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DISSERTATION

The vasculature and immune cell reconstitution of the bone marrow niche after allogeneic hematopoietic stem cell transplantation

Das Gefäßsystem und die Immunzellrekonstitution in der Knochenmarksnische nach allogener hämatopoetischer Stammzelltransplantation

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aGVHD	acute graft-versus-host disease
AID	autoimmune disorders
ALL	acute lymphoblastic leukemia
allo	allogeneic
AML	acute myeloid leukemia
APC	antigen-presenting cell
APCy	allophycocyanin
B6	C57BL/6
BDF	B6D2F1
BM	bone marrow
BMF	bone marrow failures
BMT	bone marrow transplantation
BSA	bovine serum albumin
Ca^{2+}	Calcium
CAG-DsRed	cytomegalovirus–actin–globin promoter red fluorescent protein
CAR	chimeric antigen receptor
CD	cluster of differentiation
cGVHD	chronic graft-versus-host disease
cGy	centigray
CI	confidence interval
CLL	chronic lymphoblastic leukemia
CML	chronic myeloid leukemia
CMV	cytomegalovirus
CTL	cytotoxic T lymphocyte
CXCL12	CXC-chemokine ligand 12
Cy	cyanine dye
DAPI	4',6-Diamidin-2-phenylindole
DC	dendritic cell
Diff	difference
DNA	deoxyribonucleic acid
DsRed	Actb-DsRed.T3
EBMT	European Society for Blood and Marrow Transplantation

EC	endothelial cell
ECM	extracellular matrix
EDTA	ethylenediaminetetraacetic acid
ELN	European LeukemiaNet
FACS	florescence-activated cell sorting
FITC	fluorescin isothiocyanate
G-CSF	granulocyte-colony stimulating factor
GIT	gastrointestinal tract
GVHD	graft-versus-host disease
GVL	graft-versus-leukemia
H-2	histocompatibility 2
HG	hemoglobinopathies
HL	Hodgkin's lymphoma
HLA	human leukocyte antigen
HSC	hematopoietic stem cell
HSCT	hematopoietic stem cell transplantation
HSPC	hematopoietic stem and progenitor cell
i.v.	Intravenous/ intravenously
ICAM	intracellular adhesion molecule
IDM	inherited diseases of metabolism
IFNy	interferon y
Ig	immunoglobulin
IL	interleukin
ITIM	immunoreceptor tyrosine-based inhibitory motifs
LSK	$Lineage^{-}Sca\text{-}1^{+}c\text{-}Kit^{+}$
M	mean
MACS	magnetic activated cell sorting
MD	difference between means
Mdn	median
MDS	myelodysplastic syndrome
mem	memory
Mem B cells	memory B cells
MHC	major histocompatibility complex

MiHA	minor histocompatibility antigen
MPN	myeloproliferative neoplasm
NHL	Non-Hodgkin's lymphoma
PB	pacific blue
PBS	phosphate-buffered saline
PBSC	peripheral blood stem cell
PCD	plasma cell disorder
PE	phycoerythrin
PECAM	platelet endothelial cell adhesion molecule
PerCP	peridin chlorophyll protein complex
PFA	paraformaldehyde
PID	primary immune disease
PVP	polyvinylpyrrolidone
SD	standard deviation
SHP-2	Src homology 2 domain-containing phosphatase
syn	syngeneic
TBI	total body irradiation
Tcm	central memory T cells
Tem	effector memory T cells
Tn	naive T cells
TNFα	tumor necrosis factor α
vWF	von Willebrand Factor
w/o	without
WT	wildtype
XRT	radiation therapy

1 ABSTRACT

For a variety of malignant diseases of the hematopoietic system, allogeneic hematopoietic stem cell transplantation (allo-HSCT) is the only curative treatment option. Unfortunately, more than half of the patients receiving allo-HSCT die within the first two years. A major reason for mortality after allo-HSCT is acute graft-versus-host disease (aGVHD), a systemic inflammatory disease in which activated donor T cells damage the host tissue. Patients suffering from aGVHD often develop severe prolonged cytopenia with serious complications such as heavy bleedings, fatal infections, and tumor relapse. This association suggests that the bone marrow (BM) niche, which is critical for hematopoiesis, plays a decisive role in the outcome after allo-HSCT. However, little is known about the role of the BM niche as an aGVHD target tissue, and the underlying pathophysiology of aGVHD-mediated BM niche damage is unclear.

This study aimed to unravel the influence of aGVHD on the BM vasculature and BM immune cell reconstitution as selected BM niche features. For this purpose, a clinically relevant allo-HSCT model for BM aGVHD based on the transplantation of purified hematopoietic stem cells ($Lineage^{-}Sca\text{-}1^{+}c\text{-}Kit^{+}$ cells) was established. Experimental allo-HSCT led to profound engraftment and induced typical features of aGVHD. The descriptive overview of the above-mentioned BM niche characteristics revealed an increased BM vessel density at day+20 after allo-HSCT and a typical delayed immune cell reconstitution in the BM of allo-HSCT recipients during aGVHD. Further, the effect of $CD31^{+}$ cells as a therapeutic approach to aGVHD clinics, BM vessel density, and BM immune cell reconstitution after allo-HSCT was tested. The intravenous $CD31^{+}$ cell transfer resulted in improved clinical appearance shown by lower aGVHD scores at day+23 after allo-HSCT, and alterations of the BM immune cells towards reduced $CD4^{+}$ T cells as well as $CD4^{+}$ effector memory T cells.

The findings obtained underline that aGVHD affects the BM and add preliminary data of $CD31^{+}$ cell transfer as a possible therapeutic option for aGVHD. This study provides the foundation from which further development, such as mechanistic analyses as well as translational development of therapeutic approaches, can progress.

1.1 Zusammenfassung

Für eine Vielzahl von bösartigen Erkrankungen des hämatopoetischen Systems stellt die allogene hämatopoetische Stammzelltransplantation (allo-HSZT) die einzige kurative Behandlungsmöglichkeit dar. Leider sterben mehr als die Hälfte der Empfänger einer allo-HSZT innerhalb der ersten zwei Jahre. Ein Hauptgrund für die Mortalität nach allo-HSZT ist die akute graft-versus-host Krankheit (aGVHD). Die aGVHD ist eine systemische Entzündungskrankheit, bei der aktivierte T-Zellen des Spenders das Wirtsgewebe schädigen. Patienten, die an aGVHD leiden, entwickeln häufig eine schwere, langanhaltende Zytopenie mit erheblichen Komplikationen wie starken Blutungen, fatalen Infekten und Tumorrezidiven. Diese Assoziation deutet darauf hin, dass die Knochenmarksnische, welche von wesentlicher Bedeutung für die Hämatopoese ist, eine entscheidende Rolle für das Ergebnis nach allo-HSCT spielt. Über die Bedeutung und Wirkung der Knochenmarksnische als aGVHD-Zielgewebe ist jedoch wenig bekannt, und die zu Grunde liegende Pathophysiologie der aGVHD-vermittelten Knochenmarksnischenschädigung ist unklar.

Ziel dieser Studie war es, den Einfluss der aGVHD auf die Gefäßdichte und die Immunzellrekonstitution im Knochenmark (KM) als ausgewählte KM-Nischenmerkmale zu untersuchen. Hierfür wurde zunächst ein klinisch relevantes allo-HSZT-Modell für die KM aGVHD etabliert, welches sich durch die Transplantation von aufbereiteten hämatopoetischen Stammzellen (Lineage$^-$Sca-1$^+$c-Kit$^+$ Zellen) auszeichnet. Die experimentelle allo-HSZT führte zu einem sicheren Engraftment und induzierte typische Merkmale der aGVHD. Die Aufstellung eines beschreibenden Überblicks über die genannten KM-Nischenmerkmale ergab eine erhöhte KM-Gefäßdichte an Tag+20 nach allo-HSZT und eine typisch verzögerte Immunzellrekonstitution im KM von allo-HSZT Empfängern während der aGVHD. Darüber hinaus wurde der Einfluss von CD31$^+$ Zellen als therapeutischer Ansatz auf die aGVHD Klinik, die KM-Gefäßdichte und die KM-Immunzellrekonstitution nach allo-HSZT getestet. Der intravenöse Transfer von CD31$^+$ Zellen führte zu einem verbesserten klinischen Erscheinungsbild, das sich durch niedrigere aGVHD Scores an Tag+23 nach allo-HSZT darstellte, zu reduzierten CD4$^+$ T Zellen, sowie reduzierten CD4$^+$ T Effektor-Gedächtnis Zellen.

Die gewonnenen Erkenntnisse unterstreichen, dass die aGVHD das KM beeinflusst und zeigen erste Daten von einem CD31$^+$ Zell-Transfer als möglicher Therapieoption. Diese Studie liefert die Grundlage, auf der weitere Entwicklungen, wie mechanistische Analysen sowie die Entwicklung von therapeutischen Ansätzen, entstehen können.

2 INTRODUCTION

2.1 Allogeneic hematopoietic stem cell transplantation

Allogeneic hematopoietic stem cell transplantation (allo-HSCT) is an established procedure to replace hematopoietic stem cells (HSCs) and the immunological repertoire of a recipient by the transplant of the donor.(1) Allo-HSCT provides a crucial curative treatment for a variety of malignant and nonmalignant hematologic diseases, solid tumors, autoimmune disorders, amyloidosis, and inherited genetic hematological disorders.(2) Over the last years, allo-HSCT has seen rapid expansion (Figure 1A). Reasons for the increased numbers of allo-HSCT are, inter alia, technological evolution, extension of HSC sources, new conditioning regimes, and novel indications.(3) These achievements have also changed the donor type distribution towards unrelated and haploidentical transplantations (Figure 1B).

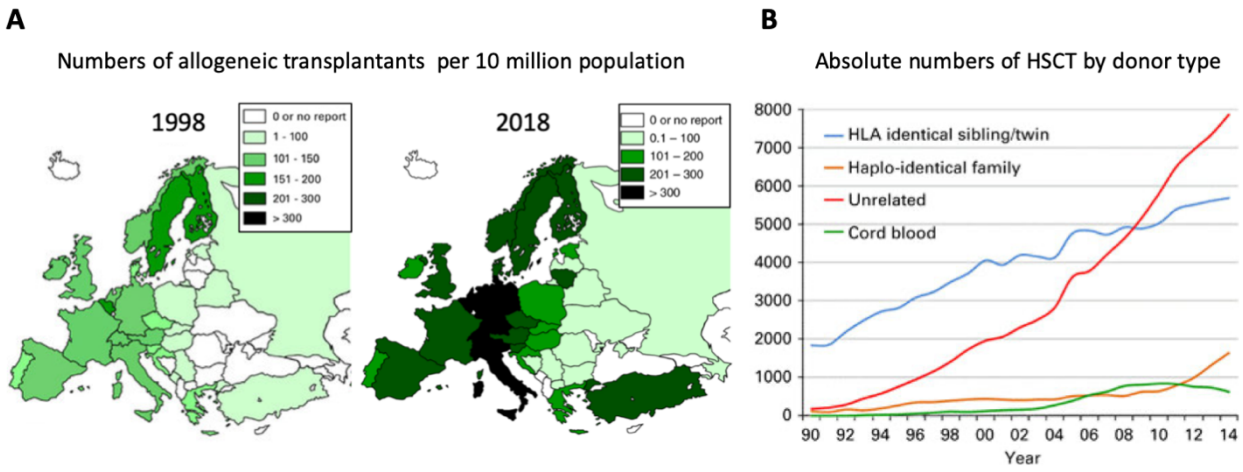


Figure 1. Numbers of allo-HSCTs performed over the last years in Europe. **(A)** Allogeneic transplant rates in Europe per 10 million population in 1998 and 2018. Modified according to Passweg et al., 2016,(4) 2020.(5) **(B)** Development of HSCTs by donor type in Europe 1990 – 2014. HLA: human leukocyte antigen. From Passweg et al., 2016.(4)

The therapeutic success of allo-HSCT is based on the intensive conditioning regimen and the antileukemic property of the graft itself (graft-versus-tumor effect).(6) Unfortunately, more than half of the patients receiving allo-HSCT die within the first two years. Major reasons for mortality after allo-HSCT are acute graft-versus-host disease (aGVHD), tumor relapse, and infections.(2)

2.1.1 History of allo-HSCT

The history of allo-HSCT dates back to World War II and the detonation of nuclear weapons in Nagasaki and Hiroshima.(6) Bone marrow (BM) failures of survivors led to experimental attempts to protect the BM and resulted in the development of allo-HSCT as a treatment for leukemia.(7)

The first successful allo-HSCT in mice was reported in 1956.(8) Only one year later, Thomas et al. reported treatment of acute leukemia in humans by supralethal irradiation and BM infusion from cadavers.(9) However, until 1969 all allo-HSCTs had resulted in no long-term survivors, and until the discovery of human leukocyte antigen (HLA) typing, no allo-HSCT could be translated into clinical use. Since then allo-HSCT has evolved from a highly experimental technique to the standard care for many hematological diseases and pioneered the concepts of stem cell therapy and immunotherapy as valuable tools against cancer (Figure 2).(6)

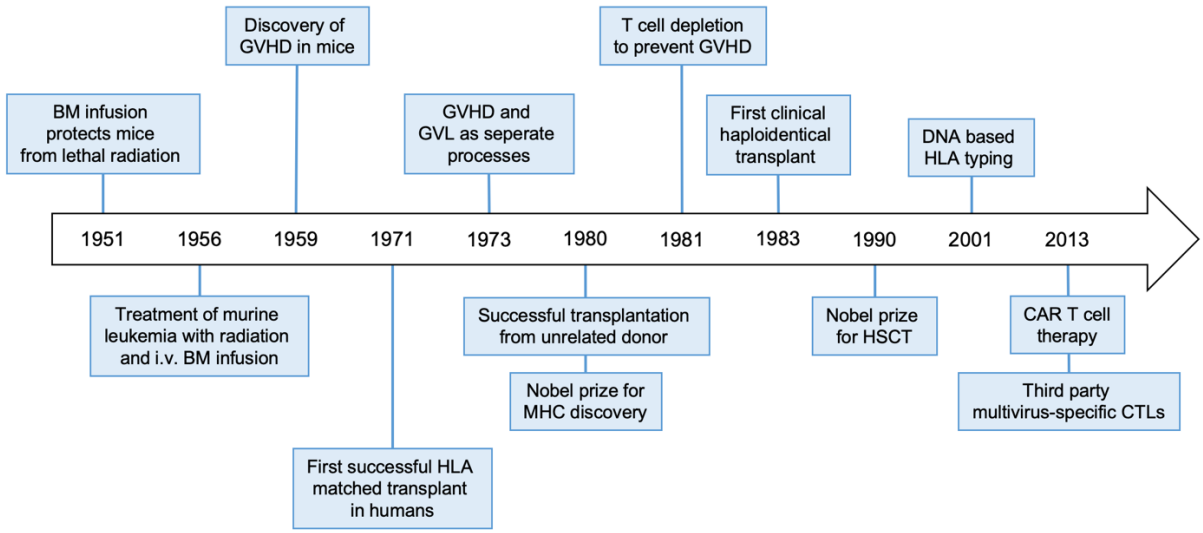
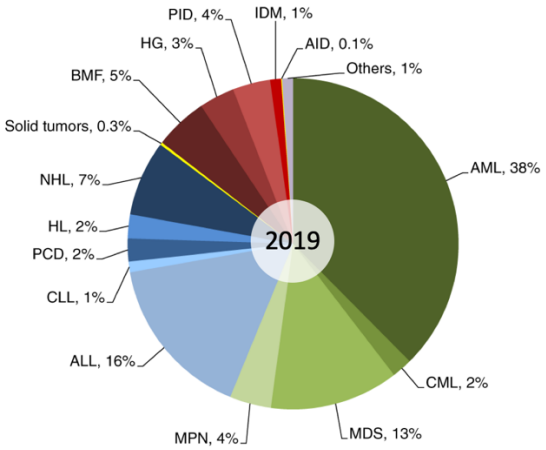


Figure 2. Milestones in allo-HSCT. BM: bone marrow, CAR: chimeric antigen receptor, CTL: cytotoxic T lymphocytes DNA: deoxyribonucleic acid, GVHD: graft-versus-host disease, GVL: graft-versus-leukemia, HLA: human leukocyte antigen, HSCT: hematopoietic stem cell transplantation, MHC: major histocompatibility complex. Own representation based on Singh et al., 2016.(6)

2.1.2 Indications for allo-HSCT

The main indications for allo-HSCT remain hematologic malignancies and predominantly acute myeloid leukemia (Figure 3).(5) Alternative treatment options have partly replaced allo-HSCT for some diseases (e.g. chronic myeloid leukemia and chronic lymphoblastic leukemia), but for other indications, allo-HSCT numbers continue to increase (Figure 3).(10-14)



	proportion of allo-HSCT over time		
	2019	2007	1997
AML	38	32.5	24.5
CML	2	4.3	25.3
MPN + MDS	17	13.4	6.8
ALL	16	17.7	20.0
CLL	1	3.2	0.7
PCD	2	5.0	3.2
HL	2	2.8	0.7
NHL	7	8.5	4.8
solid tumors	0.3	0.6	0.3
BMF	5	5.2	5.0
HG	3	2.5	2.6
PID	4	2.7	1.7
IDM	1	0.8	2.8
AID	0.1	0.1	0.1
Others	1	0.7	1.6

Figure 3. Relative proportion of indications for allo-HSCTs in Europe. AID: autoimmune disorders, ALL: acute lymphoblastic leukemia, AML: acute myeloid leukemia, BMF: bone marrow failures, CLL: chronic lymphoblastic leukemia, CML: chronic myeloid leukemia, HG: Hemoglobinopathies (thalassemia and sickle cell disease), HL: Hodgkin's lymphoma, IDM: inherited diseases of metabolism, MDS: myelodysplastic syndrome, MPN: myeloproliferative neoplasm, NHL: Non-Hodgkin's lymphoma, PCD: plasma cell disorders, PID: primary immune diseases. Modified according to Passweg et al., 2019,(15) 2020.(5)

2.1.3 Immune biology of allo-HSCT

The HLA genes on chromosome 6p21 encode the human major histocompatibility complex (MHC) and are inherited from each parent as a haplotype in classical Mendelian fashion.(16) HLA-typing is the basis for donor selection and compares HLA-A, -B, -C, -DR, and to a lesser extent -DQ as the most important genes for transplantation.(17) 8/8 or 10/10 is considered as a matched donor. HLA-identical sibling donor transplantations remain the gold standard,(18) but due to growing BM donor registries, the rates of HLA matched unrelated donor transplantations have overtaken those of matched related donor transplantations (Figure 1B).(4) Clinical studies have demonstrated no differences in overall survival between matched related or matched unrelated donor transplantations.(18, 19) However, the likelihood of finding an optimal matched unrelated donor varies among racial and ethnic groups.(20) While for Caucasians the probability is given by 75%, ethnic minorities have only a 20% chance of finding a matched unrelated donor in the required time period.(21) When a fully matched donor (either matched related or matched unrelated donor) is not available, haploidentical transplantations provide plausible alternatives.(22) Haploidentical transplantations refer to any family members with two or more loci mismatch within the loci HLA-A, -B, -C, -DR, and -DQ in graft versus host and/or host versus graft direction.(13) Given the mismatches, haploidentical transplantations carry a higher risk for graft failure and graft-versus-host disease (GVHD).(23, 24) Yet an intensified conditioning regimen as well as advances in the T cell depletion of grafts have made this practice a clinical reality that provides similar outcomes to matched unrelated donor transplantations.(25-28)

Haploidentical transplantations have become a rapidly rising procedure, and the rates are predicted to increase even further as falling fertility rates reduce the chances to find a matched related donor in the future.(29)

2.1.4 Requirements for allo-HSCT and allo-HSCT implementation

Central to the preparation of the recipient is the conditioning therapy by a combination of chemotherapy, total body irradiation (TBI), and/or immunotherapy. The primary goals of conditioning are: 1) immunosuppression to decrease the risk of rejection, 2) antileukemic activity to eradicate the disease, and 3) myeloablation to create a space for donor HSCs to engraft.(25) Each indication, as well as the patient's constitution, requires different weighting of the listed goals to attain the individual best-case scenario. Unfortunately, apart from the beneficial effect, conditioning leads to direct apoptotic cell damage of epithelia and other tissues, as well as to unspecific inflammatory activation and thus directly contributes to dreaded complications after allo-HSCT.(25)

The HSCs used for allo-HSCT are characterized by the expression of cluster of differentiation (CD) 34 paired with the absence of CD38 and known myeloid and lymphatic markers (Lineage-negative).(25) Their ability of homing, engraftment, self-renewal, and differentiation into all hematopoietic lineages is the basis for the success of allo-HSCTs. Donor HSCs can be obtained from the BM, the peripheral blood, or the umbilical cord. Nowadays, granulocyte-colony stimulating factor (G-CSF) mobilized peripheral blood stem cells (PBSCs) have become the predominant graft source, constituting worldwide 65% of all allo-HSCTs.(30)

After intravenous (i.v.) transplantation, the donor HSCs migrate to the BM and initiate hematopoiesis. Allo-HSCT leads to a hematopoietic transient mixed and, consequently, full donor chimerism with central tolerance between graft and recipient.(25) The recovery of the BM aplasia is of utmost importance for the overall survival after allo-HSCT.(31) The restoration of hematopoietic cells usually follows predictable kinetics post-transplant: Reconstitution of the innate immunity out of myelomonocytic progenitor cells occurs rapidly within 20-30 days post allo-HSCT, followed by reconstituted platelets and erythrocytes.(31) Reconstitution of the adaptive immunity arising from lymphoid progenitor cells requires several months up to one year after allo-HSCT and even after years there is often incomplete recovery.(31, 32)

2.1.5 Barriers to allo-HSCT

Despite constant therapy improvement, allo-HSCT still harbors significant complications, and more than half of the patients receiving allo-HSCT die within the first two years.(2, 33, 34) Prolonged post-transplant cytopenia and aGVHD remain the predominant problems after allo-HSCT. Due to their critical importance, they will be described in detail in chapters 2.2 and 2.3. The toxicity of conditioning regimens and the use of immunosuppressive agents may lead to further associated complications. In the short-term, this explicitly includes endothelial damage and a resultant transplant-associated microangiopathy.(25, 35, 36) In the long-term, nearly all tissues can be damaged, but the bone has turned out to be the most frequently affected: One-third of allo-HSCT patients develop substantial bone damage (osteopenia and osteoporosis).(25, 37, 38) These associated complications promote enduring cytopenia and aGVHD progression. Further common complications after allo-HSCT are secondary malignancies and a reduced quality of life.(39, 40)

2.2 Cytopenia after allo-HSCT

The temporary or long-term decrease of one or more blood cell lineages after allo-HSCT plays a significant role in major concerns such as fatal infections, tumor relapse, and serious bleedings.(41) Cytopenia is not only attributed to the conditioning regimen, but also to stem cell course and dose, graft manipulation, microangiopathy, alloimmunization and the development of aGVHD.(31, 42-45) Particularly leukopenia has emerged as a general concern in allo-HSCTs, as the timing and quality of the immunological reconstitution have profound impacts on morbidity and mortality post-transplantation.(7, 46-48) Immune deficiencies during the reconstitution phase are associated with a high risk for life-threatening bacterial, viral, fungal, and protozoic infections.(25, 49, 50) According to the rebuilding kinetics of the innate and adaptive immunity, the host defense weakness can be divided into three phases (Figure 4):(31)

1) The pre-engraftment phase (<30 days post allo-HSCT) is characterized by the conditioning-dependent neutropenia and holds the risk for bacterial and fungal infections.(31, 51-53)

2) In the early post-engraftment phase (30-100 days post allo-HSCT), the weakened cellular immunity (particularly B and T cells) and aGVHD-induced risks can lead to fatal viral and fungal infections.(49, 51, 52, 54)

3) In the late post-engraftment phase (>100 days post allo-HSCT) encapsulated bacteria and reactivation of previous viral infections are significant risk factors.(49, 55)

In the long term, persistent immunodeficiency also contributes to tumor relapse due to an insufficient graft-versus-tumor effect.(56)

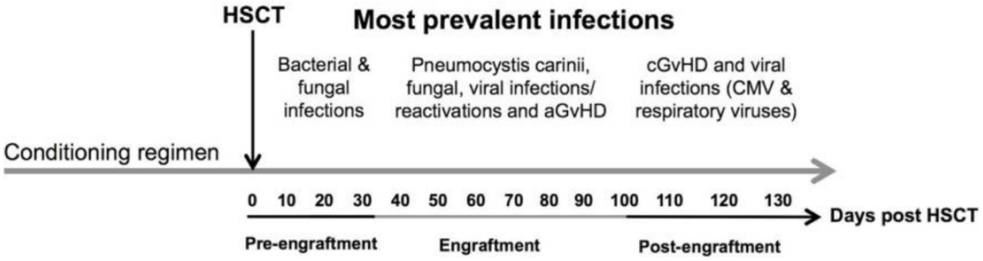


Figure 4. Timeline of the most prevalent infections after allo-HSCT. CMV: cytomegalovirus, aGVHD: acute graft-versus-host disease, cGVHD: chronic graft-versus-host disease. From Ogonek et al., 2016.(31)In addition to the dreaded leukopenia, thrombocytopenia and anemia are frequent issues after allo-HSCT. Thrombocytopenia is associated with an increased risk of serious bleeding and the consumption of significant resources.(57) Post-transplant anemia (often autoimmune hemolytic anemia) rarely causes death, but is associated with other complications and considerable morbidity.(58, 59)

2.3 Graft-versus-host disease

Despite advances in prophylaxis and treatment, GVHD remains a challenging issue after allo-HSCT.(60) GVHD mortality is still high and limits the use of allo-HSCT therapy.(61) The HLA disparity between donor T cells and recipient tissues is the key factor for GVHD development (62, 63); however, genetic differences outside the MHC loci (known as minor histocompatibility antigens (MiHAs)) can also induce severe GVHD.(7, 64, 65) Given the current trend, matched unrelated donor and haploidentical transplantations are expected to significantly increase within the next years, which in turn is predicted to provoke a considerable rise in patients with GVHD.(61)

Clinical GVHD occurs in acute and chronic forms with differences in pathophysiology and clinical development. This work will only center on acute GVHD (aGVHD). aGVHD is a systemic inflammatory disease caused by alloreactive T cells attacking the host tissue. The skin, liver, and gastrointestinal tract (GIT) are the principal target organs of aGVHD, which can be affected to varying degrees.

2.3.1 Pathophysiology of aGVHD

Extensive previous research has proposed that aGVHD development includes five basic steps (Figure 5).(7, 62, 66)

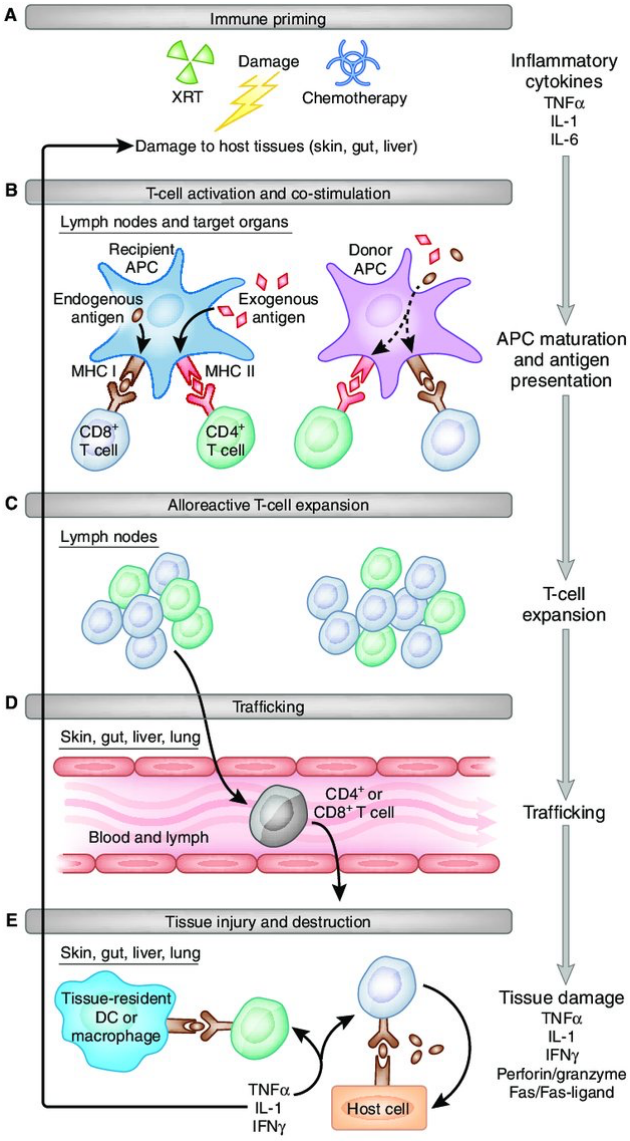


Figure 5. Illustration of the 5-step cascade of aGVHD. APC: antigen-presenting cell, DC: dendritic cell, IFNy: interferon y, IL-1: Interleukin-1, TNFα: tumor necrosis factor α, XRT: radiation therapy. From Schroeder et al., 2011.(110)

1) Priming of the immune response: Tissue damage by conditioning results in the release of a pro-inflammatory cytokine storm, which in turn promotes activation and maturation of antigen-presenting cells (APCs) and rapid amplification of donor T cells.(67)

2) T cell activation and costimulation: Donor T cells proliferate and differentiate in response to T cell receptor activation by minor and MHC antigens presented by host or graft APCs.(68, 69) The T cell activation is augmented by increasing expression of costimulatory molecules in response to the cytokine storm of the first stage.(70, 71)

3) Alloreactive T cell expansion and differentiation: Alloreactive T cells undergo expansion and differentiation into $CD4^+$ T helper and $CD8^+$ cytotoxic cells.

4) Activated T cell trafficking: Activated cells migrate into aGVHD target tissues and recruit other effector leukocytes.(72)

5) Destruction of the target tissue: Cytotoxic cells destroy tissues via cell surface and soluble immune effector molecules. Increased inflammatory signals perpetuate and augment the disease process by contributing to the cytokine storm that fuels aGVHD.(7, 73) However, alloreactive T cells also attack the tumor and induce the beneficial graft-versus-tumor effect.(74, 75)

Besides these basic steps, it has been recognized that the endothelium plays an important role in the pathophysiology of aGVHD.(76-78) Endothelial injury by the conditioning regimen, the pro-inflammatory agents used during transplantation, the translocation of endotoxins across the damaged GIT, and the engraftment(36, 79) has been proposed as a significant contributor to the pro-inflammatory, pro-coagulant and pro-apoptotic phenotype after allo-HSCT, which further stimulates the aGVHD cascade.(35, 78, 80, 81) Recent studies have described endothelial involvement and angiogenesis in aGVHD as a sequence of endothelial damage by conditioning, neovascularization and alloreactive T cells.(78, 82) The association of aGVHD and the formation of new blood vessels was confirmed in murine as well as human biopsies by an increased vessel density in the skin, liver and GIT.(83-86)

2.3.2 Clinical manifestations of aGVHD

The skin, liver, and GIT are the principal target organs of aGVHD. Most commonly, the skin becomes clinically apparent first and presents a characteristic maculopapular exanthema with subsequent blistering and epidermolysis.(61, 87) Hepatic aGVHD is manifested by an isolated hyperbilirubinemia and elevated alkaline phosphatase in peripheral blood testing.(61, 87) Clinical manifestations of gastrointestinal aGVHD include secretory diarrhea, abdominal pain, nausea, and anorexia.(7, 88) An organ stage is defined by the extent of damage to the individual organ, and the overall clinical grades result from the combination of organ stages (Table 1). The overall clinical aGVHD grades are closely associated with the prognosis of transplant-related mortality and overall survival.(61) While grades III and IV still carry dismal prognoses with 25% and 5% long-term survival,(89) in grade I the chances for overall survival are better due to less immunosuppressive treatment and a subsequently decreased relapse risk.(90, 91)

Table 1. *Organ staging and overall clinical grading of aGVHD.*

Clinical stage	**Skin**	**Liver-Bilirubin (mg/dl)**	**Gut**
1	Rash <25% body surface	2-3	Diarrhea 500-100 ml/d
2	Rash 25-50% body surface	3-6	Diarrhea 100-1500 ml/d
3	Generalized erythroderma	6-15	Diarrhea >1500 ml/d
4	Desquamation and bullae	> 15	Ileus
Overall clinical grade	**Skin stage**	**Liver stage**	**Gut stage**
I	1-2	0	0
II	1-3	1	1
III	1-3	2-3	2-3
IV	2-4	2-4	2-4

Note 1. aGVHD: acute graft-versus-host disease. From Iravani et al., 2005.(92)

Recent work has postulated additional targets of aGVHD: The lung,(87, 93, 94) the thymus,(95, 96) and the BM niche (25, 97, 98) were shown to be infiltrated, attacked and damaged by alloreactive T cells.

2.3.3 Prophylaxis and treatment of aGVHD

All patients undergoing allo-HSCT receive prophylactic treatment, but there is a considerable heterogeneity in approaches amongst centers worldwide.(99) The European Society for Blood and Marrow Transplantation (EBMT) and the European LeukemiaNet (ELN) working group recommend the usage of a calcineurin-inhibitor (Cyclosporin A or Tacrolimus) paired with an antimetabolite (methotrexate or mycophenolate mofetil) for patients with a standard-risk disease from matched related or matched unrelated donor transplantations.(99, 100) When prophylaxis fails or remains insufficient, the use of corticosteroids is the first-line therapy for aGVHD.(101) However, only 70% of patients respond to steroid treatment, and a complete remission is obtained in less than 50%.(102, 103) The outcome of the remaining 50% suffering from the so-called steroid-refractory aGVHD is inferior, with mortality rates of 70-100%.(103-105)

In both prevention and treatment of aGVHD, the effects are dependent on reduced T cell activation and systemic immune suppression, and work at the expense of higher complication rates. The weakened graft-versus-tumor effect leads to higher rates of relapses,(99) and the immune suppression harbors significant risks of life-threatening infections.(34, 101, 106) Preventing and treating aGVHD without interfering with the graft-versus-tumor effect and the immunological reconstitution remains a major challenge.

2.4 Experimental models of allo-HSCT

E. Donnal Thomas emphasized in his Nobel lecture (Dec 8th, 1990) that "*marrow grafting could not have reached clinical application without animal research, first in inbred rodents and then in outbred species, particularly the dog.*"(107) In 1990, together with Joseph E. Murray, he received the Nobel Prize in Medicine or Physiology for his contributions to the preclinical and clinical development of allo-HSCT as a therapy.(108) Over the last 50 years, preclinical models of allo-HSCT have provided essential insights into the pathophysiology of aGVHD, which has led to the current gold standards for aGVHD prophylaxis and therapy and thereby gave rise to the global use of allo-HSCT.(109-111) The vast majority of preclinical allo-HSCT studies have used and still use inbred mice, which allows researchers to systematically assess individual variables in the

setting of a controlled environment with the exclusion of extraneous factors.(112, 113) The organization of the murine MHC is very similar to that of the human (114) and aGVHD development occurs, just as in humans, via the mentioned five-step cascade of 1) immune priming, 2) T cell activation, 3) T cell expansion, 4) T cell trafficking and 5) host tissue injury (see chapter 2.3.1).(7, 110) A wide variety of murine models has been established over the last years, including full MHC-matched models, MiHA-mismatched models, and xenotransplantation models (Table 2).(110, 115, 116) Most murine aGVHD models follow the same scheme: 1) myeloablative conditioning via TBI, 2) i.v. injection of whole BM grafts from the tibia and femur of 8-12 week old donor mice, 3) i.v. injection of whole splenocytes or already selected T cell subsets to induce aGVHD.(110, 116) Owing to the many advantages of mouse models, investigators forecast that they will continue as the predominant preclinical platform.(113) Their translation into clinical use, however, remains questionable.(109, 110, 112, 117) Recent debates emphasize that further development, the appropriate choice, and appropriate application are essential in ensuring continued progress and therapeutic improvements in the field of allo-HSCT.(109, 112, 115, 118)

Table 2. *Selection of available mouse models of aGVHD.*

Donor strain	Recipient strain	Conditioning regimen	Genetics	Main T-cell type contributing to phenotype	Cell type and dose	Outcome
MHC mismatched						
C57/Bl6 ($H2^b$)	BALB/c ($H2^d$)	900 cGy	Mismatched for MHCI, MHCII and miHAs	$CD4^+$ and $CD8^+$	Splenic T cells (0.5-2×10^6) and TCD donor BM cells	Systemic disease by 10-21 days; lethal
C57/Bl6 ($H2^b$)	B6D2F1 ($H2^{b/d}$)	No XRT or 1100-1300 cGy	Mismatched for MHC I, MHC II and miHAs	$CD4^+$ and $CD8^+$	Whole splenocytes: 1-10×10^7 cells if no XRT; 2-5×10^6 purified T cells with XRT	Systemic disease; lethal by 30 days if XRT used; symptoms in 30-50 days if no XRT
miHA mismatched						
B10.BR ($H2^k$)	CBA or BALB.K ($H2^k$)	750 cGy	miHA mismatches	$CD8^+$	8×10^6 TCD BM cells and 1×10^7 whole splenocytes or 5×10^6 $CD4^+$ or $CD8^+$ T cells	Systemic disease; symptoms include tail scaling, ear erosions, diarrhea, hunching
B10.D2 ($H2^d$)	DBA/2 ($H2^d$)	800 cGy	miHA mismatches	$CD4^+$ (minor contribution by $CD8^+$)	4×10^6 TCD BM cells and 1×10^6 T cells, or purified $CD4^+$ or $CD8^+$ T cells	Systemic disease; lethal by day 80
Xenotransplantation models						
Human PBMCs	NOD/SCID IL2rγ-null (NSG)	No XRT or 200-250 cGy	Mistmatched for MHC I, MHC II and miHAs	$CD4^+$	1×10^7 or 5-10×10^6	Death from GvHD in 30-50 days

Note 2. aGVHD: acute graft-versus-host disease, BM: bone marrow, CD: cluster of differentiation, cGy: centigray, H2: histocompatibility 2, MHC: major histocompatibility complex, miHA: minor histocompatibility antigen, PBMCs: peripheral blood mononuclear cells, XRT: radiation therapy. Adapted from Schroeder et al., 2011.(110)

2.5 The bone marrow niche

The BM niche is a complex network of cellular and noncellular components, which provides crucial and indispensable factors for HSC self-renewal and differentiation.(119-121) It is composed of multiple different niches, that regulate the maintenance, proliferation and quiescence of HSCs during hematopoiesis (Figure 6).(122-125) The cellular compartment includes endothelial cells (ECs), osteoblasts, osteoclasts, osteocytes, adipocytes, sympathic neurons, nonmyelinating Schwann cells, mesenchymal stem cells, reticular cells, as well as macrophages and megakaryocytes (Figure 6).(120, 126) In the past decade, the BM niche has been recognized as a vibrant and complex tissue,(120) and the in-depth understanding of the interacting components has become the subject of intense research for hematopoietic malignancies,(127-130) as well as inflammatory diseases.(131-133)

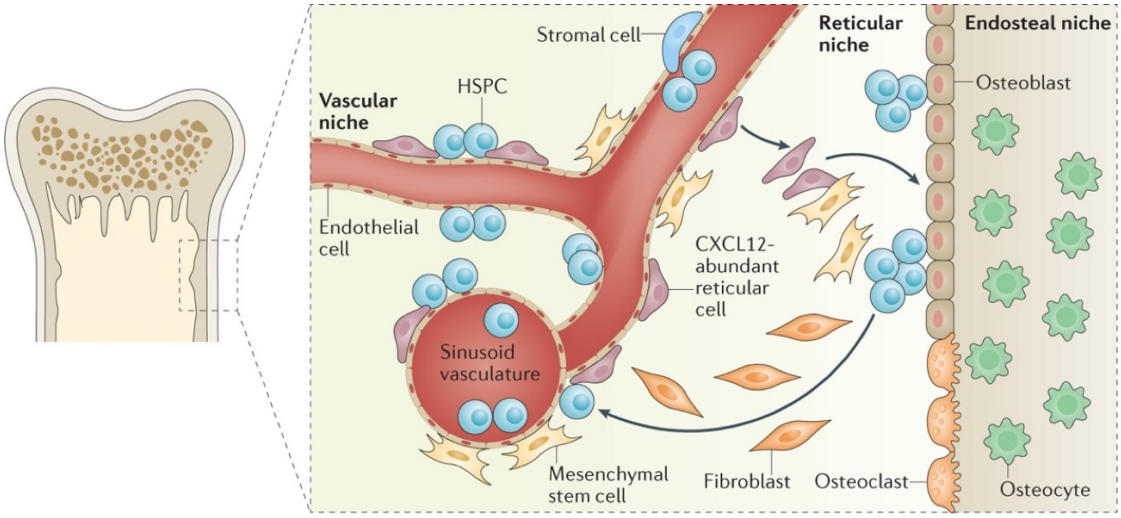


Figure 6. Stem cell niches in normal bone marrow. HSPC: hematopoietic stem and progenitor cell, CXCL12: CXC-chemokine ligand 12. From Ghobrial et al., 2018.(120)

2.5.1 The BM niche as a juncture for aGVHD and cytopenia after allo-HSCT

The BM microenvironment provides key characteristics for the success of allo-HSCT: it is critical for homing and engraftment of transplanted HSCs,(134) and also indispensable for the required reconstitution of hematopoiesis after allo-HSCT.(135, 136) Besides the known BM damage caused by the conditioning regimen,(137) the BM has recently been discovered as an additional target of aGVHD.(138-141) Preclinical studies showed the destruction of niche-forming cells by alloreactive aGVHD T cells and BM damage from conditioning linked with an impaired hematopoiesis.(139, 142) Mensen et al. were able to translate these findings into the clinical setting and reported a dramatic reduction of osteoblasts and delayed B cell regeneration in allo-HSCT patients, which was significantly associated with systemic aGVHD and a full-intensity

conditioning regimen.(97) The BM niche emerged as a central player for transplant success, and first indications of the niche as an aGVHD target with profound consequences for hematopoiesis have been made.(139, 143, 144) Still, little is known about the underlying pathophysiology of BM aGVHD. Also, the mechanisms and interplay between aGVHD-caused damage of the BM niche and prolonged cytopenia are unknown, and extensive preclinical and clinical data is lacking.

2.6 Cluster of differentiation 31

CD31, also known as platelet endothelial cell adhesion molecule (PECAM) 1, is a cell surface glycoprotein expressed on hematopoietic cells and ECs where it functions as a homophilic and heterophilic adhesion and signaling receptor.(145, 146) CD31 is a member of the immunoglobulin (Ig) gene superfamily with six heavily glycosylated extracellular Ig folds, a 19-residue transmembrane domain, and 118 residue cytoplasmic tail (Figure 7A).(147) Numerous functions have been attributed to CD31, including involvement in endothelial junction integrity, transendothelial leukocyte migration, angiogenesis, cytokine release, and T cell activation.(147-151) Because of its crucial contribution to transcellular leukocyte migration, (152-155) CD31 was initially assigned a pro-inflammatory role.(148) However, a growing number of studies reported a prominent dampening of the inflammatory response in a variety of acute and chronic inflammatory conditions.(156-158) CD31 was consequentially declared as vital to the regulation of inflammatory responses with both pro- and anti-inflammatory functions.(159)

2.6.1 Anti-inflammatory and pro-vasculogenic effects of CD31

CD31 has been found to confer protection against inflammatory damage through three main mechanisms:

1) Dampening of leukocyte activation: CD31 has been shown to work as an inhibitory receptor that limits cellular activation responses by immunoreceptor tyrosine-based inhibitory motifs-(ITIMs-) mediated inhibitory signaling (Figure 7B).(146, 160) Particularly T cell activation was shown to be hampered by CD31.(157, 158, 160) Besides lymphocytes, dampening of activation appeared to apply to mast cells and macrophages as well.(161, 162)

2) Maintenance of the vascular barrier integrity.(163-165)

3) Dampening of pro-inflammatory cytokine production.(156, 159, 163, 166)

The mechanisms of the latter two are, to date, poorly understood. In addition to the central mechanisms, Kim et al. discovered that CD31 stimulates the expression of anti-inflammatory factors, including interleukin-(IL-)10.(167)

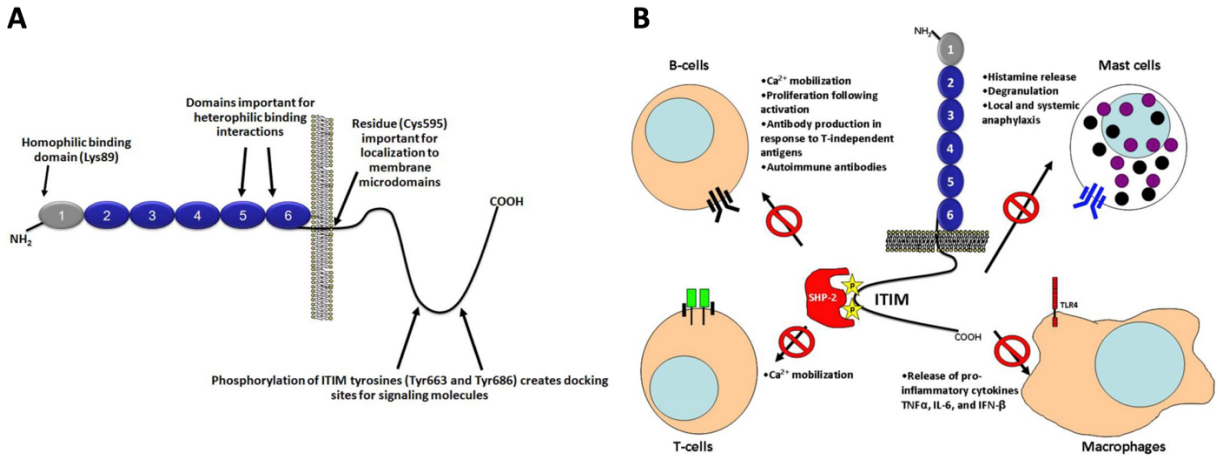


Figure 7. PECAM-1. **(A)** The structure and function of PECAM-1. From Privratsky et al., 2010.(159) **(B)** PECAM-1 interactions inhibit activation of B cell, T cell, mast cell, and macrophage function. Ca^{2+}: calcium, ITIM: immunoreceptor tyrosine-based inhibitory motifs, SHP-2: Src homology 2 domain-containing phosphatase 2. Modified according to Privratsky et al., 2010.(159)

Apart from its contribution to inflammatory responses, CD31 expression has been shown to contribute to angiogenesis, enhance adhesion and promote vessel formation.(150, 168-170) Kim et al. recently demonstrated that CD31-expressing BM-derived or peripheral blood-derived mononuclear cells are enriched with angiovasculogenic properties.(171, 172) $CD31^+$ cell treatment of murine hind limb ischemia models resulted in the effective improvement of ischemia by augmentation of neovascularization.(172) Further cardiovascular studies confirmed that $CD31^+$ cell treatment was effective for peripheral and cardiac vascular repair.(167, 171, 173, 174)

2.7 Objective of the work

This project aims at unraveling the influence of aGVHD on the BM vasculature and BM immune cell reconstitution as selected BM niche features after allo-HSCT. Patients suffering from aGVHD often develop severe prolonged cytopenia with significant consequences such as serious bleedings, fatal infections, and tumor relapse. The association of these dreaded complications suggests a decisive role of the BM niche for the outcome after allo-HSCT. First indications of the BM niche as an aGVHD target tissue with profound consequences for hematopoiesis have been found, but little is known about the underlying pathophysiology, and extensive preclinical and clinical data is missing. The present work is intended to lay the foundation for investigating this matter in more detail. The results of this study should be of help to develop a better understanding of aGVHD-induced BM damage and pave the way for targeted mechanistic and translational investigations.

For this purpose, the project is divided into two parts:

1) The aim of the first part is to establish a clinically relevant allo-HSCT model for BM aGVHD and to obtain a descriptive overview of the effects of aGVHD on the BM vasculature and BM immune cell reconstitution in this model.

2) The aim of the second part is to test a regenerative approach for aGVHD and aGVHD-induced damage of the BM niche in the established experimental model. To achieve this, it was to be investigated whether $CD31^+$ cell transfer could ameliorate aGVHD clinics and alterations in BM vasculature and BM immunological reconstitution.

3 MATERIAL AND METHODS

3.1 *In vivo* methods

3.1.1 Mice

Animal experiments were approved by the respective regional agency (Landesamt für Gesundheit und Soziales Berlin, G0119/15 (approval letter 16 June 2015) and G0081/19 (approval letter 12 August 2019)). Female C57BL/6 (B6) (H-2kb) and B6D2F1 (BDF) (H-2kb/d) mice were purchased from Charles River Laboratories (Sulzfeld, Germany). Female Actb-DsRed.T3 (DsRed) transgenic mice (B6.Cg-Tg(CAG-DsRed*MST)1Nagy/J) were bred in the Charité University Hospital animal facility. All mice were housed in the Charité University Hospital animal facility under specific pathogen-free controlled conditions and a 12-hour light/dark cycle with ad libitum access to food and water. Mice used in the experiments were 10 to 12 weeks old. Health monitoring was performed daily.

3.1.2 Experimental hematopoietic stem cell transplantation protocol

In short, the experimental hematopoietic stem cell transplantation (HSCT) protocol consisted of the following steps:

1) Donor cell isolation: On day+0 of the HSCT, donor mice (B6 for allo- and BDF for syngeneic (syn-) HSCT) were sacrificed by cervical dislocation; bones (femora and tibiae) and spleens were harvested, and surrounding tissue was removed; Lineage$^-$Sca-1$^+$c-Kit$^+$ (LSK) cells were isolated out of the bone marrow (BM); cluster of differentiation (CD) 3$^+$ cells were isolated out of the spleen.

2) Conditioning: On day+0 of the HSCT, recipient mice (BDF for both groups) received myeloablative total body irradiation (TBI).

3) Transplantation: On day+0 of the HSCT, irradiated recipient mice (BDF) were injected intravenously (i.v.) with isolated LSK cells and the allo-HSCT group additionally with CD3$^+$ cells.

4) CD31$^+$ cell transfer: On day+2 after HSCT, donor mice (DsRed) were sacrificed by cervical dislocation; bones (femora and tibiae) were harvested, and surrounding tissue was removed; CD31$^+$ cells were isolated out of the BM and injected i.v. into recipient mice (BDF).

3.1.3 Lineage$^-$Sca-1$^+$c-Kit$^+$ cell isolation

BM cells from B6 mice (allo-HSCT) or BDF mice (syn-HSCT) were flushed out of the tibia and femur, and a single-cell suspension was prepared in phosphate-buffered saline (PBS) (Thermo Fisher Scientific, Waltham, MA, USA) /2% fetal calf serum (Invitrogen, Carlsbad, CA, USA)/1mM Ethylenediaminetetraacetic acid (EDTA) (Sigma-Aldrich, St. Louis, MO, USA) by gently passing the solution through a 23-G needle (B. Braun Melsungen, Melsungen, Germany) and over a 70 µm cell strainer (BD Biosciences, San Jose, CA, USA). After lysis of erythrocytes with ammonium chloride (8.3 g/L NH4Cl in 0.01 M Tris-HCl buffer, Sigma-Aldrich), cells were passed through another 70 µm cell strainer. BM cells were lineage depleted by Magnetic Activated Cell Sorting (MACS) separation (Milteny Biotec, Bergisch Gladbach, Germany), using CD5, CD45R (B220), CD11b, GR-1 (Ly-6G/C), 7-4, Ter-119 as lineage markers, and subsequently selected by fluorescence-activated cell sorting (FACS) for the markers Sca-1$^+$ and c-Kit$^+$. Cell viability was quantified using Trypan blue (Sigma-Aldrich) staining, and cells were counted with a Neubauer chamber (Marienfeld Superior, Germany).

3.1.4 T cell isolation

Spleens from B6 donor mice were grounded with a sterile syringe plunger (B. Braun Melsungen) and passed through a 40 µm cell strainer (BD Biosciences). After lysis of erythrocytes with ammonium chloride (8.3 g/L NH4Cl in 0.01 M Tris-HCl buffer, Sigma-Aldrich), cells were passed through another 40 µm cell strainer and resuspended in MACS buffer. Splenic T cell suspension was obtained using the Pan T cell Isolation Kit II (Miltenyi Biotec). The kit is based on a cocktail of biotin-conjugated antibodies against CD11b, CD11c, CD19, CD45R (B220), CD49b (DX5), CD105, Anti-MHC-class II, and Ter-119. The isolation of T cells is achieved by depletion of magnetically labeled cells. Cell viability was quantified using Trypan blue staining (Sigma-Aldrich), and cells were counted with a Neubauer chamber (Marienfeld Superior). T cell purity was evaluated by CD3$^+$ staining and flow cytometry analysis. The number of injected T cells was adjusted according to the acquired purity.

3.1.5 DsRed$^+$ CD31$^+$ cell isolation

BM cells from transgenic DsRed mice were flushed out of the tibia and femur, and a single-cell suspension was prepared as described before. After lysis of erythrocytes with ammonium chloride (8.3 g/L NH4Cl in 0.01 M Tris-HCl buffer, Sigma-Aldrich), CD31$^+$ cells were isolated from the BM by MACS separation (Miltenyi Biotec). Cell viability was quantified using Trypan Blue staining (Sigma-Aldrich), and cells were counted with a Neubauer chamber (Marienfeld Superior).

3.1.6 Conditioning regimen

BDF recipient mice received myeloablative TBI with 1100 centigray from a 137Cs source (GSR D1, Gamma Service Medical, Leipzig, Germany) as a split dose with a four-hour interval.

3.1.7 LSK cell and T cell transplantation, $CD31^+$ cell transfer

BDF mice were subsequently injected i.v. with different cells and cell numbers according to the experimental set-up (Table 3). For i.v. tail vein injection mice were placed in an immobilization tube (TV-150 small, Braintree Scientific, Braintree, MA, USA) after being under a heat lamp for 10 minutes. LSK cells and $CD3^+$ cells from donor mice were injected i.v. at day+0 of HSCT four hours after the second radiation dose. The total injection volume was 200 µl, divided into 100 µl of LSK cells and 100 µl of T cells in the allo-HSCT set-up and 100 µl of LSK cells and 100 µl of PBS (Thermo Fisher Scientific) in the syn-HSCT set-up. For $CD31^+$ cell treatment, $DsRed^+$ $CD31^+$ cells were injected i.v. at day+2 after allo-HSCT with an injection volume of 100 µl.

Table 3. *Cells injected in this study.*

Cell type	Day		LSK		LSK + CD31
			Allo-HSCT	Syn-HSCT	Allo-HSCT
LSK cells	+0	Number	$1x10^4$	$1x10^4$	$0,7x10^4$
		Donor	B6	BDF	B6
$CD3^+$ cells	+0	Number	$1x10^6$		$1x10^6$
		Donor	B6		B6
$CD31^+$ cells	+2	Number			$1x10^{6\ treat}$
		Donor			DsRed

Note 3. All cells were injected into the tail vein intravenously. ^{treat} only treatment group. Allo-HSCT: allogeneic hematopoietic stem cell transplantation, B6: C57BL/6, BDF: B6D2F1, CD: cluster of differentiation, DsRed: Actb-DsRed.T3, LSK: $Lineage^-Sca\text{-}1^+c\text{-}Kit^+$, Syn-HSCT: syngeneic hematopoietic stem cell transplantation.

3.1.8 Acute graft-versus-host disease monitoring

Mice were individually scored twice a week for five clinical parameters (weight loss, posture, activity, fur texture and skin integrity) on a scale from 0 to 2 and the clinical acute graft-versus-host disease (aGVHD) score was assessed by summation of individual score-numbers according to the established Cooke grading system (Table 4).(175, 176) Animals were sacrificed when their total score exceeded 6, or if one parameter reached a score of 2.

Table 4. a*GVHD scoring system according to Cooke*

Criteria	Grade 0	Grade 1	Grade 2
Weight loss	< 10%	> 10% to < 25%	> 25%
Posture	Normal	Hunching noted only at rest	Severe hunching impairs movement
Activity	Normal	Mild to moderately decreased	Stationary unless stimulated
Fur texture	Normal	Mild to moderate ruffling	Severe ruffling/ poor grooming
Skin integrity	Normal	Scaling of paws/tail	Obvious areas of denuded skin

Note 4. aGVHD: acute graft-versus-host disease. Own representation based on Cooke et al., 1996.(175)

3.2 *Ex vivo* methods

3.2.1 Flow cytometry

To quantify cell subsets in the peripheral blood and BM via FACS, recipient mice (BDF) were sacrificed by cervical dislocation at day+20, day+40, or day+60 post HSCT. Blood was collected via retro-orbital bleeding, and bones (humeri) were harvested and cleaned from surrounding tissue. Single-cell suspensions of blood and BM were prepared as described above. After lysis of erythrocytes with ammonium chloride (8.3 g/L NH4Cl in 0.01 M Tris-HCl buffer, Sigma-Aldrich), single-cell suspensions were washed twice and stained for 20 minutes at 4°C in PBS (Thermo Fisher Scientific)/0.5mM EDTA (Sigma-Aldrich)/0.5% bovine serum albumin (BSA) (Carl Roth, Karlsruhe, Germany) with the antibodies listed in Table 5. Afterward, all samples were washed twice with PBS (Thermo Fisher Scientific), collected in MACS buffer, and subsequently measured with the BD FACSCanto II (BD Biosciences). Samples were analyzed using FlowJo Software (TreeStar Inc, Ashland, OR, USA). The cell percentages were obtained from the percentage of cells in the relevant gate out of all live cells (4′,6-Diamidin-2-phenylindole (DAPI) negative cells) unless otherwise indicated. The cell count was obtained by the absolute numbers of cells in the relevant gate. The cell count per µl was obtained by using the formula cell count / sample volume in µl. The cell count per BM was obtained using the formula cell count/µl x µl FACS sample.

Table 5. *Antibodies for fluorescence staining of single-cell suspensions.*

Name	Clone	Fluorochrome	Company, Country
CD3	145-2c11	APCy-Cy7	BD Bioscience, USA
CD4	RM4-5	PE Cy7	BD Bioscience, USA
CD8a	53-6.7	PerCP Cy5.5	BD Bioscience, USA
CD11b	M1/70	APCy-Cy7	BD Bioscience, USA
CD19	1D3	PE	BD Bioscience, USA
CD31	MEC13.3	APCy	BD Bioscience, USA
CD38	90	PE-Cy7	BD Bioscience, USA
CD44	IM7	APCy	BD Bioscience, USA
CD45	30-F11	PerCP Cy5.5	BD Bioscience, USA
CD45R	RA3-6B2	PerCP Cy5.5	BD Bioscience, USA
CD62L	MEL-14	PE	BD Bioscience, USA
CD80	16-10AA	PE	BD Bioscience, USA
CD86	GL1	APCy	BD Bioscience, USA
c-Kit	2B8	PE/APCy/PE-Cy7	BD Bioscience, USA
Gr1	RB6-8C5	PE Cy7	BioLegend, USA
H-2Kb	AF6-88.5.5.3	FITC	BD Bioscience, USA
H-2Kd	SF1-1.1	FITC	BD Bioscience, USA
F4/80	BM8	APCy-Cy7	BioLegend, USA
MHC II	OX-6	PerCP Cy5.5	BD Bioscience, USA
Sca-1	D7	FITC/PE-Cy7	BD Bioscience, USA

Note 5. APCy: allophycocyanin, CD: cluster of differentiation, C-Kit: protein kinase KIT, Cy: cyanine dye, F4/80: macrophage marker, FITC: fluorescin isothiocyanate, Gr1: granulocytic marker 1, H-2: histocompatibility 2, MHC: major histocompatibility complex, PB: pacific blue, PE: phycoerythrin, PerCP: peridin chlorophyll protein complex.

3.2.2 Histology of murine bones – endomucin staining

Mice were sacrificed by cervical dislocation at day+20, day+40, or day+60 after HSCT. Femora and tibiae were harvested, cleaned from surrounding tissue, and fixed in 4% paraformaldehyde (PFA) (Sigma-Aldrich) at 4°C on a shaker (Heidolph Instruments, Schwabach, Germany) overnight. Then they were washed in PBS (Thermo Fisher Scientific), decalcified in 0.5 M EDTA

(Sigma-Aldrich) in H_2O, pH 8, at 4°C on a shaker (Heidolph Instruments) for three days and incubated in 20% Sucrose/2% polyvinylpyrrolidone (PVP) (all Sigma-Aldrich)/H_2O at 4°C on a shaker (Heidolph Instruments) overnight. Finally, they were embedded in a solution of 8% gelatin/20% sucrose/2% PVP (all Sigma-Aldrich) in H_2O. 60 µm thick sections were cut with an NX78 cryotome (Thermo Fisher Scientific), placed on microscope slides (VWR International, Radnor, PA, USA), dried for 15 min at room temperature, and stored at -20°C until further preparation. For staining, sections were permeabilized in 0.5% Triton-X100 (Sigma-Aldrich) in H_2O, blocked in 5% donkey serum/0.5% Triton X-100 (Sigma-Aldrich) in H_2O and stained in a humid chamber overnight at 4°C with the primary goat monoclonal antibody against endomucin (PA5-47648, 1:200, Thermo Fisher Scientific). Sections were stained in a humid chamber for four hours at room temperature with a secondary donkey anti-goat antibody conjugated with cyanine dye (Cy) 3 (Sigma-Aldrich) for the LSK experiment and conjugated with Alexa Fluor 488 (Sigma-Aldrich) for the LSK CD31 experiment, and DAPI (BD Bioscience). Sections were then mounted using ROTI Mount (Carl Roth), covered by coverslips (VWR International) and stored at 4°C until analysis.

For high-resolution imaging, an acquisition of Z-stacks was performed with a Zeiss ApoTome.2 microscope (Carl Zeiss Microscopy, Thornwood, NY, USA) and 35 images per Z-stack were taken. A 3D projection was generated out of the stacks, and the Endomucin$^+$ area was quantified with a predetermined threshold using Fiji Software (http://fiji.sc/Fiji). For each sample, four to eight representative projections of different BM areas were obtained, measured, and the mean was calculated. Vessel density was reported as the percentage of aggregated pixel area to total area.

3.3 Statistics

This study was planned and ran as an explorative study. All values are to be interpreted as strictly explorative. The data was not adjusted for multiple testing, and the results do not allow generalization. All statistical analyses were performed using GraphPad Prism software (GraphPad Software Inc., La Jolla, CA, USA). An alpha level of .05 was defined as statistically significant. Scoring data and weight change were analyzed by the nonparametric unpaired Mann-Whitney U Test and values are presented as median, 25th and 75th percentile and differences between medians. Histological and remaining FACS data were analyzed by the parametric unpaired two-tailed Student's T-Test with values presented as mean ± standard deviation, difference between means, and the 95% confidence interval.

3.4 Bias-reducing methods

To reduce the selection bias, each mouse was given a random internal number, on which the later group assignment was based. To reduce the performance bias, all mice were held in the same housing, diet, and location conditions. Mixed housing (mice of different groups were kept together in one cage) excluded cage-related deviations and dynamics. To reduce the performance and detection bias in the histological analysis, assumed blinding was conducted by assigning each mouse a letter. After the initial assignment, the blinding codes were kept under lock and key during the data collection and outcome assessment. The blinding codes were broken only after the data analysis had been completed.

3.5 Material

3.5.1 Instruments

Table 6. *Instruments used.*

Name	Company	Country
BD FACSCanto II	BD Bioscience	USA
Fixation tube TV-150 small	Braintree Scientific	USA
GSR D1	Gamma Service Medical	Germany
Neubauer chamber	Marienfeld Superior	Germany
NX78 cryotome	Thermo Fisher Scientific	USA
Objective inverter	LSM Tech	USA
Pipettes	Thermo Fisher Scientific	USA
Shaker	Heidolph instruments	Germany
Zeiss ApoTome.2	Carl Zeiss Microscopy	USA

3.5.2 Consumables

Table 7. *Consumables used.*

Product	Company	Country
23 G needle	B. Braun Melsungen	Germany
4',6-Diamidino-2-phenylindole	Sigma-Aldrich	USA
Cell strainer, 40 µm, 70 µm	BD Bioscience	USA
Ammonium chloride	Sigma-Aldrich	USA
Bovine serum albumin	Carl Roth	Germany
Coverslips	VWR International	USA
4´,6-Diamidino-2-phenylindole (DAPI)	BD Bioscience	USA
Donkey serum	Sigma-Aldrich	USA
Dulbeccos phosphate-buffered saline	Thermo Fisher Scientific	USA
Ethanol	Carl Roth	Germany
Ethylenediaminetetraacetic acid (EDTA)	Sigma-Aldrich	USA
Falcon 15 ml, 50 ml	VWR International	USA
Fetal calf serum	Invitrogen	USA
Gelatin	Sigma-Aldrich	USA
MACS separation columns	Milteny Biotec	Germany
Microscope slides, SuperFrost	VWR International	USA
Pan T cell isolation KIT	Milteny Biotec	Germany
Paraformaldehyde	Sigma-Aldrich	USA
Pasteur pipettes	VWR International	USA
Pipette Tips	Thermo Fisher Scientific	USA
Polyvinylpyrrolidone	Sigma-Aldrich	USA
ROTI Mount	Carl Roth	Germany
Serological pipettes 5 ml, 10 ml, 25 ml	Sarstedt	Germany
Sucrose	Sigma-Aldrich	USA
Syringes 1 ml, 5 ml	B. Braun Melsungen	Germany
Triton X-100	Sigma-Aldrich	USA
Trypan blue	Sigma-Aldrich	USA

3.5.3 Software

Table 8. *Software used.*

Name	Company	Country
Fiji software	National Institutes of Health	USA
FlowJo 7.6.5 software	Treestar Inc.	USA
GraphPad Prism software	GraphPad Software Inc.	USA
ImageJ	National Institutes of Health	USA

4 RESULTS

4.1 Lineage$^-$Sca-1$^+$c-Kit$^+$ cell transplantation model

4.1.1 Lineage$^-$Sca-1$^+$c-Kit$^+$ cell transplantation model

To enable the investigation of the influence of acute graft-versus-host disease (aGVHD) on the bone marrow (BM) vasculature and BM immune cell reconstitution, as well as the influence of CD31$^+$ cell transfer on these, I adapted the established haploidentical murine allogeneic (allo) bone marrow transplantation (BMT) model of the "Penack lab" for the transplantation of Lineage$^-$Sca-1$^+$c-Kit$^+$ (LSK) cells (Figure 8A). After lethal irradiation and LSK cell transplantation, allo hematopoietic stem cell transplantation (HSCT) recipients showed an initially slower engraftment, indicated by a lower count of live cells in the BM of allo- (M=33.34(32.96)) compared to syngeneic (syn-) HSCT recipients (M=11.03(9.95)); MD=-22.31, 95% CI=-36.36 to -08.26, t(5)=3.19, p=.003 at day+20 post HSCT (Figure 8B). Chimerism analysis was performed by H-2kb and H-2kd chimerism markers. Allogeneic donors (C57BL/6) exhibit only H-2kb, whereas syngeneic donors and recipients (B6D2F1) exhibit H-2kb/d. Allo-HSCT recipients had already developed a mixed chimerism, indicated by donor-specific absence of H-2kd in 76% of all blood and 54% of all BM cells at day+20 post HSCT with increasing percentages over time (Figure 8C). All transplanted mice regained weight after conditioning and HSCT, but allo-HSTC recipients presented a significant weight loss at day+5, +9, and day+16 (Table 9). From day+26 post allo-HSCT on, their weight change data successively approached the values of syn-HSCT recipients (Table 9). Weight loss is a typical feature of murine aGVHD and the initial weight loss corresponded to increased aGVHD scores, displayed from day+9 until day+36 post allo-HSCT. In detail, allo-HSCT recipients exhibited typical clinical symptoms of murine aGVHD, including a hunched posture paired with decreased activity, ruffled fur, and scaly paws and tails. Syn-HSCT recipients did not exhibit these clinical symptoms, shown by low aGVHD scores post HSCT (Table 9, Figure 8D). An aGVHD phase with significantly increased aGVHD scores in allo-HSCT recipients occurred until day+36 (Table 9, Figure 8D). From day+43 post HSCT aGVHD attenuated and the aGVHD scores of allo-HSCT recipients aligned to those of syn-HSCT recipients (Table 9, Figure 8D). Figure 8D illustrates the aGVHD scores throughout the experiment in addition to the tabular output to demonstrate the dynamic progress.

In summary, these findings demonstrate the successful establishment of a novel experimental haploidentical model of aGVHD with the use of LSK cells as transplantation material. The set-up

obtained sufficient engraftment and typical clinical features of aGVHD in an allogeneic setting with additional allogeneic T cell transfer. The present model, therefore, is a suitable and clinically relevant tool for the characterization and closer investigation of aGVHD after haploidentical transplantations.

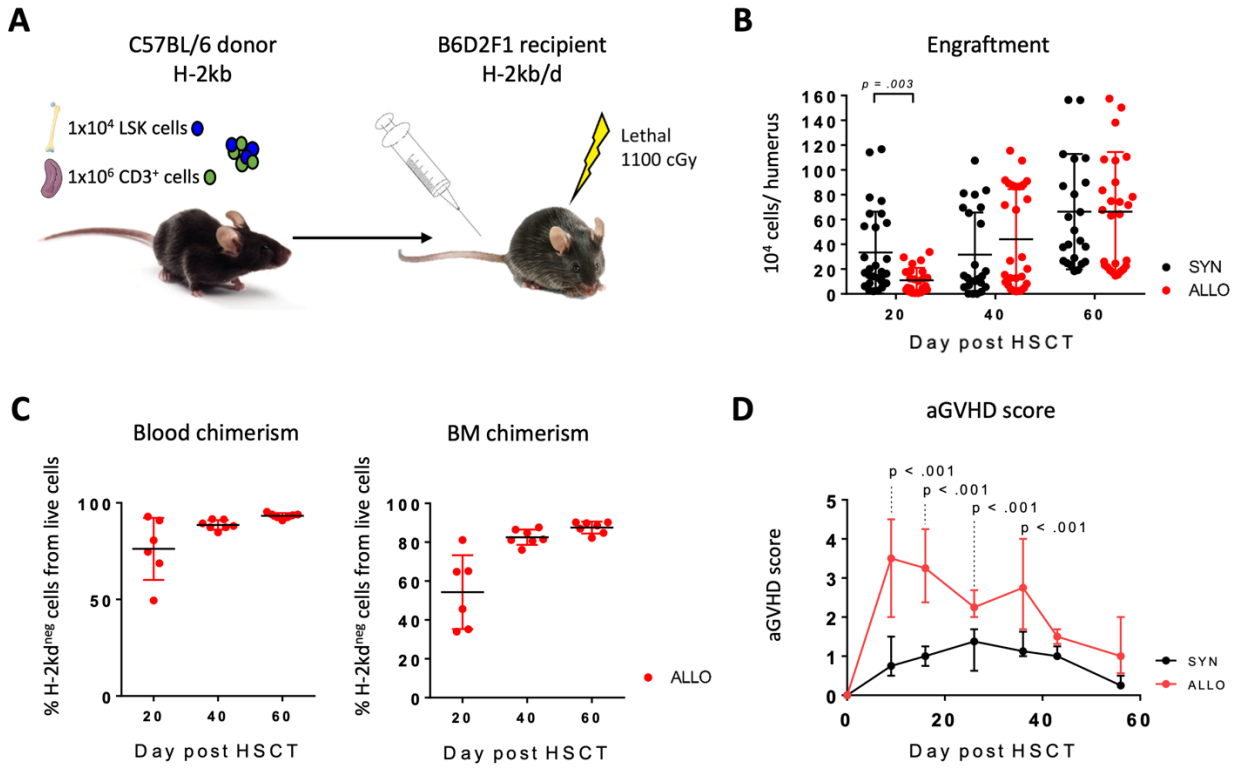


Figure 8. Characterization of the LSK transplantation model. (A) Allogeneic set-up for the LSK transplantation aGVHD mouse model. (B) Absolute counts of live cells in the BM (N=28 per group and date); scatter plots with error bars indicate mean ± standard deviation; analysis by unpaired, two-tailed Student's T-test. (C) Chimerism of allo-HSCT recipients in blood and BM over time (day+20 N=6; day+40 and day+60 N=7); scatter plots with error bars indicate mean ± standard deviation. (D) aGVHD scores of syn- and allo-HSCT recipients (syn day+9, +16 N=19, day+26, +36 N=12, day+43, +56 N=3; allo day+9, +16 N=21, d+26 N=16, day+36 N=14, day+43,56 N=4); points with error bars indicate median ± interquartile range; analysis by unpaired, two-tailed Mann-Whitney U Test. For all graphs, data pooled from two independent experiments. aGVHD: acute graft-versus-host disease, allo: allogeneic-transplanted mice, allo-HSCT: allogeneic hematopoietic stem cell transplantation, BM: bone marrow, cGy: centigray, HSCT: hematopoietic stem cell transplantation, LSK: Lineage−Sca-1+c-Kit+, syn: syngeneic transplanted mice, syn-HSCT: syngeneic hematopoietic stem cell transplantation.

Table 9. *Mann-Whitney U Test results of weight change and aGVHD scores comparing syn- to allo-HSCT recipients.*

Parameter	Day	Syn-HSCT			Allo-HSCT			Mdn Diff	U	p
		N	Median	25-75 Percentile	N	Median	25-75 Percentile			
Weight change	5	19	97.17	92.41 – 100.40	21	90.98	84.01 – 97.14	- 6.19	103.5	.008
	9	19	100.40	94.51 – 103.10	20	86.50	83.04 – 95.06	- 13.90	68.5	< .001
	16	19	102.90	94.05 – 106.70	20	90.29	80.42 – 99.08	- 12.61	72	< .001
	26	15	102.90	94.09 – 108.10	17	96.11	90.73 – 103.30	- 6.83	85	.114
	36	15	109.30	98.98 – 119.60	17	98.39	87.56 – 106.40	- 10.88	67	.022
	43	9	112.10	98.50 – 120.70	11	104.70	92.66 – 111.10	- 7.40	31	.175
	56	9	113.50	98.50 – 123.20	11	104.70	92.66 – 114.90	- 8.79	34	.253
aGVHD score	9	19	0.75	0.50 – 1.50	21	3.50	2.00 – 4.50	2.75	22	< .001
	16	19	1.00	0.75 – 1.25	21	3.25	2.38 – 4.38	2.25	5.5	< .001
	26	12	1.38	0.63 – 1.69	16	2.25	2.00 – 2.69	0.88	23	< .001
	36	12	1.13	1.00 – 1.63	14	2.75	1.69 – 3.44	1.63	14.5	< .001
	43	3	1.00	1.00 – 1.25	4	1.50	1.31 – 1.69	0.50	0.5	.086
	56	3	0.25	0.25 – 0.50	4	1.00	0.56 – 2.00	0.75	0.5	.086

Note 9. Analysis by unpaired, two-tailed Mann-Whitney U Test. Data pooled from two independent experiments. aGVHD: acute graft-versus-host disease, allo-HSCT: allogeneic hematopoietic stem cell transplantation, Mdn Diff: difference between medians, syn-HSCT: syngeneic hematopoietic stem cell transplantation. Significant p values marked with a grey background.

4.1.2 BM vessel density after LSK cell transplantation

To address the question whether the density of blood vessels in the BM was altered during aGVHD in the present model, BM sections were stained with the endothelial marker endomucin (Figure 9A). Endomucin expression is described to be uniquely endothelial cell- (EC-) specific and therefore it can be used as a distinct marker for ECs.(177) At day+20 after transplantation, the BM of allo-HSCT recipients showed an increased vessel density (M=7.40 (1.04)) compared to syn-HSCT recipients (M=4.57 (1.80)); MD=2.83, CI=0.99 to 4.67, t(11)=3.38, p=.006 (Figure 9B). However, in the follow-up of 40 and 60 days post HSCT, no differences between both groups could be observed (Figure 9B).

In sum, compared to syn-HSCT recipients, allo-HSCT recipients revealed a tighter vascular network in the BM at day+20 and an equal BM vessel density at day+40 and day+60 post HSCT.

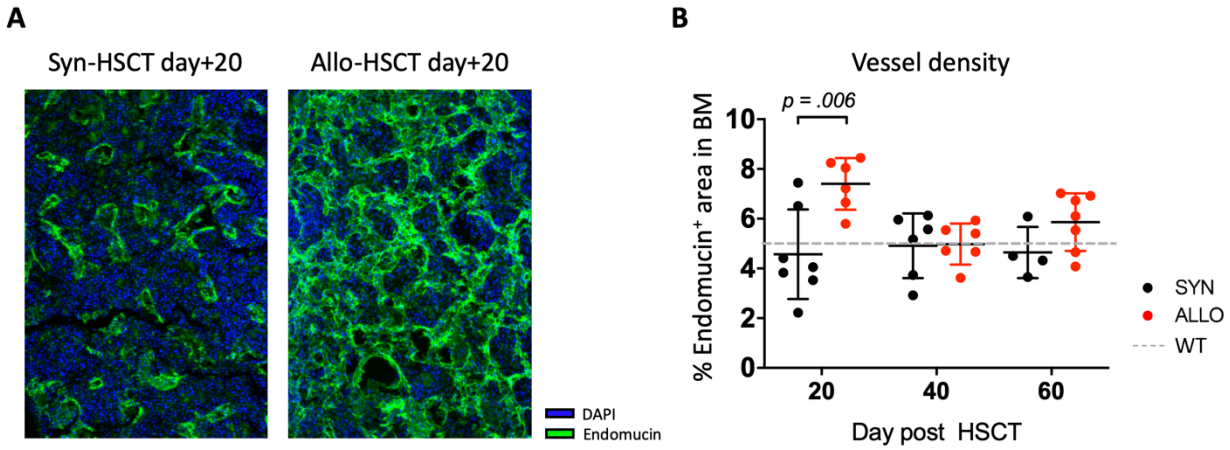


Figure 9. BM endomucin staining. **(A)** Representative pictures of BM sections stained with endomucin (green) on day+20 post HSCT. Nuclei were counterstained with DAPI (blue). **(B)** Quantification of BM vessel density (day+20 syn N=7, allo N=6; day+40 N=6 per group; day+60 syn N=4, allo N=7); scatter plots with error bars indicate mean ± standard deviation; grey dotted line indicates endomucin$^+$ percentages of B6D2F1 WT mice; analysis by unpaired, two-tailed Student's T-test. Data pooled from two independent experiments. Allo: allogeneic-transplanted mice, allo-HSCT: allogeneic hematopoietic stem cell transplantation, BM: bone marrow, syn: syngeneic transplanted mice, syn-HSCT: syngeneic hematopoietic stem cell transplantation, WT: B6D2F1 wild type mice.

4.1.3 Immune cell reconstitution after LSK cell transplantation

To obtain information about the immune cell reconstitution in the BM after HSCT, flow cytometric immunophenotyping of major immune cell subsets in the BM of syn- and allo-HSCT recipients was performed. More specifically, percentages and absolute counts of granulocytes, monocytes and dendritic cells, B cells, T cells, memory B, and memory T cells at day+20, day+40 and day+60 post HSCT of syn- and allo-HSCT recipients were quantified and compared. The data output of all tests is displayed in Table 10, and significant p values are marked with a grey background.

4.1.3.1 Myeloid cells: granulocytes, monocytes and dendritic cells

Granulocytes were defined as cluster of differentiation (CD) 11b$^+$ and Gr-1$^+$, and monocytes and dendritic cells were defined as CD11b$^+$ and Gr-1$^-$ without further subdivision. Fluorescence-activated cell sorting (FACS) analysis of BM cells revealed at no time differences in percentage and cell counts of granulocytes, monocytes and dendritic cells between syn- and allo-HSCT recipients (Table 10).

4.1.3.2 Lymphoid cells: B and T cells

B cells were defined as B220$^+$ cells. Quantification of B cells in the BM of allo- compared to syn-HSCT recipients indicated fewer B cell percentages at all time points and a trend towards a decreased cell count at day+20 post allo-HSCT (Table 10).

$CD3^+$ T cell percentages were elevated at day+40 and day+60 post HSCT, but $CD3^+$ T cell counts did not differ at any time in the BM of allo- compared to syn-HSCT recipients (Table 10). Analysis of the BM for $CD4^+$ T helper cells revealed higher percentages at all time points and an elevated cell count at day+60 post HSCT in allo- compared to syn-HSCT recipients (Table 10). Analysis of the BM for $CD8^+$ cytotoxic T cells revealed higher percentages at day+40 and day+60 as well as a higher cell count at day+60 post HSCT in allo- compared to syn-HSCT recipients (Table 10).

4.1.3.3 Immunological memory: memory B and memory T cells

Next, the BM of syn- and allo-HSCT recipients was investigated with regard to subsets of immunological memory. For B cells, BM cell samples were stained with B220, IgD, CD38 and CD19, and $B220^+IgD^-CD38^+CD19^+$ cells were defined as memory B cells. The BM of allo-HSCT recipients showed diminished memory B cell percentages, but no differences in memory B cell counts at all time points (Table 10).

For T cells, BM cell samples were stained with CD4, CD8, CD44 and CD62L. Naïve T cells (Tn) were defined as $CD44^-CD62L^+$, effector memory T cells (Tem) as $CD44^+CD62L^-$ and central memory T cells (Tcm) as $CD44^+CD62L^+$. The BM of allo-HSCT recipients exhibited increased $CD4^+$ Tem percentages at day+40 and +60, increased $CD4^+$ Tem counts at day+60, a decreased $CD4^+$ Tcm percentage at day+60, and an increased $CD4^+$ Tcm count at day+20 post HSCT (Table 10). Analysis of the subdivision of $CD8^+$ T cells in the BM of allo-HSCT recipients revealed increased Tem percentages at all time points, higher Tem counts at day+40 and day+60, decreased Tcm percentages at day+20 and day+60, and a lower Tcm count at day+20 post HSCT (Table 10).

Table 10. *T-test results of immune cell subsets in the bone marrow comparing syn- to allo-HSCT recipients.*

Cell subset	Unit	Day	Syn-HSCT			Allo-HSCT			MD	95% CI	t	df	p
			N	M	SD	N	M	SD					
Myeloid cells													
Granulocytes	%	20	4	13.99	2.46	3	10.40	3.91	-3.59	-9.72 to 2.54	1.51	5	.192
		40	3	52.25	15.71	3	71.18	7.52	18.93	-9.02 to 46.88	1.88	4	.133
		60	3	28.47	3.42	3	35.54	3.76	7.07	-1.07 to 15.22	2.41	4	.074
	Cell count	20	4	1.23	1.03	3	0.34	0.33	-0.9	-2.52 to 0.73	1.42	5	.215
	x 10^4/ BM	40	3	5.93	3.86	3	10.12	9.22	4.19	-11.84 to 20.21	0.73	4	.508
		60	3	9.07	3.76	3	6.91	0.36	-2.15	-8.21 to 3.91	0.99	4	.379
Monocytes/ Dendritic cells	%	20	4	0.17	0.14	3	0.14	0.14	-0.02	-0.29 to 0.24	0.23	5	.826
		40	3	0.25	0.17	3	0.1	0.02	-0.15	-0.42 to 0.12	1.59	4	.187
		60	3	0.14	0.03	3	0.09	0.03	-0.06	-0.12 to 0.01	2.48	4	.068
	Cell count	20	4	0.06	0.06	3	0.01	0.01	-0.05	-0.15 to 0.05	1.34	5	.238
	x 10^4/ BM	40	3	0.25	0.02	3	0.34	0.34	0.1	-0.45 to 0.64	0.5	4	.646
		60	3	0.37	0.2	3	0.21	0.02	-0.16	-0.47 to 0.16	1.38	4	.239
Lymphoid cells													
B cells	%	20	7	19.03	6.01	6	2.13	1.26	-16.90	-22.44 to -11.37	6.73	11	< .001
		40	6	29.47	10.84	7	6.12	4.24	-23.35	-33.08 to -13.61	5.28	11	< .001
		60	6	16.78	4.88	7	8.68	2.76	-8.11	-12.85 to -03.36	3.76	11	.003
	Cell count	20	7	6.9	8.38	6	0.24	0.27	-6.66	-14.24 to 0.92	1.93	11	.079
	x 10^4/ BM	40	6	12.61	16.41	7	3.53	4.26	-9.23	-23.34 to 4.83	1.45	11	.176
		60	6	10.81	9.66	7	5.82	5.07	-4.99	-14.20 to 4.21	1.20	11	.257
$CD3^+$ T cells	%	20	7	47.76	8.81	6	44.35	14.08	-3.41	-17.5 to 10.69	0.53	11	.605
		40	6	39.05	10.49	7	62.54	7.99	23.49	12.22 to 34.77	4.59	11	< .001
		60	6	43.75	2.79	7	52.81	5.03	9.06	3.97 to 14.16	3.91	11	.002
	Cell count	20	7	11.01	6.88	6	5.78	6.41	-5.23	-13.39 to 2.95	1.41	11	.187
	x 10^4/ BM	40	6	18.90	24.70	7	29.11	29.49	10.20	-23.37 to 43.77	0.67	11	.517
		60	6	21.81	12.08	7	25.52	13.47	3.72	-12.03 to 19.46	0.52	11	.614
$CD4^+$ T cells	%	20	7	0.79	0.67	6	6.47	5.82	5.68	0.84 to 10.52	2.58	11	.025
		40	6	0.44	0.26	7	1.72	0.81	1.28	0.52 to 2.05	3.69	11	.004
		60	6	0.64	0.13	7	2.05	0.64	1.42	0.83 to 02.0	5.33	11	< .001

Table 10 continued on the next page.

Table 10 (continued). *T-test results of immune cell subsets in the bone marrow comparing syn- to allo-HSCT recipients.*

Cell subset	Unit	Day	Syn-HSCT			Allo-HSCT			MD	95% CI	t	df	p
			N	M	SD	N	M	SD					
Lymphoid cells													
$CD4^+$ T cells	Cell count	20	7	1.49	0.97	6	2.79	1.72	1.3	-0.38 to 2.97	1.71	11	.116
	x 10^3/ BM	40	6	2.38	3.17	7	5.30	3.84	2.92	-1.43 to 7.27	1.48	11	.167
		60	6	3.12	1.75	7	9.31	4.3	6.2	2.05 to 10.35	3.29	11	.007
$CD8^+$ T cells	%	20	7	3.12	2.32	6	8.45	7.6	5.34	-1.27 to 11.96	1.78	11	.103
		40	6	1.30	0.82	7	3.70	0.70	2.40	1.48 to 3.32	5.73	11	< .001
		60	6	1.41	1.0	7	3.12	1.23	1.72	0.32 to 3.11	2.71	11	.02
	Cell count	20	7	4.65	2.85	6	4.72	2.33	0.07	-3.14 to 3.29	0.05	11	.96
	x 10^3/ BM	40	6	4.74	5.41	7	16.52	15.99	11.78	-3.35 to 26.92	1.71	11	.115
		60	6	5.04	2.58	7	12.65	3.55	7.61	3.76 to 11.46	4.35	11	.001
Memory cells													
Memory B	%	20	7	14.32	6.53	6	0.31	0.17	-14.01	-19.92 to -08.10	5.22	11	< .001
cells		40	6	21.85	6.78	7	4.23	3.84	-17.62	-24.21 to -11.03	5.90	11	< .001
		60	6	11.97	4.91	7	5.23	1.97	-6.56	-10.99 to -02.13	3.26	11	.008
	Cell count	20	7	5.68	7.54	6	0.07	0.1	-5.61	-12.43 to 1.2	1.81	11	.097
	x 10^4/ BM	40	6	10.44	13.63	7	2.47	3.34	-7.97	-19.62 to 3.69	1.51	11	.161
		60	6	8.03	7.96	7	3.32	2.88	-4.71	-11.78 to 2.36	1.47	11	.171
$CD4^+$ naïve	% of $CD4^+$	20	7	1.93	1.98	6	0.15	0.21	-1.78	-3.58 to 0.03	2.17	11	.053
T cells	cells	40	6	1.97	1.75	7	1.20	1.78	-0.77	-2.93 to 1.39	0.78	11	.451
		60	6	5.5	3.14	7	7.59	7.03	2.1	-4.77 to 8.96	0.67	11	.515
	Cell count	20	7	0.02	0.01	6	0.01	0.01	-0.01	-0.02 to 0.00	2.10	11	.059
	x 10^3/ BM	40	6	0.07	0.12	7	0.04	0.06	-0.03	-0.14 to 0.09	0.53	11	.608
		60	6	0.15	0.08	7	0.52	0.32	0.37	0.07 to 0.66	2.73	11	.02
$CD4^+$ T	% of $CD4^+$	20	7	29.34	32.48	6	37.48	21.87	8.15	-26.34 to 42.63	0.52	11	.613
effector	cells	40	6	17.01	21.42	7	58.23	14.68	41.22	19.10 to 63.34	4.10	11	.002
memory cells		60	6	29.31	26.68	7	64.43	8.52	35.12	11.64 to 58.60	3.29	11	.007
	Cell count	20	7	0.49	0.59	6	1.07	0.92	0.58	-0.35 to 1.51	1.38	11	.195
	x 10^3/ BM	40	6	0.96	1.47	7	3.28	2.36	2.32	-0.14 to 4.77	2.08	11	.062
		60	6	1.25	1.36	7	6.24	3.44	5.00	01.69 to 08.30	3.33	11	.007

Table 10 continued on the next page.

Table 10 (continued). *T-test results of immune cell subsets in the bone marrow comparing syn- to allo-HSCT recipients.*

Cell subset	Unit	Day	Syn-HSCT			Allo-HSCT			MD	95% CI	t	df	p
			N	M	SD	N	M	SD					
Memory cells													
$CD4^+$ T	% of $CD4^+$	20	7	19.48	10.88	6	19.32	7.62	-0.16	-11.84 to 11.51	0.03	11	.976
central	cells	40	6	19.75	19.12	7	27.93	17.50	7.64	-14.72 to 29.99	0.75	11	.468
memory cells		60	6	20.28	1.11	7	11.7	2.24	-8.59	-10.81 to -06.36	8.51	11	< .001
	Cell count	20	7	0.23	0.17	6	0.46	0.17	0.23	00.02 to 00.43	2.47	11	.031
	x 10^3/ BM	40	6	0.95	1.43	7	1.29	1.22	0.34	-1.28 to 1.96	0.46	11	.652
		60	6	0.63	0.36	7	1.09	0.47	0.46	-0.06 to 0.99	1.95	11	.077
$CD8^+$ naïve	% of $CD8^+$	20	7	1.98	3.1	6	1.0	0.78	-0.98	-3.86 to 1.31	0.75	11	.47
T cells	cells	40	6	3.25	2.32	7	1.60	1.89	-1.65	-4.22 to 0.93	1.41	11	.187
		60	6	11.61	7.02	7	5.95	3.32	-5.66	-12.18 to 0.87	1.91	11	.083
	Cell count	20	7	0.06	0.1	6	0.04	0.04	-0.02	-0.12 to 0.08	0.47	11	.65
	x 10^3/ BM	40	6	0.18	0.22	7	0.18	0.23	-0.01	-0.28 to 0.28	0.02	11	.988
		60	6	0.48	0.19	7	0.79	0.56	0.31	-0.22 to 0.85	1.29	11	.222
$CD8^+$ T	% of $CD8^+$	20	7	1.80	1.49	6	34.92	13.48	33.11	21.90 to 44.33	6.50	11	< .001
effector	cells	40	6	2.98	3.2	7	27.04	10.68	24.96	14.95 to 34.97	5.48	11	< .001
memory cells		60	6	6.69	5.97	7	26.84	14.46	20.15	06.18 to 34.13	3.17	11	.009
	Cell count	20	7	0.07	0.04	6	1.68	1.21	1.61	00.61 to 02.61	3.55	11	.005
	x 10^3/ BM	40	6	0.28	0.41	7	5.45	6.16	5.18	-0.41 to 10.76	2.04	11	.066
		60	6	0.26	0.10	7	3.67	2.48	3.41	01.16 to 05.66	3.34	11	.007
$CD8^+$ T	% of $CD8^+$	20	7	73.94	6.62	6	20.72	10.71	-53.23	-63.91 to -42.55	10.97	11	< .001
central	cells	40	6	56.30	6.38	7	32.30	6.38	-24.10	-48.62 to 0.43	2.16	11	.053
memory cells		60	6	59.92	10.43	7	36.36	16.06	-23.56	-40.44 to -06.68	3.07	11	.011
	Cell count	20	7	3.49	2.23	6	1.01	0.65	-2.48	-04.57 to -00.40	2.62	11	.024
	x 10^3/ BM	40	6	3.39	3.98	7	5.87	6.16	2.49	-3.97 to 8.95	0.85	11	.415
		60	6	3.18	2.13	7	4.23	1.42	1.05	-1.14 to 3.23	1.06	11	.314

Note 10. Analysis by unpaired, two-tailed Student's T-test. Data pooled from two independent experiments. %: percentage of all live cells, unless indicated otherwise, allo-HSCT: allogeneic hematopoietic stem cell transplantation, BM: bone marrow, CI: confidence interval, df: degrees of freedom, CD: cluster of differentiation, M: mean, MD: difference between means, SD: standard deviation, syn-HSCT: syngeneic hematopoietic stem cell transplantation. Significant p values marked with a grey background.

In brief, FACS immunophenotyping of the BM revealed 1) no changes in granulocytes, monocytes or dendritic cells, 2) diminished B cells, 3) elevated T cells including raised $CD4^+$ and $CD8^+$ T cell subsets, 4) diminished memory B cells and 5) a shift towards Tem in allo-HSCT compared to syn-HSCT recipients.

Summarizing, this first part of the work described an LSK cell transplantation model with an exemplary aGVHD development, shown by increased weight loss and higher aGVHD scores in allo- compared to syn-HSCT recipients. Histological analysis exhibited an increased vessel density in the BM during the aGVHD phase. The immunophenotyping revealed an affected immune cell reconstitution in the BM of allo-HSCT recipients.

Since allo-HSCT recipients showed significant changes in the vascular structure and immune cell reconstitution, it was possible to test the influence of $CD31^+$ cell transfer as a therapeutic approach after allo-HSCT. CD31 is an adhesion molecule expressed on ECs, hematopoietic stem cells and subsets of leukocytes.(178) $CD31^+$ cell therapy has been demonstrated to have high angio-vasculogenic activity and was already shown to be effective for cardiac and vascular repair.(167, 172) It was therefore of interest whether $CD31^+$ cell transfer could ameliorate the aGVHD-related characteristics just presented.

4.2 CD31$^+$ cell transfer after LSK transplantation

4.2.1 CD31$^+$ cell transfer model

To pursue the question if CD31$^+$ cell transfer could ameliorate aGVHD related characteristics, the allogeneic set-up of the present LSK cell transplantation model was used. The intervention group was treated with 1x10^6 BM-derived CD31$^+$ cells from transgenic Actb-DsRed.T3 (DsRed) (C57BL/6 background) mice at day+2 post allo-HSCT (Figure 10A), and the control group did not receive any cell transfer. To track the transferred cells after intravenous (i.v.) injection into the intervention group, FACS analysis of DsRed$^+$ cells in the peripheral blood and BM was performed. A successful transfer and persistence of the transferred cells was demonstrated by significantly elevated DsRed$^+$ cell counts in the peripheral blood and BM of CD31$^+$ cell transfer recipients at day+40 post allo-HSCT (Table 11). These findings were further confirmed histologically by the detection of DsRed$^+$ cells in the BM of i.v. CD31$^+$ cells transfer recipients (Figure 10B). The successful transfer having been ensured, in the next step the engraftment and chimerism capacity of transplanted LSK cells in the two groups was investigated. Both the intervention group and the control group showed sufficient engraftment at day+40 post allo-HSCT (Figure 10C). To evaluate the donor chimerism (H-2kb$^+$, H2kd$^-$), DsRed$^+$ cells had to be excluded from located H-2kd$^-$ cells to ensure that the detected percentages of donor C57BL/6 origin were not influenced by DsRed$^+$ cells which are also of C57BL/6 origin. Both groups developed a stable mixed chimerism, but the intervention group showed a decreased chimerism percentage in the peripheral blood and also in the BM at day+40 post allo-HSCT (Table 11).

Both the intervention group and the control group developed aGVHD with typical weight losses and elevated clinical aGVHD scores above 2 for the whole length of the experiment (Table 12, Figure 10D). However, CD31$^+$ cell transfer recipients exhibited significantly lower aGVHD scores during aGVHD phase at day+23 post allo-HSCT (Table 12, Figure 10D). In detail, the CD31$^+$ cell transfer recipients showed less hunching paired with increased activity and less weight loss between day+15 and 30 after allo-HSCT. The fur texture and skin integrity did not show any differences. At day+30 and day+37 post allo-HSCT, the aGVHD scores of the intervention and the control group converged again (Table 12, Figure 10D). Figure 10D illustrates the aGVHD scores throughout the experiment in addition to the tabular output to demonstrate the dynamic progress.

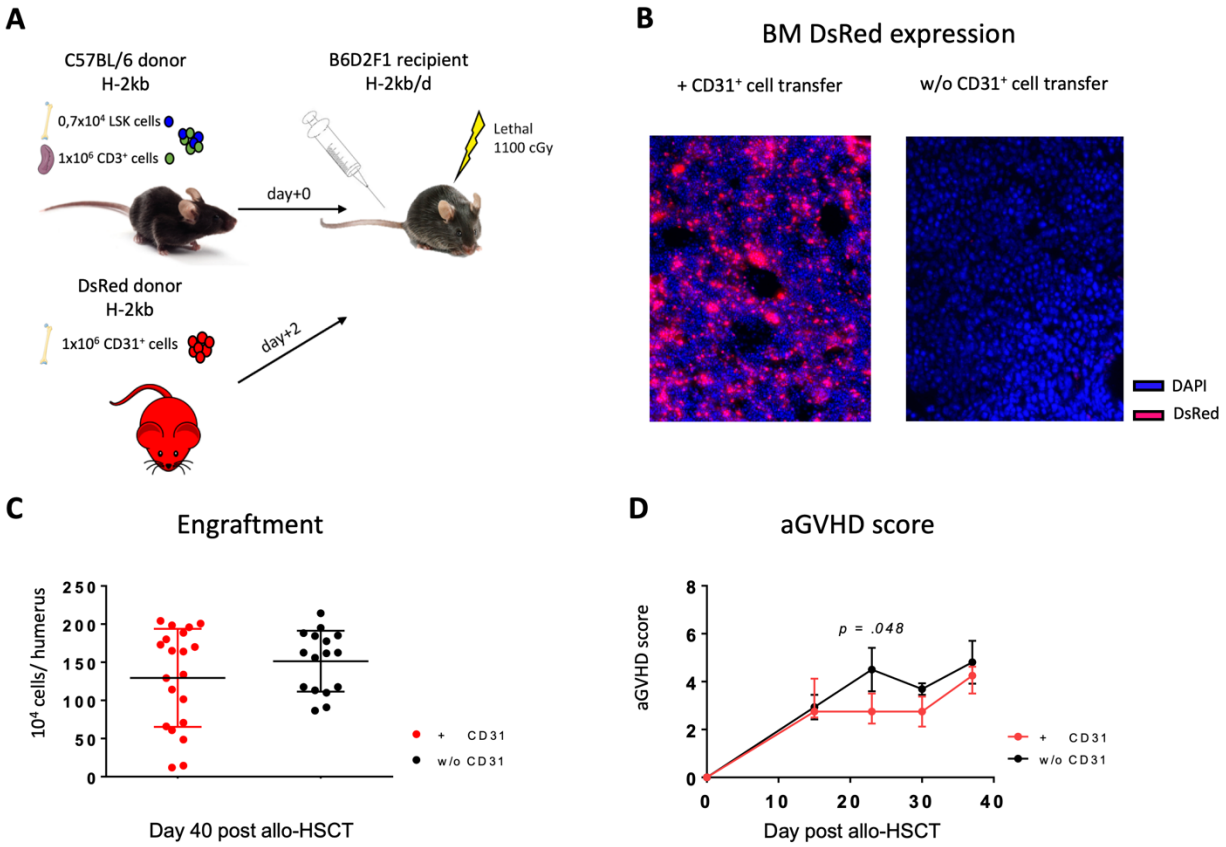


Figure 10. Characterization of the LSK cell transplantation model with CD31$^+$ cell treatment **(A)** Setting for LSK transplantation aGVHD mouse model with DsRed$^+$ CD31$^+$ cell transfer. **(B)** Representative pictures of BM sections on day+40 post allo-HSCT. DsRed$^+$ cells appear red in the Cyanine 3 channel. Nuclei were counterstained with DAPI (blue). **(C)** Absolute count of live cells in the BM (+ CD31 N=20, w/o CD31 N=16); scatter plots with error bars indicate mean ± standard deviation. **(D)** aGVHD scores of CD31$^+$ cell transfer intervention and control group (N=4-5 per group and date); points with error bars indicate median ± interquartile range; analysis by unpaired, two-tailed Mann-Whitney U Test. For all graphs, data from one experiment. aGVHD: acute graft-versus-host disease, BM: bone marrow, CD: cluster of differentiation, HSCT: hematopoietic stem cell transplantation, LSK: Lineage$^-$Sca-1$^+$c-Kit$^+$. + CD31: allogeneic transplanted mice with CD31$^+$ cell transfer, w/o CD31: allogeneic transplanted mice without CD31$^+$ cell transfer.

Table 11. *T-test results of different markers in the blood and bone marrow comparing CD31$^+$ cell transfer to no transfer in allo-HSCT recipients at day+40 post HSCT.*

Marker	Material	Unit	+ CD31$^+$ cell transfer			w/o CD31$^+$ cell transfer			MD	95% CI	t	df	p
			N	M	SD	N	M	SD					
DsRed$^+$	Blood	%	5	43.40	22.89	4	6.82	5.10	-36.49	-64.44 to – 8.53	3.09	7	.018
		Cell count x 10^4/ BM	5	385.10	259.80	4	38.83	20.48	-346.20	-658.40 to -34.05	2.62	7	.034
	BM	%	4	29.78	17.27	4	0.11	0.04	-29.66	-50.79 to -8.53	3.44	6	.014
		Cell count x 10^4/ BM	4	51.42	34.33	4	0.17	0.07	-51.25	-93.26 to -9.25	2.99	6	.025
Chimerism H-2kd$^-$ and DsRed$^-$	Blood	%	15	44.57	24.42	12	75.86	23.70	31.29	12.06 to 50.52	3.35	25	.003
	BM	%	15	64.40	20.12	12	83.74	16.47	19.37	4.51 to 34.18	2.69	25	.013
CD31$^+$	Blood	%	5	19.02	4.02	4	22.63	5.22	3.61	-3.65 to 10.86	1.18	7	.278
		Cell count x 10^4/ BM	5	204.6	86.95	4	200.1	110.9	-4.47	-159.80 to 150.90	0.07	7	.948
	BM	%	5	17.46	3.68	4	20.60	1.58	3.14	-1.56 to 7.84	1.58	7	.158
		Cell count x 10^4/ BM	5	32.45	9.32	4	36.44	9.38	3.98	-10.84 to 18.81	0.64	7	.545

Note 11. Analysis by unpaired, two-tailed Student's T-test. Data from one experiment. %: percentage of all live cells, allo-HSCT: allogeneic hematopoietic stem cell transplantation, BM: bone marrow, CI: confidence interval, CD: cluster of differentiation, df: degrees of freedom, HSCT: hematopoietic stem cell transplantation, M: mean, MD: difference between means, SD: standard deviation, w/o: without. Significant p values marked with a grey background.

Table 12. *Mann-Whitney U Test results of weight change and aGVHD scores comparing CD31$^+$ cell transfer to no transfer in allo-HSCT recipients*

Parameter	Day	+ CD31$^+$ cell transfer			w/o CD31$^+$ cell transfer			Mdn Diff	U	p
		N	Median	25-75 Percentile	N	Median	25-75 Percentile			
Weight change	15	5	81.55	78.31 – 84.69	4	82.89	73.93 to 84.19	-1.34	10	> .999
	23	5	81.95	81.30 – 84.94	4	70.16	66.25 – 79.39	11.80	2	.064
	30	5	87.28	82.67 – 90.23	4	83.38	77.27 – 83.72	3.90	4	.167
	37	5	80.77	76.04 – 85.08	4	77.12	70.47 – 81.76	3.65	6	.413
aGVHD score	15	5	2.75	2.50 – 4.13	4	3.00	2.44 – 3.38	-0.25	9	.865
	23	5	2.75	2.25 – 3.50	4	4.50	3.63 – 5.38	-1.75	1	.048
	30	5	2.75	2.13 – 3.38	4	3.63	3.50 – 3.94	-0.88	2.5	.079
	37	5	4.25	3.50 – 4.63	4	5.13	3.88 – 5.44	-0.88	4	.191

Note 12. Analysis by unpaired, two-tailed Mann-Whitney U Test. Data from one experiment. aGVHD: acute graft-versus-host disease, allo-HSCT: allogeneic hematopoietic stem cell transplantation, CD: cluster of differentiation, Mdn Diff: difference between medians, w/o: without. Significant p values marked with a grey background.

In summary, these findings indicate a successful transfer and engraftment of DsRed$^+$ CD31$^+$ cells in the allogeneic setting of the novel established LSK transplantation model. All mice developed classical features of aGVHD, but the CD31$^+$ cell transfer recipients revealed significantly lower aGVHD scores around day+23 post allo-HSCT. The data obtained supports this model as a suitable set-up for following investigations of CD31$^+$ cell transfer with regard to aGVHD.

4.2.2 CD31 expression after CD31$^+$ cell transfer

After the confirmation that the i.v. injection of DsRed$^+$ CD31$^+$ cells led to a successful transfer and engraftment, the CD31 expression in the BM between the intervention and the control group was compared by flow cytometry to find out if the DsRed$^+$ CD31$^+$ cell engraftment led to an increase of CD31$^+$ cells. Surprisingly, neither the percentage nor the absolute count of CD31$^+$ cells in the BM revealed any differences between CD31$^+$ cell transfer recipients and control mice at day+40 post allo-HSCT (Table 11). Similar results were obtained in the peripheral blood (Table 11). Deciphering DsRed expression in the CD31$^+$ cell subset of DsRed$^+$ cell transfer recipients revealed the majority of cells to be DsRed$^-$ (64,02% in the peripheral blood (Figure 11A) and 64,00% in the BM (Figure 11B)).

Summing up, the transfer of DsRed$^+$ CD31$^+$ cells did not lead to higher CD31 expression in the peripheral blood and BM at day+40 post allo-HSCT. Furthermore, the majority of all the discovered CD31-expressing cells was found to be DsRed$^-$.

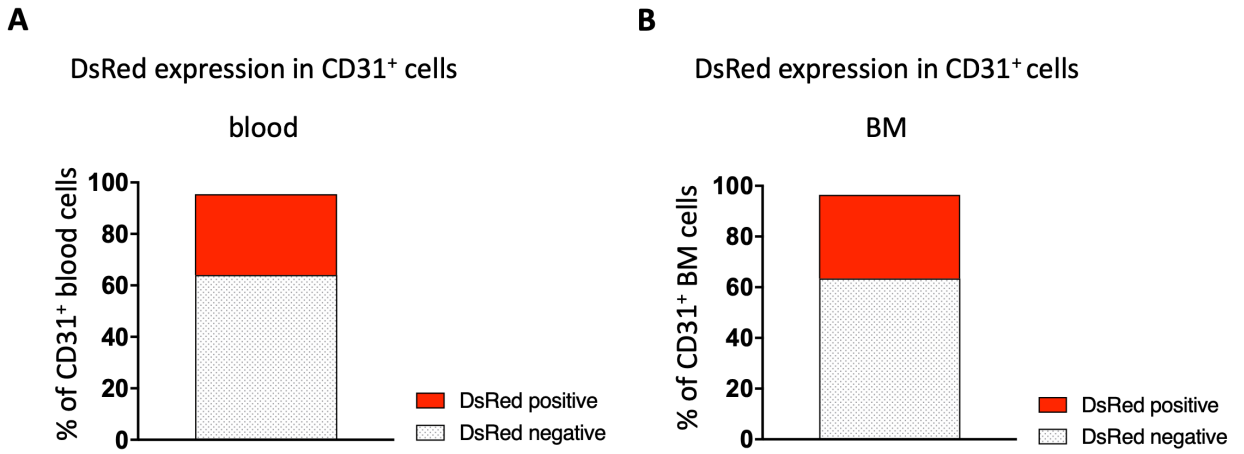


Figure 11. DsRed positive and negative percentages of CD31-expressing cells in the blood **(A)** and BM **(B)** of allogeneic-transplanted mice after $CD31^+$ cell treatment at day+40 post HSCT (N=5). Boxes indicate mean. Data from one experiment. BM: bone marrow, CD: cluster of differentiation, DsRed: Actb-DsRed.T3

In the next step, the same analyses as in the first part of this study were performed to test whether the $CD31^+$ cell transfer could influence any of the phenomena described in chapters 4.1.2 and 4.1.3.

4.2.3 BM vessel density after $CD31^+$ cell transfer

The first question was whether the transfer of $CD31^+$ cells influences the BM vessel density of allo-HSCT recipients. To determine this, BM sections were stained with the endothelial marker endomucin at day+40 post allo-HSCT as described in chapter 4.1.2 (Figure 12A). Initially, the BM vessel density of naïve wildtype (WT) B6D2F1 mice was analyzed to provide a basis for comparing observations made from the intervention and control group, which were both allo-transplanted mice. The subsequent quantification of the vessel density in the BM of $CD31^+$ cell transfer recipients and control mice at day+40 post allo-HSCT revealed an increase in contrast to the WT mice, but values did not differ between the two allo-HSCT groups (Figure 12B). Thus, $CD31^+$ cell transfer did not affect the BM vessel density in allo-transplanted mice at day+40 post HSCT.

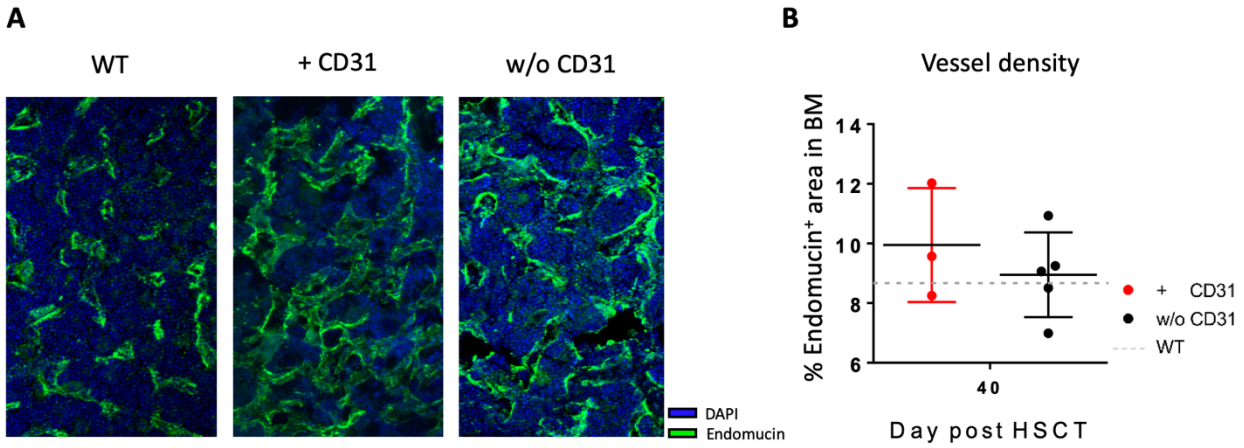


Figure 12. BM endomucin staining after CD31$^+$ cell transfer **(A)** Representative pictures of BM sections stained with endomucin (green) on day+40 post allo-HSCT. Nuclei were counterstained with DAPI (blue). **(B)** Quantification of bone marrow vessel density at day+40 post allo-HSCT (+CD31 N= 3, w/o CD31 N=5); scatter plots with error bars indicate mean ± standard deviation; grey dotted line indicates endomucin$^+$ percentages of B6D2F1 WT mice; analysis by unpaired, two-tailed Student's T-test. Data from one experiment. Allo-HSCT: allogeneic hematopoietic stem cell transplantation, BM: bone marrow, CD: cluster of differentiation, + CD31: allogeneic transplanted mice with CD31$^+$ cell transfer, w/o CD31: allogeneic transplanted mice without CD31$^+$ cell transfer, WT: B6D2F1 wild type mice.

4.2.4 Immune cell reconstitution after CD31$^+$ cell transfer

The next step was to investigate whether the CD31$^+$ cell transfer has an impact on the immunological reconstitution. For this, BM immune cell subsets were analyzed as described in chapter 4.1.3, but only at day+40 post allo-HSCT. All following observations therefore refer to day+40 post allo-HSCT. The data output of all tests is displayed in Table 13, and significant p values are marked with a grey background.

4.2.4.1 CD31$^+$ cell transfer and myeloid cells: granulocytes, monocytes and dendritic cells

First, granulocytes, monocytes and dendritic cells were investigated. Analysis of granulocytes revealed an increased percentage in the BM of CD31$^+$ cell transfer recipients compared to control mice (Table 13). However, the granulocyte count as well as the percentage and count of monocytes and dendritic cells did not vary between the BM of CD31$^+$ cell transfer recipients and control mice (Table 13).

4.2.4.2 CD31$^+$ cell transfer and lymphoid cells: B and T cells

Next, the B cell percentage and count in the BM of CD31$^+$ cell transfer recipients were compared with those in the BM of the control mice, but no differences between the two groups were found (Table 13).

Also, $CD3^+$ BM T cells showed no differences in the percentage or absolute count between $CD31^+$ cell transfer recipients and control mice (Table 13). Analysis of the subsets of $CD3^+$ T cells revealed a decreased $CD4^+$ T helper cell count and a trend towards a reduced $CD4^+$ T helper cell percentage in the BM of $CD31^+$ cell transfer recipients compared to control mice (Table 13). $CD8^+$ cytotoxic T cell percentage and cell count did not vary in the BM of $CD31^+$ cell transfer recipients compared to control mice (Table 13).

4.2.4.3 $CD31^+$ cell transfer and the immunological memory: memory B and memory T cells

To address the question if $CD31^+$ cell transfer influences the reconstitution of the immunological memory, memory B and memory T cells in the BM of $CD31^+$ cell transfer recipients and control mice were studied. Memory B cells showed no differences in distribution or absolute count (Table 13).

Analysis of BM $CD4^+$ T cells revealed no differences in Tn, but a decreased Tcm and Tem count in $CD31^+$ cell transfer recipients compared to control mice (Table 13). $CD8^+$ cells showed an increased Tn count in the BM of the $CD31^+$ cell transferred recipients; counts and percentages of Tem and Tcm in the BM, however, did not differ between intervention and control mice (Table 13).

Table 13. *T-test results of immune cell subsets in the bone marrow comparing CD31$^+$ cell transfer to no transfer in allo-HSCT recipients at day+40 post HSCT.*

Cell subset	Unit	+ CD31$^+$ cell transfer			w/o CD31$^+$ cell transfer			MD	95% CI	t	df	p
		N	M	SD	N	M	SD					
Myeloid cells												
Granulocytes	%	5	39.38	0.9	4	29.73	7.56	-9.66	-17.58 to -1.73	2.88	7	.024
	Cell count x 10^4/BM	5	53.80	50.19	4	49.61	12.77	-4.19	-65.82 to 57.44	0.16	7	.877
Monocytes/ Dendritic cells	%	5	1.22	0.35	4	1.2	0.04	-0.02	-0.44 to 0.40	0.12	7	.910
	Cell count x 10^4/BM	5	1.95	2.16	4	2.00	0.19	0.05	-2.55 to 2.66	0.05	7	0.96
Lymphoid cells												
B cells	%	5	3.66	2.14	4	4.14	2.95	0.47	-3.53 to 4.47	0.28	7	.788
	Cell count x 10^4/BM	5	7.70	6.12	4	10.24	8.01	2.53	-8.56 to 13.62	0.54	7	.606
CD3$^+$ T cells	%	5	35.68	8.06	4	34.0	7.69	-1.68	-14.22 to 10.86	0.32	7	.761
	Cell count x 10^4/BM	5	70.37	16.17	4	88.03	16.23	17.66	-15.84 to 51.17	1.25	7	.253
CD4$^+$ T cells	%	5	1.02	0.45	4	1.52	0.23	0.5	-0.09 to 1.09	2.0	7	.086
	Cell count x 10^4/BM	5	1.99	0.82	4	3.9	0.72	1.91	0.68 to 3.15	3.66	7	.008
CD8$^+$ T cells	%	5	6.29	3.09	4	3.64	1.02	-2.66	-6.51 to 1.2	1.63	7	.147
	Cell count x 10^4/BM	5	4.49	2.61	4	3.09	0.84	-1.4	-4.65 to 1.85	1.02	7	.342
Memory cells												
Memory B cells	%	5	5.12	2.77	4	5.48	3.89	0.36	-4.87 to 5.59	0.16	7	.877
	Cell count x 10^4/BM	5	10.69	8.06	4	13.56	10.54	2.87	-11.73 to 17.47	0.47	7	.656
CD4$^+$ naïve T cells	% of CD4$^+$ cells	5	0.46	0.32	4	0.32	0.21	-0.14	-0.58 to 0.30	0.77	7	.474
	Cell count x 10^4/BM	5	0.01	0.01	4	0.02	0.01	0.01	-0.00 – 0.02	1.42	7	.200
CD4$^+$ T effector memory cells	% of CD4$^+$ cells	5	71.36	9.24	4	66.38	3.60	-4.99	-16.68 to 6.71	1.01	7	.347
	Cell count x 10^4/BM	5	1.45	0.67	4	2.58	0.41	1.13	0.22 to 2.03	3.94	7	.022
CD4$^+$ T central memory cells	% of CD4$^+$ cells	5	14.64	6.31	4	16.7	2.44	2.06	-5.91 to 10.04	0.61	7	.560
	Cell count x 10^4/BM	5	0.28	0.15	4	0.64	0.03	0.36	0.17 to 0.08	1.58	7	.002

Table 13 continued on the next page.

Table 13 (continued). *T-test results of immune cell subsets in the bone marrow comparing CD31$^+$ cell transfer to no transfer in allo-HSCT recipients at day+40 post HSCT.*

Cell subset	Unit	+ CD31$^+$ cell transfer			w/o CD31$^+$ cell transfer			MD	95% CI	t	df	p
		N	M	SD	N	M	SD					
Memory cells												
CD8$^+$ naïve T cells	% of CD8$^+$ cells	5	1.10	0.97	4	0.49	0.10	-0.62	-1.79 to 0.55	1.25	7	.253
	Cell count x 10^4/BM	5	0.04	0.01	4	0.01	0.01	-0.02	-0.04 to -0.01	3.25	7	.014
CD8$^+$ T effector memory cells	% of CD8$^+$ cells	5	17.35	13.41	4	28.68	5.30	11.32	-5.66 to 28.30	1.58	7	.159
	Cell count x 10^4/BM	5	0.60	0.35	4	0.86	0.17	0.26	-0.19 to 0.71	1.37	7	.213
CD8$^+$ T central memory cells	% of CD8$^+$ cells	5	16.88	5.72	4	17.78	6.01	0.90	-8.37 to 10.16	0.23	7	.826
	Cell count x 10^4/ BM	5	0.67	0.27	4	0.54	0.17	-0.13	-0.5o to 0.24	0.85	7	.426

Note 13. Analysis by unpaired, two-tailed Student's T-test. Data from one experiment. %: percentage of all live cells, unless indicated otherwise, allo-HSCT: allogeneic hematopoietic stem cell transplantation, BM: bone marrow, CD: cluster of differentiation, CI: confidence interval, df: degrees of freedom, HSCT: hematopoietic stem cell transplantation, M: mean, MD: difference between means, SD: standard deviation, w/o = without. Significant p values marked with a grey background.

In sum, FACS immunophenotyping of BM cells at day+40 post allo-HSCT revealed 1) an increased granulocyte percentage accompanied by no changes in monocytes or dendritic cells, 2) no changes in B cells, 3) reduced $CD4^+$ T helper cells, but no differences of $CD3^+$ T cells or $CD8^+$ cytotoxic T cells, 4) no differences of memory B cells, and 5) decreased $CD4^+$ Tem and Tcm and elevated $CD8^+$ Tn after $CD31^+$ cell transfer compared to no transfer.

This second part of the study demonstrated a successful transfer of $DsRed^+$ $CD31^+$ cells in the allogeneic setting of the newly established LSK cell transplantation model. The findings confirmed the set-up of the pilot study described here and, further, delivered first results: $CD31^+$ cell transfer recipients showed an improved aGVHD score at day+23 post allo-HSCT, $CD31^+$ cell transfer did not affect the BM vessel density at day+40 post allo-HSCT, and $CD31^+$ cell transfer indicated a few differences in the BM immune cell reconstitution at day+40 post allo-HSCT.

5 DISCUSSION

The first part of this project described a murine model of haploidentical allogeneic hematopoietic stem cell transplantation (allo-HSCT) which is based on the transplantation of purified Lineage^{-}Sca-1^{+}c-Kit^{+} (LSK) cells. Allogeneic LSK cell transplantation led to profound engraftment and induced typical features of acute graft-versus-host disease (aGVHD). Investigation of the bone marrow (BM) vasculature and immune cell reconstitution as selected BM niche characteristics revealed an increased bone marrow (BM) vessel density as well as a typically affected immune cell reconstitution during aGVHD.

The second part of this project described the influence of cluster of differentiation (CD) 31^{+} cell transfer on aGVHD clinics, BM vessel density, and BM immune cell subsets in the allogeneic set-up of the present HSCT model. The CD31^{+} cell transfer resulted in an improved aGVHD score, no alterations of the BM vessel density, but alterations of the BM immune cell subsets towards reduced CD4^{+} T cells as well as CD4^{+} effector memory T cells.

5.1 Murine models of allo-HSCT as a tool for understanding aGVHD

In the last years, murine and canine models of aGVHD have provided significant insights and enhanced our understanding of the immunobiology of aGVHD. Nowadays, with the availability of a wide variety of models, it is of particular importance to choose an appropriate model and adjust it to one's study objective. This work is based on an adapted version of the established murine bone marrow transplantation (BMT) model of the Penack group. To facilitate the specific analysis of CD31^{+} cell transfer effects, the transplant material was exchanged for purified LSK cells, which correspond to most primitive mouse hematopoietic stem cells (HSCs).(179) In whole BM grafts, 10-20% of all cells are CD31^{+}, which consequently prevents studying consequences of CD31^{+} cell transfer apart from transplantation effects in general. Purified LSK cells, however, do not contain CD31^{+} cells and thus allow a separate investigation of CD31^{+} cell transfer effects. In addition, the transplantation of LSK cells is closer to the clinical situation. Most experimental aGVHD models use whole BM grafts,(110, 111, 117, 180) but patients, on the other hand, receive purified HSCs.(25) To mimic the ongoing trend of haploidentical transplantations in clinical use, all analyses were performed in a haploidentical set-up (C57BL/6→B6D2F1), mismatched for major histocompatibility complex (MHC) class I, MHC class II and minor histocompatibility

antigens (MiHAs). The number of haploidentical transplantations has increased by 250% since 2010 and is predicted to rise even more in the future.(13, 15, 20, 181, 182)

By modifying the model with the amendments described above, a novel murine allo-HSCT model based on the use of LSK cells as transplantation material was established. Characterization of the model revealed profound engraftment, a stable mixed donor chimerism and the development of aGVHD in an allogeneic setting with additional allogeneic T cell transfer. The clinical presentation of aGVHD was manifested, as seen in classical experimental aGVHD models, by decreased weight, hunching, reduced activity, and changes of the fur and skin. The data obtained indicates that this novel LSK transplantation model is suitable and hence recommendable for aGVHD-related investigations in a clinically relevant haploidentical setting.

Experimental aGVHD models offer powerful tools for studying the complex scenario of allo-HSCT and aGVHD in a controlled, reproducible, and simplified environment.(110, 183) Advantages include the detailed dissection of mechanistic pathways, *in vivo* analysis of genetic modifications through the availability of transgenic and knockout lines, and *in vivo* live imaging of cell trafficking, to name only a few. Of particular value is that experimental modeling of aGVHD is very close to the clinical reality.(109, 110, 183)

Withal, some limitations need to be considered. Murine models provide a controlled experimental system and it is known that not all findings may be directly extrapolated into clinical applications.(183, 184) For the present work, healthy and young mice housed in specific-pathogen-free conditions were used. In contrast, most patients are adult or elderly humans who exhibit genetic and phenotypic diversity, different exposure to microorganisms and variations in health status.(117) In the experimental aGVHD model presented here, the influence of age, health status, comorbidities as well as the microbiome is mostly disregarded, although all these parameters were found to have a substantial impact on the severity of aGVHD in mice and humans.(61, 117, 185-188) Moreover, unlike the clinical approach, the recipient mice only received lethal total body irradiation (TBI) as conditioning therapy. In the clinic, allo-HSCT recipients receive a combined, intensified conditioning regimen of TBI, chemotherapy and immune therapy.(25) A further limitation is the T cell origin. To induce aGVHD, recipient mice were supplemented with additional T cells isolated from secondary lymphoid organs of donor mice. In contrast, in clinical allo-HSCTs allogeneic T cells are transmitted as “contamination” of peripheral blood grafts or purified as a controlled adoptive T cell transfer to improve the immunological ability after transplantation. Different T cell origins have been described to have varying homing capacities as

well as varying functional characteristics, which in turn might affect the aGVHD phenotype.(110, 115, 183)

However, despite all limitations, aGVHD mouse models are still valuable tools for studying the complex scenario of allo-HSCT and aGVHD. The allogeneic haploidentical LSK transplantation model established and described in this study presents a new set-up that takes its place alongside a range of existing, well-functioning models for aGVHD. Its main feature is the transplantation of LSK cells, which could be advantageous for specific analyses apart from the general effects of transplantation. The use of this model might help to gain a better understanding of underlying pathogenic mechanisms as well as to develop and translate new therapeutic approaches into the growing field of haploidentical HSCTs. However, as pointed out in this chapter, caveats need to be considered very seriously when translating preclinical results into the clinical setting.

5.2 aGVHD targets the BM

The bone and the BM are of significant interest after allo-HSCT because of their critical importance for hematopoiesis, leukemia growth and immunity. Analysis of T cells in the BM of allo-HSCT recipients revealed increased $CD3^+$ T cell percentages at day+40 and day+60 post HSCT. $CD4^+$ and $CD8^+$ T cell subsets were already elevated at day+20 and remained increased throughout the entire experimental term. Activated T cell migration into aGVHD target tissues has been described to be the fourth step within the widely accepted five-step model of aGVHD development.(7, 115) After priming of the immune response (step one), T cell activation (step two), and alloreactive T cell expansion and differentiation (step three), the migration of activated T cells into target tissues (step four) eventually leads to the destruction of those tissues (step five).(7, 72) The elevated T cell rates in the BM of allo-HSCT recipients represent a characteristic allogeneic T cell infiltration into the BM. The data obtained hence 1) suggests the BM as an aGVHD target tissue and 2) indicates the usability of this specific mouse model to investigate the bone and BM with respect to aGVHD. Shono et al. identified the BM as a novel aGVHD target in 2010.(138, 139) Recent studies have strengthened their findings and described the subsequent destruction of niche-forming cells, including osteoblasts and vascular endothelial cells (ECs).(97, 98)

5.3 Structural changes of the BM vascular density and bone substance during aGVHD

Following the findings and literature mentioned above, I hypothesized that due to the vulnerability of the BM to aGVHD, bone and BM structure could also be affected. Regarding vascular ECs, BM sections were stained with the EC-specific marker endomucin and revealed an increased vessel density at day+20 post allo-HSCT during established aGVHD. These findings are in line with previous studies of the principal aGVHD target organs skin, liver and gastrointestinal tract, in which an increased vessel density has also been described at an early stage of the disease.(86, 189, 190) Riesner et al. recently observed that angiogenesis precedes leukocyte infiltration and plays a fundamental role in the initiation of aGVHD.(85) An association between aGVHD and the formation of new blood vessels has not only been reported in preclinical models, but also in clinical studies.(82-84, 86) However, previous research regarding the vessel density has been restricted to changes in principal aGVHD target tissues in delimitation to an absence of changes in nonclassical target tissues such as skeletal and cardiac muscle or joints.(85) To my knowledge, this was the first study of BM vessel density in a haploidentical experimental model of aGVHD. The data obtained gives first insights into vascular alterations in the BM during established aGVHD and therefore strongly supports the hypothesis of a correlation between angiogenesis and aGVHD in the BM. Accordingly, later time points contemporaneously with reduced aGVHD scores revealed no differences in the BM vessel density.

Withal, it has to be considered that these results are only descriptive. The endomucin staining served the purpose of illustrating ECs in the BM and further allowed for quantifying. For future studies, it would be interesting to examine the BM vascular changes in more detail. The analysis should be complemented by precise structural and organizational examination of the vessels to gain information about diameter, length, straightness, and branching. Also, investigation of endothelial activation (e.g. by soluble intracellular adhesion molecule-(ICAM-)1(191) and von Willebrand Factor (vWF)(192)), and endothelial damage should be followed up. For the latter, previous research has identified circulating ECs as a suitable biomarker.(193, 194) Analysis of pericyte coverage, tight junctions (e.g. staining of ZO-1), vascular perfusion (e.g. with FITC lectin perfusion), endothelial leakage (e.g. with an Evans Blue Assay or leakage or microbeads) as well as electron microscopy to detect microstructural changes of ECs are further useful options to characterize the endothelial damage of BM ECs.(191, 195)

Regarding the destruction of osteoblasts previously described,(97, 98) a collaboration with the "Duda lab" of the "Julius Wolff Institute for Biomechanics and Musculoskeletal Regeneration" was established. By histological analysis of BM sections from allo-transplanted mice with the osteoblast marker osteocalcin, they found a disrupted osteoblast lining and displacement of osteoblasts into the BM.(196) They further performed microCT analyses of the bones and discovered increased trabecular bone substance paired with reduced cortical thickness – a process contrary to frequent structural changes that come with age, glucocorticoid excess, or atherosclerosis.(196) Both observations were made at day+20 post allo-HSCT during manifested aGVHD, and both observations led to significant bone loss.(196) Osteoblast reduction and osteopenia are well-known phenomena after allo-HSCT and have been described in mice and patients as resulting from multiple factors.(38, 97, 197) The concurrence of vascular and spatial changes, together with significant aGVHD severity, suggests a connection between these components. It is known that angiogenesis is closely associated with bone development, regeneration and remodeling, and previous studies have proposed that vessel density is directly linked to architectural renovation in the bone.(198-200) The data from the "Duda lab" adds preliminary critical findings with regard to the impairment of the bone after allo-HSCT. However, to date, their observations are restricted to day+20 post allo-HSCT and do not allow for generalization. Further collaborative research is mandatory to enhance our understanding.

5.4 BM immune cell reconstitution during aGVHD

Recent studies have highlighted the importance of osteoblasts and the vasculature in maintaining HSC-renewal and differentiation.(124, 201, 202) I hypothesized that the observed structural alterations of the bone and BM therefore might interfere with engraftment, differentiation and survival of hematopoietic cells. The fluorescence-activated cell sorting (FACS) quantification provided a descriptive overview of major immune cell subsets in the BM after syngeneic (syn)- and allo-HSCT at the early (day+20), intermediate (day+40) and late (day+60) phases of aGVHD in the newly established model. The use of distribution and absolute count calculations provided a more accurate picture of the alterations observed, which is important for the design of future mechanistic investigations. In this study, however, the two calculations were not directly related. The aim of this overview was to characterize the model more closely and to establish a basis for future analyses. It is very important to note, however, that the cellular immune cell rates and counts

give no information on the functional capabilities since numbers do not speak for competence. As mentioned before, this is only a descriptive overview.

Analyses of granulocyte, monocyte and dendritic cell rates and counts did not reveal any differences in the BM of allo- compared to syn-HSCT recipients at any time. Previous studies have reported a rapid reconstitution of the innate immune system in syn- as well as allo-HSCT recipients within 20-30 days post HSCT.(31, 203-205) In addition, an increase of granulocytes and dendritic cells during the initiation phase of aGVHD has been described.(7, 61, 206) This rise was explained as due to their role in aGVHD pathogenesis, where the recruitment of granulocytes, amongst other cytotoxic cells, causes a considerable portion of the destruction of target tissues.(72) The selection of analysis dates may explain the missing increase in this work, as the initial evaluation of the early phase was set at day+20 post allo-HSCT, when aGVHD had already been manifested for several days. Future studies should therefore focus on earlier dates during the acute inflammation phase. Also, natural killer (NK) cells should be included in the investigation of immune subsets in the present LSK cell transplantation model to obtain an enlarged picture of the innate immunity in this model after allo-HSCT.

Analyses of B cells revealed decreased percentages and trends towards B cell count diminution at all time points in allo- compared to syn-HSCT recipients. These findings are in line with several preclinical studies using different mouse models, in which impaired B lymphopoiesis after allo-HSCT was described.(139, 142) Mensen et al. were recently able to translate these findings into the clinical setting and discovered a dramatic reduction of osteoblasts and delayed B cell regeneration in allo-HSCT patients which were significantly associated with systemic aGVHD and a full-intensity conditioning regimen.(97) Diverse mechanisms for decreased B lymphopoiesis have been discussed, out of which the “graft-versus-stroma reaction” has evolved to be one of the most essential ones.(98, 207-209) The “graft-versus-stroma reaction” describes the destruction of the BM stroma, including the osteoblastic and EC niche. As brought up in chapter 5.3, the “Duda lab” showed disrupted osteoblast lining in allo-transplanted mice.(196) Their observations are in line with the above-mentioned publications and could be of help to explain the reduced B cells in this LSK transplantation model.

As mentioned in chapter 5.2, increased $CD3^+$, $CD4^+$, and $CD8^+$ cells were found in the BM of allo-transplanted mice for the entire term (60 days) of the experiment. Increased T cells have been consistently described to appear in aGVHD target tissues early after transplantation, enduring until the late phase of aGVHD, and also as a feature of chronic GVHD.(210) The increased T cells

confirmed the findings provided by previous studies of the bone as an aGVHD target tissue,(97, 98, 141) as well as the general observation of infiltrating alloreactive T cells during aGVHD.(211, 212) Further, allo- and syn-HSCT recipients displayed an inverted CD4/CD8 ratio at all time points post HSCT. This observation is also consistent with previous research, in which a rapid expansion of $CD8^+$ T cells and consequently inverted CD4/CD8 ratio with a predisposition towards opportunistic infections has been reported.(213, 214)

The analysis of memory B cells revealed reduced percentages and trends towards diminution in absolute counts in the BM of allo-transplanted mice at all time points. This observation is not surprising, as memory B cell reconstitution has been reported to take 1 to 5 years after allo-HSCT (31, 215-218) and decreased memory B cells have been described in a variety of experimental aGVHD models as well as in patients.(218, 219)

Analysis of memory T cells revealed increased $CD4^+$ and $CD8^+$ effector memory T cells (Tem) and reduced $CD4^+$ and $CD8^+$ central memory cells (Tcm) in allo- compared to syn-HSCT recipients. Consistent with this study, increased $CD4^+$ and $CD8^+$ Tem paired with decreased $CD4^+$ and $CD8^+$ Tcm have been reported in preclinical(220) and clinical(210, 221) studies of aGVHD.

Again, one has to consider that the percentages and numbers obtained are strictly descriptive and furthermore restricted to the BM. The findings were, for the most part, in line with previous studies of immune cell subset quantifications of the BM in murine aGVHD models as well as in clinical studies of aGVHD and thereby confirmed that this novel model is usable for further clinically relevant research.

5.5 Limitations and future aspects of the LSK cell transplantation model

An overall limitation of the first part of this study was its limited power. Although the evaluation is based on two independent experiments, the sample size was very small. The experimental data was obtained exclusively in the described major mismatch, haploidentical C57BL/6→B6D2F1 model. To increase their significance, the observations regarding BM vessel density and BM immunological reconstitution described here should be confirmed in additional aGVHD experimental models with bigger group sizes. Analyses should be repeated in other MHC mismatched models (e.g. C57BL/6→BALB/c), MHC matched but MiHA mismatched models (e.g. 129→C57BL/6) and also xenograft transplantation models (e.g. human peripheral blood mononuclear cells→NOD scid gamma). Then as now, as pointed out in chapter 5.1, one has to

consider that murine aGVHD models are valuable tools, but their translation into the clinical situation remains unsatisfactory. For this, human data on BM vessel density and BM immunological reconstitution is required. Longitudinal studies on human BM biopsies after haploidentical as well as after matched related and matched unrelated donor transplantations would be of help to examine these parameters more closely. The embedding method and BM vasculature staining used here have already been successfully applied to patient's BM biopsies in preliminary studies of the "Penack lab", providing a basis to perform the human studies mentioned above.

The observations of this study should be further complemented by the simultaneous investigation of immune cell subsets in the peripheral blood. Studies of this are currently running in the "Penack lab". Moreover, the immune cell subsets listed in this study should be examined by immunofluorescence imaging to illustrate their location in the BM and their relation to each other. The immune cell histology should then be combined with the established vessel mapping. In addition, bone cells should be included in the staining and analysis. Tokoyoda et al. recently discovered an important link between stromal cells and memory immune cells: they found memory B and $CD4^+$ memory T cells to be maintained on stromal cells near ECs and assigned a prominent role for the conversion and maintenance of these cells to the BM stroma.(222-226) An extension of the established embedding and staining of the BM could be of help to confirm and reveal connections between the components listed.

More detailed analyses of the BM vasculature should be implemented as described in chapter 5.3. Apart from the descriptive improvements, mechanistic and functional analyses are required in order to create an improved basis for further research.

In conclusion, I believe that the haploidentical LSK cell transplantation model described here may be a good option for studying the bone and BM in connection with aGVHD more closely. This study adds descriptive data to the little known field of the association of stromal, vascular and hematopoietic cells in the BM during aGVHD.

Since allo-HSCT recipients indicated alterations in BM vessel density, bone structure, and BM immune cell subsets, it was possible to implement a regenerative approach. Therefore, in the second part of this work a therapeutic option, namely $CD31^+$ cell transfer, was tested on the described parameters.

5.6 CD31$^+$ cell transfer as an approach to ameliorating aGVHD

CD31, also known as platelet endothelial cell adhesion molecule-1 (PECAM-1), is a cell adhesion and signaling molecule that has been reported to play a role in platelet biology,(147) signal transduction,(227) leukocyte transendothelial migration and inflammation,(228) as well as in maintaining and restoring the vascular permeability barrier following inflammatory challenges.(229) CD31 signaling is further described as playing a significant role in T-cell activation and angiogenesis.(230, 231) Kim et al. identified CD31$^+$ cells in murine and human BM as highly pro-angiogenic, vasculogenic, and anti-inflammatory.(167, 171, 172) In an ischemic setting, they demonstrated that BM CD31$^+$ cells induce cardiac and vascular repair through enhanced angiogenic, adhesion, and inflammatory effects.(167) To date, studies of CD31 have been limited predominantly to cardiovascular research.

I assumed that BM CD31$^+$ cell transfer could also ameliorate characteristics of aGVHD, considering that the inflammatory environment, T cell activation, and endothelial damage are significant features of both research fields. The LSK cell transplantation model described in chapter 5.1 facilitated the specific analysis of CD31$^+$ cell transfer by excluding the CD31$^+$ cells from the transplantation material. CD31$^+$ cell transfer was thus separately testable. This study was the first CD31$^+$ cell transfer study running in the “Penack lab”. I used a very small sample size, therefore allowing one analysis time point only. Kim et al. described the most striking effects in ischemic repair around day+14 to day+21, but also proved sustainment of cell retention and endothelial transdifferentiation one year post CD31$^+$ cell transfer.(167, 171) I chose day+40 post allo-HSCT (=day+38 post CD31$^+$ cell transfer) to be the endpoint. At this time point there is significant aGVHD in murine models as well as in human patients. Further, it enabled the investigated mice to develop a typical course of aGVHD and evaluation of the memory immunity to be performed.

The assumed amelioration of aGVHD clinics by CD31$^+$ cell transfer was confirmed at day+23 post allo-HSCT, when CD31$^+$ cell transfer recipients exhibited significantly lower aGVHD scores compared to control mice. However, aGVHD scores of the intervention and control group converged at day+30 and day+37 again. Reruns of this experiment to obtain bigger sample sizes are required to draw a reasonable conclusion from this dynamic. For now, the data obtained indicates a definite sign of aGVHD improvement in the period of day+15 and day+30 post allo-HSCT.

The $CD31^+$ cell transfer recipients showed a decreased chimerism percentage at day+40 post allo-HSCT. The engraftment of $CD31^-$ transplanted LSK cells might be hampered by better engrafting capacities of co-transferred $CD31^+$ cells. Kim et al. observed a significantly enhanced engraftment of $CD31^+$ cells compared to $CD31^-$ cells in ischemic hindlimb models.(171, 174)

The successful engraftment of the transferred $DsRed^+$ $CD31^+$ cells could be confirmed by the location of $DsRed^+$ in the BM of the intervention group. Surprisingly, neither the BM nor the peripheral blood indicated higher expression of CD31 at day+40 post $CD31^+$ cell transfer. Locatable $DsRed^+$ cells must, therefore, have somehow lost their CD31 expression. Consistent with this observation, neutrophils have been reported to lose their CD31 expression after transendothelial migration or extravasation *in vitro*.(232, 233) Further, Liu et al. discovered that ECs induce CD31 pattern changes after exposure to inflammatory cytokines.(231) The lack of increase in CD31 expression might ergo be explainable by the migration of transferred cells and the highly inflammatory environment during aGVHD. However, this observation is restricted to day+40. Other timepoints could reveal different results and need to be investigated to gain a better understanding of this process.

5.7 BM vessel density after allo-HSCT and $CD31^+$ cell transfer

$CD31^+$ cell transfer did not affect the vascular density in the BM of allo-HSCT recipients at day+40 post HSCT. The question behind this investigation was if $CD31^+$ cells could prevent or ameliorate endothelial damage by their vasculogenic characteristics and thus prevent reactive neovascularization. According to the observations on day+20 post allo-HSCT in chapter 5.3, both allo-transplanted groups indicated an increased vessel density during severe aGVHD manifestation in comparison to healthy B6D2F1 wildtype (WT) mice. Again, more time points need to be examined. As suggested in chapter 5.3, the investigation of the BM blood vessels should be expanded by detailed structure, endothelial activation, and endothelial damage analyses. Because $CD31^+$ cells are described as incorporating into ischemic vessels as ECs,(171) functional and mechanistic studies should also follow.

5.8 BM immune cell subsets after allo-HSCT and CD31$^+$ cell transfer

This study further provided data on immune cell reconstitution in the BM of allo-HSCT recipients after CD31$^+$ cell transfer.

The granulocyte percentage was elevated in the BM of CD31$^+$ cell transfer recipients at day+40 post allo-HSCT. Kim et al. deciphered the composition of CD31$^+$ cells and found 29% to be granulocytes, monocytes and dendritic cells.(171) Increased percentages might therefore be effects of the CD31$^+$ cell transfer.

Kim et al. further identified 63% of CD31$^+$ cells to be B cells,(171) but contrary to the increased granulocyte percentages, in this study CD31$^+$ cell transfer did not lead to increased BM B cell rates or counts at day+40 post allo-HSCT.

Analysis of BM T cells revealed reduced CD4$^+$ T cells in the BM of CD31$^+$ cell transfer recipients at day+40 post allo-HSCT. Soluble CD31 has been described as inducing reduced frequency of activated T cells and contributing to T cell tolerance.(234) Moreover, CD31 deficiency has been reported to influence T cell-mediated inflammatory responses with enhancement of disease severity described in autoimmune encephalomyelitis and arthritis models.(157, 158) Reduced CD4$^+$ cells might therefore be a consequence of less T cell activation. However, CD3$^+$ and CD8$^+$ T cells remained unaffected and further research is indispensable to draw significant conclusions out of this.

CD31$^+$ cell transfer did not influence BM memory B cell rates or counts at day+40 post allo-HSCT. Analysis of memory T cells revealed decreased CD4$^+$ Tem counts in the BM of CD31$^+$ cell transfer recipients. Thus, CD31$^+$ cell transfer seemed to counteract the aGVHD induced shift towards CD4$^+$ Tem described in chapter 5.4. To examine if the decrease is a primary effect of CD31$^+$ cell transfer or rather a reaction to aGVHD development, I am currently working on analyses of CD31$^+$ cell transfer in an LSK transplantation setting without aGVHD progression. In contrast to decreased CD4$^+$ Tem in the aGVHD set-up described here, preliminary results of the non-GVHD set-up have revealed increased CD4$^+$ Tem in the BM of CD31$^+$ cell treated mice. For this reason, I assume that the CD31$^+$ cell transfer prevents the aGVHD-dependent increase rather than reducing Tem cells as a general effect. However, CD8$^+$ T cells did not indicate alterations of Tem.

5.9 Limitations and future aspects of the CD31$^+$ cell transfer

In sum, the second part of this study allowed first insights into CD31$^+$ cell transfer effects on aGVHD clinics, BM vasculature, and BM immune cell reconstitution after allo-HSCT. The findings confirmed the feasibility of a therapeutic approach in the novel established haploidentical LSK transplantation model. The absence of elevated CD31 expression in CD31$^+$ cell-transferred mice raises the questions of why and from whom cells CD31 disappears.

The acquired data is strictly descriptive, and the power is very limited. Only one experiment with a very small sample size was performed. Moreover, one has to consider that the reported findings are restricted to day+40 post allo-HSCT. At this time, though, the aGVHD scores did not significantly differ between the CD31$^+$ cell transfer recipients and control mice. The comparisons of BM vessel density and BM immune reconstitution are consequently not based on a clinical difference. The analyses described here need to be repeated in studies with more endpoints. Particular emphasis should be placed on an early endpoint around day+20 post allo-HSCT, as this time point revealed a significant amelioration of aGVHD and significant alterations in the BM vessel density and bone structure, and is also described as an essential date for obtaining striking findings in cardiovascular research.(167, 171, 172, 174) Also, the late phase of aGVHD around day+60 should be included to get an idea of transfer effects throughout the course of aGVHD.

The limitations and improvements pointed out in chapter 5.5 should also be applied to this experiment. Repetitive experiments in additional aGVHD mouse models are required to increase the significance of the results. Complementary analyses of cell subsets in the blood are necessary and are currently running in the “Penack lab”. Expanded histological analyses of immune cell subsets in correlation to vasculature mapping should be investigated. With respect to the attributed vascular barrier protection of CD31, closer examination of BM vessels as described in chapter 5.3 is needed. All vascular investigations following CD31$^+$ cell transfer should also be applied to the principal aGVHD target organs skin, liver and gastrointestinal tract, as they are known to develop angiogenesis and endothelial damage post allo-HSCT.

Moreover, CD31$^+$ cell transfer experiments in murine HSCT models without aGVHD development should be performed. In preliminary studies, I was able to successfully establish such a model: haploidentical, allogeneic transplantation of C57BL/6 LSK cells into B6D2F1 mice without additional allogeneic T cell transfer resulted in low aGVHD scores throughout the entire experimental period. In this model, I was furthermore able to exclude the toxicity of transferred CD31$^+$ cells. Analyses of the study described here should be carried out in aGVHD-absent allo-

HSCT recipients to compare and consequently file results of $CD31^+$ cell transfer into primary or reactive effects, as already applied for $CD4^+$ Tem and described in chapter 5.8.

5.10 Outlook

This work emphasizes that aGVHD targets and damages the BM niche after allo-HSCT. The established experimental haploidentical LSK cell transplantation model for aGVHD might be advantageous for future BM niche-related analyses and is feasible for therapeutic approaches.

This study yields the results and provides the foundation from which further development can progress. Additional preclinical research should investigate underlying mechanisms. For this, special attention should be paid to the osteoblasts and their interaction with the immunological reconstitution after allo-HSCT. Also, $CD31^+$ cell transfer as a therapeutic approach should be followed up as it was shown to improve aGVHD clinics and to reduce alloreactive T cells in the aGVHD-targeted bone.

The methods used should be applied to human tissues to enable longitudinal studies of patient BM biopsies. Further preclinical as well as clinical histological and structural investigations, paired with mechanistic analyses, will be of help to gain a better understanding of the immunoskeletal interface in the BM niche after allo-HSCT. In the long-term, this could lead to a translational development of novel therapeutic approaches to improve the dreaded cytopenia, bone weakness, and the overall outcome after allo-HSCT.

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EIDESSTATTLICHE VERSICHERUNG

„Ich, Constanze Sophia Schwarz, versichere an Eides statt durch meine eigenhändige Unterschrift, dass ich die vorgelegte Dissertation mit dem Thema: „The vasculature and immune cell reconstitution of the bone marrow niche after allogeneic hematopoietic stem cell transplantation“/ „Das Gefäßsystem und die Immunzellrekonstitution in der Knochenmarksnische nach allogener hämatopoetischer Stammzelltransplantation“ selbstständig und ohne nicht offengelegte Hilfe Dritter verfasst und keine anderen als die angegebenen Quellen und Hilfsmittel genutzt habe.

Alle Stellen, die wörtlich oder dem Sinne nach auf Publikationen oder Vorträgen anderer Autoren/innen beruhen, sind als solche in korrekter Zitierung kenntlich gemacht. Die Abschnitte zu Methodik (insbesondere praktische Arbeiten, Laborbestimmungen, statistische Aufarbeitung) und Resultaten (insbesondere Abbildungen, Graphiken und Tabellen) werden von mir verantwortet.

Ich erkläre ferner, dass ich mich zur Einhaltung der Satzung der Charité – Universitätsmedizin Berlin zur Sicherung Guter Wissenschaftlicher Praxis verpflichte.

Weiterhin versichere ich, dass ich diese Dissertation weder in gleicher noch in ähnlicher Form bereits an einer anderen Fakultät eingereicht habe.

Die Bedeutung dieser eidesstattlichen Versicherung und die strafrechtlichen Folgen einer unwahren eidesstattlichen Versicherung (§§156, 161 des Strafgesetzbuches) sind mir bekannt und bewusst.“

Datum Unterschrift

CURRICULUM VITAE

Mein Lebenslauf wird aus datenschutzrechtlichen Gründen in der elektronischen Version meiner Arbeit nicht veröffentlicht.

Mein Lebenslauf wird aus datenschutzrechtlichen Gründen in der elektronischen Version meiner Arbeit nicht veröffentlicht.

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STATISTIKBESCHEINIGUNG

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Bescheinigung

Hiermit bescheinige ich, dass Frau Constanze Schwarz innerhalb der Service Unit Biometrie des Instituts für Biometrie und klinische Epidemiologie (iBikE) bei mir eine statistische Beratung zu einem Promotionsvorhaben wahrgenommen hat. Folgende Beratungstermine wurden wahrgenommen:

- Termin 1: *14.02.2020*
- Termin 2: *03.03.2020*

Folgende wesentliche Ratschläge hinsichtlich einer sinnvollen Auswertung und Interpretation der Daten wurden während der Beratung erteilt:

- Angabe von mittlerer Differenz mit 95% Konfidenzintervall für die verschiedenen Gruppenunterschiede metrischer Daten
- Bitte explizit in den Methoden schreiben, dass alle Auswertungen rein explorativ sind und die p-Werte entsprechend keinen konfirmatorischen Charakter haben, und dass keine Adjustierung für multiples Testen vorgenommen wurde.
- Wahl der Abbildungen: Boxplots bei Scores nach Möglichkeit mit individuellen Datenpunkten sowie Dotplots mit Mittelwert und SD für Gefäßdichteparameter.
- Scoring Daten: Deskription mittels Median sowie 25. und 75. Perzentilen, Gruppenvergleich mittels Mann-Whitney –U test.

Diese Bescheinigung garantiert nicht die richtige Umsetzung der in der Beratung gemachten Vorschläge, die korrekte Durchführung der empfohlenen statistischen Verfahren und die richtige Darstellung und Interpretation der Ergebnisse. Die Verantwortung hierfür obliegt allein dem Promovierenden. Das Institut für Biometrie und klinische Epidemiologie übernimmt hierfür keine Haftung.

Datum: 29.4.2020 Name der Beraterin: Dr.rer.nat. Sophie K. Piper

Unterschrift BeraterIn, Institutsstempel

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