSD:3β-Hydroxysteroid dehydrogenase.

sis, the collaboration between various tissues that ensures a balance between cholesterol absorption (in the intestine) and cholesterol biosynthesis (mostly in the liver) with its release into the bloodstream and subsequent uptake (and removal if necessary) by cells in the body is necessary so that sufficient cholesterol is produced for cell growth and function, but, at the same time, excess cholesterol accumulation is avoided. Thus, many various components, such as SREBP complex, INSIGs, HMGCR, ACATs, LXRs, and the recently identified nuclear factor erythroid 2-related factor 1 (NRF1) [465], are able to directly sense sterol fluctuations and trigger adaptive responses.

Chapter 4

The aim of the thesis

Taking into consideration the high incidence of CV morbidity in post-allo-HCT survivors [7], as well as the shown incidence of ECs damage during the course of cGvHD [14], the main aim of this thesis is to elucidate the impact of GvHD, as the syndrome that affects a significant amount of patients (approximately 40% of related to even 60% of URD transplants [466]), on the development and progression of atherosclerosis, as the onset disease of severe CV events. $CD8^+$ T cells play a significant role in the development of atherosclerotic lesions and stability of formed plaque [346, 347, 328], and their infiltration was noticed around the microvessels of the skin in patients facing skin cGvHD[14]. Thus, we were interested specifically in the role of $CD8^+$ in the context of the impact of GvHD on the development and severity of atherosclerosis. The preliminary data obtained during the establishment of the GvHD-atherosclerosis model suggested a prominent impact of WD feeding on the severity and, therefore, survival of mice facing aGvHD when the diet was given concomitantly with pre-conditioning regimen and HCT. This striking phenotype drew our attention, and led to separating this project into two projects that will be discussed in two separated chapters. The methods are common for both projects; thus, they will be explained together.
Chapter 7 and 9 describe the impact of subclinical GvHD, shown in miHAg allo-HCT mouse model, on atherosclerosis and the impact of $CD8^+$ T cells as one of emerged T cell subtype responsible for the phenotype.
Chapter 8 and 10 focus on the impact of short-term WD feeding on the severity of aGvHD.

Part II

Materials and Methods

This part presents detailed information on the materials and methods that were used in both of the projects. All experiments (apart from the ones stated in the figures) are done by the author of this thesis.

Chapter 5

Methods

5.1 Mouse models

C57BL/6 (B6.WT)(H-2b) and FVB (H-2q) mice were purchased from Janvier Laboratories (St. Berthevin Cedex, France). B6.LdLr$^{-/-}$(H-2b), FVB.L2G85 (H-2q, CD45.1, CD90.1), BALB/b (H-2b) were bred and housed under specific pathogen-free controlled conditions and 12h-light/dark cycle at the Zentrum für Experimentelle Molekulare Medizin, Würzburg. Mice had access to food and water ad libitum. Male and female animals of the same age (8 to 12 weeks) were equally employed in experiments. All experiments were approved by the Regional Ethics Committee for Animal Research (Regierung von Unterfranken, Würzburg, Germany, Akt.-Z 2-559, 2-705 and 2-1292).

5.1.1 Transplantation

5.1.1.1 Preconditioning Regimen

B6.WT (H-2b) and B6.LdLr$^{-/-}$(H-2b) as recipient mice were treated with 9 gray (Gy) for 14,4 min as one dosage of myeloablative TBI using a Faxitron CP-160 X ray irradiation system. The treatment was done in Zentrum für Experimentelle Molekulare Medizin, Würzburg.

5.1.1.2 Donor BM Cells Isolation

BM cells were isolated from the hind legs (femura and tibiae) of 8- to 12-week-old sex-matched donor mice (see Table.5.1). Cell numbers were determined by trypan blue exclusion, and the cell concentration was adjusted to 50 x 10^7 cells/mL. BM cell yields was dependant on the donor mouse strain, with BALB/b 5–7 x 10^7, FVB 1-1.5 x 10^8 and B6.WT 8-9 x 10^7 cells per mouse.

5.1.1.3 Donor T Cell Enrichment

T cells were enriched from the spleen of 8- to 12-week-old sex-matched donor mice by using Dynabeads™ Untouched™ Mouse T Cells Kit (Thermo Fisher Scientific). After erythrolysis (see subsubsection 5.3.1.3, splenocyte suspensions were enriched for T cells according to the manufacturer's protocol, counted by trypan blue exclusion, and adjusted to the appropriate cell number in PBS (see Table.5.1). Typical T cell yields depended on the mouse strain of the donor and lay between 15 and 30% of the splenocyte input with a final T cell purity of 85–95%.
When both cell preparation processes are done, BM cell suspension is diluted with PBS or T cell suspension in a 1:1 ratio depending on the transplanted group. Both the T cells and the BM cell suspensions were stored at 4°C for up to 2 h until transplantation.

Table 5.1: GvHD models.

Model	Recipient	Donor	BM cells concentration	T cells concentration
Minor mismatch	B6.LdLr$^{-/-}$(H-2Kb)	Balb.B (H-2Kb)	5 x 10^6	5 x 10^6
Major mismatch	C57BL/6 (B6)(H-2Kb)	FVB(H-2Kq, CD45.1 or CD45.2)	5 x 10^6	6 x 10^5
Major mismatch	C57BL/6 (B6)(H-2Kb)	FVB(H-2Kq, CD45.1 or CD45.2)	5 x 10^6	5 x 10^4

5.1.1.4 Allo-HCT

At a maximum of 4h after myeloablative irradiation, mice are subjected to transplantation. Recipient mice should ideally have more than 20 g body weight before the preconditioning regimen and transplantation in order to be sufficiently resilient to partial body weight loss. Before the transplantation, mice were anesthetized by ip. injection of anesthetic composed of Xylazin (16 mg/kg) and Ketamine (80 mg/kg) dissolved in PBS. Cell suspensions are injected intravenously via the retro-orbital venous plexus in a total volume of 200μL/mouse. In the first week after the transplantation, the drinking water was supplied with Baytril (Enrofloxacin, 0.05%) for seven days after transplantation to avoid infections. The occurrence of GvHD clinical symptoms (changes in posture, activity, fur, skin and weight loss) was monitored daily, from the day of transplantation until the end of the experiment, and scored 0-2 per symptom. The score was assessed by summing up the individual score numbers for each parameter. Animals were sacrificed when exceeding a total score of 6.

5.1.2 Induction of atherosclerosis

In order to induce atherosclerosis, mice were put on the fat-enriched diet, WD (21% milk fat, 0,15% cholesterin, from Altromin) 10 days after transplantation and fed for eight weeks. The schematic overview of the GvHD-atherosclerosis model is shown in the Figure.5.1.

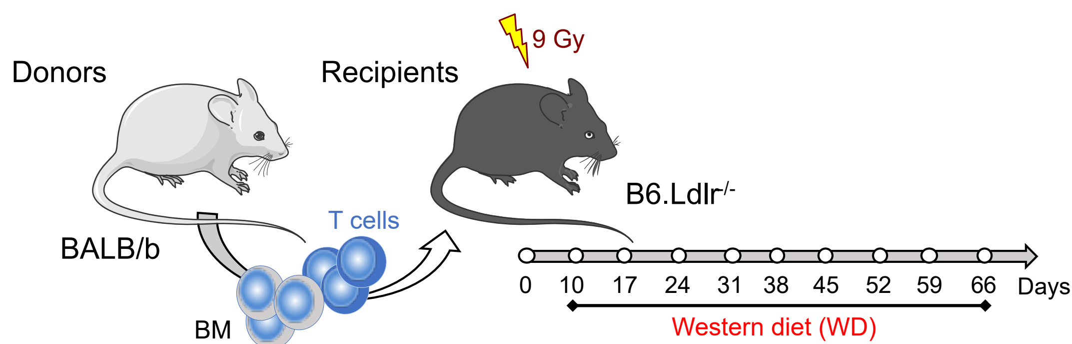

Figure 5.1: GvHD-atherosclerosis experimental design. Scheme made by using smart.servier.com. BM:bone marrow.

5.1.3 Western Diet feeding

In order to elucidate the impact of HFD feeding on the onset of aGvHD, mice were fed with fat-enriched food, WD (21% milk fat, 0,15% cholesterin). The feeding started one day before the transplantation and lasted up to day 60 post-HCT, depending on the experiment.

5.1.4 Anti – CD8β treatment

Rat anti-mouse CD8β antibody (referred to as anti-CD8β) or isotype-matched irrelevant rat-anti-Phyt1 (referred to as isotype) were injected peritoneally once per week starting on day ten post-HCT in the amount of 250 µg.

5.1.5 Euthanization of mice

Isoflurane anaesthetized mice were euthanised by cervical dislocation. When the aorta and heart were isolated, mice were initially flushed with PBS with followed perfusion with 4% PFA in PBS, if the organs were prepared for histological analysis. After the perfusion, organs were collected, weighed if necessary, and processed to histological or flow cytometry protocols, as described in the Section. 5.3.

5.1.6 Serum collection and analysis

Blood was collected in serum tubes and kept on ice. After the collection of all samples, serum tubes were brought to room temperature during 30 minutes and then centrifuged at 10.000 rpm for 5 minutes to separate serum from coagulated blood. The serum was aliquoted into 1.5 ml tubes and kept at -80°C until use.

5.1.6.1 Serum cholesterol, triglycerides, and transaminases measurements

Total cholesterol (Amplex Red Cholesterol Assay Kit, Invitrogen) and triglycerides (Enzy-Chromtm Triglyceride Assay Kit, Bioassay Systems) were analysed in serum according to the manufacturer's protocol. The measurements of fluorescence and absorbance, the end-point in cholesterol and triglycerides, respectively, were done on the Infinite M200 PRO microplate reader (Tecan Life Sciences). Transaminases (AST, ALT), LDH, urea and creatinine were analysed on cobas c701 (Roche) in the laboratory for clinical diagnostics, Laboklin (Bad Kissingen).

5.2 Histology

5.2.1 Aorta and Aortic root

To study lesion development, the heart and whole aorta were post-fixed with 4% PFA in PBS. After overnight incubation, PFA was replaced with fresh PBS. The aorta was cut longitudinally, opened and cleaned of adventitia. Thereafter, aortas were stained for the lipid content according to the Oil-red-O staining protocol given below (Section.5.2.1.1). PFA fixed hearts were embedded in Tissue Tek, frozen, and cut into 5μm transverse sections. The section collection started when all three valves were clearly visible. Three sections covering the region of the valves (cut from the different parts of the aortic root) were collected on a slide and left at 4°C until use. Aortic root sections were used for the

assessment of plaque size and plaque phenotype according to the protocols shown below (Sections:5.2.1.2, 5.2.1.3).

5.2.1.1 Oil-red-O staining

After washing the aortas in PBS for 5 min, they were dipped ten times in 60% 2-propanol. Sections were then stained in freshly prepared Oil-red-O working solution for 15 min and dipped in 60% 2-propanol afterwards. After it, sections were finally washed for 5 min in clean PBS and mounted with Kaiser´s glycerin jelly. Images were recorded using Leica DM 4000B fluorescence microscope and JVC KY-F75U camera. Lesion size was assessed in three separate sections of the aorta (abdominal, thoracic and arch) by marking red-stained areas using the Image J analysis software. The results are expressed as a percentage of the total size of each aortic section and the sum of all sections, shown as the total lesion formation.

5.2.1.2 Aldehyde-Fuchsin and Sirius Red staining

Aortic root sections were assessed for atherosclerotic plaque size after staining of elastic fibers with Gabe's Aldehyde-Fuchsin solution. Briefly, sections were stained in Aldehyde-Fuchsin working solution for 15 min and then dipped in 70% ethanol. The differentiation was stopped by washing with distilled water for 5 min. Sections were, afterwards, stained for collagen with Picrosirius Red solution for 90min and placed in 0.01N HCl for 1 min. In the next step, sections were dehydrated by dipping them from distilled water to xylene in the following order: 1) distilled water (short dip) 2) 70% ethanol (3x/1min) 3) 96% ethanol (3x/1min) 4) 100% ethanol (3x/1min) 5) Xylene (3x/5min) Finally, sections were covered with VectaMount and sealed with nail polish. Lesion size was quantified as the average of all three sections per mouse. Images were recorded using Leica DM 4000B fluorescence microscope and JVC KY-F75U camera, while quantification was performed using the Image J analysis software. Picrosirius Red staining was quantified with polarized light by Leica DM 4000B fluorescence microscope and JVC KY-F75U camera and quantified by Image J software. Briefly, images were turned into 8-bit type, the area of plaque was labelled, and collagen inside the plaque was measured with the threshold function and expressed as a percentage of plaque area.

5.2.1.3 Macrophage and SMC staining

PFA fixed aortic root sections were assessed for plaque phenotype by staining of macrophage and SMC content. Sections were firstly subjected to citric, heat treatment antigen retrieval protocol. Briefly, slides were placed in a slide holder containing 150 ml of working solution and heated for 15 minutes in a water bath. Thereafter, samples were taken out and left to cool down to room temperature for 30 min, followed by a final two-step wash with PBS for 5 min. Afterwards, sections were dried and blocked in the wet incubation chamber with Mac Staining Buffer for 30 min. After the incubation period, sections were covered with the primary antibody mix (anti-mouse Mac-2 and anti-mouse α-actin-Cy3, Table.6.2) diluted in Macs buffer and left at 4°C overnight. The next day, slides were washed 2X in clean PBS for 10 minutes, and the secondary detection antibody was applied and incubated for 1 hour at room temperature. Slides were then washed 2X in clean PBS for 10 minutes and dried before adding one drop of DAPI containing Vectashield and coverslips. Borders were sealed with nail polish and slides were stored in the dark at 4°C until analysis. Images of slides containing three sections per mouse were recorded with

Leica DM 4000B fluorescence microscope and JVC KY-F75U camera. Quantification of the macrophage and SMC content was calculated as a percentage of the total lesion per section using the Image J analysis software.

5.2.1.4 Hematoxylin and Eosin (H&E) staining

The necrotic core was quantified in H&E stained PFA fixed aortic root sections. Briefly, sections were washed in PBS for 5 min and stained in Hematoxylin for 6 min. Thereafter, sections were left to differentiate in running tap water for 5 min and stained with Eosine for 6 min. After the new wash step with distilled water, sections were subjected to dehydration protocol, as explained before (Section. 5.2.1.2). Slides were finally covered with VectaMount and coverslips. Images of slides containing three sections per mouse were recorded with Leica DM 4000B fluorescence microscope and JVC KY-F75U camera, and results are calculated as a percentage of the total lesion per section using the Image J analysis software.

5.2.2 Liver, colon, small intestine and skin

Tissues were fixed in 4% PFA in PBS overnight. Afterwards, tissues were embedding in paraffin and sectioned at 5 μm, slides mounted and stained with (H&E). Histopathological scoring of GvHD was done by professionals at the Institute of Pathology (University Hospital, Würzburg/Erlangen).

5.3 Flow cytometry

5.3.1 Preparation of single-cell suspension

5.3.1.1 Aorta and Aortic root

Fat was carefully removed throughout the aorta and separated from the aortic root during the tissue extraction. Both tissues were separately placed in 1.5 mL tubes containing 250 μL of RPMI1640 and kept on ice. When the extraction of all samples was done, both tissues were minced, and 250μL of digestion solution (450 U/mL collagenase I, 125U/mL collagenase XI and 60U/mL hyaluronidase) was added to each tube (total volume of 500μL). Samples were incubated for 1 hour at 37°C, 1200 rpm. After the incubation, tissues were filtered through 70μm cell strainer, and cells were centrifuged at 400 Gs for 5 minutes at 4°C. The cell pellet was resuspended in 200-250 μL of PBS/1%FCS containing 2mM EDTA and transferred to a round 96-well plate for FACS staining.

5.3.1.2 Small intestine

Small intestinal tissue in the size of 8 cm from the caecum was excised for analysis. Fat and mesenteric tissue, as well as Peyer's patches, were removed and the intestine was washed with PBS. After removal of all fecal material, the tissue was cut into 1,5 cm pieces and transferred to a 50 mL falcon tube filled with 30 mL of Ca^{2+}/Mg^{2+}-free HBSS medium containing 5% FCS and 2mM EDTA. When all samples were collected, tubes were incubated for 20 min at 37°C with a rotation of 250rpm. After the incubation, the supernatants were recycled through a metal mesh, and the intestine pieces were transferred to a new 50mL conical tube containing 30mL of pre-warmed Ca^{2+}/Mg^{2+}-free HBSS medium (2 mM EDTA, 5% FCS). The samples were again incubated for 20 min at 37°C

with a rotation of 250rpm. After the second incubation round, the content of each 50 mL conical tube was poured through the 100μm cell strainer, and 1.5 cm pieces of intestine were transferred to a small plastic weigh boat. The intestine pieces were rapidly minced by scissors and transferred to gentleMACS tubes filled with 10mL of digestion solution (1.5 mg/mL Type VIII Collagenase dissolved in pre-warmed HBSS (Ca^{2+}/Mg^{2+}) with 40 μg/mL of DNase I). Tubes were placed horizontally into an orbital shaker and digested at 200 rpm for 20 min at 37 °C. Thereafter, the samples were filtered through 100μm strainer into a new 50mL falcon and centrifuged at 1500 rpm for 5 min at 4°C. The supernatant was discarded and the pellet was resuspended in 30mL ice-cold Ca^{2+}/Mg^{2+}-free HBSS medium containing 10%FCS followed by additional filtration through a new 100μm strainer. The samples were centrifuged again at 1500 rpm for 5 min at 4°C, and the pellet was resuspended in 5mL of ice-cold Ca^{2+}/Mg^{2+}-free HBSS containing 10% FCS. Cells were counted and kept at 4°C until further analysis.

5.3.1.3 Spleen

Spleen was disrupted, washed with PBS 1%FCS and passed through a 70 μm cell strainer. The cell suspension was centrifuged at 400g for 5 minutes at 4°C. The supernatant was recycled, and the pellet was resuspended in 3 mL of Ery-Lysis-Buffer and incubated at RT for 7 minutes to allow proper lysis of erythrocytes. After the incubation period, 7 mL of cold PBS 1%FCS was added to stop the reaction, and samples were centrifuged at 400g for 5 minutes at 4°C. The cell pellet was resuspended in 10 mL of PBS 1%FCS and filtered again in 70 μm cell strainer to remove possible cell clumps. Cells were counted and kept at 4°C until further analysis.

5.3.1.4 Peripheral blood

Blood was collected in EDTA-coated tubes (mixed gently to prevent clotting) and kept on ice until all samples were collected. 150 μL EDTA blood was transferred to 1.5 mL eppendorf and mixed with 850 μL of Ery-Lysis-Buffer. Samples were left at 4°C for 10 minutes to allow erythrolysis. After the incubation period, samples were centrifuged at 400 Gs for 10 minutes at 4°C, when the clear white pellet could be observed. The supernatant was carefully removed by aspiration, and cells were resuspended in ice-cold PBS 1%FCS and kept at 4°C until further analysis. 20μL of EDTA blood was separately taken, diluted 1:3 with PBS and ran on Sysmex to obtain the cell counts.

5.3.1.5 Bone marrow

BM cells were isolated from hind legs (femur and tibia). Briefly, one side of the bone was cut and placed downwards in a 0.5mL eppendorf tube with a hole on the bottom of the tube made with a 12G needle. The 0.5mL tube was then placed in a 1.5 mL eppendorf tube and centrifuged at 10000g for 15 seconds. BM cells were then collected in the 1.5 mL eppendorf tubes, and right after, resuspended in 5mL PBS/1% FCS. Cells were counted and kept at 4°C until further analysis.

5.3.1.6 Lymph nodes

Inguinal and mesenteric LNs were excised and processed separately. All samples were disrupted, washed with PBS 1%FCS and passed through a 70 μm cell strainer. The cell suspension was centrifuged at 400 Gs for 5 minutes at 4°C. The supernatant was recycled,

and the pellet was resuspended in 300-500μL and proceeded to cell counting and FACS staining.

In the case of the aGvHD model, because of the low yield of cells, the digestion protocol of LNs was applied. Briefly, cervical, axillary, mediastinal and inguinal LNs were excised and pooled together. Mesenteric LNs were processed separately. The samples were kept on ice in 1.5 mL tubes filled with 0.5 mL of RPMI. When all samples were collected, LNs were rapidly minced and transferred to 15 mL falcon filled with 2 mL of digestion mix (Collagenase A (0.2 mg/mL), Collagenase B (0.2 mg/mL), and DNAse 1(0.1 mg/mL)). Samples were incubated at 37°C in a water bath for 20 min with gentle inversion every 5 min to ensure proper mixing of the content. After the incubation period, the liquid phase was aspirated and transferred through PBS/1% FCS pre-wetted 70μm cell strainer to 50 mL falcon without disturbing precipitated tissue fragments. The digestion process was repeated two more times with the addition of 2 mL of digestion mix and incubation for 10 min with proper mixing of the content every 5 min. Finally, the whole content was passed through the cell strainer to the 50 mL falcon, where all previous aspirates were transferred. The tissue was disrupted with a syringe plunger through the cell strainer, and the content was centrifuged for 5 min at 1200rpm and 4°C. Cells present in the pellet were resuspended in 300-500μL of PBS/1% FCS. Cells were counted and kept at 4°C until further analysis.

5.3.2 Flow cytometry protocol

5.3.2.1 Staining of surface antigens

Single-cell suspensions were prepared differently depending on the organ in use, as described above. Cells were transferred to round 96-well plates and centrifuged at 400 Gs for 5 minutes at 4°C, and the supernatant was removed by inverting the plate. Cells were then resuspended in 50 μL of PBS 1%FCS containing an Fc Block and incubated for 20 min at 4°C. After washing with 150 μL of PBS 1%FCS, cells were again resuspended in 50 μL of FACS buffer containing the appropriate antibody mixture and incubated for 30 min at 4°C. Cells were washed once more and resuspended in 200 μL of PBS 1%FCS for flow cytometry analysis. Antibodies used in the experiments are shown in the Table.6.2.1

5.3.2.2 Staining of intracellular cytokines or transcription factors

For the study of transcription factors such as a FoxP3, the FoxP3/Transcription Factor Staining Buffer kit was used, whereas to study intracellular cytokines such as IFNγ, TNFα and IL-17A, the Fixation/Permeabilization Solution Kit was selected. When intracellular cytokines were measured, 1 x 10^6 cells were transferred to round 96-well plates and resuspended in 200 µL of RPMI containing the cytokine stimulation cocktail (Subsection.6.1.3) to induce the production and accumulation of cytokines. Cells were incubated for 4 hours at 37°C and 5% CO_2. Thereafter, cells were washed once with PBS 1%FCS and stained for surface antigens, as explained in the subsection.5.3.2.1. Afterwards, cells were fixed and permeabilized using 100 µL of the corresponding buffer for 20 min at 4°C. Cells were washed twice using the washing buffer provided for each kit separately, resuspended in 50 µL of washing buffer containing the antibodies against intracellular antigens, and incubated for 30 min at 4°C. After a final wash, cells were finally resuspended in 200 μL of PBS 1%FCS and left at 4°C until the measurement.

5.4 Quantitative Real-Time Polymerase Chain Reaction (RT-qPCR)

Part of the liver and spleen were excised, cleaned, snap-frozen in liquid nitrogen, and kept in -80°C until the processing for RNA isolation. RNA isolation was done with trizol (Thermo Fisher) according to the manufacturer's protocol. RNA concentration was measured with Nanodrop 2000C, normalized to 1000ng, and subjected to reverse transcription (cDNA synthesis) in Thermocyclers. cDNA was diluted depending on the expected gene expression in the organ, and qPCR analysis were done on QuantStudio 6 Flex Thermal Cycler. Primers that were used during experiments are shown in the Table.6.8. Values were normalized by usage of HPRT (spleen) or Rplp0 (liver) as housekeeping genes.

Bacterial DNA was analysed from the ileum and mesenteric LNs. Mesenteric LNs and small intestine (1 cm of tissue measured from the caecum and regarded as ileum) were excised and cleaned from fat and fecal content in the case of ileum. Bacterial and genomic DNA was isolated using the QIAamp Pathogen Mini Kit according to the manufacturer's instructions, using pre-treatment T2 for enzymatic digestion of tissue and pre-treatment B1 for difficult-to-lyse bacteria. The quantitative polymerase chain reaction was performed using the Thermocycler, peqSTAR SYBR Green Master Mix. Universal 16S primers (926F and 1062R) were used for the standardization of bacteria per tissue, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) primers were used to determine levels of mouse genomic DNA, which was used as a reference. Primer sequences are shown in the Table.6.8.

5.5 Intestinal permeability assay – FITC-Dextran(4kDa)

Total intestinal permeability was measured indirectly by measuring the difference in fluorescence in plasma pre- and post-fluorescently-labelled small molecule, FITC-Dextran treatment. Briefly, after fasting for 4h mice are given 150 μL of 80 mg/mL FITC-Dextran through oral gavage. 4h hours post-treatment, the difference in fluorescence pre- and post-gavage is measured in plasma. As a post-gavage fluorescence control, we used a non-irradiated, healthy mouse with the same treatment, and as a pre-treatment, a negative control mouse without treatment was used. Plasma samples were diluted according to the manufacturer's protocol, and fluorescence was measured on Infinite M200 PRO microplate reader.

5.6 Bioluminiscence ex vivo imaging and analysis

Mice were injected intraperitoneally with 300 mg/kg D-luciferin (Biosynth, Staad, Switzerland). Ten minutes later, animals were killed and selected tissues were prepared and imaged in the next 3-5 minutes. Images were taken with an IVIS Spectrum imaging system (Perkin-Elmer/Caliper Life Sciences, Mainz, Germany). Imaging data were analyzed and quantified with Living Image Software (Xenogen).

5.7 Aortic CD45$^+$ cell isolation for scRNA-seq

Previously anaesthetized mice received an intravenous injection of 2.5μg of anti-CD45.2 APC to label all circulating leukocytes. Aortic cells were enzymatically isolated as previously described in the Section.5.3. Briefly, cells were incubated with TruStain FcX to remove unspecific staining for 5 min on ice and then labelled with anti- CD45.2-Alexa488, Fixable Viability Dye e780 and anti-mouse TotalSeqA-Hashtag Antibodies (1:250) as follows: BMT WD (Hashtag 1-4), BM WD (Hashtag 5 to 8). Cells from both conditions were then washed twice with PBS/1% FCS and pooled. Viable CD45.2-APC(i.v.) negative CD45.2-Alexa488 positive cells were sorted using a FACS Aria III (BD Biosciences) with a 100µm nozzle. Cells were, afterwards, labelled post-sort with a panel of TotalSeq-A CITE-seq antibodies against surface markers. CITE-seq mix list is provided in separated tables (6.10). Cells were then washed twice in PBS supplemented with 0.04% ultrapure BSA,counted and loaded in the 10x Genomics Chromium with the aim to recover 10,000 cells. Libraries were generated with the Chromium Single Cell 3' Reagents Kit v3.

5.7.1 scRNA-seq data analysis

5.7.1.1 Pre-processing

By using Cell Ranger software (version 3.0.1) transcriptome data, HTO and ADT libraries were demultiplexed. Mouse GRCm38 reference genome was used for the alignment and counting steps. The –feature-ref flag of Cell Ranger software was used to generate counts of gene expression matrix and cell surface protein expression. The obtained gene-barcode matrix was further analysed using Seurat v3 package from R (www.satijalab.org , version 4.1.1).

5.7.1.2 Demultiplexing

To identify the origin of samples, multiplet exclusion and cells with undetectable hashtag signal, demultiplexing was performed in Seurat v3. Hashtag demultiplexing was processed according to software developers' instructions. Analysis of cell surface epitopes with CITE-seq was performed using the standard Seurat workflow.

5.7.1.3 Clustering analysis

By using the standard Seurat workflow, clustering analysis was performed based on RNA levels. Briefly, low quality cells were removed (with more than 5% mitochondrial transcripts), data were normalized and "vst" method was used to identify 2000 variable features. Furthermore, the "ScaleData" unction was applied, Principal Component Analysis (PCA) was performed, and significant principal component (PC) was discriminated using the JackStraw method. 20 PCs were used to perform clustering analysis and Uniform Manifold Approximation and Projection (UMAP) dimensional reduction. Immune cell populations were identified by combining transcript and CITE-seq surface marker expression of canonical markers.

5.8 Statistical analysis

The data are expressed as mean ± SD. The comparisons were made using the unpaired Student's t-test in Prism software (GraphPad Prism 9). Log-rank tests were used to anal-

yse differences in animal survival (Kaplan-Meier survival curves). Statistically significant differences were considered when $p<0.05$ was reached.

Chapter 6

Materials

6.1 Buffers, solutions and media

6.1.1 Aldehyde-Fuchsin solution

Stock solution

2.5 g Basic-Fuchsin in 500 mL of 70% ethanol (high grade).

Working solution

Mix 50 mL stock solution + 2.5 mL acid aldehyde + 1 mL concentrated HCl and leave overnight. Filter prior to use.

6.1.2 Antigen retrieval solution

Stock solution

- Solution A: 21.01 g citric acid in 1L distillated water
- Solution B: 29.41 g tri-sodium citrate dihydrate in 1L distillated water

Working solution

1.8 ml solution A + 8.2 ml solution B + 90 mL distilled water + 50 µg Tween-20

6.1.3 Cytokine stimulation cocktail

In order to measure intracellular cytokines, cells were stimulated with 50 ng/mL PMA, 750 ng/mL ionomycin and 2,5 μg/mL BFA in DC Medium for 4 hours.

DC Stimulation Medium

- RPMI 1640 with 2 mM L-glutamine
- 0.5% heat-inactivated FCS
- 100 U/mL penicillin/streptomycin
- 50 µM 2-Mercaptoethanol

6.1.4 Erythrocyte Lysis Buffer (Ery-lysis-buffer)

- 150 mM NH_4Cl
- 10 mM $KHCO_3$
- 0,1 M Na_2EDTA
- 50 µM 2-Mercaptoethanol

6.1.5 FACS Buffer

PBS supplemented with 2% mouse serum, 2% rabbit serum, 2% BSA.

6.1.6 Heat-inactivation of FCS

Bottles of FCS were incubated at 56 C for 30 minutes, inverting them every 10 minutes.

6.1.7 Kaiser´s glycerin jelly

Stock solution

4 g gelatin + 21 mL distilled water + 25 mL glycerol

Working solution

3 parts stock solution + 7 parts distilled water

6.1.8 Mac staining buffer

Following compounds were mixed in 1X PBS:

- 2% mouse serum
- 2% rabbit serum
- 2% horse serum
- 1% BSA
- 0.1% Triton X100

6.1.9 Oil-Red-O Solution

Stock solution

1 g Oil-Red-O in 200 mL 99% 2-Propanol (isopropanol)

Working solution

160 mL Stock solution mixed with 120 mL milli-Q water (1 hour at room temperature). Solution was filtered prior use.

6.1.10 Picrosirius red solution

0.1% Sirius Red/Direct Red 80 in saturated aqueous picric acid (pH 2.0).

6.2 Antibodies

6.2.1 Primary antibodies used in flow cytometry

Table 6.1 shows the antibodies used in flow cytometry experiments.

Table 6.1: Anti-mouse antibodies used in flow cytometry experiments.

Name	Fluorophore	Dilution	Clone	Ref Number	Company
CD11b	V500	300	M1/70	562127	BD Bioscience
CD11b	Brilliant Violet 605	300	M1/70	101257	Biolegend
CD11b	PerCP/Cyanine5.5	300	M1/70	45-0112-82	Thermo Fisher Scientific
CD11c	APC/Cyanine7	300	HL3	561241	BD Bioscience
CD11c	Alexa Fluor 647	400	N418	117312	Biolegend
CD11c	Brilliant Violet 650	300	N418	117339	Biolegend
CD115	PE	300	AFS98	12-1152-83	Thermo Fisher Scientific
CD25	PerCP/Cyanine5.5	300	PC61	102030	Biolegend
CD25	APC	300	PC61:5	17-0251-82	Thermo Fisher Scientific
CD25	Alexa Fluor 488	400	PC61.5	53-0251-82	Thermo Fisher Scientific
CD274 (B7-H1, PD-L1)	APC	400	10F.9G2	124312	Biolegend
CD274 (B7-H1, PD-L1)	Brilliant Violet 421	200	10F.9G2	124315	Biolegend
CD3	PE	300	145-2C11	12-0031-82	Thermo Fisher Scientific
CD3	APC-eFluor 780	300	145-2C11	47-0031-80	Thermo Fisher Scientific
CD4	eFluor506	300	RM4-5	69-0042-82	Thermo Fisher Scientific
CD4	FITC	300	GK1.5	11-0041-82	Thermo Fisher Scientific
CD4	PerCP/Cyanine5.5	400	RM4-5	100540	Biolegend
CD44	PerCP/Cyanine5.5	300	IM7	45-0441-82	Thermo Fisher Scientific
CD44	PE	300	IM7	12-0441-83	Thermo Fisher Scientific
CD44	Brilliant Violet 421	300	IM7	103040	Biolegend
CD45	APC	300	30-F11	2093725	Thermo Fisher Scientific
CD45	Alexa Fluor 700	400	30-F11	103128	Biolegend

CD45	APC/Cyanine7	300	30-F11	557659	BD Bioscience
CD45	PerCP/Cyanine5.5	300	30-F11	45-451-80	Thermo Fisher Scientific
CD45	PE/Cyanine7	300	30-F11	25-0451-81	Thermo Fisher Scientific
CD45	FITC	300	30-F11	11-0451-82	Thermo Fisher Scientific
CD45.2	APC/Cyanine7	400	104	109824	Biolegend
CD45.2	PE	400	104	109808	Biolegend
CD45.2	eFluor 450	400	104	48-0454-82	Thermo Fisher Scientific
CD45.2	eFluor506	300	104	69-0454-82	Thermo Fisher Scientific
CD45.2	APC	200	104	109814	Biolegend
CD62L	FITC	300	MEL-14	553150	BD Bioscience
CD62L	PE/Cyanine7	300	MEL-14	2055158	Thermo Fisher Scientific
CD62L	Brilliant Violet 605	300	MEL-14	104438	Biolegend
CD8a	Brilliant Violet 785	300	53-6.7	100749	Biolegend
CD8a	PE/Cyanine7	300	53-6.7	100722	Biolegend
CD80	PE/Cyanine7	300	16-10A1	104734	Biolegend
CD90.1 (Thy 1.1)	APC-eFluor 780	400	HIS51	47-0900-82	Thermo Fisher Scientific
CD90.1 (Thy 1.1)	APC	400	HIS51	17-0900-82	Thermo Fisher Scientific
F4/80	PE	300	BM8	12-4801-82	Thermo Fisher Scientific
F4/80	PE/Cyanine7	50	BM8	123114	Biolegend
F4/80	Alexa Fluor 488	300	BM8	123120	Biolegend
FoxP3	PE	300	FJK-16s	12-5773-80	Thermo Fisher Scientific
FoxP3	Alexa Fluor 488	300	FJK-16s	53-5773-82	Thermo Fisher Scientific
IFN$^{\gamma}$	Alexa Fluor 488	400	XMG1.2	53-7311-82	Thermo Fisher Scientific
Integrin a4ß7 (LPAM-1)	APC	400	DATK32	120608	Biolegend
Integrin a4ß7 (LPAM-1)	PE	400	DATK32	120606	Biolegend
Ly6C	PE/Cyanine7	500	HK1.4	25-5932-80	Thermo Fisher Scientific
Ly6C	APC	300	HK1.4	17-5932-80	Thermo Fisher Scientific
Ly6C	Brilliant Violet 510	400	HK1.4	128033	Biolegend
Ly6G	Alexa Fluor 488	400	1A8	127626	Biolegend
Ly6G	Alexa Fluor 700	300	1A8	127622	Biolegend

MHC II	V500	300	MP/114:15:2	562366	BD Bioscience
MHC II	Brilliant Violet 785	400	M5/114:15:2	107645	Biolegend
MHCII (I-Ab)	PerCP/Cyanine5.5	300	AF6-120.1	116415	Biolegend
PD-1	Brilliant Violet 421	200	29F:1A12	135217	Biolegend
Siglec F	PE	100	1RNM44N	12-1702-82	Thermo Fisher Scientific
TCRb	PerCP/Cyanine5.5	300	H57-597	45-5961-82	Thermo Fisher Scientific
TCRb	Brilliant Violet 421	300	H57-597	562839	BD Bioscience
TNFa	PE	1600	MP6-XT22	506306	Biolegend
TruStain FcX		200	93	101320	Biolegend

6.2.2 Antibodies used for other purposes

Table 6.2 shows the antibodies used for depletion experiments as wells as for histology stainings.

Table 6.2: Antibodies not used for flow cytometry.

Name	Clone	Nature	Company
Anti-mouse α-Actin Cy3	1A4	Primary	Sigma Aldrich
Goat anti-rat IgG – Alexa Fluor 488		Secondary	Thermo Fisher Scientific
Rat anti-mouse Mac-2	M3/38	Primary	Cedarlane
Rat anti-mouse– CD8β antibody	YTS156.7.7		Bioceros
Rat-anti-Phyt1	AFRC-MAC51		Bioceros

6.3 Instruments

Table 6.3 presents a list of instruments used in all experiments.

Table 6.3: List of used instruments.

Name	**Company**
FACSCelesta^{TM}	BD Biosciences
FACSAria^{TM} III	BD Biosciences
QuantStudio 6 Flex Thermal Cycler	Applied Biosystems
Leica CM3050 S Research Cryostat	Leica Biosystems
Leica DM 4000B Fluorescence Microscope	Leica Biosystems
Nanodrop 2000C	Thermo Fisher Scientific
Thermocycler, peqSTAR	VWR
Infinite M200 PRO microplate reader	Tecan Life Sciences
10X Genomics Chromium	10x Genomics
IVIS Spectrum imaging system	Perkin-Elmer/Caliper Life Sciences
Faxitron CP-160 X ray irradiation system	Faxitron Bioptics

6.4 Software

Table 6.4 lists the software used for analysis.

Table 6.4: List of used software.

Name	**Company**
FlowJo (V10.4)	Tree Star / BD Biosciences
GraphPad Prism (V9)	GraphPad
Image J	Open source
i-control™ Microplate Reader Software	Tecan Life Sciences
R (Seurat v3 package)	Satija Lab
Cell Ranger software (version 3.0.1)	10x Genomics
Living Image Software	Xenogen

6.5 Kits

Table 6.5 shows the different kits used in all experiments.

Table 6.5: List of used kits.

Name	**Fluorophore**	**Dilution**	**Company**
eBioscience™ Fixable Viability Dye	eFluor™ 780	1000	Invitrogen
LIVE/DEAD™ Fixable Violet Dead Cell Stain Kit	405 nm	1000	Invitrogen
eBioscience™ Annexin V Apoptosis Detection Kits	eFluor 450	2.5uL/sample	Invitrogen

eBioscience™ Fixation/Permeabilization Concentrate			Invitrogen
eBioscience™ Fixation/Permeabilization Diluent			Invitrogen
Fixation and Permeabilization Solution			BD Bioscience
Perm/Wash Buffer			BD Bioscience
eBiosciencesTM FoxP3/Transcription Factor Staining Buffer Set			Thermo Fisher Scientific
AmplexTM Red Cholesterol Assay Kit			Thermo Fisher Scientific
EnzyChromTM Triglyceride Assay Kit			Bioassay Systems
First strand cDNA Synthesis Kit			Thermo Fisher Scientific
QIAamp Pathogen Mini Kit			Qiagen

6.6 Reagents and consumables

Tables 6.6 and 6.7 give an overview of reagents and consumables used in all experiments, respectively.

Table 6.6: Chemical, Reagents and Enzymes.

Chemical	**Company**
100% Ethanol	Carl-Roth
70% Ethanol	Carl-Roth
2-Propanol (Isopropanol)	Sigma-Aldrich
Acid aldehyde	Sigma-Aldrich
Ammonium Chloride (NH4Cl)	Sigma-Aldrich
Basic-Fuchsin	Sigma-Aldrich
Citric acid (C6H8O7)	Carl Roth
Paraformaldehyde (PFA)	Carl Roth
Hydrogen chloride (concentrated) (HCl)	Sigma-Aldrich
Oil-Red-O	Sigma-Aldrich
Potassium bicarbonate (KHCO3)	Sigma-Aldrich
Sirius Red (Direct Red 80)	Sigma-Aldrich
Tween 20	Carl Roth
Trisodium citrate	Carl Roth
Xylen	Carl Roth
2-Mercaptoethanol (50 mM)	Thermo Fisher Scientific
Brefeldin A (BFA)	Sigma-Aldrich
Bovine serum albumin	Sigma-Aldrich
Fetal calf serum (FCS), low in endotoxin	Sigma Aldrich
Ionomycin	Sigma-Aldrich
Penicillin-Streptomycin (10,000 U/ml)	Thermo Fisher Scientific
Phorbol 12-myristate 13-acetate (PMA)	Sigma-Aldrich
RPMI-1640 (with 2 mM L-Glutamine) (RPMI)	Thermo Fisher Scientific
Maxima SYBR Green/ROX qPCR Master Mix (2x)	Thermo Fisher Scientific

Collagenase I (C1030)	Sigma Aldrich
Collagenase XI (C7657)	Sigma Aldrich
Collagenase A (COLLA-RO)	Sigma Aldrich
Collagenase D (COLLD-RO)	Sigma Aldrich
DNAse 1 (D5025)	Sigma Aldrich
Wester Diet (21% fat, 0.15% Cholesterin)	Altromin
Hyaluronidase type I-S (H3506)	Sigma Aldrich
Tissue-Tek O.C.T Compound	Sakura
Vectashield with DAPI	Vector laboratories
VectaMount® Permanent Mounting Medium	Vector laboratories
Gelatin (from bovine skin – type B)	Sigma-Aldrich
Glycerol	Applichem
Trizol	Thermo Fisher Scientific
Power up Sybr Green	Thermo Fisher Scientific
Fluoresceinisothiocyanat-Dextran	Sigma
D-luciferin	Biosynth
HBSS (w/o $Ca^{2+}Mg^{2+}$)	Thermo Fisher Scientific
HBSS ($Ca^{2+}Mg^{2+}$)	Thermo Fisher Scientific
ultrapure BSA	Thermo Fisher Scientific

Table 6.7: List of used consumables.

Material	**Company**
384well Multiply PCR plate	Sarstedt
5 ml Polystyrene Round-Bottom Tube (FACS Tubes)	Corning
FalconR 70µm Cell Strainer	Corning
Micro-test plate 96 well (round and bottom)	Sarstedt
Microtube 1.3 ml K3E (EDTA-coated tubes)	Sarstedt
Microtube 1.1 ml Z-Gel (Serum tubes)	Sarstedt
Syringe (BD PlastipakTM)	BD Biosciences
1,5mL Eppendorf Safe-Lock Tubes	Eppendorf
0.5mL Eppendorf Safe-Lock Tubes	Eppendorf

6.7 Primers

Table 6.8 presents the primers used in all qPCR experiments.

Table 6.8: List of used primers.

Gene name	**Forward primer**	**Reverse primer**
Rplp0	CCTATAAAGGCACACGCGG	CACGCGGGGTTTAAAGACG
HMGCR	CTT GTG GAA TGC CTT GTG ATT G	AGC CGA AGC AGC ACA TGA T
Abca1	AGTGATAATCAAAGTCAAAGGGACAC	AGCAACTTGGCACTAGTAACTCTG
Abcg5	CTGCTGAGGCGAGTAACAAGAA	GACGCATAATCACTGCCTGCT

Abcg8	ACTTCAGGATGCTTCGCAGG	TGCTCAAACCAAGGCACCTG
Srebf2	GACCTAGACCTCGCCAAAGGT	AGCACGGATAAGCAGGTTTGTAG
Cyp7a1	AACGGGTTGATTCCATACCTGG	GTGGACATATTTCCCCATCAGTT
Cyp27a1	TTTTGGCTGGGGTGGACA	GGGCACCACACCAGTCACTT
Lxra	GTCAACTGGGGTTGCTTTAGG	GACGAAGCTGTGTGGGCTC
Acat1	CAGGAAGTAAGATGCCTGGAA	TTCACCCCCTTGGATGACATT
IFNγ	GCTGTTTCTGGCTGTTACTGC	TCACCATCCTTTTGCCAGTTCC
IL-6	GTGGCTAAGGACCAAGACCA	ACCACAGTGAGGAATGTCCA
mIL-10	ATTTGAATTCCCTGGGTGAGAAG	CACAGGGGAGAAATCGATGACA
GAPDH	AGTATGACTCCACTCACGGC	ATGTTAGTGGGGTCTCGCTC
16S	AACTCAAATGAATTGACGG	TCACAACACGAGCTGAC
HPRT	TCCTCCTCAGACCGCTTTT	CCTGGTTCATCATCGCTAATC

6.8 Hashtag antibodies

Table 6.9 presents the hashtag antibodies used in scRNA sequencing.

Table 6.9: List of used hashtag antibodies.

TotalSeq Antibody	Catalog Number	Company
Hashtag 1	155801	Biolegend
Hashtag 2	155803	Biolegend
Hashtag 3	155805	Biolegend
Hashtag 4	155807	Biolegend
Hashtag 5	155809	Biolegend
Hashtag 6	155811	Biolegend
Hashtag 7	155813	Biolegend
Hashtag 8	155815	Biolegend

6.9 CITE-seq antibodies

Table 6.10 presents the CITE-seq antibodies used in scRNA sequencing.

Table 6.10: List of used CITE-seq antibodies.

CITE-seq Antibody	Catalog Number	Company
Ly6G	127655	Biolegend
CD11b	101265	Biolegend
CD62L	104451	Biolegend
IAIE	107653	Biolegend
ICAM1	116127	Biolegend
Ly6C	128047	Biolegend
CXCR4	146520	Biolegend
MSR1	154703	Biolegend
CD64	139325	Biolegend

MAR1	134333	Biolegend
CCR3	144523	Biolegend
CD80	104745	Biolegend
CD117	105843	Biolegend
Sca1	108147	Biolegend
CD11c	117355	Biolegend
TIMD4	130011	Biolegend
CX3CR1	149041	Biolegend
XCR1	148227	Biolegend
F4/80	123153	Biolegend
CD86	105047	Biolegend
CD135	135316	Biolegend
CD103	121437	Biolegend
CD169	142425	Biolegend
CD8a	100773	Biolegend
SiglecH	129615	Biolegend
CD19	115559	Biolegend
CD3	100251	Biolegend
CD63	143915	Biolegend
CD9	124819	Biolegend
CD163	155303	Biolegend
NK1.1	108755	Biolegend
CD279	109123	Biolegend
CD127	135045	Biolegend
CD68	137031	Biolegend
Sirpa	144033	Biolegend
CD274	153604	Biolegend
ITGB7	321227	Biolegend
CD4	100569	Biolegend
TCRgd	118137	Biolegend
MGL2	146817	Biolegend
CD26	137811	Biolegend

Part III

Results

This part is separated into two separate chapters composed of two different projects, presenting and discussing obtained data separately

Chapter 7

Eludicating the interaction between GvHD and atherosclerosis after allo-HCT

Chapter 7 is planned to be submitted as original research article (Ivana Jorgacevic, Haroon Shaikh, Maja Bundalo, Sarah Schäfer, Maike Büttner-Herold, Clement Cochain, Antoine-Emmanuel Saliba, Melanie Rösch, Giuseppe Rizzo, Estibaliz Arellano Viera, Juan Gamboa Vargas, Friederike Berberich-Siebelt, Louis Boon, Andreas Rosenwald, Andreas Beilhack and Alma Zernecke: "Allogeneic hematopoietic cell transplantation promotes atherosclerosis in mice and is driven by donor CD8$^+$ T cells". The article is based on the work of the author of this thesis, Ivana Jorgacevic, who planned and performed the experiments and data analysis. Manuscript is submitted.

7.1 Subclinical GvHD aggravate atherosclerosis

To address the possible interconnection between GvHD and atherosclerosis, we established a MHC-matched, miHAg-mismatched allo-HCT mouse model, BALB/b (H-2b) $\rightarrow$B6.LdLr$^{-/-}$ (H-2b) that enabled monitoring of the onset of GvHD, but also the lesion formation that is formed during the prolonged period of time. GvHD was induced in atherosclerosis-prone mice (B6.LdLr$^{-/-}$) by myeloablative TBI and allogeneic transplantation of either BM or BM and CD3^{+} enriched, T cells derived from sex-matched MHC compatible donor (BALB/b). We started atherosclerosis induction ten days after transplantation by feeding the recipient mice with WD and monitored them for 66 days post-allo-HCT (Figure.7.1.A). Interestingly, even though the clinical GvHD score as well as histopathological scoring of GvHD target organs indicate subclinical GvHD in both groups fed WD, BM, and BMT (Figure.7.1.B,G), we could clearly see a significant increase in lesion formation in the aorta (in total: 3.45 ± 0.19 versus 2.20 ± 0.17) and aortic root (7.27 ± 1.27 versus 4.10 ± 0.67) (Figure.7.1.E-F) in BMT transplant. Noteworthy, the cholesterol level in the serum of BMT transplanted mice and fed with WD was significantly increased in comparison to BM transplant with the same diet treatment (9.28 ± 0.83 versus 6.83 ± 0.76) without the change in triglycerides levels (Figure.7.1.C-D). Control groups (BM and BMT) that were fed SD did not obtain increased lesion formation as an impact of T cell transplant (without the presence of high cholesterol levels that are induced by WD). Therefore, we did not further analyse these two groups (Figure.7.2.A-B). These data point out the immunological interplay and impact of GvHD on the severity of atherosclerosis and emphasize GvHD as a risk factor for CVD.

7.2 GvHD does not have an impact on the number of circulating monocytes and plaque phenotype

During atherosclerosis, blood monocytes are recruited into intima and subintima of blood vessels [467, 468, 469] where, in the presence of high cholesterol levels, they take up oxLDL and other lipids [470] resulting in the accumulation of lipid-laden monocytes/macrophages in the forming lesions [467]. Uncontrolled lipid accumulation is followed by the death of macrophages, deposition of collagen, and migration of SMCs into intima that, finally, increases the plaque size [471]. In B6.LdLr$^{-/-}$ mice, as recipients in our miHAg allo-HCT mouse model, we did not observe significant differences in the number of circulating or BM residing monocytes (Figure.7.3.A-B). On the other hand, the plaque phenotype in regard to infiltration of macrophages, SMC and collagen, as well as the size of the necrotic core in a formed plaque, was also not impacted by alloreactivity (Figure.7.3.C-F).

7.3 GvHD impact on T cell/ Tregs infiltration in atherosclerotic model

The main drivers of GvHD pathology are activated alloreactive, donor T cells. On the other hand, T cells are abundant in the shoulder region, the fibrous cap, adventitial tissue and the intima of human atherosclerotic plaque, where they play a significant role in the development and progression of atherosclerosis. Thus, increased amount of circulating alloreactive T cells that follow GvHD in BMT transplant, could induce higher infiltration of these cells through irradiation and alloreactivity damaged endothelial layer of blood

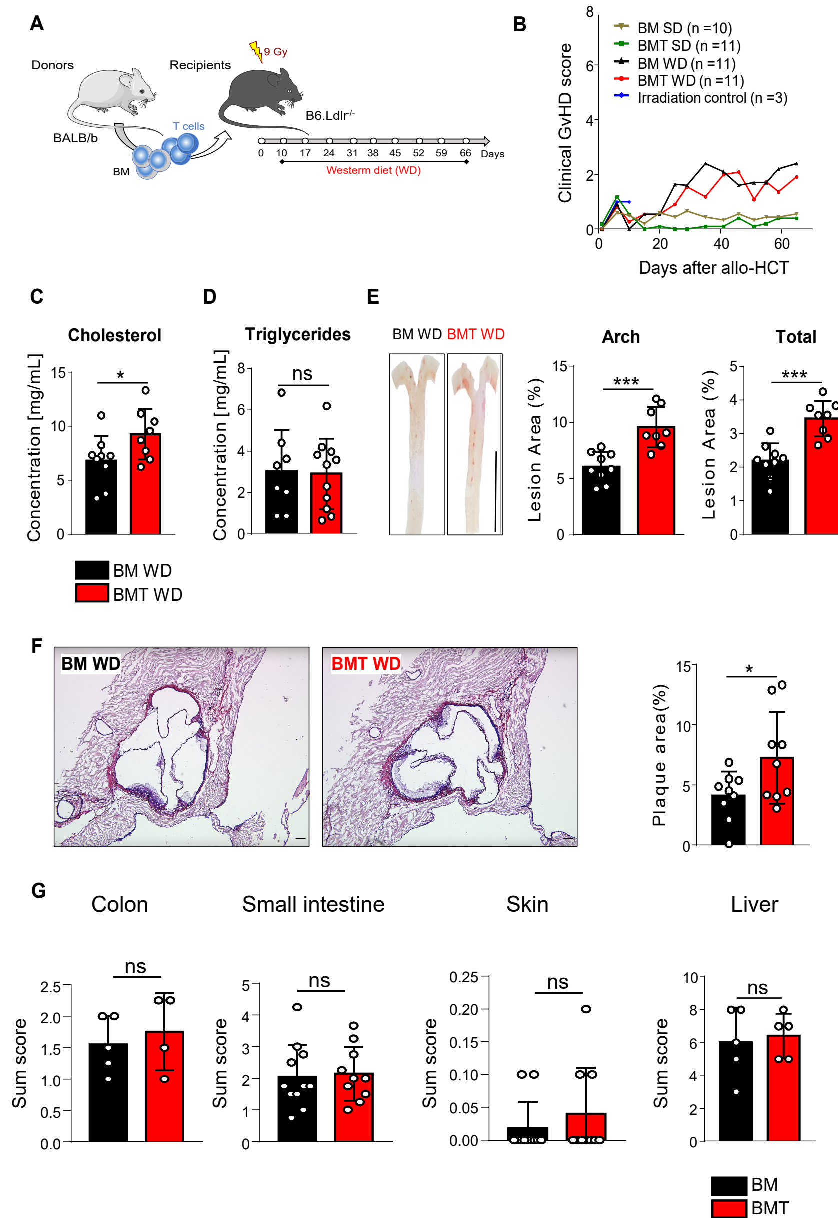

Figure 7.1: Subclinical GvHD aggravates atherosclerosis. (A-F) GvHD was induced in B6.LdLr$^{-/-}$ (H-2b) by TBI (9 Gy) and allogeneic transplantation of 5x10^{6} BM cells and 5x10^{6} of purified T cells from sex-matched donor BALB/b (H-2b). Recipient mice B6.LdLr$^{-/-}$ were fed with SD or WD for eight weeks, starting on day 10 post-HCT. Combined data from two independent experiments are presented, n=8-11. (A) Experimental design of miHag allo-HCT WD-induced atherosclerosis model. B) Clinical GvHD score. (C-D) Quantification of cholesterol and triglycerides serum levels. (E) Quantification of Oil-Red-O-stained aortas. Enface images of the aorta, scale bars 500 μm. F) Representative images and quantification of plaque area in aldehyde-fuchsin stained aortic root sections, scale bar 100μm. G) Histopathological scoring of the colon, small intestine, skin and liver sections. Statistical significance was determined by an unpaired t-test. Data are presented as a mean ± SD. *p<0.05, ***p<0.001, ns, non-significant. BM: bone marrow, BMT: bone marrow plus T cells, SD: standard diet, WD: Western diet. *Histopathological GvHD scoring was done by experienced pathohistologists.*

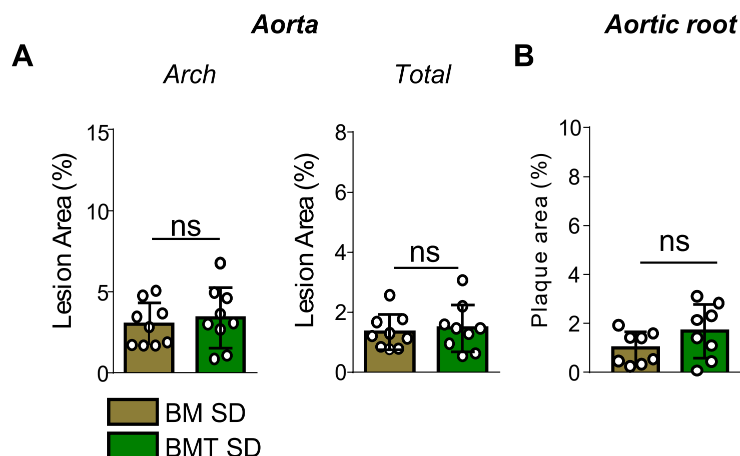

Figure 7.2: Low plaque formation in mice fed SD. (A-B) MiHAg-mismatched allo-HCT induced atherosclerosis model. Combined data from two independent experiments are shown, n=8-11. (A-B) Quantification of Oil-Red-O-stained aortas (A) and aldehyde-fuchsin stained aortic root sections (B) in mice fed with SD. Statistical significance was determined by an unpaired t-test. Data are presented as a mean ± SD, ns, non-significant. BM: bone marrow, BMT: bone marrow plus T cell, SD: standard diet.

vessels [13, 149, 472, 151] and stimulate the progression of atherosclerosis in our model. Relative number of circulating T cells in BMT was significantly increased in comparison to BM transplant (23.06 ± 1.40% versus 15.94 ± 1.50%) with significant increase in $CD4^+$ (17.11 ± 1.58% versus 12.21 ± 1.32%) and $CD8^+$ cells (3.18 ± 0.32% versus 1.91 ± 0.32%), presented in total leukocytes (Figure.7.4.A). During the course of GvHD, T cells were shown to have fluctuations in their abundance in different organs starting from the day of the transplantation (day 0) to day 100 post-HCT [473]. Mesenteric LNs are one of the main priming sites during the onset of GvHD, from where activated alloreactive T cells migrate to the intestine as the first target organ of GvHD. On day 66 post-HCT, in our miHAg allo-HCT atherosclerosis mouse model, ∼60% of mesenteric LN infiltrating leukocytes were T ($CD3^+$) cells, of which ∼10% were $CD8^+$ and ∼50% were $CD4^+$. However, there was no difference in regard to the presence of GvHD, BM WD vs. BMT WD (Figure.7.5.C). Of note, among aorta infiltrating leukocytes, we noticed increase in T cell infiltration after BMT transplantation in comparison to BM transplant (38.48 ± 3.87% versus 22.57 ± 2.45%, respectively), which was also noticed in peripheral (in our case inguinal) LNs (66.37 ± 1.01% versus 57.78 ± 0.90%) (Figure.7.4.B-C). In both organs, infiltration of $CD4^+$ and $CD8^+$ cells was similarly increased, while there was no significant difference in the CD4/CD8 infiltration of the spleen and aortic root (Figure.7.5.A-B). Noteworthy, aorta infiltrating $CD8^+$ T cells showed higher expression of cell-surface glycoprotein CD44, suggesting an effector phenotype of these cells, while there was no difference among $CD4^+$ T cells (Figure.7.4.D). Lastly, even though the amount of $CD4^+$ T cells was not changed in the presence of GvHD in mesenteric LN or was even increased in inguinal LN, flow cytometry analysis revealed a significant decrease in the relative number of Treg in both lymphoid organs, suggesting systemic immune dysregulation (Figure.7.5.D-E).

To better characterize the phenotype of lesion infiltrating cells, we employed scRNA sequencing (scRNA seq) of $CD45^+$ cells in aorta (Figure.7.6.A). The most prominent changes in gene expression were present in the $CD8^+$ cluster of T cells. These cells showed increased expression of genes regulating cytotoxicity, such as NFATc1, Prf1, Gzmk, and

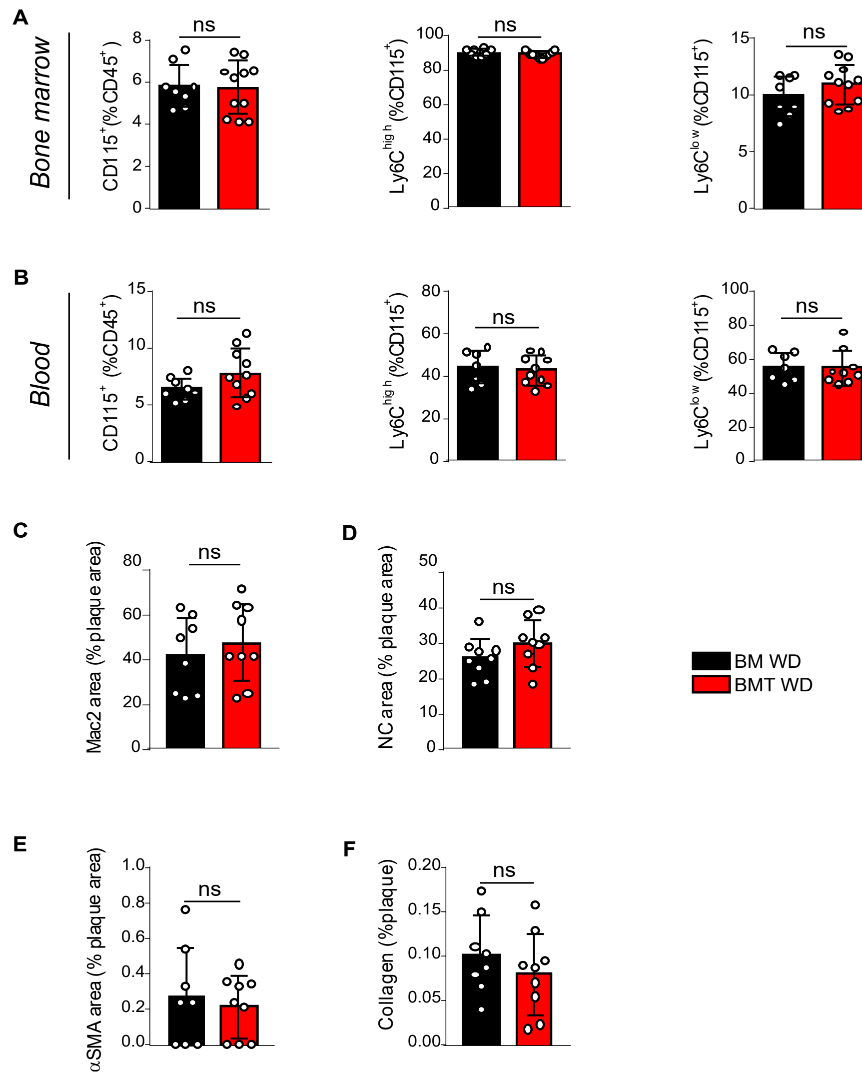

Figure 7.3: Subclinical GvHD does not impact the number of circulating monocytes and the plaque phenotype. (A-D) MiHAg-mismatched allo-HCT induced atherosclerosis model. Combined data from two independent experiments are shown, n=8-11. (A-B) BM and blood cell suspensions were analysed by flow cytometry. The relative number of monocytes, Ly6C^{high} and Ly6C^{low} in BM (A) and blood (B) are shown. (C-F) Quantification of the area positive for Mac-2 (C), necrotic core (D), α-SMC actin (E), and collagen (F) relative to the total plaque area is shown. Statistical significance was determined by an unpaired t-test. Data are presented as a mean $\pm$ SD, ns, non-significant. BM: bone marrow, BMT: bone marrow plus T cell, WD: Western diet, NC:necrotic core

higher expression of genes regulating their activation and differentiation towards CTLs, such as Tnfrsf9, Traf1, and CD27 (Figure.7.6.B-C). High expression of activation and CTL-favoring genes was followed by increased expression of exhaustion markers such as Tox and Pdcd1 (Figure.7.6.D), which was already seen in lesional effector memory T cells of symptomatic CV patients [263]. These data suggest the significant role of alloreactive T cells, and especially CD8$^+$ T cells, in dysregulation of the immune response post-HCT, which is followed by increased lesion formation.

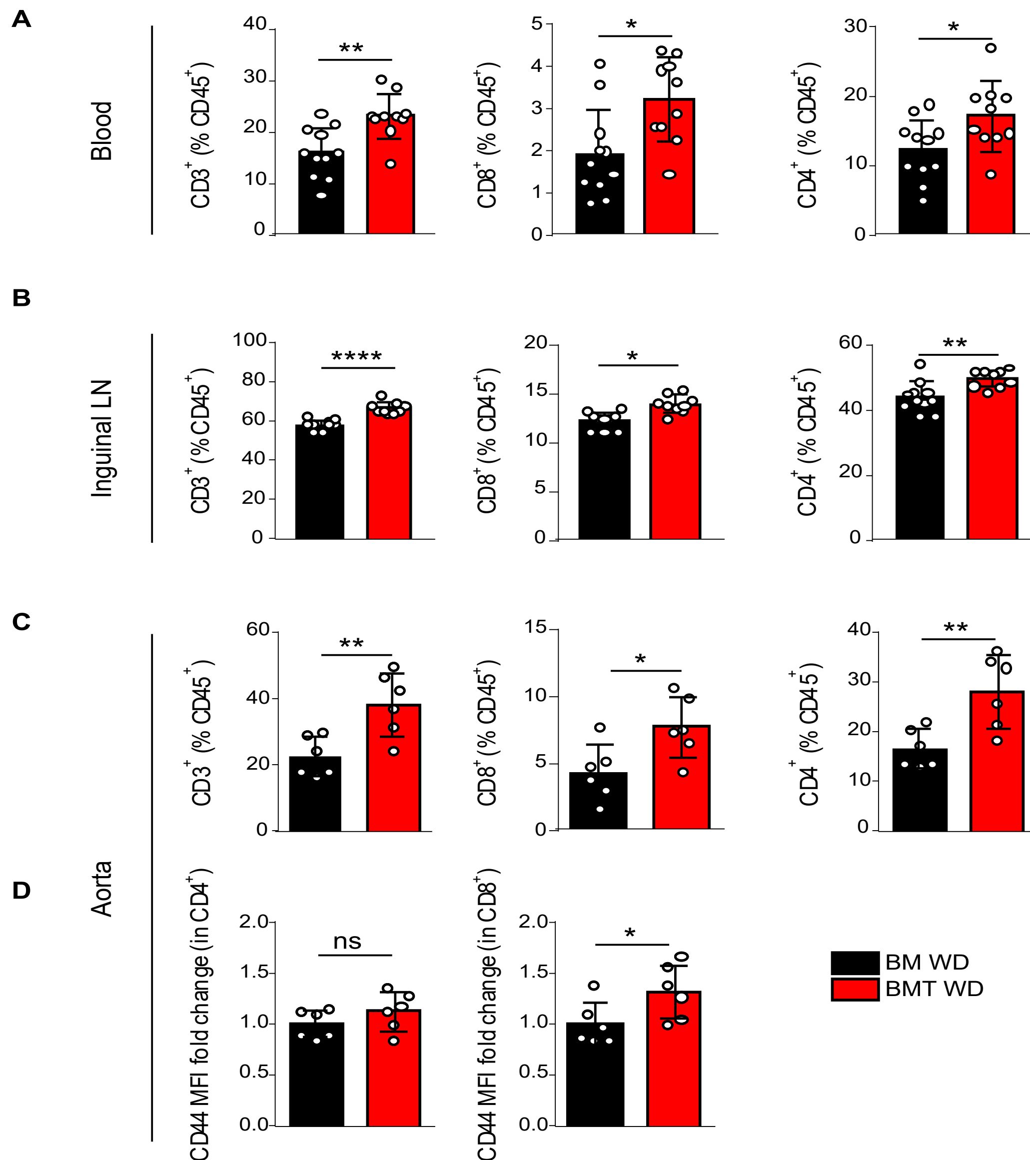

Figure 7.4: GvHD increases the relative number of circulating T cells and their infiltration in the aorta and inguinal LNs in atherosclerosis miHAg-mismatched allo-HCT mouse model. (A-D) Aorta, inguinal LNs, and blood were analysed by flow cytometry. Combined data from two independent experiments are shown, n=6-11. (A) Quantification of infiltrating T cells ($CD3^+$), $CD8^+$ and $CD4^+$ in the blood (A), inguinal LNs (B), and aorta (C) is shown. (D) Expression of CD44 by $CD4^+$ and $CD8^+$ T cells in the aorta, quantified as MFI fold change (normalized to the control, BM group). Statistical significance was determined by an unpaired t-test. Data are presented as a mean ± SD. *p<0.05, **p<0.01, ****p<0.0001 ns, non-significant. BM: bone marrow, BMT: bone marrow plus T cell, WD: Western diet, LN: lymph node.

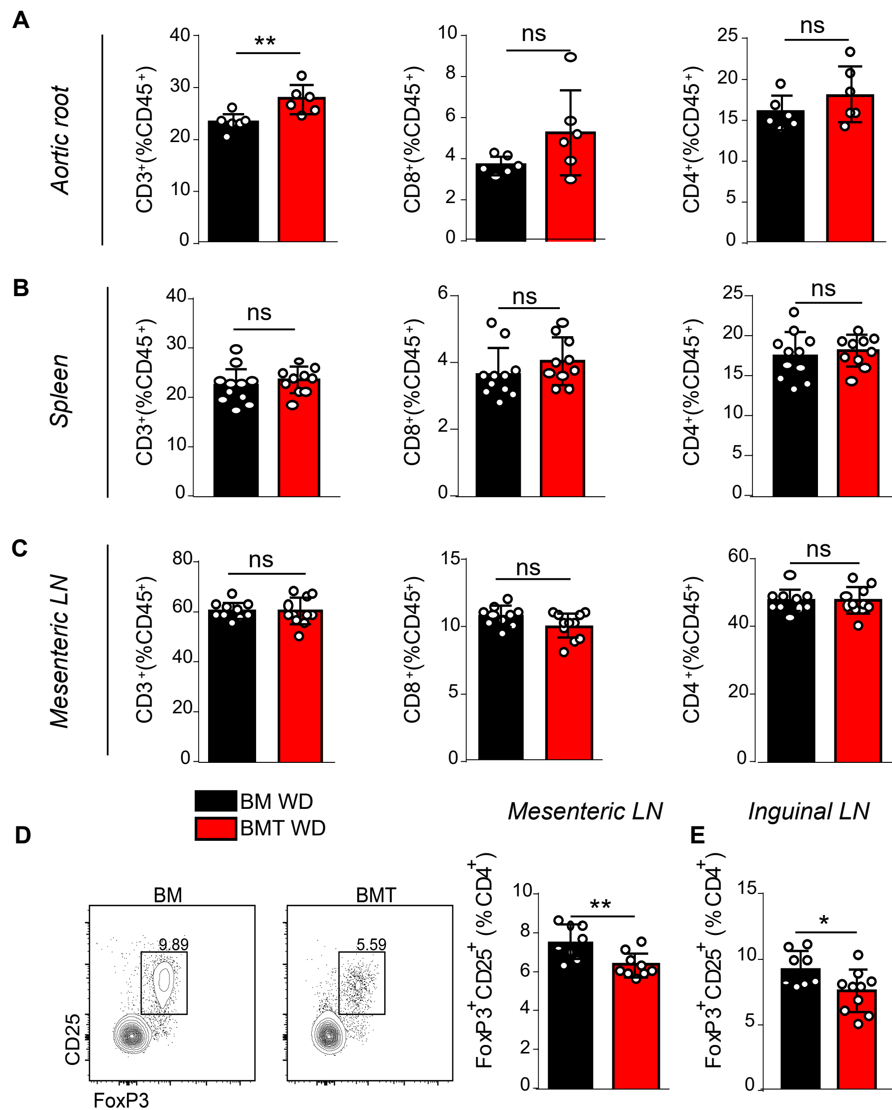

Figure 7.5: T cells and Tregs in post-HCT-induced atherosclerosis. (A-E) The aortic root, spleen, and mesenteric LNs were analysed by flow cytometry. Combined data from two independent experiments are shown, n=6-11. (A) Quantification of infiltrating T cells (CD3$^+$), CD8$^+$ and CD4$^+$ in the aortic root (A), spleen (B), and mesenteric LNs (C) is shown. (D-E) Quantification of Tregs in mesenteric (D) and inguinal (E) LNs with corresponding dot plots. Statistical significance was determined by an unpaired t-test. Data are presented as a mean ± SD. *p<0.05, **p<0.01, ns, non-significant. BM: bone marrow, BMT: bone marrow plus T cell, WD: Western diet, LN: lymph node.

7.4 CD8β depletion decreases plaque formation in the aorta and aortic root in BMT recipients after allo-HCT

Studies have shown that CD8$^+$ T cells play an important role in the pathogenesis of atherosclerosis by secretion of IFNγ that impact monopoiesis and consequently capability of plaque formation [346] on the one hand, but also they secrete Prf and Gmzb, known to induce apoptosis of plaque infiltrating cells resulting in the increased necrotic core [347].

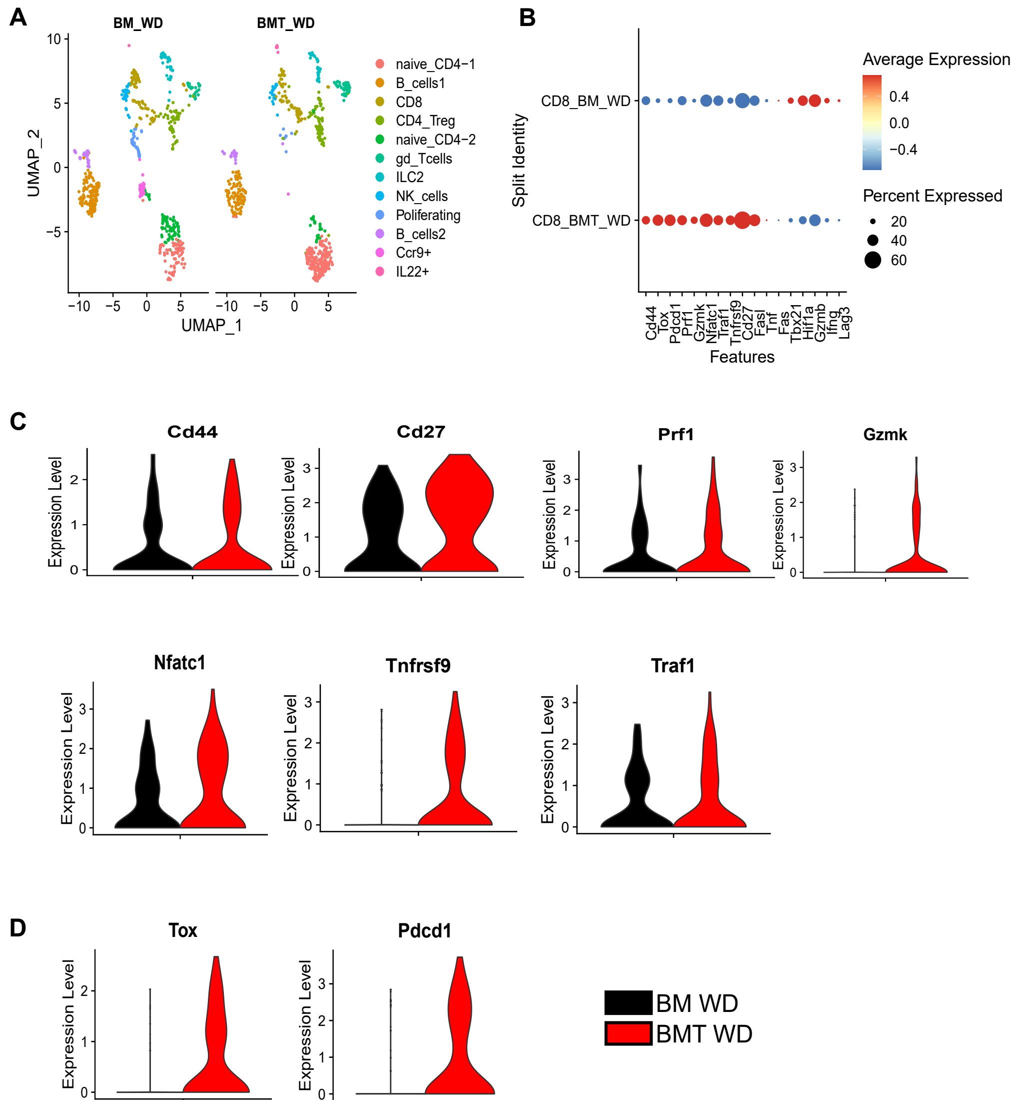

Figure 7.6: Aorta infiltrating CD8$^+$ T cells show a highly cytotoxic gene expression profile. (A-D) Aorta infiltrating CD45$^+$ cells are analyzed by scRNA sequencing, n=3. (A) UMAP clustering of CD45$^+$ cells, (B) Dot plot showing expression of the selected transcripts in CD4 and CD8 cluster, (C-D) Violin plots of selected transcripts in CD8 cluster analysed in (B), shown as comparison BM WD versus BMT WD. BM: bone marrow, BMT: bone marrow plus T cell, WD: Western diet, Prf: perforin, Gzmk: granzyme k, Nfatc1: nuclear factor of activated T-cells, cytoplasmic 1, Tnfrsf9: TNF receptor superfamily member 9, Traf1: TNF receptor-associated factor 1, Tox: thymocyte selection-associated high mobility group box, Pdcd1: gene encoding PD-1 protein. *Analysis of the scRNA seq data was done by Clement Cochain and Sarah Schäfer.*

Biederman et al. [14] showed specific localization of CD8$^+$ T cells around microvessels in the skin of patients suffering from skin GvHD, suggesting their important role in endothelial damage and the loss of microvessels in GvHD, while studies implementing CD8$^+$ depletion in patients treated with donor lymphocyte infusion (DLI) showed decreased incidence of GvHD [474, 475, 476]. As we observed cytotoxic CTL phenotype in aorta infiltrating cells, we hypothesized that depletion of CD8$^+$ T cells in our model might ameliorate atherosclerosis with even possible improved impact on the present subclinical

GvHD. Using the same mouse model (BALB/b (H-2b) $\rightarrow$ B6.LdLr$^{-/-}$ (H-2b)), we treated BMT transplanted mice with anti-CD8β antibody (as explained before [346]) starting at day 10 post-HCT (along with the WD) (Figure.7.7.A). After eight weeks of WD feeding and anti-CD8β treatment, we could confirm the decreased number of CD8$^+$ T cells present in the spleen of treated mice (Figure.7.7.B). The treatment did not show any impact on GvHD severity, observed through survival and clinical score (Figure.7.7.C-D). Interestingly, even in this incomplete depletion model, we could notice significantly lower serum cholesterol levels in anti-CD8β treated group in comparison to the isotype-treated BMT transplant (5.47 $\pm$ 0.40 versus 8.96 $\pm$ 0.05) without the change in the level of triglycerides (Figure.7.8.A-B). This decrease in circulating cholesterol levels was followed by a significant reduction in the lesion formation in the aorta (1.002 $\pm$ 0.20, versus 1.754 $\pm$ 0.14, in total) as well as in plaque size in the aortic root (7.403$\pm$ 0.87 versus 15.04 $\pm$ 0.62) when anti-CD8β treatment was applied (Figure.7.8.C-D). These data suggest that CD8$^+$ T cells have a prominent impact on plaque formation, possibly through the influence on cholesterol levels.

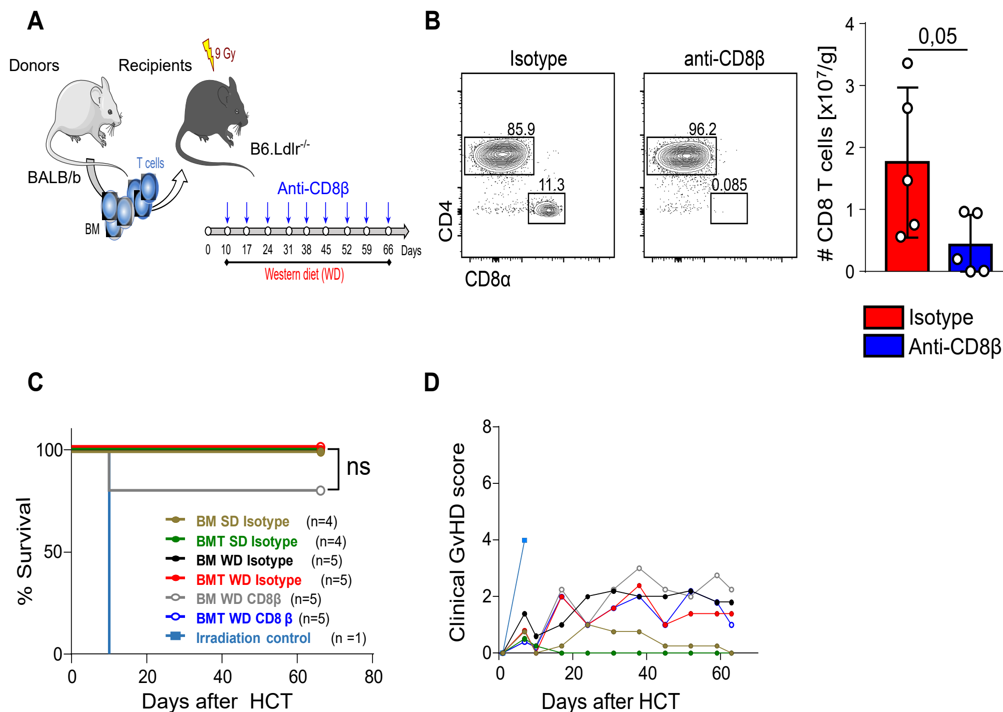

Figure 7.7: CD8$^+$ depletion in miHAg-mismatched allo-HCT induced atherosclerosis mouse model. (A-D) In established atherosclerosis - GvHD model, mice were injected with anti-CD8β antibody or isotype control once per week starting along with the WD feeding, ten days post-HCT (n=4-5). (A) Experimental design. (B) Quantification of CD8$^+$ T cells in spleen analysed by flow cytometry with representative dot plots. (C) Kaplan-Meier survival curve. (D) Clinical GvHD score. Data are presented as a mean $\pm$ SD. Statistical significance was determined by log–rank test (C) and an unpaired t-test (B). ns, non-significant. BM: bone marrow, BMT: bone marrow plus T cells.

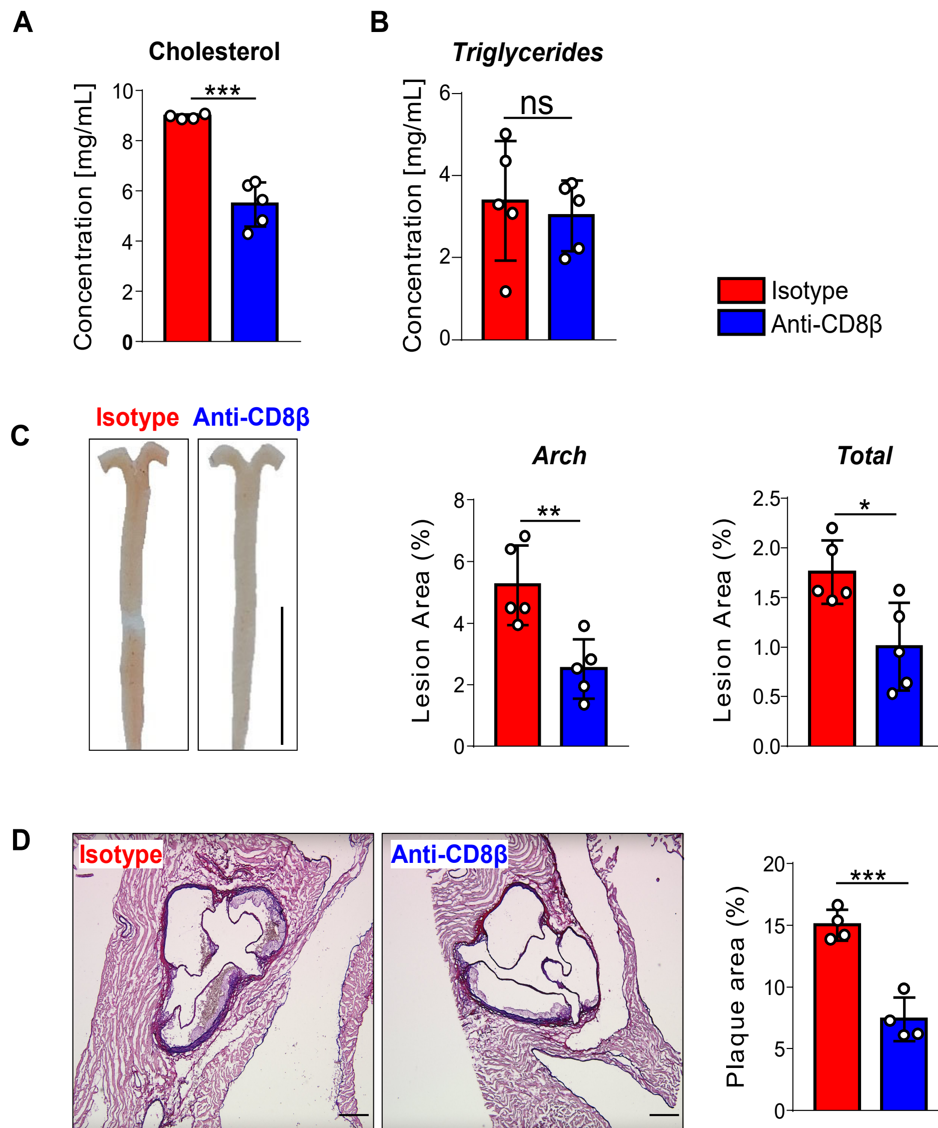

Figure 7.8: CD8$^+$ depletion decreases the plaque formation in aortic root and aorta in atherosclerosis - miHAg-mismatched allo-HCT model. (A-E) In established atherosclerosis - GvHD model, mice were injected with anti-CD8β antibody or isotype control once per week starting along with the WD feeding, ten days post-HCT (n=4-5). (A-B) Quantification of cholesterol and triglyceride levels in serum. (C) Quantification of Oil-Red-O-stained aortas. Enface images of the aorta, scale bars 1 cm. (D) Representative images and quantification of plaque area in aldehyde-fuchsin stained aortic root sections, scale bar 100μm. Data are presented as a mean ± SD. Statistical significance was determined by an unpaired t-test, *p<0.05, **p<0.01, ***p<0.001, ns, non-significant.

7.5 CD8β depletion improves plaque phenotype and its stability in BMT recipients without the impact on monopoiesis and circulating levels of monocytes

To further elucidate the impact of CD8$^+$ T cells on the severity of atherosclerosis, we characterized the plaque phenotype in the aortic root of mice after eight weeks of WD feeding and anti-CD8β treatment. We could not notice any difference in the infiltration of plaque by macrophages (Figure.7.9.A). This non-changeable trend was also observed in the levels of monocytes in BM and blood (Figure.7.10.A-B). However, in accordance with previous studies investigating the impact of CD8$^+$ T cells in atherosclerosis [347, 346], a significant decrease in the necrotic core was observed after anti-CD8β treatment (Figure.7.9.B), confirming the role of these cells in the induction of cell apoptosis in plaque. On the other hand, we could observe a trend towards increased infiltration of SMC and no change in the relative amount of collagen (Figure.7.9.C-D) in the plaque of treated animals. Overall, these changes in the cell composition of plaque seem to improve the plaque stability when anti-CD8β-antibody treatment is applied (Figure.7.9.E).

7.6 Changed liver lipid metabolism in GvHD-athersoclerosis mouse model

As we showed above (Figure.7.1.C-D), BMT transplantation increased serum cholesterol levels in atherosclerosis-prone recipients, and this was followed by increased plaque formation in the aorta and aortic root (Figure.7.1.E-F). When mice were treated with an anti-CD8β antibody, cholesterol levels were significantly decreased (5.47 ± 0.40 versus 8.96 ± 0.05) in comparison to isotype-treated control (Figure.7.8.A), getting to a level similar to the one observed in BM WD (Figure.7.1.C). During cGvHD, CD8$^+$ T cells were shown to be infiltrated in areas of tissues facing lesional changes, such as skin and liver [14, 477]. Liver and intestine are the main organs responsible for the regulation of cholesterol metabolism and in this, subclinical GvHD model, we did not observe histopathological differences in these tissues (Figure.7.1.G). However, assessment of serum levels of transaminases (aspartate aminotransferase (AST) and alanine aminotransferase (ALT)) as a measure of induced liver damage, showed a tendency towards increased levels in BMT recipients (Figure.7.11.A-B) Quantification of expression of the main genes involved in the regulation of cholesterol metabolism, LXR and FXR, seem not to be different between BM and BMT (Figure.7.11.C-D). However, the expression of subsequent genes involved in the cholesterol metabolism in the liver showed changes in the sense of reducing the high serum cholesterol levels: the expression of genes regulating cholesterol synthesis, HMGCR and SREBF2, was downregulated (Figure.7.11.E) whereas the expression of genes regulating bile acid production, CYP7A1 (Figure.7.11.F), cholesterol esterification, ACAT1 (Figure.7.11.G) and efflux, ABCG5 (Figure.7.11.H) was increased. Overall, it seems that the liver tries to compensate for the increased cholesterol levels. However, it remains under-compensated.

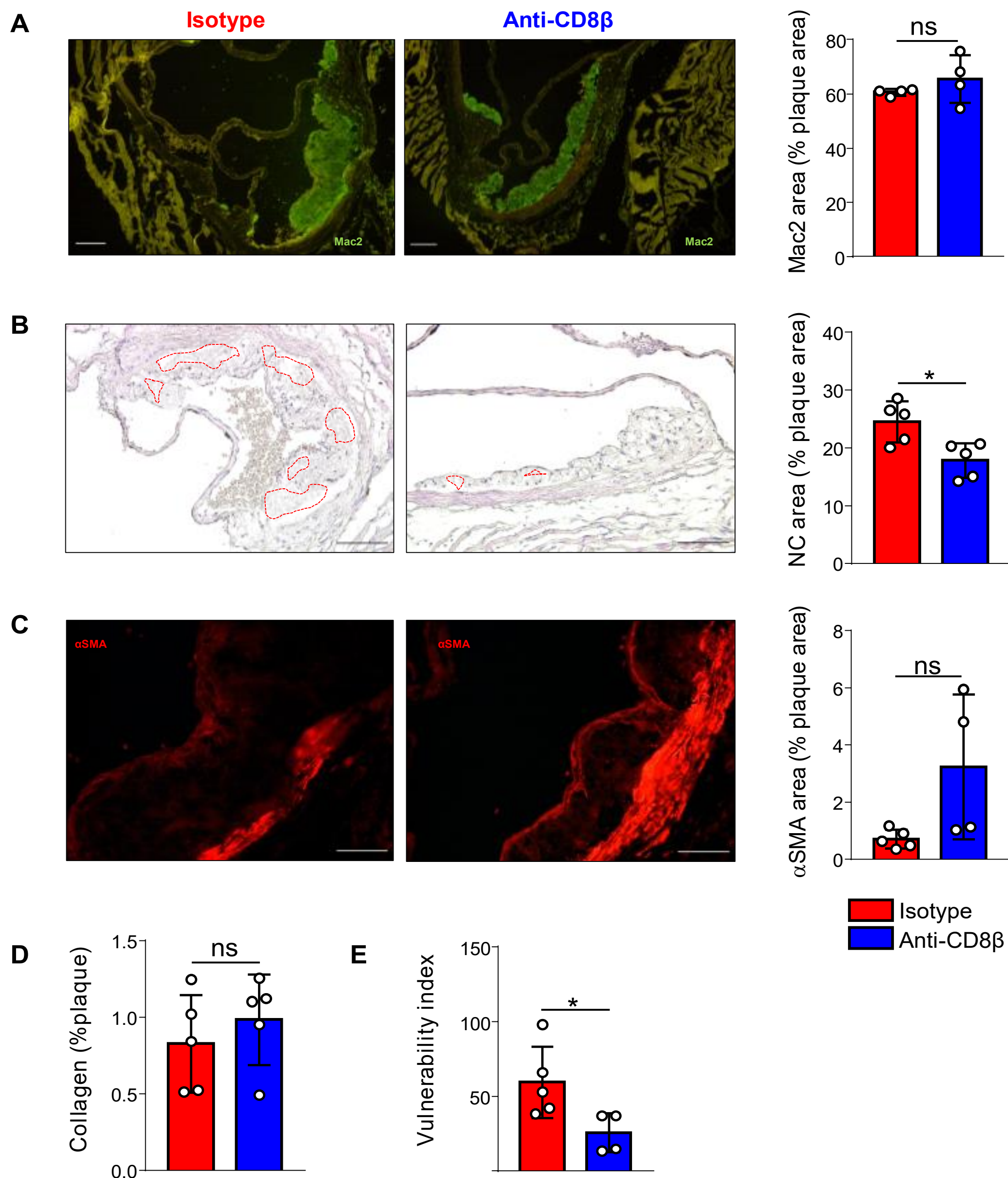

Figure 7.9: CD8$^+$ depletion improves plaque stability in atherosclerosis - GvHD mouse model. (A-E) In the established atherosclerosis - GvHD model, mice were injected with anti-CD8 β antibody or isotype control once per week starting along with the WD feeding, ten days post-HCT (n=4-5). (A-C) Quantification of the area positive for Mac-2 (A), necrotic core (B), α-SMC actin (α-SMA) (C), as a percentage of total plaque area with corresponding images are shown, scale bar 50μm. (D, E) Quantification of collagen in the plaque area (D) and vulnerability index (E). Data are presented as a mean ± SD. Statistical significance was determined by an unpaired t-test ,*p<0.05, ns, non-significant. NC:necrotic core

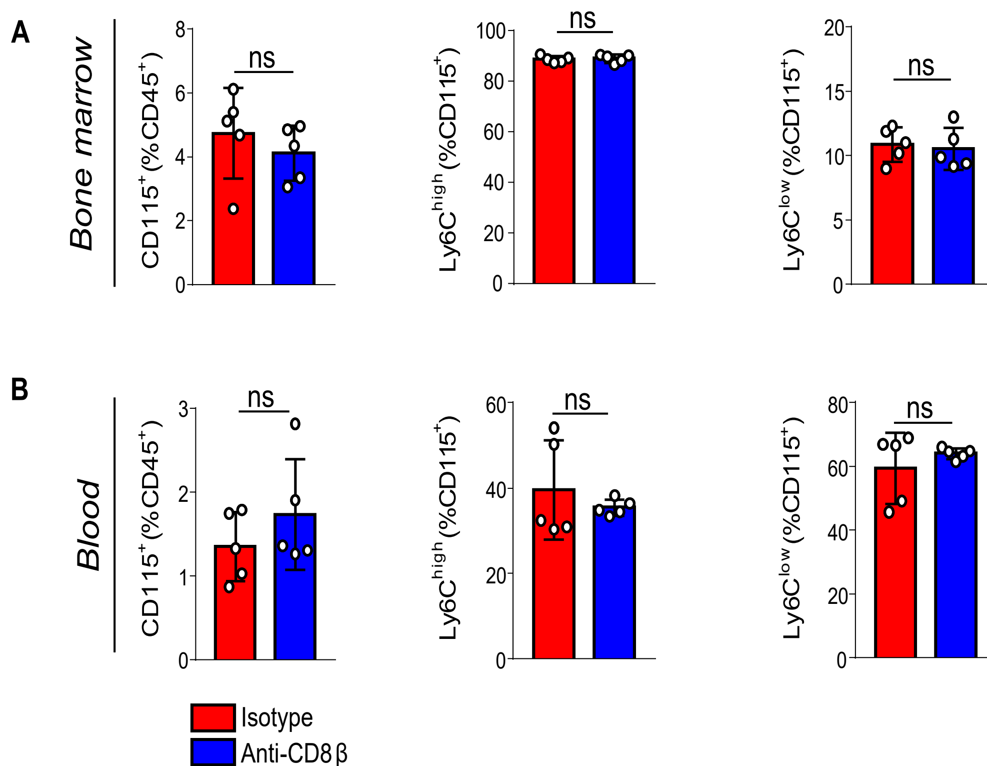

Figure 7.10: CD8β depletion does not have an impact on monopoiesis or the number of circulating monocytes in GvHD-atherosclerosis model. (A-B) Anti-CD8β treatment in atherosclerosis - GvHD mouse model (n=5). Monocytes were analysed by flow cytometry in blood and bone marrow. The relative number of total (CD115$^+$), as well as Ly6C^{high} and Ly6C^{high} monocytes subsets in bone marrow (A) and blood (B) are shown. Statistical significance was determined by an unpaired t-test. Data are presented as a mean ± SD. ns, non-significant.

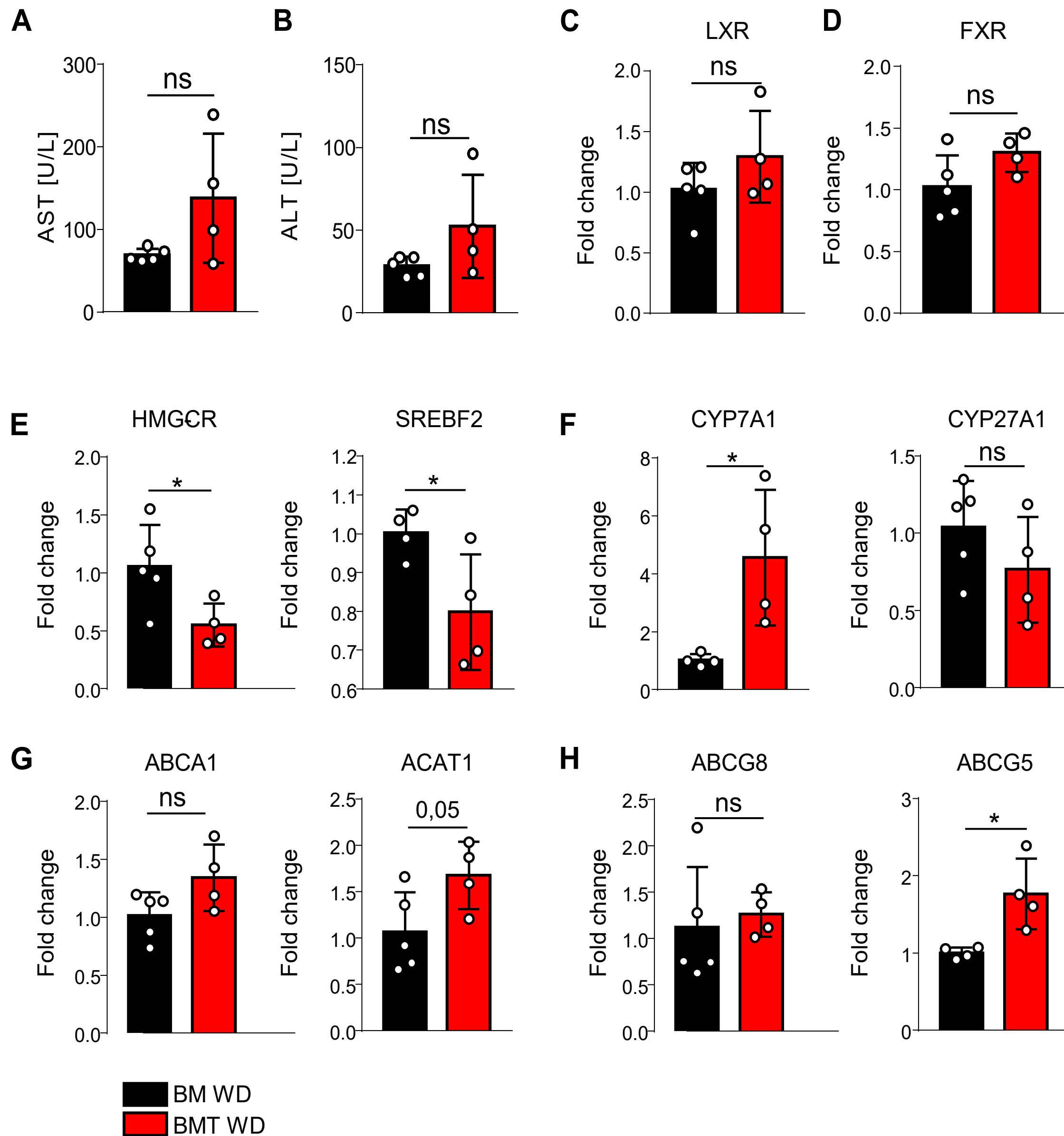

Figure 7.11: High cholesterol levels induce changes in liver lipid metabolism. (A-I) MiHAg-mismatched allo-HCT induced atherosclerosis model. GvHD was induced in B6.LdLr$^{-/-}$ (H-2b) by TBI (9 Gy) and allogeneic transplantation of 5x10^{6} BM cells and 5x10^{6} of purified T cells from sex-matched donor BALB/b (H-2b). Recipient mice B6.LdLr$^{-/-}$ were fed with SD or WD for eight weeks, starting on day 10 post-HCT, n=4-5. (A-B) Serum levels of transaminases. (C-H) Relative mRNA fold change is shown. Expression of LXR, FXR (C-D), HMGCR, SREBF2 (E), CYP7A1, CYP27A1 (F), ABCA1, ACAT1 (G) and ABCG5, ABCG8 (H) are analysed. Statistical significance was determined by an unpaired t-test. Data are presented as a mean ± SD. *$p<0.05$, ns, non-significant. AST: aspartate aminotransferase, ALT: alanine aminotransferase, LXR: liver X receptor, FXR: farnesoid X receptor, HMGCR: 3-hydroxy-3-methylglutaryl coenzyme A reductase, SREBF2: sterol regulatory element-binding transcription factor 2, CYP7A1: cytochrome P450 family 7 subfamily A Member 1, CYP27A1: cytochrome P450 family 27 subfamily A Member 1, ABCA1: ABC subfamily A member 1, ACAT1: acyl coenzyme A-cholesterol acyltransferase 1, ABCG8/5: ABC subfamily G 8/5, BM: bone marrow, BMT: bone marrow plus T cells, WD: Western diet.

Chapter 8

Impact of short-term WD on the development of aGvHD

Chapter 8 presents data that is part of an ongoing project whose publication is planned at a later stage. The article is based on the work of the author of this thesis, Ivana Jorgacevic, who planned and performed the experiments and data analysis.

8.1 Short-term WD feeding aggravate aGvHD

For the purpose of elucidating this phenotype, we used an MHC major mismatched allo-HCT mouse model, FVB/N(H-2q) → B6.WT (H-2b), where GvHD was induced in B6.WT (H-2b) mice by myeloablative irradiation and allo-transplantation of BM derived from FVB/N and $CD3^{+}$ enriched, T cells from FVB.L2G85 mice. Recipient mice were put on WD starting one day before irradiation and BM and T cell transplantation (Figure.8.1.A). Strikingly, BMT transplanted mice fed WD exhibited significantly reduced survival in comparison to mice fed SD (Figure.8.1.B), followed by significantly increased clinical GvHD score, including significant weight loss in comparison to the mice, fed SD (Figure.8.1.B-D). The survival was improved by a decrease in the amount of transplanted T cells, but the noticeable impact of WD feeding still remained (Figure.8.1.B).

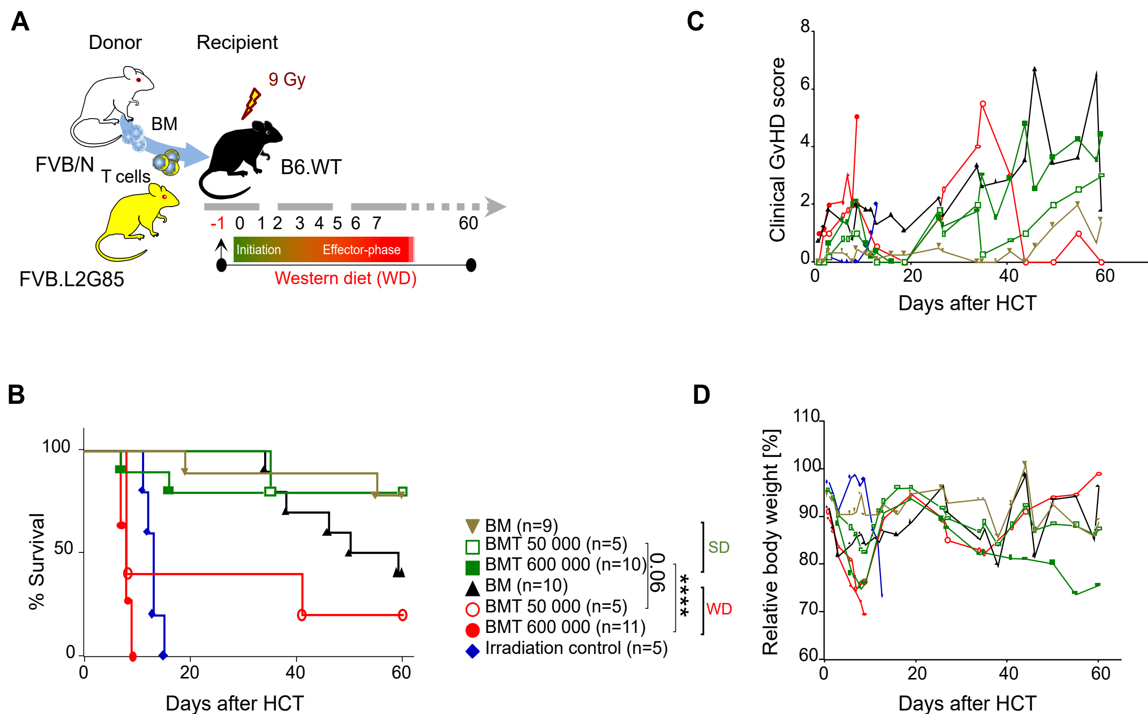

Figure 8.1: Short term WD feeding decrease survival post-HCT. (A-D) aGvHD was induced in B6.WT recipient mice by TBI (9 Gy), and allo-transplantation with $5x10^{6}$ BM cells from FVB/N and $6x10^{5}$ $CD3^{+}$ enriched T cells from FVB.L2G85 mice. Recipient mice were fed with SD or WD for 60 days, starting one day pre-HCT. Combined data from two independent experiments are presented n=5-11. (A) Experimental design. B) Kaplan-Meier survival curve. (C-D) Clinical GvHD score (C) and relative body weight (D) are shown. Statistical significance was determined by log-rank test. ****$p<0.0001$. BM: bone marrow, BMT: bone marrow plus T cells, WD: Western diet, SD: standard diet.

8.2 Short-term WD feeding leads to increased infiltration of alloreactive T cells in spleen and GIT post-HCT

aGvHD manifests when alloreactive T cells recognize disparate histocompatibilty antigens and attack GIT, liver, lung and skin and cause significant tissue damage in these target organs. Our first observation that decreased number of transplanted donor T cells manages to improve the survival rate of WD-fed animals suggests that alloreactive T cells play a significant role in the inflammatory response induced by this diet regimen. In order to answer this question lethally-irradiated recipient B6.WT (H-2b,CD45.2$^+$CD90.2$^+$) mice where transplanted with BM from FVB (H-2q,CD45.2$^+$CD90.2$^+$) and purified T cells from donor, FVB.L2G85 (H-2q,CD45.1$^+$CD90.1$^+$) mice. The transplantation of Luciferase positive (Luc$^+$) T cells (derived from FVB.L2G85) enabled us to track the migration of donor T cells during the course of aGvHD by BLI, as described before [478]. In the mouse model of aGvHD, it was shown that during the initiation phase (considered three days post-HCT), T cells are mainly present in the SLOs, where is the highest possibility of encountering APCs [478], while during the effector phase (observed on day 6 post-HCT), cells are migrating and infiltrating target organs. Thus, we did the screening of T cell infiltration and their phenotype on these two different days by ex vivo BLI imaging post-HCT. On day 3, we could observe higher infiltration of alloreactive T cells in the spleen and GIT (Peyer's patches) of mice fed WD, implying a higher possibility for the activation by APCs in the SLOs (Figure.8.2.A). On day 6 the trend remained, with a noticeable increase in the number of alloreactive T cells infiltrating GIT as one of the target organs (Figure.8.2.B).

8.3 WD stimulate migration of alloreactive T cells towards intestine

Noticeably increased infiltration of alloreactive T cells in GIT on day 6 indicate upregulation of chemokine receptors during the initiation phase of aGvHD that are responsible for their migration to GIT during the effector phase. Thus, by flow cytometry, we further defined the phenotype of infiltrating alloreactive T cells (CD90.1$^+$) on day 3 post-HCT. As explained before [478], during the first days, post-HCT alloreactive T cells are mainly infiltrating SLOs, such as Peyer's patches, mesenteric LNs, peripheral LNs and spleen. In our aGvHD model, we observed predominant CD4$^+$ infiltration in the peripheral LNs ($\approx$70%), while CD8$^+$ accounted for around 25% of total alloreactive, CD90.1$^+$ T cells. We could not observe any difference in the relative number of infiltrating CD4$^+$ or CD8$^+$ alloreactive T cells between different diet regimens (Figure.8.3.A-B). Even though the studies suggest that the main source of gut-homing alloreactive T cells comes from Peyer's patches and mesenteric LNs, in our model, the main differences occurred in peripheral LNs (the pool composed of axillary, mediastinal, inguinal and cervical LNs) suggesting rather systemic than local effect. After WD feeding, the populations highly expressing the gut-homing receptor, $\alpha4\beta7$ integrin, were increased among CD4$^+$ as well as CD8$^+$ T cells (Figure.8.3.C-E). These data suggest an impact of WD on the migration capability of alloreactive T cells through upregulation of chemokine receptor $\alpha4\beta7$.

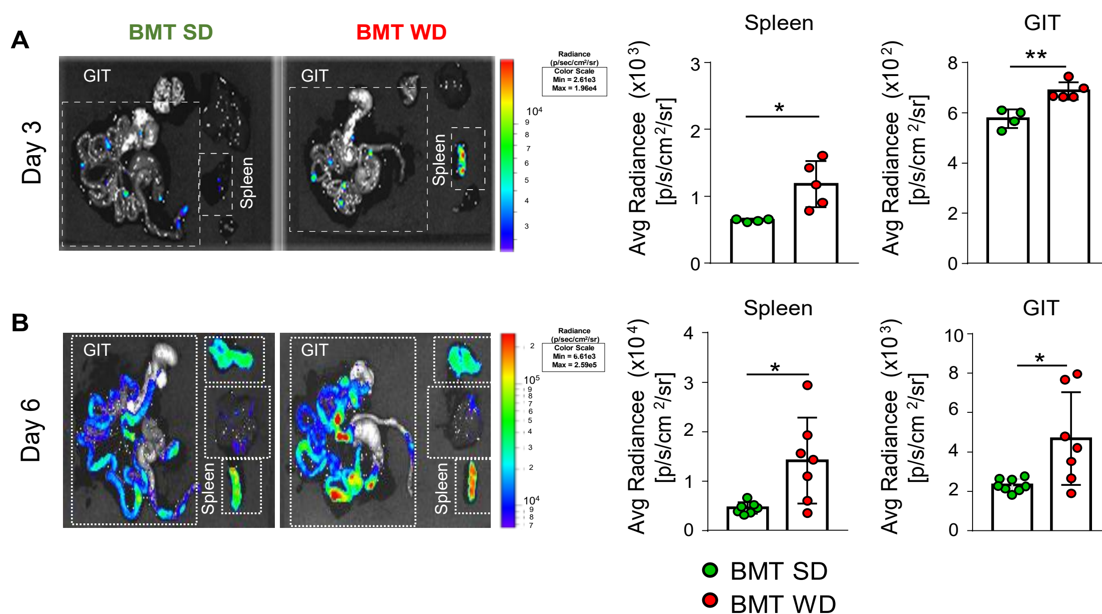

Figure 8.2: WD induce spleen and GIT infiltration by alloreactive T cells. (A-B) GvHD was induced in a MHC-mismatched allo-HCT mouse model (FVB(H-2q) → B6.WT(H-2b)) by TBI (9 Gy) and allo-transplantation with 5x10^6 BM cells derived from FVB/N and 6x10^5 of enriched T cells from FVB.L2G85 mice. Recipient mice were fed with SD or WD beginning on day -1. Combined data from two independent experiments are presented n=4-8. Quantification (p/s/cm2/sr) of bioluminescence signals emitted from Luc^+ donor derived T cells in GIT and spleen with the corresponding IVIS ex vivo images on day 3(A) and day 6(B) post-HCT. Statistical significance was determined by an unpaired t-test. Data are presented as a mean ± SD. *$p<0.05$, **$p<0.01$. BMT: bone marrow plus T cells, WD: Western diet, SD: standard diet, GIT: gastrointestinal tract.

8.4 WD promotes activation of $CD4^+$ alloreactive T cells in SLOs

The predominance of $CD4^+$ T cells during the first days after transplantation was shown in the mouse model of aGvHD [478]. The same study showed the shift in subpopulations starting from day 4 in the spleen through mesenteric LNs on day 5 in favour of $CD8^+$ T cells. However, in our model, we could still observe the prevalence of $CD4^+$ on day 6 post-HCT in mesenteric LNs (Figure.8.4.A,E), peripheral LNs (Figure.8.4.B,F), small intestine (Figure.8.4.C,G) and spleen (Figure.8.4.D,H), reaching up to 60% in all mentioned organs. Noteworthy, $CD4^+$ T cells infiltrating peripheral LNs, small intestine and spleen of WD fed recipients exhibited higher expression of CD44 (in comparison to $CD8^+$, where the expression seemed to be similar) (Figure.8.4.J-P). There was no change in the expression of CD44 by $CD4^+$ T cells in mesenteric LNs (Figure.8.4.I), suggesting WD impact through stimulation of rather systemic than local $CD4^+$ T cells activity.

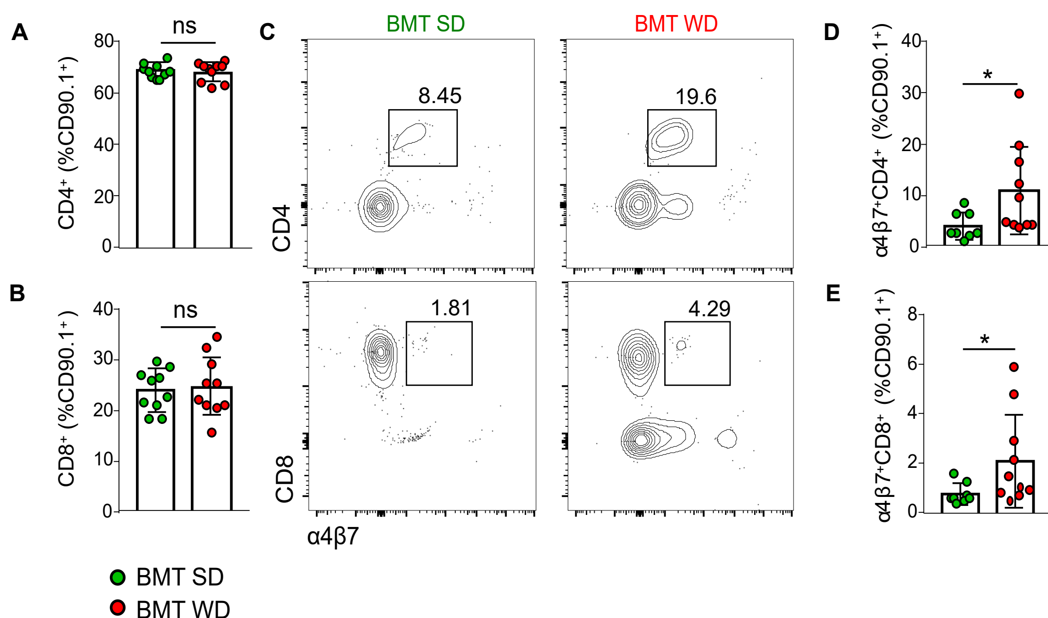

Figure 8.3: Alloreactive T cells upregulate $\alpha 4\beta 7$ in peripheral LNs under the impact of WD. (A-E) GvHD was induced in a MHC-mismatched allo-HCT mouse model (FVB(H-2q) → B6.WT(H-2b)) by TBI (9 Gy) and allo-transplantation with 5×10^6 BM cells derived from FVB/N and 6×10^5 of enriched T cells from FVB.L2G85 mice. Recipient mice were fed a SD or WD starting one day before TBI and reconstitution. (A-B) Pooled peripheral LNs were analysed by flow cytometry for T cells infiltration on day 3 post-HCT. The relative number of $CD4^+$ (A) and $CD8^+$ (B) T cells. (C-E) The relative number of $\alpha 4\beta 7$ expressing $CD4^+$ (D) and $CD8^+$ (E) T cells among total donor T cells ($CD90.1^+$) with corresponding dot plots (C) is shown. Combined data from two independent experiments are shown (n=8-10). Data are presented as a mean ± SD. Statistical significance was determined by an unpaired t-test. *$p<0.05$, ns, non-significant. BMT: bone marrow plus T cells, WD: Western diet, SD: standard diet.

8.5 WD changes spleen morphology and elevate inflammation

Since we observed in this model that mice are not able to recover one week post-HCT if fed WD, we paid specific attention to morphological features of organs on day 6. Interestingly, the main significant difference was observed in the spleen, which looked quite smaller in comparison to SD-fed animals (Figure.8.5.A). However, as we showed above (Figure.8.4.D,L), the remaining prevailing cells in the spleen seemed to be effector alloreactive $CD4^+$ T cells. Cytokine secretion, as shown by flow cytometry analysis of these cells, showed increased TNFα, but not IFNγ response by both T cell subtypes, $CD4^+$ and $CD8^+$ (Figure.8.5.B-E). On the other hand, quantitative analysis of gene expression in spleen yielded increased pro-inflammatory cytokines such as IL-6, and IFNγ, with concomitant increased expression of IL-10 but no differences in TNFα were observed (Figure.8.5.F-I). These data suggest a potential involvement of other cell types that could show increased expression of TNFα apart from T cells, such as macrophages (as they also secrete IL-6) and NK cells, B cells, or $\gamma\delta$ T cells that can, on the other hand, secrete IFNγ, that can lead to significant pro-inflammatory environment observed after WD feeding.

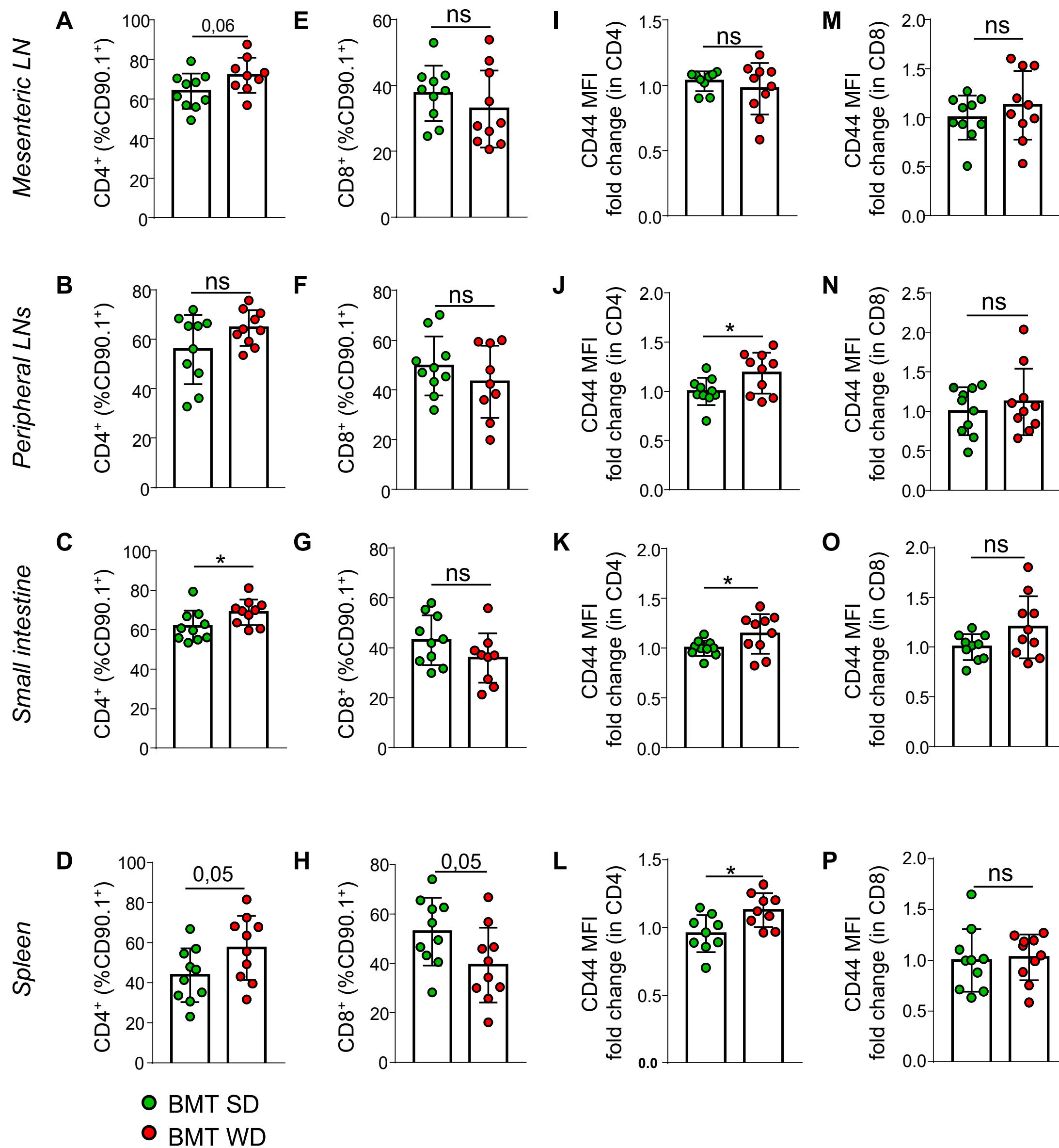

Figure 8.4: Alloreactive T cells on day 6 post-HCT. (A-P) GvHD was induced in MHC-mismatched allo-HCT mouse model (FVB(H-2q) → B6.WT(H-2b)). Recipient mice were fed with SD or WD beginning on day -1. Mesenteric LNs, pooled peripheral LNs, small intestine and spleen were analysed by flow cytometry for T cells infiltration on day 6 post-HCT. Relative number of $CD4^+$ (A-D) and $CD8^+$ (E-H) among total donor ($CD90.1^+$) T cells. (I-P) CD44 MFI fold change (normalized to SD, control group) in $CD4^+$ (I-L) and $CD8^+$ (M-P) donor T cells. Combined data from two independent experiments are shown (n=8-10). Data are presented as a mean ± SD. Statistical significance was determined by an unpaired t-test. *$p<0.05$, ns, non-significant. BMT: bone marrow plus T cells, WD: Western diet, SD: standard diet, LN: lymph node.

8.6 WD changes intestinal innate immune response during the course of aGvHD

The preconditioning regimen is the primary event that induces tissue damage and subsequent innate immune response activation and inflammation during the initiation phase of

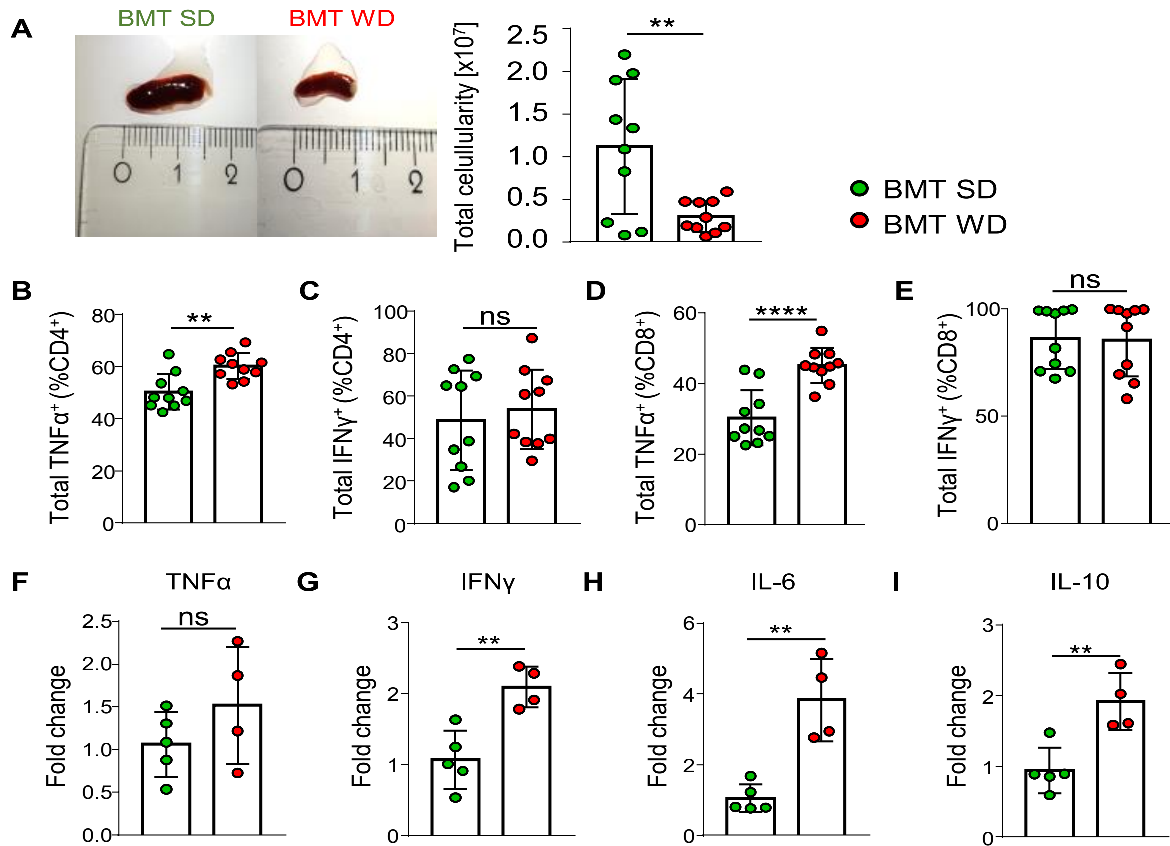

Figure 8.5: WD promotes a pro-inflammatory environment in the spleen during aGvHD effector phase. (A-J) MHC-mismatched allo-HCT mouse model. Recipient mice were fed with SD or WD beginning on day -1. (A) Total cellularity of the spleen with corresponding ex vivo images. (B-E) Spleenocytes were assessed by flow cytometry after four hours stimulation with PMA, Ionomycin, and Brefeldin A. Relative number of TNFα and IFNγ producing CD4$^+$ (B-C) and CD8$^+$ (D-E) donor T cells. Combined data from two independent experiments are shown (n=10). (F-J) RT qPCR analysis of spleen. Gene expression of TNFα (F), IFNγ (G), IL-6 (H) and IL-10 (I) is shown as fold change (normalized to a control, SD fed group), n=4-5. Data are presented as a mean $\pm$ SD. Statistical significance was determined by an unpaired t-test. **<0.01, ****<0,0001, ns, non-significant. BMT: bone marrow plus T cells, WD: Western diet, SD: standard diet.

GvHD. Damages in the intestinal epithelial barrier lead to the translocation of bacteria from the intestinal lumen, leading to an additional increase in an inflammatory response but also to increased LPS circulating levels. LPS accumulates in the liver and spleen of animals with GvHD prior to its appearance in systemic circulation [479], but also chronic HFD feeding increases circulating endotoxin levels through increased intestinal permeability [480]. Thus, in the same mouse model (Figure.8.1.A), we indirectly measured the intestinal permeability on day 2 post-HCT by measuring fluorescence derived from orally administered FITC dextran (see Section.5.5). However, there was no significant difference in comparison to the group fed with SD (Figure.8.6.A). Hülsdünker et al. [481] showed that induction of aGvHD is followed by increased bacterial translocation to the ileum, as the closest part to caecum and colon, followed by translocation to mesenteric LNs. This prominent abundance of bacteria activates innate immune response - infiltration of neutrophils and increased production of ROS that lead lastly to the activation and co-

ordination of adaptive immune response, but also to severe damage of the tissue. We isolated mesenteric LNs and part of ileum on day 2 and day 3 post-HCT, respectively, and quantified the abundance of bacteria by 16S RT qPCR. Along with it, we performed flow cytometry analysis of infiltrating neutrophils on different days post-HCT. Interestingly, even though both organs showed a tendency towards increased bacterial abundance after WD feeding (Figure.8.6.B-C), we did not observe any significance in regard to WD consumption. These results correlated with the infiltration of ileal neutrophils on these first days post-HCT (Figure.8.6.D). However, on day 6, even though the main force of neutrophils had already left the intestine, a relative number of remaining neutrophils became significantly higher when WD was consumed in comparison to SD ($2.55 \pm 0.60\%$ vs. $0.64 \pm 0.15\%$ of leukocytes respectively). Noteworthy, the significant amount of these neutrophils showed, specifically on day 6 post-HCT (when their number becomes significantly increased), high expression of Siglec F (Figure.8.6.E). Lastly, we quantified the relative number of host-derived macrophages in the small intestine. In our mouse model, independently of the day post-HCT, the relative number of macrophages was decreased under the WD treatment (Figure.8.6.F-G). We, therefore, defined their phenotype by quantifying their expression of PDL-1 and CD80 by flow cytometry. Expression of CD80 was significantly upregulated on day 3 and day 6 post-HCT, while the expression of PDL-1 was changing through the GvHD phases - from significantly decreased on day 3 to an increased expression on day 6 (Figure.8.6.H-I). Overall, these data suggest that WD has a prominent impact on innate immune response, likely accumulating effect with a continued diet feeding as the main differences occur on day 6 post-HCT.

8.7 Significant impact of WD on post-HCT response increases liver damage

Even though we managed to characterize and track the main pathways of response in SLOs as well as in the ileum as GvHD target one, we have not managed to find the main reason that would explain the detrimental impact of WD during the onset of aGvHD. To get an overview of the systemic functioning of the organism just before the onset of increased mortality, we tested blood samples of mice on different biochemical parameters. Overall, we could notice an increased amount of transaminases, AST, and ALT (Figure.8.7.A-B), but also highly increased levels of LDH when mice are fed WD (Figure.8.7.C). On the other hand, there was an opposite trend in the concentration of urea and creatinine, which possibly can yield because of the smaller size of WD mice and therefore decreased muscle volume, which is followed by such creatinine levels (Figure.8.7.D-E). There was no change in the levels of total protein and albumin (Figure.8.7.F-G), ruling out dehydration or undernourishment as a reason for such a phenotype. Moreover, hemoglobin values were also not changed (Figure.8.7.J), whereas the levels of RBCs and PLTs were increased (Figure.8.7.I,K), but blood leukocytes were significantly reduced (Figure.8.7.H) in WD treated group.

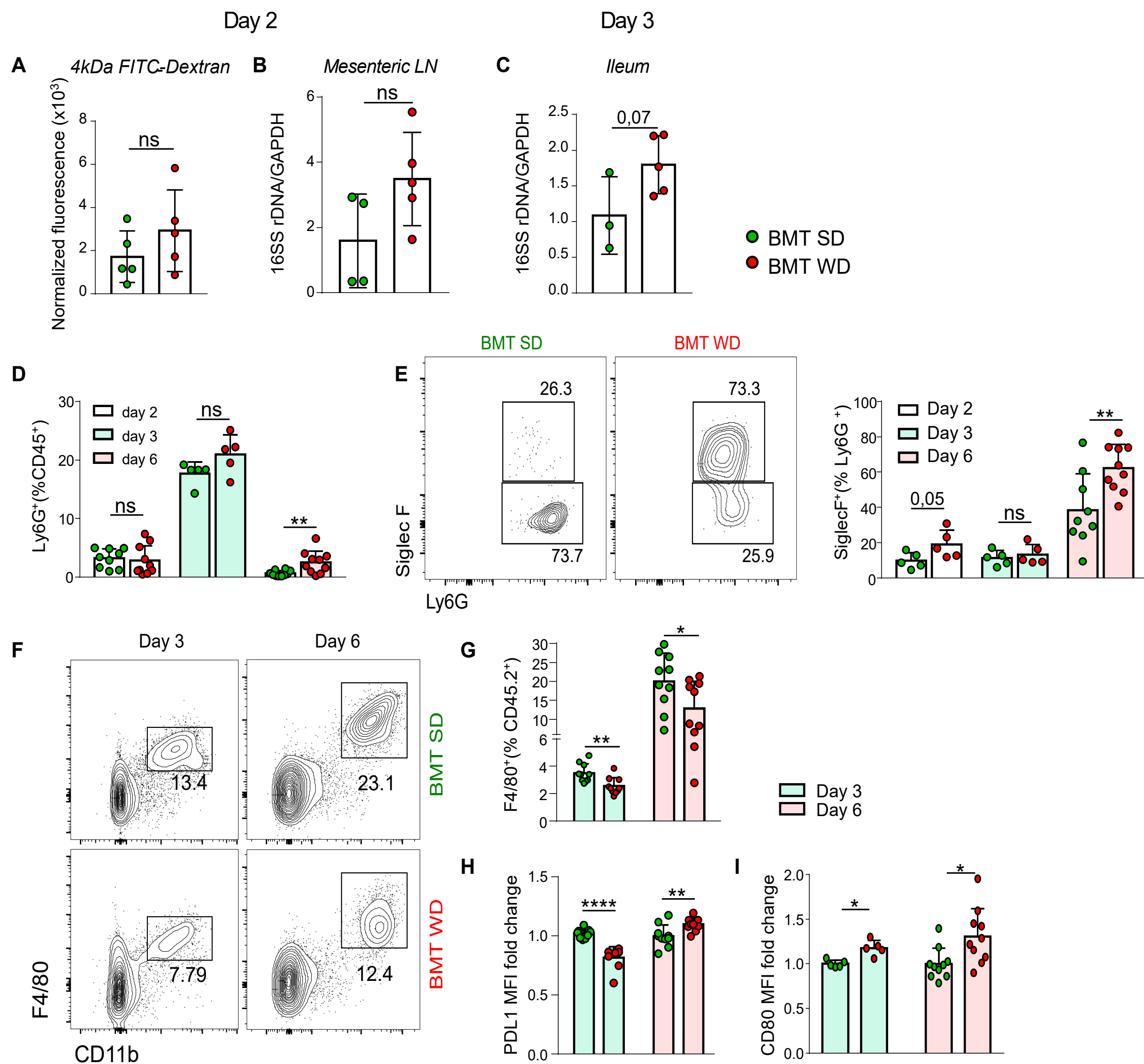

Figure 8.6: WD impact on intestinal innate immune response during aGvHD.(A-J) MHC-mismatched allo-HCT mouse model. Recipient mice were fed with SD or WD beginning on day -1. (A) Intestinal permeability measured on day 2 post-HCT, n=5. (B-C) Bacterial load in mesenteric LNs on day 2 (B) and ileum on three days (C) post-HCT was analysed by RT qPCR. 16S rDNA content was normalized to mouse genomic DNA (glyceraldehyde-3-phosphate dehydrogenase (GAPDH)), N=3-5. (D-I) Flow cytometry quantification of neutrophils and macrophages in small intestine on different days post-HCT is shown. (D-E) The relative number of neutrophils (D) and Siglec F expressing population among them with corresponding dot plots (E) is presented. (F-I) The relative number of macrophages (G) with corresponding dot plots (F) and their expression of PD-L1 (H) and CD80 (I) shown as MFI fold change (normalized to the control, SD fed group). Combined data from two independent experiments are shown (n=5-10). Data are presented as a mean ± SD. Statistical significance was determined by an unpaired t-test. *p<0.05, **<0.01, ****<0,0001, ns, non-significant. LN: lymph node, BMT: bone marrow plus T cells, WD: Western diet, SD: standard diet.

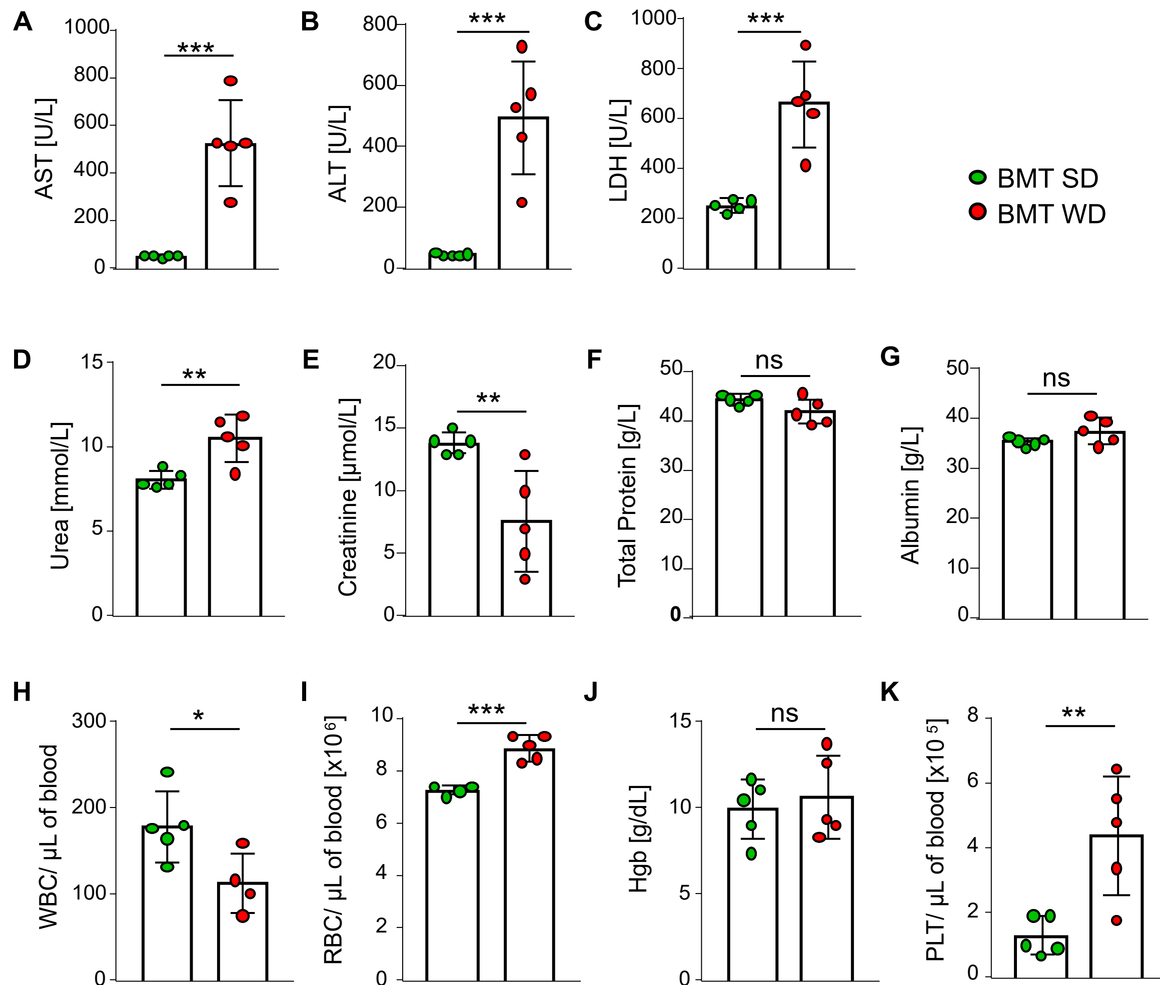

Figure 8.7: Systemic effects of short-term WD feeding during HCT. (A-G) Quantification of serum levels of AST, ALT, LDH, urea, creatinine, total protein, and albumin, n=5. (H-K) Analysis of complete blood count (CBC), n=5. Data are presented as a mean ± SD. Statistical significance was determined by an unpaired t-test. *p<0.05, **<0.01, ***<0,001, ns, non-significant. BMT: bone marrow plus T cells, WD: Western diet, SD: standard diet, AST: aspartate aminotransferase, ALT: alanine aminotransferase, LDH: lactate dehydrogenase, WBC: white blood cell, RBC: red blood cell, Hgb: haemoglobin, PLT: platelets.

Part IV

Discussion

This part is separated into two separate chapters composed of two different projects, discussing the obtained data and giving future perspectives separately

Chapter 9

Discussion I

GvHD still remains the main obstacle to successful HCT therapy. However, patients who survive more than two years post-HCT are considered to be at a high risk of CV morbidity and mortality, especially after allo-HCT [482, 7, 8]. Studies have shown that there is a prominent impact of allo-HCT and cGvHD on the onset of dyslipidaemia, hypertension, DM, and the incidence of metabolic syndrome, which are considered the main risk factors for severe CV morbidity [19, 20, 21, 22, 23]. On the other hand, allo-HCT was shown to be followed by a significant increase in endothelial damage depending on the preconditioning regimen in use, shown through the increased number of CEC [483]. As atherosclerosis is considered a chronic inflammatory disease whose onset is believed to start with the damage of the endothelial layer of blood vessels, it is inevitable to question the potential relation between allo-HCT and GvHD on one hand and atherosclerosis and severe CV post-HCT morbidity on the other. Our study aimed to address this interconnection.
We here propose a novel atherosclerosis- miHAg allo-HCT mouse model that enables monitoring of the development of both diseases. In this model (BALB/b (H-2b) $\rightarrow$ B6.LdLr$^{-/-}$ (H-2b)) we show that miHAg allo-HCT of BMT into atherosclerosis-prone mice fed WD increase serum cholesterol levels and the lesion formation in aortic arch and root even without the apparent clinical GvHD signs compared to BM transplant.
First, the very prominent impact that was noticed in this model was the impact of WD itself on the onset of GvHD, which was already suggested in the obesity model of previous studies [29]. Both groups fed WD, with or without the addition of T cells, showed similar levels in clinical GvHD scoring that slightly increases 20 days post-HCT. This trend points out the impact of WD on the development of aGvHD even when the feeding starts ten days post-allo-HCT, when irradiation-induced damage of intestinal barrier is subsided [484]. The WD impact is further discussed in the Chapter.10.
Atherosclerosis is mediated by an inflammatory cascade [485] - starting with increased expression of adhesion molecules on the surface of ECs [486] that stimulate recruitment of monocytes and T cells to sites of endothelial injury, secretion of different cytokines and chemokines that increase recruitment of additional leukocytes, their activation in the media, causing final recruitment and proliferation of SMCs [487]. Quantification of the total, as well as pro-inflammatory Ly6C^{high} and 'patrolling', Ly6C^{low}, monocytes showed no difference in both groups in BM as well as in blood, excluding the impact of alloreactivity on myelopoiesis and mobilization of monocyte in our model. Furthermore, there were no significant differences observed in the phenotype of the formed plaque (amount of SMCs, necrotic core or collagen).
The incidence of increased levels of CEC, was shown to coincide with an increase in the level of circulating alloreactive T cells [17] and T cell depletion, by which the allogenicity of the graft is reduced, leads to a decrease in the incidence of endothelial complications, suggesting the involvement of alloreactive T cells in the damage of the endothelium[151]. Our data goes along with the previous studies. We observed increased frequencies of

circulating alloreactive T cells in the blood of BMT recipients in comparison to BM. Moreover, infiltration of alloreactive T cells in inguinal LNs showed a similar trend as in the aorta, while there were no major differences observed in spleen or mesenteric LNs. High T cell infiltration of the aorta after BMT transplantation was followed by upregulation of CD44 expression by infiltrating $CD8^+$ but not $CD4^+$ T cells, suggesting a specific $CD8^+$ T cell accumulation in aorta after their activation.

In regard to GvHD, $CD8^+$ T cells were also shown to be involved in the loss of subcutaneous microvessels in patients suffering from cGvHD, which coincided with a high concentration of circulating vWF [14, 9]. Studies [346, 347] showed the presence of $CD8^+$ T cells in the plaque of carotid arteries in humans and the significant impact of $CD8^+$ T cells on the plaque size and the size of the necrotic core, suggesting their influence on apoptosis and instability of plaque as well as on myelopoiesis and monocyte numbers.

ScRNA-seq of $CD45^+$ aorta infiltrating cells revealed striking differences in expression of genes associated with T cell activation and cytotoxic effector functions present in $CD8^+$ T cells in BMT recipients, which could not be detected in $CD4^+$ T cells cluster. Studies of atherosclerosis have shown the specific phenotype of T cells infiltrating the lesions, pointing out the role of TNFRSF9 (or CD137) expression in the stimulation of atherosclerosis development and progression, especially through increased infiltration of $CD8^+$ T cells and elevated levels of pro-inflammatory cytokines [24, 25, 26]. Specifically, $CD137^+$ effector $CD8^+$ T cells were shown to promote lesion formation by stimulating infiltration of endogenous IFNγ-producing $CD8^+$ T cells, whereas CD137-deficient $CD8^+$ T cells showed reduced vessel wall infiltrating capability with minimal IFNγ production [351]. On the other hand, TNF receptor-associated factor 1 (Traf-1) was shown to be overexpressed in lesions, fibrous atheromatous, aneurysmal atherosclerotic lesions of carotid arteries, and in neointima formation after arterial injury [27, 28], and its deficiency led to significantly smaller atherosclerotic lesions after 8 or 18 weeks of high cholesterol diet, suggesting its pro-atherogenic role [384]. Gmzb and FasL are genes associated with the induction of apoptosis of target cells and they promote necrotic core formation and plaque vulnerability in atherosclerosis [346, 347]. All these above-mentioned genes exhibited upregulation in comparison to non-T cell transplant in our model, suggesting the responsible phenotype for the present increased lesion formation. T cells from human atherosclerotic plaque express high levels of PD-1, and in symptomatic CV patients, lesional effector memory T cells showed T cell exhaustion, as suggested by expression of PD-1 and lower levels of Prf [263]. Likewise, lesion infiltrating $CD8^+$ T cells in our model revealed slightly increased expression of Prf1, but a decrease in Gmzb, along with increased expression of thymocyte selection-associated high mobility group box (Tox) gene and gene encoding PD-1 protein (PDCD1), suggesting the beginning of an exhausted lesional $CD8^+$ T cell phenotype, that can be induced by environmental high cholesterol levels[488]. This specific phenotype of lesion-infiltrating $CD8^+$ T cells could promote enhanced atherosclerotic lesion formation in our GvHD-atherosclerosis model. Indeed, introduction of anti-CD8β antibody treatment once per week, along with the start of WD, ameliorated the BMT-allo-HCT-induced increase in atherosclerotic lesion formation, demonstrating the critical contribution of $CD8^+$ T cells to the observed phenotype. Improved plaque stability, shown through the trend towards increased plaque SMCs and a reduction in the size of necrotic core further emphasize the important role of $CD8^+$ T cell-driven cytotoxicity towards plaque cells in this mouse model. Interestingly, in ou hands, we did not notice any impact of $CD8^+$ T cell depletion on monopoiesis or the number of circulating monocytes, which might be explained by incomplete $CD8^+$ depletion (because of the prolonged injection time period, as mice might start producing antibodies against anti-CD8β depleting an-

tibody), but also with a different systemic cytokine environment present during GvHD, that is not only IFNγ dependant.

Tregs are consideed atheroprotective and low Tregs to CD4$^+$ T cell ratio correlate with the increased rate of CV events in patients [304]. Interestingly, in our mouse model we observed reduction in frequencies of Tregs in mesenteric and inguinal LNs, that could as an systemic effect also contribute to the increased aortic lesion formation.

A striking observation was a significant increase in cholesterol levels in BMT transplant in comparison to BM, which was reversed after CD8$^+$T cell depletion implying an impact of alloreactive T cells on the metabolism of lipids. As the earliest stages of atherosclerosis are initiated in response to turbulent flow in the unfavourable setting of high serum cholesterol levels, alloreactive T cell-induced hypercholesterolemia would be an additional risk factor post-HCT. Dyslipidaemia is a common post-HCT condition [166, 190] that is thought to be induced by liver cGvHD [189] and/or different immunosuppressive therapies used post-HCT for a prolonged period of time, such as sirolimus (mammalian target of rapamycin (mTOR) inhibitor) [177], CsA (calcineurin inhibitor) [183, 184] and glucocorticoids [188, 184]. Severe elevations in serum cholesterol levels during cGvHD of the liver are caused by the inability of bile salts and cholesterol to be cleared through the bile duct. Therefore, cholesterol is backed up into the serum and transported by abnormal lipoprotein particles called LpX, whose concentration is, in this case, also increased [189]. As the liver is one of the main GvHD target organs but also the organ responsible for the synthesis and regulation of circulating cholesterol levels, we assessed this organ for alloreactivity-induced damages. We observed a trend towards increased levels of transaminases in BMT transplant, but major pathological changes were not present on the tissue level, suggesting mild increase in liver damage. Cholesterol levels are controlled through de novo synthesis (regulated by sterol regulatory element-binding transcription factor 2 (SREBF2) and HMGCR), secretion in the form of bile acids (regulated by CYP7A1 and CYP27A1 that are responsible for bile acid synthesis and ABCG5 and ABCG8, regulating the secretion) and cholesterol efflux (regulated by ABCA1) and through intestinal reabsorption. The expression of main genes sensing a high level of oxysterols (LXR) and bile acid level (farnesoid X receptor (FXR)) did not differ, but subsequently regulated genes (HMGCR reductase and SREBF2) showed significant changes in their expression responding to the high circulating cholesterol levels, while genes regulating esterification (ACAT1), bile acid synthesis and secretion (CYP7A1, ABCG5) seemed to be upregulated, pointing out counter-regulatory mechanism in the sense of reducing cholesterol levels.

Increased expression of CYP7A1, as the main regulator of bile acid production, leads to increased production of 7α-hydroxycholesterol, one of the oxysterols identified in the human atherosclerotic aorta [489, 490], that was shown to cause foam cell formation from macrophages and lead to atherosclerosis in humans [491]. As CYP27A1, one of the important enzymes involved in the alternative pathway of bile acid production [445], remained the same in both groups, it is questionable if the subsequent metabolization of oxysterols such as 7α-hydroxycholesterol and bile acid synthesis are efficient. On the other hand, the bile acid pool was shown to be decreased during GvHD induction [492], which can be a reason for the unchanged expression of FXR, which then enables the increased function of CYP7A1. Lastly, cholesterol excretion into the bile is regulated by ABCG5 and ABCG8 transporters in the liver, where both of them are indispensable for the normal functioning of this process [493, 431]. Our data suggest upregulated expression of ABCG5, but no change in the ABCG8 expression, questioning its functionality and implying potential retention of cholesterol that overall can justify such increase that we observe in our model. In addition, development of lipodystrophy leads to increased cholesterol levels and was

suggested as a consequence of GvHD in the adipose tissue [494, 495] that could be additional reason for observed outcome. Quantification of bile acids as well as lipoproteins and levels of cholesterol synthesis metabolites would allow getting the more clear picture about the exact mechanism responsible for obtained phenotype.
However, the exact role of $CD8^+$ T cells in the context of hypercholesterolemia that we observed in our model still remains to be elucidated. FcγR2b receptors expressed in DCs were shown to be involved in the modulation of lipid metabolism in $Ldlr^{-/-}$ mice [496]. Mice lacking this receptor specifically in DCs, showed significantly decreased serum cholesterol levels. The expression of these receptors is also present on $CD8^+$ T cells, whose activity was shown to be increased after cholesterol deprivation in the tumor microenvironment [488]. Furthermore, genetic deficiency of Fcgr2b resulted in significantly reduced tumor burden with enhanced tumor-infiltrating $CD8^+$ T cell responses [497] pointing out the impact of environmental cholesterol levels on the function of $CD8^+$ T cells.
According to our knowledge, we are the first to show GvHD-dependent atherosclerosis phenotype in a mouse model, even though many clinical studies have shown an increased incidence of CV events post-allo-HCT [7, 16, 9, 22], pointing out the involvement of endothelial damage and CTLs co-localization in the pathogenesis [14, 128, 149, 498].
GvHD studies are usually involving few different models in order to confirm the obtained phenotype. Inclusion of additional miHAg-mismatched allo-HCT mouse model would improve the quality of this study. Major mismatch mouse models are more severe GvHD models, where monitoring of atherosclerosis is challenging as its induction requires many weeks of HFD feeding that increases the severity of GvHD and decrease mice survival.
Even though our study has limitations in translatability to clinical usage, it shows a very significant impact of GvHD on the severity of atherosclerosis. We could show that even when atherosclerosis is induced ten days after irradiation and transplantation, the consequence of high plaque formation emerges. It would be of immense importance to establish the model where atherosclerotic lesions would be already established before allo-HCT. The phenotype would be probably even more severe as WD was shown to induce 'trained immunity' where myeloid cell-induced innate immune responses remain augmented contributing to increased inflammatory responses [499].
Considering that the vast of patients subjected to allo-HCT are aged with already formed vessel lesions, it is of immense importance to find a way to decrease the number of risk factors as well as to control the growth and stability of already formed plaques. We suggest with this study that modulation of $CD8^+$ T cell response can be one of the ways to tackle this problem. However, further studies are needed to test the pros and cons, considering the important role that these cells play in gaining desirable GvL effect.

9.1 Schematic summary

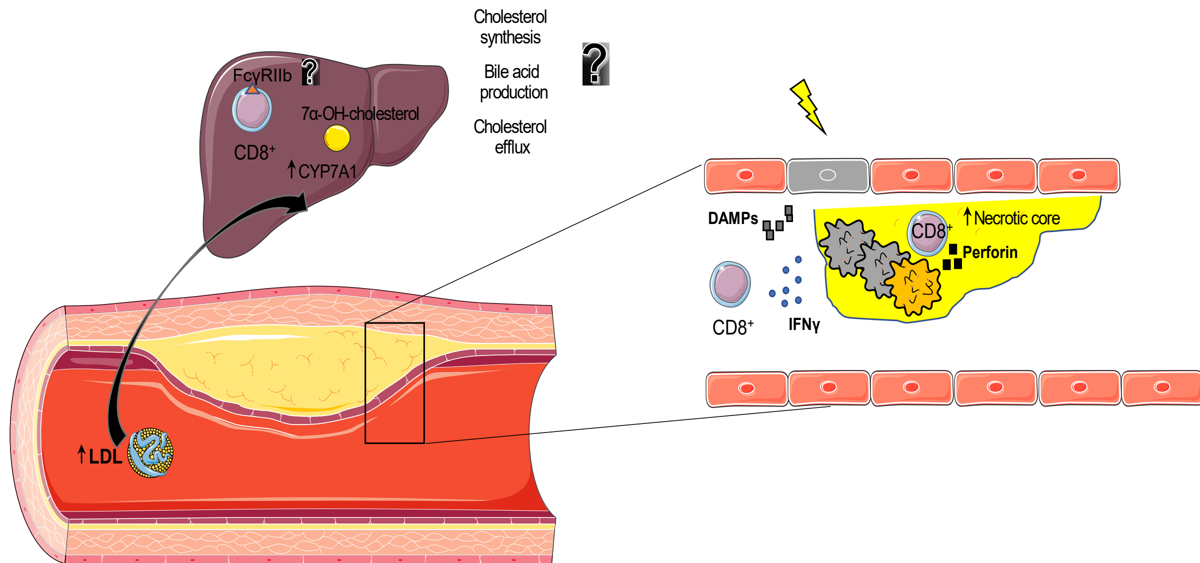

Figure 9.1: GvHD aggravate the severity of atherosclerosis. Scheme is made by using smart.servier.com. CYP7A1: cytochrome P450 family 7 subfamily A Member 1, DAMP: danger-associated molecular pattern, LDL: low-density lipoprotein.

Chapter 10

Discussion II

Chronic HFD feeding followed by obesity was shown to increase the risk of the onset of severe GvHD in mice and humans. The mechanism for such severe impact leans on the increased release of pro-inflammatory cytokines as well as on the changes of microbiome induced by the diet itself as an addition to already changed composition because of the preconditioning regimen [29]. This very elaborate study was one of the first done in mice, showing the prominent impact of obesity and HFD feeding on the development of GvHD and confirming the effect also in clinics. Keeping in mind how important impact diet has on the body's function in a steady state, let alone in the context of HCT, we addressed in this study the impact of short-term WD regimen on the onset of aGvHD. Of note, WD, that we used in our study, contains ≈21% of fat and is suggested as the representative amount used nowadays in the western world (in comparison to other studies using a diet enriched in fat and containing ≈60% of it, with the purpose of obesity induction).

We could show that WD has a detrimental impact on post-HCT recovery, which was alleviated by decrease in the concentration of transplanted alloreactive T cells. T cells are considered the main players in the effector phase of GvHD, which migrate to the target tissues, depending on the chemokine signals that they receive, where they induce tissue damage. WD seems to induce systemic inflammation by impacting the migration and activity of alloreactive T cells in SLOs. $CD4^+$ T cells seem to be the main T cell subset responsible for this phenotype, as we observed the trend towards their prevalence in SLOs, that was previously noticed also in an obesity-aGvHD model [29].

HFD changes the composition and decreases diversity of microbiome, leading to domination by a single taxa [500, 501] that, together with pre-conditioning-induced increase in gut permeability, lead to severe, specifically gut localized, aGvHD. Specific prevalence of Firmicutes, Proteobacteria and Bacteroidetes [502] promotes inflammatory environment in the gut and is caused by WD consumption, but also correlates with the incidence of aGvHD [501]. Dextran sulfate sodium (DSS)-induced colitis in mice receiving WD is associated with significantly greater body weight loss, presence of diarrhea, appearance of fecal blood, and presence of higher amount of pro-inflammatory cytokines, such as IL-6, CXCL1 and IL-1β in colonic mucosa compared with mice fed a conventional diet [502]. WD feeding for two weeks significantly increases susceptibility to oral L. monocytogenes infection at day 3 post-infection. The microbiome changes are followed by decreased expression of the short-chain fatty acids (SCFAs) receptor G-protein-coupled receptor 43 (GPR43) in colonic mucosal membranes, reduced production of SCFAs, and therefore alterations in Treg population in mesenteric LNs, but it also induces defects in Paneth cells leading to decreased production of antimicrobial peptides [502, 503, 504]. Taurocholate-induced necrotising pancreatitis in WD fed mice leads to increase in mortality rate, systemic inflammation and bacteria dissemination followed by a loss of microbiome diversity and an altered metabolic profile with butyrate depletion. Butyrate supplementation (both, oral and systemic) in this model decreased mortality, bacterial dissemination, and reversed

the microbiota alteration [505]. The treatment with GPR43 agonist prolongs survival and reduces GvHD in mice [506] and low concentrations of propionate and butyrate correlate with GvHD development [507]. In this context, it is worthwhile to quantify the abundance of microbiome produced metabolites, but also to pay closer attention to changes in colonic mucosal environment and microbiota composition in our model in order to get a further insight into the mechanism behind the present immune dysregulation.

Chronic HFD consumption is well known to induce endotoxemia [480]. After eight weeks of WD consumption defects in intestinal Paneth cells occur that are induced by enhanced signalling of FXR in myeloid cells and subsequent activation of type I IFN response and inhibition of any of these pathways prevents their dysfunction [503]. The transcription of genes encoding tight junction proteins, that can be used as an assessment of barrier function, is reduced in HFD-fed mice [504]. Levels of claudin-7, a protein involved in the formation of tight junctions, are reduced following 4 weeks HFD [508], whereas no changes in Zo-1 at 3 days [509] or 1 week [510] of HFD feeding are observed, suggesting time-dependent alterations. As pre-conditioning induced damages in the intestinal barrier also lead to the migration of microbiota, it would be worthwhile quantifying the LPS levels in serum, liver, and spleen as subsequent stimulation of APCs to secrete type I IFN and other pro-inflammatory cytokines, such as IL-12, indirectly stimulate and promote the survival of activated T cells [511] that seemed to be kept for a longer than expected in SLOs. On the other hand, LPS is recognized as the most potent microbial mediator implicated in the pathogenesis of sepsis and septic shock. The sudden release of large quantities of LPS into the bloodstream is deleterious to the host as it initiates the release of a dysregulated and potentially lethal array of inflammatory mediators and procoagulant factors in the systemic circulation generating diffuse endothelial injury, tissue hypoperfusion, disseminated intravascular coagulation and refractory shock [512].

We did not observe any difference in the intestinal permeability on day 2 post-HCT as an effect of WD, as well as in the abundance of bacteria infiltrating mesenteric LNs or ileum. However, day 3 post-HCT is possibly a late time point as ileum is the first location where bacteria migrate after post-conditioning. In addition, FITC-dextran shows overall permeability that is possibly too early to be expected only 3 days after the beginning of diet. HFD conditions were more specifically associated with paracellular permeability detected through the measurement of albumin leakage into the feces [513].

On the other hand, the relative number of neutrophils had risen on day 6 post-HCT, on the day when the majority of them had left to mesenteric LNs (as explained in [481]), and interestingly, these remaining subsets seem to upregulate Siglec F expression. The presence and the exact role of Siglec F$^+$ neutrophils have not been defined in the context of GvHD. However, they were observed in lung tumors and in the ischaemic heart after MI, and they are defined as a long-lived, mature subset that is highly phagocytotic, secrete ROS, and has pro-inflammatory function [514, 515, 516]. In contrast to other cells types, neutrophils release nuclear chromatin, called NETs into the extracellular space [517], whose role is to eliminate bacteria. However, it can induce excessive inflammation through the release of its intracellular components, such as nucleic acids, proteins, and proteases, which act as intrinsic ligands affecting natural immunity and tissue damaging enzymes [518].

Interestingly, independently on the day when the analysis was done post-HCT, the relative number of macrophages was significantly decreased when mice were fed with WD. It has been observed that reduction of the host macrophage pool in recipient mice leads to increased donor T cell expansion and aggravated GvHD mortality after allo-HCT and that pre-transplant administration of colony stimulating factor 1 (CSF-1) expands host

macrophage pool, reduces donor T cell expansion, and improves GvHD survival [519]. We could observe that under the WD treatment, these macrophages significantly upregulate expression of CD80 on day 3 and day 6 post-HCT, suggesting their prominent activity during initiation as well as effector phase that subsequently activate alloreactive T cells in small intestine on day 6 post-HCT. Furthermore, the expression of PD-L1 is specifically upregulated on day 6, which could suggest an attempt for immune regulation even with decreased numbers of macrophages on that day. At this point we could speculate that increased ROS production by neutrophils as well as overstimulation of TLRs by environmental increase in PAMPs and DAMPs could easily induce uncontrolled cell death of these macrophages. Components of WD, such as cholesterol or saturated fatty acids, induce activation of NLRP3 inflammasome through deposition of cholesterol crystals which induce lysosomal damage or through cytotoxic effects [520] and induction of ER stress [521], respectively, that leads to pyroptosis.

Spleen has been shown to be the only indispensable organ responsible for the onset of intestinal aGvHD, as mice deficient in the lymphotoxin-alpha chain (B6.LT$\alpha^{-/-}$) that do not develop SLOs, but only morphologically altered spleen managed to obtain lethal aGvHD, while splenectomy in these mice, B6.LT$\alpha^{-/-}$, saved mice from GvHD onset by day 6 [522]. Increased T cell infiltration of spleen that is present in our model go along with severe aGvHD. Moreover, unexpected morphological changes as well as the significant inflammatory conditions present in spleen of WD fed mice, followed by striking increase in blood levels of transaminases and LDH could additionally support the speculated uncontrollable activation of programmed cell death pathways. As liver is the first organ to encounter not only absorbed nutrients but also gut-derived PAMPs (as majority of the venous blood from GIT is drained into the portal circulation) it is not surprising that liver diseases are associated with increased intestinal barrier permeability [523, 524]. Short-term (4-14 days) HFD feeding revealed increased hepatic expression of IFNγ, TNFα, IL-10, monocyte chemoattractant protein-1 (MCP-1), TF, PAI-1 mRNAs, and fibrin/fibrinogen deposition in the liver tissues [525]. The same study showed that HFD feeding led to a significantly increased response to concanavalin A (ConA)-induced acute liver injury suggesting that metabolic alterations and ER stress induced by the HFD intake were associated with the pro-inflammatory and pro-coagulant states in the liver that increases its susceptibility to circulating inflammatory stimuli [525]. Our data goes along with this phenotype of increased susceptibility; therefore, further studies should be pursued to confirm and elucidate the involvement of liver inflammation and pro-inflammatory and pro-coagulant state in the context of WD impact on aGvHD. PLTs are considered a prognostic biomarker during GvHD, as patients with thrombocytopenia have a poor survival rate [526, 527]. As the spleen and liver are the main organs responsible for the regulation of RBCs and PLTs, mainly their disposal, it is inevitable to question whether observed changes in the spleen and liver are responsible for such unregulated blood cell numbers when short-term WD is consumed.

Lastly, WD consumption is associated with increased risk of chronic kidney disease as defined by a rapid decline in glomerular filtration rate (GFR) and/or the presence of moderate to severely increased levels of urine albumin excretion. However, dietary practices alone are usually not sufficient to induce kidney damage. Kidney is also considered as non-classical GvHD target organ [528]. Infiltration of donor T cells was observed in the kidney [529] and induction of aGvHD caused glomerular injury and tubulointerstitial nephritis, which was mediated by activation of the complement system [530]. In our aGvHD model, serum levels of urea were increased suggesting an insufficient function of the kidneys. However, creatinine levels were decreased that could be explained by de-

crease in the weight of mice fed WD, as muscle volume also decreases. Studies suggest that WD (considered as a high protein/low vegetable diet) drives a surplus of acid production inducing a slight degree renal-compensated metabolic acidosis [531, 532]. Furthermore, decreased kidney function leads to reduced acid excretion. Metabolic acidosis leads to changes in levels of electrolytes, such as hypocalcemia and hypocitraturia, but it also impact muscle metabolism, leading to the muscle loss. Such a state could be a reason for decreased survival in our animals, therefore the thorough analysis of electrolytes and acid-base state should be considered.

So far, we could suggest that WD, even in a short-term consumption period, has a detrimental role in GvHD-associated mortality. However, it is still not clear what is the main mechanism by which this severe impact of WD occurs. Additional experiments are needed in order to elucidate the exact mechanism behind this phenotype.

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LIST OF PUBLICATIONS

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AFFIDAVIT

I hereby confirm that my thesis entitled "Elucidating the interconnection of GvHD and Western diet-induced atherosclerosis" is the result of my own work. I did not receive any help or support from commercial consultants. All sources and/or materials applied are listed and specified in the thesis. Furthermore, I confirm that this thesis has not yet been submitted as part of another examination process neither in identical nor in similar form.

Place, Date **Signature**

EIDESSTATTLICHE ERKLÄRUNG

Hiermit erkläre ich an Eides statt, die Dissertation „Aufklärung des Zusammenhangs von GvHD und durch westliche Ernährung induzierter Atherosklerose." eigenständig, d.h. insbesondere selbständig und ohne Hilfe eines kommerziellen Promotionsberaters, angefertigt und keine anderen als die von mir angegebenen Quellen und Hilfsmittel verwendet zu haben. Ich erkläre außerdem, dass die Dissertation weder in gleicher noch in ähnlicher Form bereits in einem anderen Prüfungsverfahren vorgelegen hat.

Ort, Datum **Unterschrift**