From Department of Laboratory Medicine
Clinical Research Center- Experimental Cancer Medicine
Karolinska Institutet, Stockholm, Sweden

PATHOPHYSIOLOGICAL ASPECTS OF TRANSPLANTATION-RELATED COMPLICATIONS FOLLOWING BUSULPHAN/CYCLOPHOSPHAMIDE CONDITIONING REGIMEN IN MOUSE MODEL

Sulaiman Al-Hashmi

Karolinska Institutet

Stockholm 2011

العلم خير من المال
لأن المال تحرسه والعلم يحرسك
والمال تفنيه النفقة والعلم يزكو بالإنفاق
والعلم حاكم والمال محكوم عليه
مات خازنو المال وهم أحياء
والعلماء باقون ما بقي الدهر
أعيانهم مفقودة و آثارهم في القلب موجودة
(الإمام علي ابن ابي طالب)

To My Family

ABSTRACT

Hematopoietic stem cell transplantation (HSCT) is a curative treatment for several malignant and non-malignant disorders. However, transplantation related morbidity and mortality limit its use. The complications of the HSCT procedure can be caused by several factors including toxicity of the conditioning regimen and allogeneicity. Despite the fact that 50% of the transplanted patients are conditioned with chemotherapy, the majority of transplantation models are based on radiation. In the present thesis, we utilized a HSCT mouse model following conditioning using busulfan-cyclophosphamide (Bu-Cy) to explore mechanisms and factors that might affect graft versus host disease (GVHD) and/or treatment related toxicity, thus altering transplantation outcome.

Study I: Was designed to investigate early cell dynamics during the development of GVHD after allogeneic HSCT. We found an early expansion and activation of dendritic cells (DCs) that peaked by day +3 post HSCT. The T cell expansion started later and reached its peak by day +5 post HSCT. The majority of these cells were donor CD8+ cells. The inflammatory cytokines (IL-2, INF-γ and TNF-α) also reached maximum levels by day +5. The results showed the important role of donor DCs in GVHD.

Study II: We studied the early histopathological changes in several organs at different time points from conditioning, as well as during the development of GVHD, until day +21. The study showed that the liver and spleen were the most affected organs; however, no morphological effects were detected in the pancreas, heart, lungs or kidneys after the conditioning regimen. Histopathological changes such as vasculitis, inflammation and apoptotic cell forms in the liver, spleen, pancreas, lungs and heart were observed during GVHD development, however, only hypocellular spleen and extramedullar hematopoiesis were detected in syngeneically transplanted animals. No morphological changes were observed in the kidneys in either HSCT setting. These results may help in understanding mechanisms underlying the development of GVHD.

Study III: We investigated the toxicity of Bu-Cy conditioning regimen on the arteries. We found that the conditioning regimen enhanced acetylcholine relaxation in the mesenteric arteries through the increased expression of endothelial nitric oxide. In contrast, the sensitivity of the aorta to the acetylcholine was similar between the Bu-Cy treated group and the controls. However, the aortas from the treated animals had a higher sensitivity to noradrenalin. The Bu-Cy treated animals had lower blood pressure, lower hematocrit and more endothelial damage compared to the controls. These results might help in developing prophylactic treatment for cardiovascular complications.

Study IV: We studied the effect of omega-3 on the Bu-Cy conditioning regimen and on the allogeneic HSCT outcome focusing on GVHD. We used corn oil and standard food as controls. The mice that were fed omega-3 food had the lowest survival rate and showed early signs of GVHD. Omega-3 enhanced the effect of the conditioning regimen by increasing its myeloablative properties, decreasing the expression of CD4+CD25+Fox*P*3+ T cells and reducing their function. Less GVHD and a higher rejection rate were observed in the corn oil group. The higher death rates in the omega-3 fed group might be explained by a greater myeloablative effect and increased severity of acute GVHD.

Taken together, these studies increase our knowledge of GVHD and conditioning related toxicity. This may improve treatment strategies and hence the clinical outcome of HSCT.

Key words: Busulfan, cyclophosphamide, conditioning regimen, HSCT, vascular, omega-3, GVHD, mouse model, treatment related toxicity.

LIST OF PUBLICATIONS

I. Behnam Sadeghi, **Sulaiman Al-Hashmi**, Zuzana Hassan, Björn Rozell, Hernan Concha, Carin Lundmark, Kjell-Olov Grönvik, Manuchehr Valugerdi and Moustapha Hassan. Expansion and activation kinetics of immune cells during early phase of GVHD in mouse model based on chemotherapy conditioning. *Clinical and Developmental Immunology* 2010.

II. **Sulaiman Al-Hashmi**, Zuzana Hassan, Behnam Sadeghi, Björn Rozell and Moustapha Hassan. Dynamics of early histopathological changes in GVHD after conditioning regimen with busulphan and cyclophosphamide. *International journal of clinical and experimental pathology* 2011; Aug 15;4(6):596-605

III. **Sulaiman Al-Hashmi**, Piet Boels, Fahad Al-Zadjali, Behnam Sadeghi, Johan Sällström, Kjell Hultenby, Zuzana Hassan, Anders Arner and Moustapha Hassan. Busulfan-cyclophosphamide conditioning in mice leads to microarterial remodeling with enhanced endothelial relaxation and lowered systemic blood pressure. (*Submitted*).

IV. **Sulaiman Al-Hashmi**, Behnam Sadeghi, Zuzana Hassan, Magnus Lindskog and Moustapha Hassan. Omega-3 enhanced busulphan-cyclophosphamide conditioning effect and augments graft versus host disease via FoxP3 regulatory T cell suppression during HSCT. (*Manuscript*).

CONTENTS

LIST OF ABBREVIATIONS

AA	Arachidonic acid
aGVHD	Acute graft versus host disease
ALA	Alpha (α) – linolenic acid
ALL	Acute lymphoplastic leukemia
APC	Antigen presenting cell
AT	Active tension
BM	Bone marrow
Bu	Busulfan
Bu-Cy	Busulfan-cyclophosphamide
Ca0PSS	Calcium free physiological salt solution
cAMP	Cyclic adenosine monophosphate
CaPSS	Physiological salt solution with calcium
CB	Cord blood
cGMP	Cyclic guanosine monophosphate
cGVHD	Chronic graft versus host disease
CHF	Cardiac heart failure
COX	Cyclooxygenase
Cy	Cyclophosphamide
CyA	Cyclosporine A
DC	Dendritic cell
DHA	Docosahexaenoic acid
DSF	Disease free survival
EDTA	Ethylenediaminetetraacetic acid
eNOS	Endothelial nitric oxide synthase
EPA	Eicosapentaenoic acid
FBS	Fetal calf serum
FOXP3	Forkhead box P3
G-CSF	Granulocyte colony-stimulating factor
GLA	Gamma (γ) – linolenic acid
GVHD	Graft versus host disease
GVL	Graft versus leukemia
HLA	Human leukocyte antigens
HSCT	Hematopoietic stem cell transplantation
HVG	Host versus graft
ICAM-1	Intercellular adhesion molecule 1
IC_{opt}	Optimal internal circumference
IC_{ref}	Reference slack internal circumference
INF-γ	Interferon gamma
iNOS	Inducible nitric oxide synthase
iP C	Induced pluripotent stem cell
KPSS	Physiological salt solution with potassium
LA	Linoleic acid
LPS	Lipopolysacharide
LTB4	Leukotriene B4

M.A.	Microarteries
MHC	Major histocompatibility complex
MSC	Mesenchymal stem cell
NK	Natural killer cell
NKT	Natural killer T cell
nNOS	Neuronal nitric oxide synthase
NO	Nitric oxide
PB	Peripheral blood
PBS	Phosphate buffer saline
PD-1	Programmed death-1
PGD2	Prostaglandin D2
PGE-2	Prostaglandin E-2
PPARγ	Peroxisome proliferator activated receptor γ
PT	Passive tension
PUFA	Polyunsaturated fatty acid
RBC	Red blood cell
RIC	Reduced intensity conditioning
SCT	Stem cell transplantation
SNP	Sodium nitroprusside
SOS	Sinusoidal obstruction disease
TEM	Transmission electron microscopy
TNF-α	Tumor necrosis factor alpha
Treg	Regulatory T cell
TRM	Transplant related mortality
VCAM-1	Vascular cell adhesion molecule 1
VOD	Venoocclusive disease

1 INTRODUCTION

1.1 HEMATOPOIETIC STEM CELL TRANSPLANTATION

1.1.1 History

The use of the atomic bomb in World War II was the beginning of understanding radiation and cell biology and subsequently the discovery of hematopoietic stem cell transplantation (HSCT) [1-2]. After lethal radiation dose, E. Donnall Thomas in 1957 [3] was able to rescue the mice by infusing stem cells. HSCT was originally used as the last choice of treatment for malignancies. Since then, the application of stem cell transplantation has increased to cover a wide range of diseases including genetic and metabolic disorders, hematological malignancies and solid tumors. However, the progress from the experimental treatment to clinical practice was dependent on the development of several areas such as the histocompatibility system, drug development and mechanisms underlying different diseases [4-6]. The following diagram (Figure 1) illustrates the development of hematopoietic stem cell transplantation in 1950s and 1960s [7].

Timeline | History of stem cell transplantation

Snell documents the existence of major and numerous minor systems that are involved in histocompatibility[34].

Main and Prehn show that allogeneic bone-marrow infusions promote survival of skin grafts in mice[5].

van Bekkum finds that intravenous infusions are the optimal route of delivery for repopulating bone-marrow spaces[120].

Uphoff discovers that the immune reaction directed against the host is controlled by genetic factors[122].

Thomas et al. show that autologous bone-marrow infusions after total body irradiation (TBI) induce prompt recovery of bone marrow and lymphoid tissue[22,31,123].

Goodman provides the first evidence for the presence of haematopoietic stem cells in the peripheral blood of mice[124].

Recognition of the importance of cellular subpopulations in immunogenetics and transplantation biology[125-127].

Schwartz and Beldotti discover lymphoproliferative disorders in bone-marrow-grafted mice undergoing a GVH immunological reaction[128].

Epstein, Storb et al. use peripheral blood stem cells for allogeneic transplantation in dogs[32,130].

Boak and Brent show that antibodies directed against lymphocytes suppress GVHD in mice[132,133].

| 1950–1960 | 1954 | 1955 | 1956 | 1956/59 | 1957 | 1958 | 1958–1964 | 1962 | 1962–1968 | 1964 | 1965 | 1966/67 | 1967 | 1968 |

Barnes and Loutit find that recovery from lethal irradiation and spleen-cell infusions might be due to living cells[4].

Barnes treats a mouse model of leukaemia with supralethal irradiation and bone-marrow grafts[13]. Ford, Nowell and Vos report marrow transplantation in rodents, supporting the cellular hypothesis[7,8,119].

Barnes reports that bone marrow is an immunologically competent tissue that is capable of mounting an immune response, resulting in graft-versus-host disease (GVHD)[15,121].

Uphoff discovers that methotrexate prevents or ameliorates GVHD[42].

Thomas et al. report some long-term stable engraftment in dog allogeneic transplantation, despite problems with graft rejection, GVHD and engraftment[43].

Syngeneic and autologous transplantation of dogs[31].

Malnin et al. infuse leukocytes into lethally irradiated guinea pigs[129].

Steinmuller and Motulsky successfully treat haemolytic anaemia in mice by haematopoietic stem-cell transplantation[131].

Epstein, Storb et al. identify the canine major histocompatibility complex dog leukocyte antigen (DLA) and show that this determines the outcome of transplantation[35,36,39,55,58].
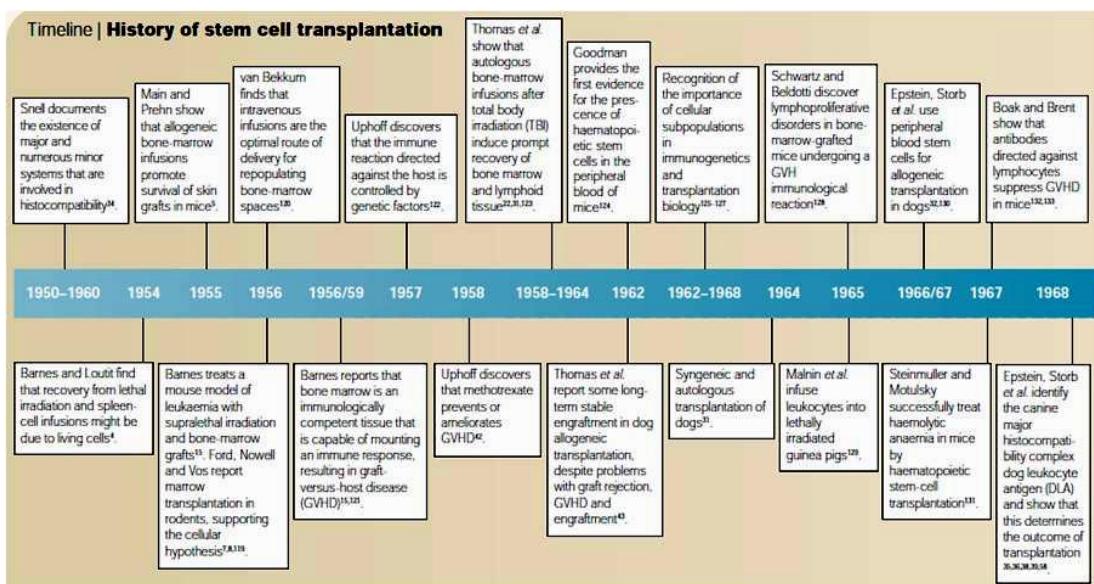
Figure 1: 1950s – 60s history of stem cell transplantation

1.1.2 Indication

The use of HSCT is continuously expanding to cover a broader range of diseases [8]. The diseases treated with HSCT are either acquired diseases or inherited disorders [9-11]. Acute and chronic leukemia, multiple myeloma and lymphomas are among the most common hematological malignancies indicated for HSCT. While, Hurler, Hunter, Goucher, SCID, thalassemia and aplastic anemia belong to the most common non malignant disorders that are indicated for HSCT.

1.1.3 Stem cell biology and cell therapy

Following the success of HSCT, stem cell therapy has become a new promising approach for the treatment of several disorders. Today, stem cell therapy has entered

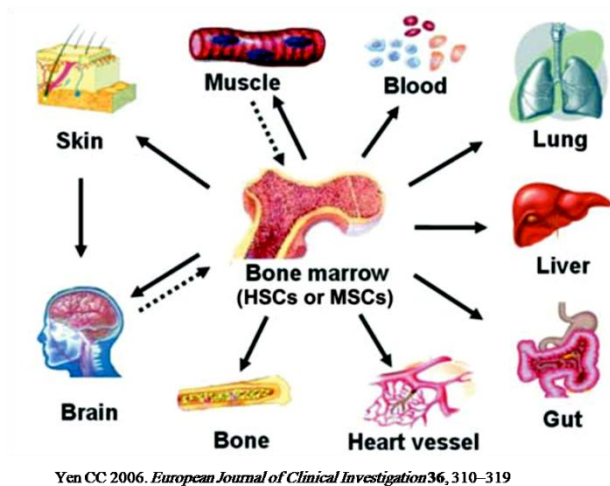Yen CC 2006. *European Journal of Clinical Investigation* 36, 310–319

Figure 2: Stem cell plasticity

clinical trials for tissue repair and other clinical applications. Mesenchymal stem cells (MSC), for example, are used in clinical trials for the treatment of cartilage damage, cancer, diabetes, neurodegenerative diseases [12-13], tissue toxicity and graft versus host disease (GVHD) [14-16]. Neural stem cells are also used in phase I and II trials for several diseases including chronic spinal cord injury, advanced Parkinson disease and stroke [17-19], however, several challenges such as adequate cell survival in the recipient and proliferation have to be solved. There are several types of stem cells depending on source, function or developmental stage. Germ cells have the ability to develop into all body cell types and they are called totipotent cells [20-21]. Embryonic stem cells are pluripotent [22] and they have the ability to produce different types of cells and tissues. Somatic or adult stem cells are more tissue specialized cells. Adult stem cells have been identified in different tissues such as brain, bone marrow, peripheral blood, blood vessels, skeletal muscle, skin, teeth, heart, gut, liver, ovarian epithelium and testis. These cells reside in a special place called a stem cell niche. Stem cells remain quiescent and leave the niche when there is a requirement from the body due to any type of stress. Neural stem cells, hematopoietic stem cells and fat stem cells are all examples of more specialized types of stem cells. Although these stem cells are tissue specific, there is enough evidence to conclude that these stem cells have trans-differentiation potential. Hematopoietic stem cells, for example, can be trans-differentiated into hepatocytes [23].

Natural trans-differentiations or reprogramming is a promising strategy in cell therapy for different kinds of injuries and diseases. Although embryonic stem cells have a higher capacity than adult stem cells to produce other tissues, their use is limited due to ethical and technical problems. Research continues to challenge and mimic the stem cell properties *in vitro* to achieve better treatment. Induced pluripotent stem cells (iPSCs) are *in vitro* modified stem cells with similar properties to mouse and human embryonic stem cells [24]. The therapeutic window for these cells is wide, as they have the potential to regenerate different tissues and thus to be used for the treatment of different types of diseases [25-28]. Somatic or adult stem cells also attract attention in scientific research, and due to their potential for so far incurable diseases. All types of stem cells have common properties in that they are capable of renewing themselves and supplying needs of the body whenever required. Stem cell self renewal is divided into long- and short-term self renewal. Long-term stem cells are not specialized, and they have the capacity to self renewal for an extended period of time, this feature is lost during specialization. The other common feature is that stem cells can differentiate to produce specific tissues. Better understanding of stem cell biology would improve the therapeutic approaches in regenerative medicine and in the treatment of cancer and

12

many other diseases. Figure 2 illustrates the plasticity of adult stem cells [29] and Figure 3 illustrates stem cell division and differentiation into different cell lineages [30].
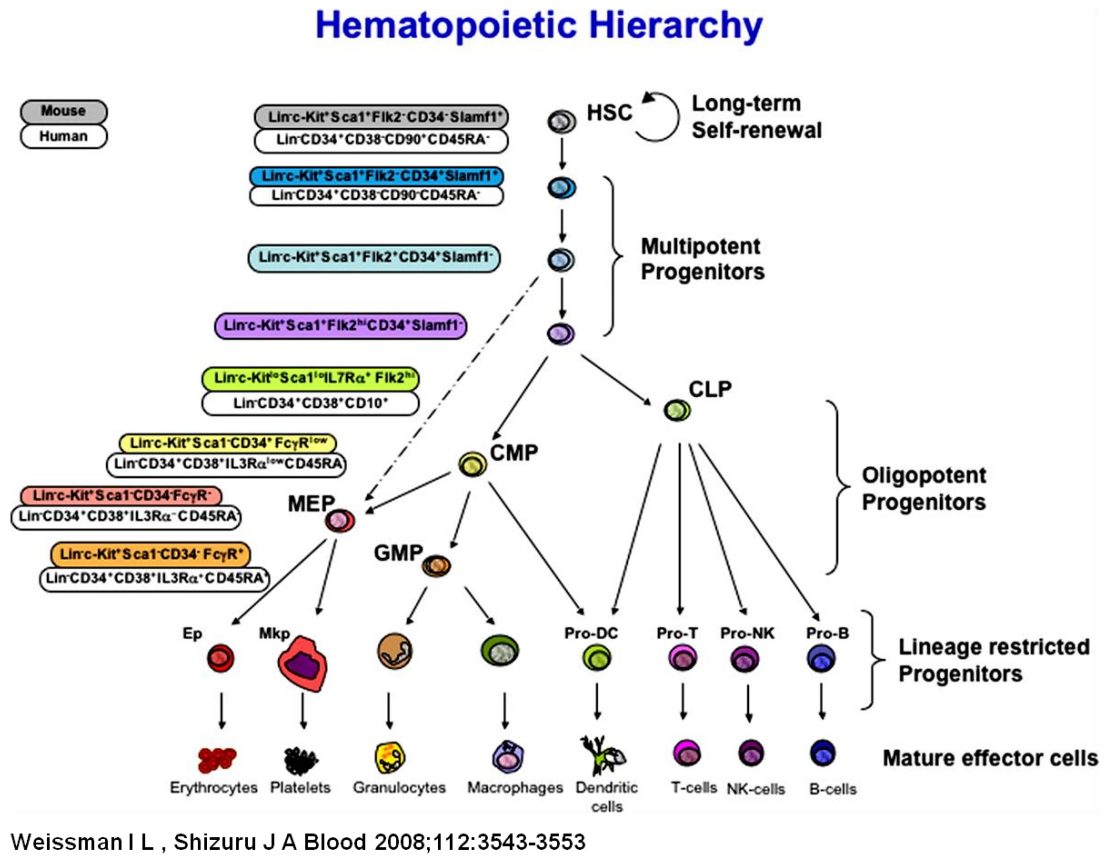

Weissman I L , Shizuru J A Blood 2008;112:3543-3553

Figure 3: Stem cell hierarchy

1.1.4 MHC system

Histocompatibility matching is one of the factors that play a major role in transplantation outcome [6]. There is a large cluster of genes defined as a major histocompatibility complex (MHC) and has a high degree of polymorphism. This cluster of genes plays very important role in transplantation, immunity, inflammation, infection and different diseases [31-32].The complex region is divided conventionally into three classes; class I, II and III, each containing a cluster of genes with related functions. Class I proteins are found on all body nucleated cells and present endogenous antigens that are processed by the proteasome to the cytotoxic CD8+ T cells. The exogenous antigens taken up by endocytosis or phagocytosis are degraded and loaded onto MHC class II, which are found in the immune cells such as B cells, dendritic cells and endothelial cells, then presented to helper CD4+ T cells. Class III genes encode the components of the complement system that target foreign cells and break their membranes. In humans, the MHC system is called a human leukocyte antigen (HLA) system and is located on the short arm of chromosome 6. It spans over four million base pairs of DNA and contains over 128 functional genes. The MHC

complex in the mouse is known as H2, and is located on chromosome 17. Class I encodes the K, D and L and class II encodes the Ia (I-A and I-E) antigens [33]. BALB/c mice express the d haplotype whereas C57Bl/6 express the b haplotype in their gene products [34]. Figure 4 A & B represent human and mouse MHC, respectively.

Due to the limited number of suitable donors and the risk of developing graft versus host disease (GVHD), MHC compatibility is still one major barrier to the success of HSCT. Consequently, there is extensive research to overcome this barrier; one approach is *in vitro* expansion of stem cells aiming to increase the number of cells, especially in cord blood transplantation [35]. Another aim is to produce cells that have less immunogenicity in order to overcome GVHD. However, there are several obstacles limiting this process, such as the function of the cells *in vivo*. Moreover, the expansion of specific required cells is still difficult according to current defined phenotype and technical limitations. [36-39].
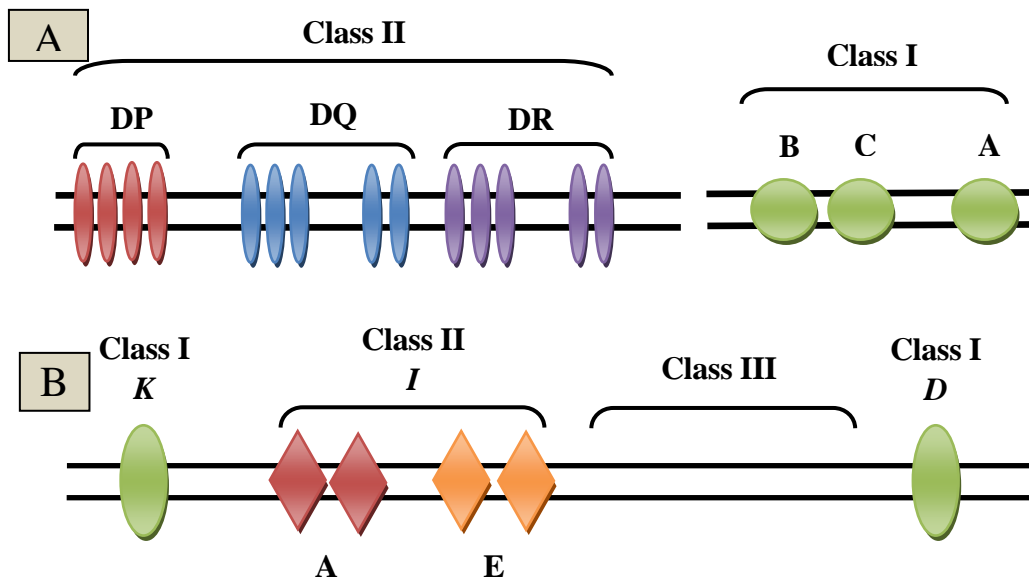

Figure 4: A represent human MHC and B represent mouse MHC

1.1.5 Hematopoietic stem cells (HSCs)

Stem cells can be retrieved from bone marrow (BM), peripheral blood (PB) and cord blood (CB) [40], as well as from other sources. Bone marrow was the first established source for stem cells followed by peripheral blood [41-44]. Cord blood as a stem cell source was established later, and is used mainly in pediatric transplantation due to the limited cell content. Cord blood is also used in adult transplantation; however, two units have to be combined for one recipient [45-47]. Numerous studies have compared the three sources of HSCs. The CB was reported to have lower GVHD intensity and treatment related mortality [48-49]. However, both Takahashi's studies related this advantage to the established set-up in their transplantation center, such as conditioning regimen, GVHD prophylaxis and genetic allo-reactivity between their studied populations. One of the advantages of the cord blood is its availability without the need

for a donor to go through the donation procedure. Moreover, cord blood transplantation required less histocompatibility matching. However, transplantation of peripheral blood or bone marrow from a living donor encounters the ability to repeatedly harvest stem cells in case of booster or re-transplantation are required. On the other hand, the availability of cord blood matching the first transplant could also be a limiting factor [50] for retransplantation after CBT.

Although no results have shown the advantage of PBSC transplantation over BMT and vice versa with regards to relapse, disease free survival, outcome and GVHD, PB donation has the advantage of being a less invasive procedure compared to the BM donation [51-52]. However, acute (aGVHD) and chronic GVHD (cGVHD) have been reported with high incidence after PBSCT [53-54]. PBSCs are more frequently used in autologous transplantations compared BM. One of the advantages of the PBSCT over the BMT is the faster engraftment, especially in malignant disorders [55-57].

New sources of stem cells such as adipose tissues are promising for the future of transplantation. Stem cells from adipose tissues have some advantages compared to other sources, such as its less complicated procedure and its capacity to produce other types of cells (for example, mesodermal and non- mesodermal lineages). The adipose tissue is also rich in mesenchymal stem cells [58-60].

1.1.6 Donor types

There are different types of transplantation depending on the donor. The cells can be obtained from the recipient (autologous transplantation). In this case the recipient's own cells are harvested and stored. The recipient then receives an intensive conditioning regimen adjusted to the disease, disease stage and co-morbidities. Afterward, the recipient receives his or her own stem cells through infusion. The G-CSF mobilized peripheral stem cell protocol is a standard procedure for an autologous transplantation [61-62]. High intensity conditioning has also been used before transplantation in the treatment of autoimmune diseases [63-64]. Syngeneic transplantation is the transplantation between genetically identical individuals with the same histocompatibility antigens, such as monozygotic twins in humans and members of the same inbred strain in animals. Because of the high polymorphism of the MHC, syngeneic transplantation in humans is rare. There is a very low incidence of GVHD in syngeneic HSCT, but there is an absence of graft versus malignant cells effect which compromises the outcome of HSCT in malignant diseases, especially in advance stages [65-69].

Allogeneic transplantation is transplantation between individuals within the same species, but with genetic disparities. This is a common type of transplantation in human due to limited number of identical twins and the benefit of allogeneic HSCT over syngeneic for cancer eradication. With a steady increase of transplantation applications to cover more diseases and patient groups, the research aims to minimize the complications and improve transplantation outcome [70-72]. One approach is to decrease the intensity of conditioning regimen and thereby reduce toxicity [73] without compromising the outcome. The success of these approaches increases the number of patients accepted for HSCT, including older patients including those who have minor organ dysfunction [74-76]. Xenogeneic transplantation is transplantation between two different species. This type of transplantation is used in special cases (such as transplantation of a heart valve), but its application in stem cell transplantation is very

limited [77]. Beside the major mismatches between the immune system of different species, infection transfer is a major concern that limits the research in this area.

1.1.7 Patient selection for HSCT

There are specific criteria to consider when determining if a patient is suitable for HSCT. These include: health status, disease stage, type of transplantation (autologous or allogeneic) and type and dose of conditioning regimen. Details on clinical criteria as well as which diseases are suitable for which type of transplantation at which stage of the disease are summarized elsewhere [78-83].

1.1.8 Conditioning regimen

The host immune system has to be suppressed in advance to permit the establishment of the donor immune system. Different conditioning regimens have been used to suppress the host immune system involving chemotherapy, radiotherapy or a combination of both. Anti-thymocyte globulin is sometimes added to the conditioning regimen [84-85]. Antineoplastic agents are classified according to cell cycle phase specificity and mechanism of action. Based on the mechanism of action, cytostatics are classified as alkylating agents [86-88], antimetabolites [89-90], anthracyclines [91], antitumor antibiotics [92], monoclonal antibodies [93] or platinum compounds [94]. Several alkylating agents are used in the conditioning regimens. The term alkylating agents arose from their ability to alkylate many nucleophilic functional groups. Alkylating agents can be divided into two groups according to their mechanism of action. The first group functions by forming covalent bonds with the amino, carboxyl, sulfhydryl and phosphate groups. The second group of alkylating agents acts by irreversibly binding to DNA and thus affecting the replication of DNA resulting in the disturbance of fundamental proliferation pathways. However, both groups of alkylating agents are cell cycle non-specific. Cisplatin, carboplatin and oxaliplatin are an example of the first group and cyclophosphamide, busulfan, chlorambucil, ifosfamide and mechlorethamine are examples of the second group.

Busulfan (Bu) and cyclophosphamide (Cy) in combination is a standard conditioning regimen [95] for stem cell transplantation. The introduction of Bu in the conditioning regimen reduced the use of TBI. The combination of TBI and Cy is still a common conditioning regimen. The combination of both drugs covers a wide spectrum of effects as Bu is myeloablative while Cy is immunosuppressive [96]. Bu is given orally or intravenously, and the advances in therapeutic dose monitoring has reduced Bu side effect [97]. However, individualizing the dose according to the patient's genetic variations is still not feasible [97]. Conditioning regimen Bu-Cy or Cy-TBI is selected based on the type and stage of the disease, since each combination has been shown to have its specific advantages. The Cy-TBI combination has been reported to have advantages in terms of less relapse, fewer transplant related mortality (TRM) and better disease free survival (DFS) in patients with acute lymphoblastic leukemia (ALL), whereas it has different effects in chronic myeloid leukemia [98-101]. However, the engraftment and GVHD occurrence is similar in both conditioning regimens [102].

The intensity and type of conditioning regimen is selected with regards to patient, disease type and stage as well as co-morbidities. Reduced intensity conditioning regimen (RIC) is usually considered for older patients [103-105], however, the use of

16

RIC in children is increasing [106-108]. One of the drawbacks after RIC is the increased risk of infections [109]. Although a high intensity conditioning regimen (myeloablative conditioning) has a higher risk for side effects, it is still the first choice in many situations (especially in advanced leukemia). However, intensive research is being done for the possible use of RIC or by a combination of conditioning regimens that has less toxicity without compromising the transplantation outcome [110]. Retrospective studies in patients with acute myeloid leukemia showed that transplant-related mortality was decreased and relapse was increased in patients treated with RIC, instead of myeloablative conditioning [111-112]. Leukemia-free survival was similar in both regimens.

1.2 COMPLICATIONS

Despite of continuous improvement in HSCT, several major complications still compromise its outcome. GVHD, sinusoidal obstruction syndrom (SOS) previously named as hepatic veno-occlusive disease (VOD) [113-116] and infections are the most common complications following HSCT [117]. Other complications after HSCT have also been reported, such as mucositis [118], dental development problems [118-120], hemorrhagic cystitis [121] and ocular GVHD [122-124].

1.2.1 Graft versus host disease

GVHD is a frequent complication that occurs after allogeneic stem cell transplantation when the new immune system derived from the donor recognizes the host tissues as foreign [125]. GVHD was first characterized in 1957 [126]. Extensive research and development of immunosuppressive drugs resulted in improvement of the prevention and the treatment of GVHD [127-130]. Nevertheless, GVHD is still the major obstacle in allogeneic HSCT whenever the transplantation is involving a high disparity between recipient and donor immune systems. Apart from the few reported cases [131], GVHD is not considered a barrier in syngeneic HSCT.

Specific conditions are required for GVHD to occur: the donor cells must be immunologically competent and the host should be immuno- compromised, and the host tissues must have histocompatibility antigens that are lacking in the donor cells [132-133]. GVHD is defined as acute or chronic according to its manifestation. The classical definition of aGVHD is that it takes place within the first 100 days post HSCT while cGVHD occurs beyond 100 days post HSCT. However, this is an arbitrary definition; acute and cGVHD onset may overlap if biology and symptoms are taken into account [134]. The onset of both types of GVHD is affected by the intensity of the conditioning regimen, graft type, graft content and immunosuppressant [135-136]. Donor lymphocyte infusion (DLI) used to enhance engraftment of donor cells or to induce an antileukemic effect may stimulate GVHD [137].

Acute GVHD is characterized by dermatitis, hepatitis and damage to the gastrointestinal tract resulting in abdominal pain and diarrhea [138]. The mechanism behind aGVHD has been extensively studied. The aGVHD takes place in three phases involving both the innate and the adaptive immune systems. [139-144]. The first phase starts when the conditioning regimen damages the host tissues and produce inflammatory signals. The gastrointestinal tract is one of the first targeted tissues. The tissue injuries translocate the lipopolysaccharides (LPS) to the circulation. LPS stimulate the tissues to secrete inflammatory cytokines such as IL-1, TNF-α and INF-γ.

The second phase of aGVHD is the stimulation of mature donor T cells to recognize the different antigens. This stimulation is achieved through the secretion of adhesion molecules and T cells attracting chemokines, as well as by the elevation of MHC expression in the inflamed tissues. The third phase is the effector part, which is consequence of the accumulation of the events from the two preceding phases. Both the innate and the adaptive immunoresponses in this step cause cytolysis. The activated macrophages and cytotoxic T lymphocytes start to attack the targeted tissues [140]. Figure 5 illustrates the pathophysiology of aGVHD [145]. However, other mechanisms that explain the pathophysiology of GVHD (such as early destruction of the host niche) have also been reported [146].

Chronic GVHD is less explored than aGVHD. cGVHD has features similar to immunological disorders and autoimmune diseases such as scleroderma, immune cytopenias and chronic immunodeficiency syndrome [147]. cGVHD can manifest in one organ or it can spread to several tissues. The patients who develop cGVHD may have a history of aGVHD. cGVHD can lead to organ dysfunction and life threatening complications. Although the mechanisms behind cGVHD are less clear than those in aGVHD, there are four suggested pathways for the development of cGVHD. Damage to the thymus and effects on the T cell negative selection, decrease in the regulatory T cells, uncontrolled production of antibodies and the formation of profibrotic lesions are the most probable pathways leading to cGVHD [148].

Donor T cells are among the cells responsible for acute GVHD through their immunological features and recognition of host antigens as foreign molecules. On the other hand, depletion of T cells from the graft either *ex vivo* or *in vivo* by treating the recipient with T cell antibodies such as anti-thymocyte globulin (ATG) can cause graft rejection and/or tumor relapse in cases where malignant disease has been the indication for transplantation [149-152]. Since donor T cells recognize malignant cells as foreign, the graft versus tumor effect is an important parameter of HSCT [153-155]. T cell subsets differ in their functional properties. Some of them have cytotoxic functions and some have been shown to play a regulatory role. However, the role of each T cell population and its involvement in cytotoxicity and regulatory effect has still not been totally identified. Within the T cells subsets, αβ T cells have been reported to be the main inducers of GVHD. CD4+ and CD8+ T cells induce GVHD by different mechanisms [156-159].

Regardless of the achievements in understanding GVHD, prediction of GVHD occurrence is still a major concern. Accordingly, all recipients undergoing HSCT receive immunosuppressive treatment (even in the case of HLA matched siblings) to prevent GVHD and host versus graft (HVG). At present, several immunosuppressive drugs are used in clinical practice. Cyclosporine A (CyA) is one of the most common used drugs for GVHD prevention after transplantation; however, these drugs also have side effects [158-159]. The golden standard for immunosuppression after HSCT today is cyclosporine combined with methotrexate [128, 160].

New strategies to prevent GVHD have been investigated either directly *in vivo* or *ex vivo* [161-162]. Soluble Fas ligand, for example, has been shown to enhance activation induced cell death when incubated with alloreactive cells before transplantation. This

method also results in proliferation reduction of allogeneic stimulator responses *in vivo*, improves the graft versus tumor effect and preserves the engraftment capacity of allogeneic cells [163]. NKT cells from recipients have been shown to prevent tissue inflammation caused by donor T cells, but retain their anti-tumor effect against B cell lymphoma. In the same study, the authors showed reduction in the expansion of donor T cells in the lymphoid tissues and the colon in aGVHD model [164]. Programmed death-1 (PD-1) protein, which belongs to the B7:CD28 superfamily diminished alloreactive T cell response after BM transplantation through its negative regulatory effect on CD4+ and CD8+ T cells. Therefore, an increase in the expression of PD-1 has been suggested to decrease GVHD [165].

Roncarolo MG et al, Nat Rev Immunol. 2007 Aug;7(8):585-98
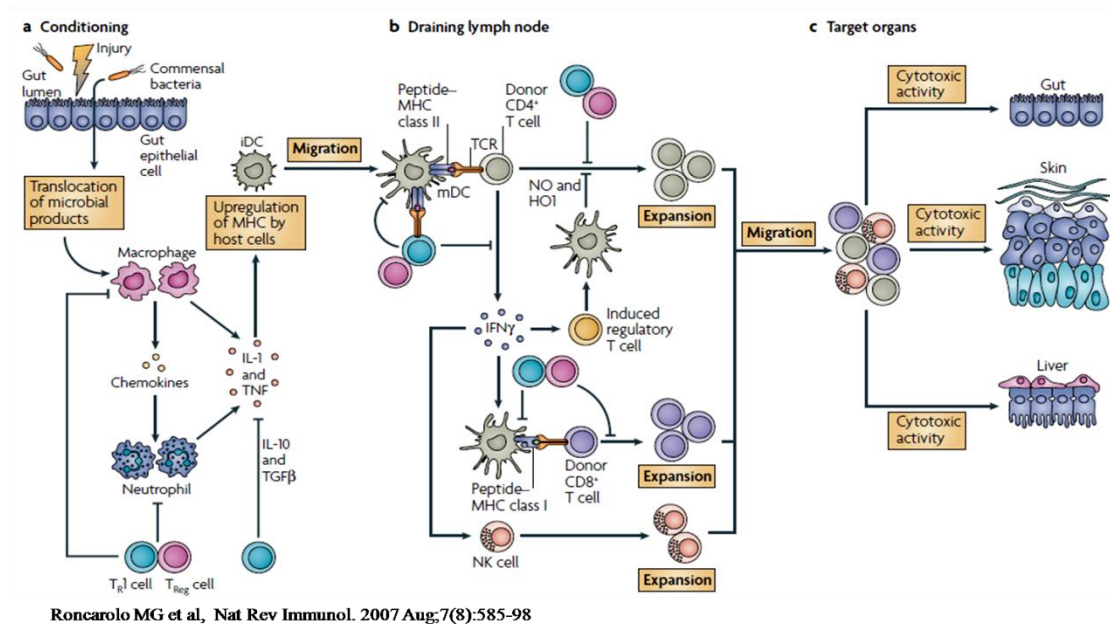
Figure 5: Pathophysiology of GVHD

1.2.2 Sinusoidal obstruction syndrome

SOS remains one of the complications following myeloablative HSCT which manifests as hyperbilirubinemia, hepatomegaly and fluid retention. SOS damages the hepatocyte and the sinusoidal cells in the centrilobular zone of the liver acinus. High doses of alkylating agents are responsible for SOS onset, which in severe cases could lead to multi organ failure [114, 116, 166-169]. Busulfan is known as one of the main risk factors for SOS [170-172]. Regardless of the long history of the disease, there is still no efficient curative therapy. Defibrotide is one of the promising therapies now in clinical trials [173]. However, since high dose chemotherapy is the initiative cause of the disease, reduced intensity conditioning can reduce the damage to tissues and result in a lower rate of SOS [174].

1.2.3 Rejection and disease relapse

Rejection occurs when the new immune system cannot compete with the existing immune system. When the conditioning regimen is not strong enough to suppress the host immune system, host immune cells start to proliferate.

Since T cells are the main cells involved in graft rejection, a lower number of T cells in the graft or a high number of residual T cells in the host might result in graft rejection. T cells from the donor are important in further eradicating the host cells. Moreover, donor T cells are necessary to kill cancer cells in case of malignancy. However, the type of donor cells or residual host T cells is also important in graft rejection. The higher number of *Treg* cells in the graft may help to develop graft rejection and disease relapse. Other host residual cells such as natural killer cells (NK) or DCs also contribute to graft rejection. An increased histocompatibility mismatch between recipient and donor increases the chance of graft rejection when the donor has MHC antigens not found in the recipient. Moreover, the RIC also results in higher residual cells and thus increases the chance for host cells to proliferate. The correct dosage and continuous administration of immunosuppressant limit T cell expansion and help to decrease GVHD and rejection [175-176]. ABO blood group matching is also critical; a mismatch increases graft failure after unrelated HSCT [177]. In identical HSCT the ABO mismatch causes some complications but do not impair the clinical outcome [178-180]. Although disease relapse goes hand in hand with graft rejection, disease relapse is more dependent on the type and stage of the disease. Moreover, second or third transplants are more likely to result in disease relapse [181] as the cancer cells develop resistance to the treatment. A greater understanding of the immune system, specific targeting of tumor cells and separating them from normal cells will help to decrease the frequency of relapse and to separate GVHD from the graft versus tumor (GVT) effect [182].

1.2.4 Infections

Infections after HSCT contribute to morbidity and mortality [117]. There is a high risk for bacterial infections during the neutropenic period when the immune system is still not recovered. Infection occurrence depends on several factors such as the aseptic procedure, type of graft, type of transplantation, the degree of mismatch between recipient and donor and the use of anti-infectious treatments. The incidence of different bacterial [183], fungal [184-187] or viral infections varies during the post HSCT period. Development of new prophylactic agents, post transplantation monitoring of infections and better aseptic techniques have resulted in a decreased incidence of several infections [188-192].

1.3 FATTY ACIDS

Nutrition is an important factor affecting health. Several food components have been shown to decrease the risk of various diseases [193-194]. Polyunsaturated fatty acids (PUFAs) have been reported to affect the immune system. PUFAs may also modify the effects of different therapeutic agents [195]. The efforts to reduce the complications of HSCT are ongoing, and food has been highlighted as a factor that affects transplantation outcome [196]. Fatty acids are linear hydrocarbon chains with a

terminal methyl group called omega (ω) and another terminal with a carboxyl (-COOH) group. The names and classifications of fatty acids are based on the number of carbon atoms and the double bonds. Fatty acids are classified according to the number of double bonds into saturated (no double bond), monounsaturated (one double bond) and polyunsaturated (two or more double bonds). The PUFAs are defined as short, medium or long chain PUFAs. Omega-3 and -6 are within the latter group as they consist of more than 16 carbon atoms. The omega-3 fatty acid has three main derivatives; α-linolenic acid (ALA), eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). The major omega-6 derivatives are: linoleic acid (LA), γ-linolenic acid (GLA) and arachidonic acid (AA). Although the human body can synthesize saturated and monounsaturated fatty acids, it is unable to produce an amount sufficient for its requirements. Thus, it is essential to obtain these acids from external sources. Moreover, the conversion of these fatty acids to polyunsaturated fatty acids is low, which makes it necessary to obtain the polyunsaturated acids from an external source as well [197-198]. ALA can be obtained from various types of vegetable oils and from some green vegetables, but the body has a limited capacity for converting this acid to DHA and EPA. Accordingly, DHA and EPA intake from other sources is rquired [199]. Nutritionists recommend that these fatty acids should be acquired through diet rather that supplemental products, since natural food also contains other beneficial elements such as proteins and vitamins [199]. Fatty fish such as tuna, salmon and halibut as well as fish oil are good sources of omega-3 EPA and DHA. The effects of omega-3 may also differ according to its source. Fish species, geographical location, water quality and other environmental factors may affect the ratio of omega-3 derivatives and thus affect their function [200]. Figure 6 demonstrates omega-3 and omega-6 synthesis process.

Figure 6: Omega-3 and omega-6 synthesis
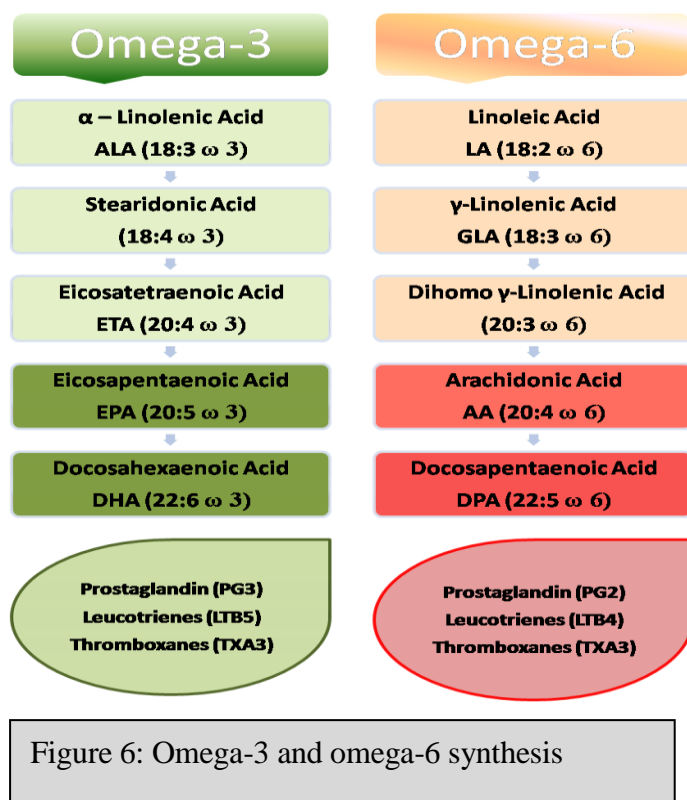
Omega-3 fatty acids have been shown to affect the immune system by altering the balance of Th1/Th2 towards Th2 by the suppression of Th1 development and reduction of IFN-γ production [201-202]. Omega-3 PUFAs have been reported to improve endothelial function, protect against oxidative DNA damage [203] and suppress CD4+ T cell function and proliferation [204-205]. Harbige et al 2003 reported that a high intake of PUFA has an inhibitory effect on adhesion molecule expression, antigen presentation and lymphocyte apoptosis whereas

a low intake could enhance some immune functions [206]. Based on these findings we hypothesized that omega-3 PUFA as dietary might affect the outcome of stem cell transplantation.

1.3.1 Omega-3 and its mechanisms of action

It is important to understand how omega-3 works inside the body in order to understand its function in different diseases and how to use it for therapeutic purposes. Several studies have explained the effect as the incorporation of omega-3 into the cell membrane, modulation of intracellular pathways and alteration of gene expression [207-208] which may result in the alteration of cell function [209-211]. Omega-3 fatty acids were reported to decrease triglycerides and very-low-density lipoprotein blood levels in hyperlipidemic individuals [212]. Omega-3 modulates cyclooxygenase (COX) activity and alters the membrane and cell fluidity, hence affecting the expression of different cell receptors [213]. Omega-3 has been reported to have an effect on prostate cancer through the COX-2 pathway [214-215]. The COX-2/prostaglandin E-2 (PGE-2) pathway has been reported to be involved in cancer promotion and progression [216]. The down regulation of COX-2 results in the decrease of its enzymatic product.

1.3.2 Omega-3 and diseases

Omega-3 plays an important role in health and disease. A shortage of omega-3 may lead to fatigue, poor memory, dry skin, heart problems, depression, and poor circulation [217].

1.3.2.1 Neurodegenerative diseases

Omega-3 has been reported to have beneficial effects on neurological complications such as Parkinson's disease [218], Alzheimer disease and other neurodegenerative diseases [219-220]. Although a low level of omega-3 may contribute to the manifestation and progress of neurological disorders [221], an extra supply of omega-3 does not eliminate them. However, there are some indications that omega-3 improves cognitive decline and dementia. Omega-3 might help to suppress disease development at an early stage, but does not seem to reverse the disease at an advanced level [222-225]. Thus, omega-3 seems to function protectively rather than as a treatment for neurodegenerative syndromes [226].

1.3.2.2 Omega-3 and cancer

The role of nutrition in cancer has become recognized during the recent years because of its impact on cell content and its interaction with several drugs, that might affect treatment efficacy. Omega-3 is one of the nutritional elements being studied, and it has been found to have potential to decrease tumor development and increase treatment efficacy [198, 200, 227]. Omega-3 has been reported to have a protective effect as well as a suppressive function toward the development of many types of solid tumors such as breast [228-230], colorectal [213] and prostate cancer [214, 231]. There is no clear evidence of an effect of omega-3 in hematological malignancies. However, some reports show that omega-3 have an effect on the growth suppression of leukemia cells in vitro [232-234].

22

1.3.3 Omega-3 and cytostatics

Omega-3 has been reported to enhance drug efficacy and to facilitate drug uptake in cancer chemotherapy. Omega-3 supplementation in combination with various chemotherapies has been shown to decrease tumor size and prolong patient survival with lower side effects [235-239]. Omega-3 may enhance tumor response to cytostatic agents in the treatment of breast cancer. The amount of omega-3 found in breast adipose tissue was correlated with the response to chemotherapy [240]. Cyclophosphamide widely used in the treatment of leukemia, lymphoma and solid tumors [241]. Cyclophosphamide is a pro-drug and is converted to active metabolites in the liver [242]. Growth inhibition of cancer cells by cyclophosphamide and/or its metabolites has been shown to be significantly enhanced by omega-3 [239]. In contrast, there is no report on the effect of omega-3 on busulfan function.

1.3.4 Omega-3 and inflammation

Omega-3 is among the most potent immunomodulatory and anti-inflammatory PUFAs. The lower incidence of autoimmune and inflammatory diseases in Greenland Eskimos (Upernavik district) and Japanese populations point out the importance of omega-3 and implicates that an increased intake of fish may protect against several diseases. In both populations, especially Greenland Eskimos, a higher ratio of long chain omega-3 PUFAs was found compared to other studied (mainly western) populations [243-245]. Inflammation is a complex biological process initiated by the innate immune system as a response to infection, tissue damage or other stimulus. Inflammation occurs in vascular tissues and the process involves various chemokines, cytokines and leukocytes. Although the process is a protective mechanism for the body, it may be harmful when it is out of control. The process requires the movement of leukocytes from the blood stream to the injured tissues through the blood vessels. In this process the recruited leukocytes migrate and adhere to the endothelial cells. The inflammation itself results in the expression of inflammatory products followed by antagonists in order to control the inflammation. Unresolved inflammation may lead to various types of diseases. Within a short time after the initiation of the inflammatory response, the process to resolve the inflammation begins. The granulocytes themselves promote prostaglandins and leukotrienes that have been derived from arachidonic acid to lipoxins. Omega-6 provokes the inflammatory process by supporting leukocyte adhesion and transmigration across the endothelial cells through the generation of the prostaglandin D2 (PGD2). Omega-3 prevents the progression of this process through the generation of prostaglandin D3 (PGD3) which antagonizes PGD2. Moreover, omega-3 reduces the expression of vascular cell adhesion molecule 1 (VCAM-1), E-selectin, the intercellular adhesion molecule 1 (ICAM-1) and inflammatory cytokines, which all promote the transmigration of the leukocyte [246-249].

1.3.5 Omega-3 and transplantation

Although omega-3 is extensively studied in many inflammatory disorders and in some cancer treatments, only a few studies have reported the effects of omega-3 on the outcome of HSCT. Thus, the benefit of omega-3 supplementation to transplanted patients is uncertain [250], due to limited data and the fact that HSCT is a complex procedure involving many factors. Transplantation involves the immune system,

immunosuppressive agents, conditioning regimen, disease type and patient status. Moreover, the amount of omega-3 used and the duration of treatment both influence the effect, something which is mirrored by the reported contradictory data. Nevertheless, some studies report benefits from omega-3 in at least some stages of the transplantation procedure inasmuch as omega-3 suppresses the inflammatory situation and may reduce drug toxicity. Apart from the two publications by Takatsuka 2001 [251] and 2002 [252], there are no further detailed studies on the effect of omega-3 on HSCT. The first study reported significant benefits from EPA supplementation and showed better survival in EPA treated patients, which was explained by reduced inflammation. The second study assessed the action of aGVHD indirectly by measuring the level of leukotriene (LT) B4 and several inflammatory cytokines. The study reported beneficial effects from EPA on aGVHD. However, both studies had a limited number of patients, and thus, the results are not fully conclusive.

1.4 REGULATORY CELLS

Many kinds of regulatory cells are naturally presented in the body or produced upon stimulation of the immune system. The conventional regulatory cells of the immune system are T cells that have the CD4+CD25+ phenotype and are developed by the thymus or in periphery. The intracellular transcription factor forkhead box P3 (FoxP3) has been reported as an essential marker for the development and suppressive properties of *Treg* cells [253]. However, natural and induced T*reg* cell populations have an important function in immune regulation [254]. *Treg* cells are also defined by other markers that differentiate them from other T cells in that they have lower or absent expression of CD127 surface antigen. However, other cells than CD4+ have also been reported to have regulatory functions, such as CD8+ *Treg* cells [255-258]. The different types of *Treg* cells have different potentials and play a major role in immune tolerance and immune homeostasis through different mechanisms. Regulatory cells have therapeutic potential, especially in transplantation tolerance [259-260]. Production of regulatory T cells in cell culture suppresses CD8+ T cell mediated cytotoxicity through the exhibited suppressive properties presented as proliferation suppression, inhibition of perforin and granzyme B lyses pathway [261].

1.4.1 Regulatory cells and diseases

Regulatory T cells play an important role in the maintenance of tolerance and are key factors in different types of diseases [262]. Hence, the impairment of *Treg* function has been reported to be involved in autoimmune [263], infectious and other inflammatory diseases. Defective ability of *Treg*s to suppress T cell proliferation was reported in several autoimmune diseases. *Treg* cells have also been reported to have defective suppressive capacity in multiple sclerosis [264-266], diabetes [267] and rheumatoid arthritis [268-269].

1.4.2 Regulatory cells and transplantation

Transplantation processes involve two immune systems and the acceptance of the transplant is dependent on the degree of histocompatibility matching. T regulatory cells are involved in tissue tolerance. The host considers the alloantigens from the donor as foreign and hence initiates an immune response against them. Immunosuppressants as external regulators protect the transplanted tissues from rejection and at the same time

protect the host from the transplanted cells. Immunosuppressive agents that have been used to suppress the immune system do not have long life supportive capacity, and they have to be continuously administered to the patient. Moreover, these drugs also have side effects and might contribute to organ failure. The long term complications of HSCT are still a major challenge; thus, there are several approaches to using *Treg* for therapeutic purposes in HSCT [270]. Consequently, it is necessary to better understand the natural regulatory mechanism of tolerance in order to lower side effects and improve transplantation outcome [271]. The immune response is controlled by regulatory cells in different ways. *Treg* cells may directly suppress the effector T cells.

The number and function of regulatory T cells affect the severity of GVHD [272]. *Treg* cells have been reported to attenuate GVHD after HSCT without compromising the graft versus tumor effect [273]. However, the effect is more pronounced in aGVHD compared to cGVHD [274]. Recently, several approaches using T*reg* cells for prevention or treatment of GVHD have been reported. The donor alloreactive T cells may cause GVHD, but the uncontrolled depletion of T cells may end in graft rejection. So, the new approach is to have selective donor T cells to preserve graft tolerance, to reduce the risk of infection and at the same time to prevent the development of GVHD. The induction of *Treg* cells in HSCT reduces GVHD while their depletion from the graft intensifies it. Figure 5 illustrates the role of *Treg* cells in the modulation of GVHD.

1.4.3 The effect of omega-3 on regulatory cells

Treg cells carry a high therapeutic potential in the transplantation field and in other immunological processes. It is important to understand factors that may affect the effectiveness of their function. Omega-3 has been reported to have an important role in different metabolic, inflammatory and autoimmune diseases, so it is of interest to study omega-3 impact on the regulation of the immune system. Omega-3 may exert its effect through the modification of various mediators or balancing of fatty acids in the cell membrane. Omega-3 has been reported to increase the induction of *Treg* cells through the activation of peroxisome proliferator activated receptor γ (PPARγ) [275-276]. The results of sveral studies on the effect of omega-3 in solid organ transplantation were not consistent, but some of the studies reported a beneficial outcome and prolonged survival [277-280]. Other studies reported no influence of omega-3 on acute rejection [281] or long term survival [281-282]. Since the majority of these studies used immunosuppressants concomitantly with omega-3, it is difficult to draw a conclusion on the direct effect of omega-3 on the immune system.

1.5 STEM CELL TRANSPLANTATION AND VASCULAR TOXICITY

Late lethal complications have been reported after stem cell transplantation [283-284]. Vascular toxicity is one of the adverse effects that may be involved in late complications such as congestive heart failure (CHF) and other cardiovascular complications [285]. It is difficult to correlate the early events to the late vascular complications after HSCT due to multiple factors that might be involved in the development of these complications. These factors include the patient individual disposition to vascular diseases, different conditioning regimens, different grafts, immunosuppressive drugs, nutrition, life style, etc. With an increased number of long

term survivors, the number of late complication is also increasing and thus better understanding of the early events in vascular toxicity is urgently required. Moreover, the numbers of elderly patients undergoing HSCT is increasing, which means that the risk of cardiovascular complications is increasing among this population as well [286-287]. A better understanding of the risk factors may help to reduce the late cardiovascular problems, especially in elderly patients and in patients at high risk of cardiovascular diseases such as hypertension or atherosclerosis.

1.5.1 Vascular system

The vascular system is made up of vessels with different functions and properties. It consists mainly of two types: the arteries and the veins. The aorta is the main artery and leads blood from the heart to the rest of the body. As the vessels branch to body tissues their size decrease. Defects in the vascular system cause different types of disease which might result in various organ defects including cardiovascular complications. Peripheral artery disease [288], pulmonary arterial hypertension [289] and renal artery stenosis [290-291] are examples of circulatory system defects.

1.5.2 Nitric oxide and the vascular system

Nitric oxide (NO) is involved in several biological processes and has been reported in cardiovascular disease [292]. NO was described in 1980 as a potent relaxant of vascular smooth muscle [293]. It interacts with different mechanisms in the body. NO is synthesized from L-arginine in a process that is catalyzed by nitric oxide synthase (NOS). NO is synthesized in different tissues such as endothelial and neuronal cells. There are three types of NOS; neuronal NOS (nNOS = NO I), inducible NOS (iNOS = NOS II) and endothelial NOS (eNOS = NOS III) [294].

1.5.3 Effect of conditioning regimen

Conditioning regimen prior to HSCT facilitates the establishment of the new donor cells. Approximately half of the patients undergoing HSCT are conditioned with chemotherapy. Chemotherapeutic agents used in cancer treatment have been correlated to serious cardiac complications [295-296]. Bu and Cy are commonly used in conditioning regimen prior to HSCT [297-298]. Cy is also used in many cancer treatment protocols [299] and in low doses in the treatment of several autoimmune diseases [300-301]. Treatment with Cy has been correlated to cardiac toxicity and other tissue damage. Although the cardiotoxicity was positively correlated with the dose of Cy [302], the symptoms usually appear 10 to 20 years after HSCT in long term survivals [286]. However, cardiac failure has also been reported within weeks of Cy exposure [303]. Bu, in contrast, has not been reported to be associated with vascular toxicity, but has been suggested to play a causative role in pericardial fibrosis [304].

2 AIMS

The present thesis aimed to increase the knowledge of the mechanisms underlying graft versus host disease in stem cell transplantation following busulfan - cyclophosphamide conditioning regimen.

SPECIFIC AIMS

- To study the early expansion of donor cells after allogeneic hematopoietic stem cell transplantation (HSCT).

- To correlate cell activation and cytokine expression with onset and intensity of graft versus host disease (GVHD).

- To study the dynamic changes of the histopathology of the liver, spleen, lung, kidney, heart and pancreas after allogeneic compared to syngeneic stem cell transplantation.

- To investigate the effect of busulfan - cyclophosphamide (Bu-Cy) conditioning regimen on the physiological properties and structure of the mesenteric arteries and aorta

- To study the effect of omega-3 on the efficacy and toxicity of Bu-Cy conditioning regimen.

- To study the effect of omega-3 on immunological response and on the onset and intensity of GVHD.

3 MATERIALS AND METHODS

3.1 ANIMALS

The mice were 8 to 12 weeks old and were purchased from Scanbur (Sollentuna, Sweden). They were maintained under pathogen-free conditions with controlled humidity ($55 \pm 5\%$), 12 hours light/dark cycle, balanced temperature ($21 \pm 2^{\circ}C$), and HEPA-filtered air. The animals were kept in individually ventilated cages and were fed autoclaved chow and tap water *ad libitum*. They were acclimatized for 1 to 2 weeks before the start of the experiment.

All animal experiments were approved by the southern ethical committee and in accordance with the Animal Protection Law, the Animal Protection Regulation and the regulation of the Swedish National Board for Laboratory Animals.

In the syngeneic setting female BALB/c ($H-2K^d$) were used as recipients and male or female BALB/c mice were used as donors. While in the allogeneic setting BALB/c mice were used as recipients and male C57Bl/6 ($H-2K^b$) were used as donors. Untreated mice were used as controls whenever appropriate.

The mice were weighed and their health status was assessed as described in detail in each study. In experiments entailing GVHD, the recipient mice were weighed every day and GVHD scored every other day. GVHD was scored using the following criteria [305] described in table 1:

Criteria	Grade 0	Grade I	Grade II
Weight lost	< 10%	10 to 25%	>25%
Posture position	Normal	Hunching but not during movement	Severe hunching effecting movement
Activity	Normal	Mildly 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

Table 1: Criteria for GVHD after HSCT

For the last study evaluating the effect of Omega 3, animals in different groups were fed one of the following diets: 1) Omega-3 (from menhaden fish oil) 10% supplement, 2) Corn oil (10%) supplement or 3) Standard food. The animals in all the groups had access to their food *ad libitum* for three or eight weeks respectively. The corn oil diet, which is the source of omega-6, was given as a control for the fat effect.

3.2 ANTIBODIES

Fluorescein isothiocyanate-(FITC) conjugated $H-2K^b$(clone: AF6-88.5), $H-2K^d$(clone: SF1-1.1), CD3 (clone: AF6-88.5), NK (clone: DX5), CD44 (clone: IM7), Ia-IE (clone: 2G9) and Phycoerythrin-conjugated (PE) conjugated $H-2K^d$(clone:SF1-1.1), CD8 (clone: 53-6.7), $H-2K^b$(clone: AF6-88.5) and PerCP-Cy5.5 conjugated CD3 (clone:

145-2C11), CD25 (clone: PC61), CD11b (clone: M1/70) and APC conjugated CD4 (clone: RM4-5), CD19 (clone: 1D3), CD62L (clone:MEL-14), CD11c (clone: HL3) and FC-receptor blocking monoclonal antibody (clone: 2.4G2) were purchased from BD Pharmingen (BD Biosciences, Stockholm, Sweden).

3.3 CONDITIONING REGIMEN

Busulfan and cyclophosphamide were purchased from Sigma-Aldrich (Sigma-Aldrich Stockholm, Sweden). Busulfan was dissolved in dimethyl sulfoxide (DMSO) (Sigma-Aldrich) at a concentration of 40mg/ml and was stored at room temperature (RT). Cyclophosphamide was dissolved in sterile water at a concentration of 10mg/ml and stored at RT. Both drugs were further diluted to working sultion using PBS . Each mouse received 20mg/kg/day x 4 days of busulfan followed by 100mg/kg/day x 2days of cyclophosphamide (Figure 7). Both drugs were administered via intra-peritoneal (IP) injection.
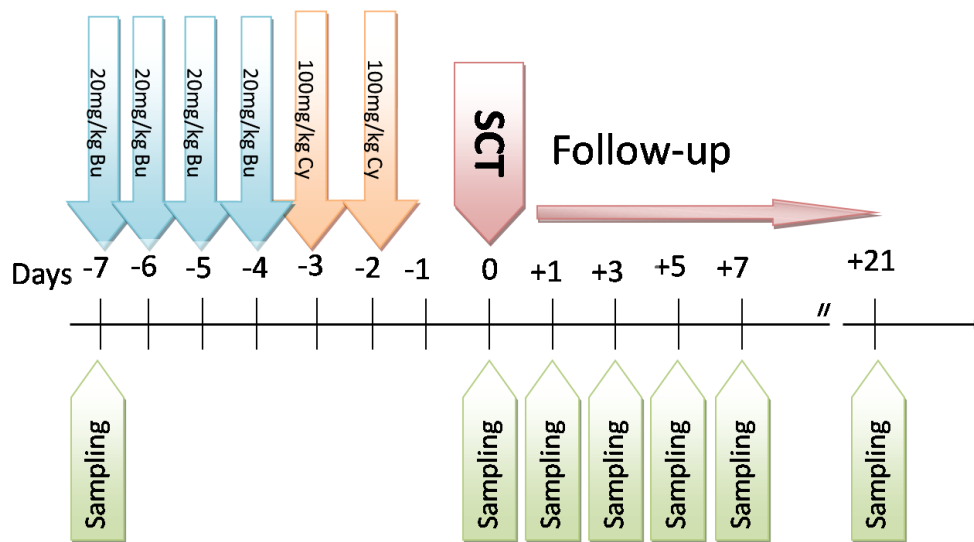
Figure 7: Schematic diagram for Bu-Cy conditioning regimen and HSCT

3.4 BONE MARROW TRANSPLANTATION PROCEDURE

3.4.1 Tissue harvesting

Donor mice were killed by cervical dislocation and then placed on their backs on clean, dry and absorbent paper. The mouse skin and fur was cleaned with 70% ethanol to disinfect the area and reduce the possibility of contamination. A mid abdominal incision was made with scissors and forceps. The skin was detached from the peritoneal wall. Using sterilized forceps and scissors, the abdomen and peritoneal area were explored carefully. The spleen was removed and placed in a cold 2% FBS/PBS (phosphate buffer saline; PBS, and fetal bovine serum; FBS), which were purchased from Invitrogen (Invitrogen AB, Stockholm, Sweden). Both femurs and tibias were aseptically removed and placed in cold 2% FBS/PBS.

3.4.2 Cell preparation

The spleens from the donor mice were placed one by one in sterile Petri-Dishes and cut. The cells were re-suspended in 2% FBS/PBS containing 0.01 mM EDTA, then passed through a 70 μm cell strainer into a 50 ml conical tube. The syringe plunger was gently used to disintegrate cell clumps.

Femurs and tibias were cleaned from soft tissues by sterile mesh and then placed in a clean Petri-Dish containing sterile PBS. The bone marrow was flushed with 5 ml 2% FBS/PBS containing 0.01 mM EDTA into 50 ml conical tube. Single cell suspension was prepared by gently mixing the cell suspension using a 14G needle attached to a 5 ml syringe.

The pooled cells from either spleens or BM were counted separately using Türk and Trypan Blue solutions to assess the total number of nucleated and viable cells. Appropriate numbers of BM and SP cells were passed through a 70 μm cell strainer before transplantation. The final volume of the graft was adjusted to 200 μl of cell suspension per mouse.

3.4.3 Transplantation

2×10^7 BM cells and 3×10^7 spleen cells in a volume of 200 μl were injected through the lateral tail vein. Transplantation day was assigned as day 0, the days before transplantation were numbered with a negative sign (-) and the days after transplantation were numbered with a positive sign (+).

3.5 TISSUE ANALYSIS

PB, spleen, femur and other studied tissues were collected for analysis at appropriate time points according to each study design.

3.5.1 Immunophenotyping

Surface markers: The mouse was placed in the mouse holder and a small cut was made in the tail vein using a clean sharp scalpel. Around 50 μl of the peripheral blood was sampled either by dropping the blood into an Eppendorf tube (pre-filled with 20 μl 0.5 mM EDTA or citrate) or by collecting the blood using a needle and syringe. Samples were then transferred into FACS tubes. Fc-blocker was added to the cells and then incubated for 15 minutes at 4^oC. The cells were incubated with the appropriate antibodies for 30 minutes at 4^oC. 1 ml of 1X RBC lysing buffer (BD Biosciences, Stockholm, Sweden) was added and then the samples were incubated at room temperature for 20 minutes in dark place. 2 ml of PBS were added and then the samples were centrifuged for 7 minutes at 350 g. The supernatant was discarded and the cells were then re-suspended in 200 μl of PBS. The samples were analyzed using FACS calibur (BD biosciences) and CellQuest software.

FoxP3 intracellular staining: Single cell suspension was prepared as above. Red blood cells (RBC) were lysed with ammonium chloride and then washed twice with PBS. The cells were incubated with Fc-blocker at 4^oC and stained with antibodies against surface markers. The cells were then washed and permeabilized in BD Perm/Wash solution

(BD Biosciences), after which they were washed with the same solution and stained with intracellular staining for PE-Fox*P3* antibody (ebiosciences, clone: FJK-16s).

3.5.2 Mixed Lymphocyte Culture (MLC)

Regulatory T cell function was assessed with MLC. Regulatory T cells were prepared from the spleens of female BALB/c mice. Red blood cells in single cell suspension were lysed by ammonium chloride. The cells were washed in PBS, after which CD4+CD25+ regulatory T cells (*Treg*) were isolated using Miltenyi Biotec (Fisher Scientific, Göteborg, Sweden) mouse isolating kit according to the manufacturer's recommendations. Spleen cell suspension from female BALB/c mice was used as responder (T*resp*) cells. The T*resp* cells were stimulated with anti-mouse CD3e (BD biosciences, Stockholm, Sweden) at two different concentrations (3µg/ml and 10µg/ml). Different ratios of T*resp* to *Treg* were used as follows; 1: 1 (1 x 10^5 T*resp*: 1 x 10^5 *Treg*), 1: 0.5 (1 x 10^5 T*resp*: 5 x 10^4 *Treg*) 1: 0.25 (1 x 10^5 T*resp*: 2.5 x 10^4 *Treg*). One µl of ^{3}H thymidine (0.037 MBq/µl) was added to each well in the mixed lymphocyte reaction (MLC) plate on day 4. After 18 hours, 100 µl of the supernatant was taken carefully from each well for cytokine measurement. The cells were harvested on filter with a Cell Harvester (Skatron Instruments Ltd, Suffolk, UK) and allowed to dry overnight at RT. The activity was measured in scintillation fluid (OptiScint Hisafe) using a liquid scintillation counter (WALLAC, EG&G Comp, Turku, Finland).

3.5.3 Histology and immunohistochemistry

For the histology staining, tissue samples were fixed in 4% formaldehyde for one day and then transferred to 70% ethanol. The tissue was dehydrated and embedded in paraffin. The tissue were sectioned and stained in Hematoxylin and Eosin solution using the Tissue Tek Prisma (Sakura Finetek Inc, Torrance, CA, USA) automated slide stainer. For the immunohistochemistry, the tissue were covered in cryo-embedding media (OCT) and frozen on dry ice cooled N-hexan. The tissue was stained with CD4 (RM4-5) and CD8 (53-6.7) antibodies from BD Pharmingen (San Diego, CA, USA). Tissue was cut into 4-5 μm slices, fixed in cold (-20^oC) acetone for 3 minutes and left to dry overnight. The tissue was rinsed with PBS, and treated with 3% H_2O_2 in methanol and blocked by 4% goat serum in PBS. Primary antibodies were diluted in the blocking solution and applied at 4^oC for one hour. After rinsing in PBS, a biotin-labeled secondary goat anti-rat antibody was applied. Sections were incubated with ABC-HRP complex (BD Pharmingen). Binding sites were visualized with diaminobenzidine/hydrogen peroxide. The slides were re-stained with hematoxylin.

3.5.4 Colony forming unit assay

Mice fed with omega-3, corn oil or standard food were killed and single cell suspensions were prepared from bone marrow. BM cell were treated in vitro with Bu in final concentration of 20, 40 or 60 µg/ml for 4 hr. Twenty thousand nucleated cells were plated in 1.1 ml MethoCult M3434 medium in 35 mm Petri dishes in duplicates. Dishes were incubated at 37°C with 5% CO_2 and 95% humidity. Colony forming unit-granulocyte-macrophage (CFU-GM) was scored at day 12 using an inverted microscope. CFU-GM was defined as consisting of 50 or more cells.

3.6 ASSESSMENT OF VASCULAR SYSTEM

Mice were conditioned with 80 mg/kg Bu for four days followed by 200 mg/kg Cy for two consecutive days. Five days after the last injection mice were sacrificed by cervical dislocation and the heart, aorta and mesentery were dissected and placed in ice-cold Calcium free physiological salt solution (Ca0PSS, composition in mmol/L: NaCl, 119; KCl, 4.7; $MgCl_2$, 1.2; KH_2PO_4, 1.2; $NaHCO_3$, 25; glucose, 11; Na_2EDTA, 0.03). The small mesenteric arteries (2-3 loops of the intestine with radial arteries up to the stub of the superior mesenteric artery intact) and the aorta were stored for a maximum of 3 hours on ice before the start of the experiment. The weight of the heart was related to the body weight as determined at the time of sacrifice. Right ventricular and left ventricular weights (including septum) were also determined.

3.6.1 Physiological study

Radial intestinal arteries (arteries running perpendicular to the surface of the intestine) or their first order branches were used in the present experiment. Using ophthalmological quality instruments, the arteries were freed by careful dissection of adhering fat, connective tissue and the accompanying vein. After making a small incision into the free wall of the micro-artery, two stainless steel wires (diameter 40μm) were introduced into the lumen of the micro-artery, taking care not to scrape the intima or to stretch the preparation longitudinally. Approximately 1 mm of preparation was thus transferred with 2 luminal stainless steel wires to a 5 ml organ bath in a small vessel myograph (DMT A/S, Skejby Science Crt, Aarhus N, Denmark). The two wires were then tightened parallel and under tension with screws on two specimen holders, one attached to a linear actuator, the other to an isometric transducer, for registration of circumferential isometric force. The organ bath was filled with 5 ml Ca0PSS and the actuator was moved carefully until a small amount of isometric force was registered. The internal circumference of the vessel at which this force was obtained was recorded and labeled "slack circumference". The next step varied according to the experimental protocol. An identical procedure was followed for the distal part of the thoracic aorta, except that rings (approx. 2 mm width) were mounted on two parallel stainless steel pins (0.2mm diameter). Solutions were freshly prepared, pre-oxygenated (95 % O_2 - 5 % CO_2) solutions and heated to 37 °C before use. The solutions in the organ baths were oxygenated with the same gas mixture and also kept at 37 °C.

3.6.2 Length-Force measurement

Having obtained slack circumference for both aorta and mesenteric micro vessels in Ca0PSS, the preparations were equilibrated in CaPSS ($CaCl_2$, 2.5mmol/L) for 30 minutes. Then the vessels were challenged every 5 min for 60 s with KPSS (CaPSS with isotonic replacement of Na^+ with K^+, final K^+-concentration 125 mmol/L). For mesenteric micro vessels, the peak of the KPSS-induced contraction (coming within 15 seconds of application of KPSS) was subtracted from the mean value of the baseline as obtained during 30 s preceding the KPSS-challenge (K_{act}). For the aorta, the value of the KPSS-induced contraction at 60 s was used for K_{act} as there was, in most instances, no clearly discernable peak early in the contraction. After the 2nd KPSS-challenge, the internal circumference was stepwise increased by 0.2 mm (0.8 mm for aorta) once approximately 2 min after the KPSS-challenge. Once K_{act} was equal to or lower than

the preceding value, the internal circumference was reduced by 0.1 mm (micro vessels) or 0.4 mm (aorta). The internal circumference at this stage was also recorded and labeled optimal internal circumference (IC_{opt}). When the complete length-force relation was not determined, the vessels were stretched to their IC_{opt} in Ca0PSS immediately after determining the slack internal circumference and were then equilibrated for at least 30 min in CaPSS before the start of the experimental protocol.

3.6.3 Contractile and relaxant agonists

U46619 noradrenalin (Sigma-Aldrich) and endothelin-1 (Bachem AG, Bubendorf, Switzerland) were applied cumulatively with log-step increases in concentrations. The zenith at every concentration was subtracted from the baseline and expressed as % of the K_{act} at IC_{opt} obtained in the same vessel. Acetylcholine-, sodium nitroprusside- and forskolin- (Sigma-Aldrich) induced relaxations were initiated from a stable contraction induced by noradrenalin (between 1 and 30 µmol/L). Concentrations were applied cumulatively with log-steps increases. From the nadir at each concentration, the stable contraction obtained by noradrenalin before the applications of the vasodilators was subtracted. This value was then normalized to the maximal relaxation (obtained by adding 1 mmol/L sodium nitroprusside and 0.2 mmol/L papaverine (Sigma-Aldrich) in Ca0PSS at the end of the experiment and expressed as % of this maximal relaxation. The following diagram is an example from a Bu-Cy treated mouse. Figure 8 shows length-force measurements followed by noradrenalin steps contractions.
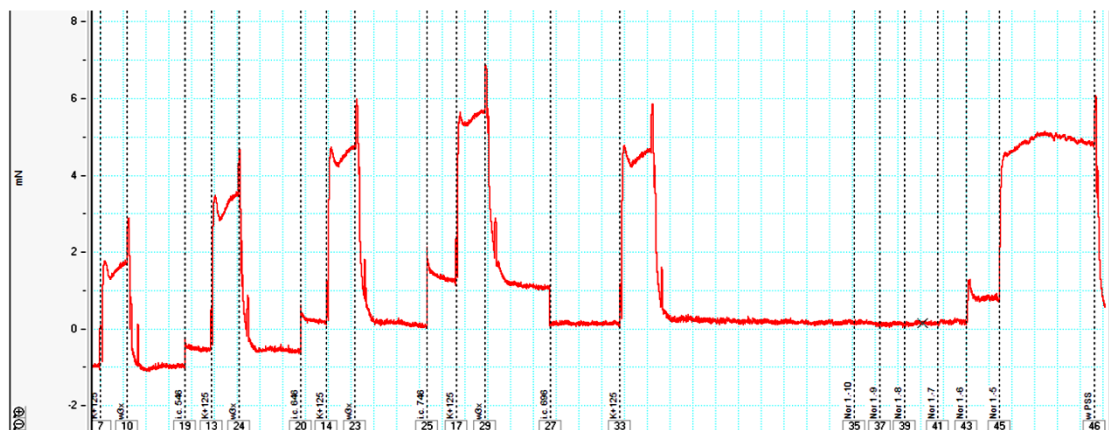
Figure 8: Myograph record of length-force relation followed by noradrenalin step contractions.

3.6.4 Blood pressure

The mice were anaesthetized with isofluran (2.6 %; Univentor 400, AgnThos, Stockholm, Sweden) and placed on a servo-controlled heating pad maintaining body temperature at 37.5 ºC. Blood pressure was measured with a fiberoptic transducer (Samba 420/360, Samba sensors AB, Västra Frölunda, Sweden) inserted in the left carotid artery. After 15 minutes of stabilization, the pressure was continuously sampled
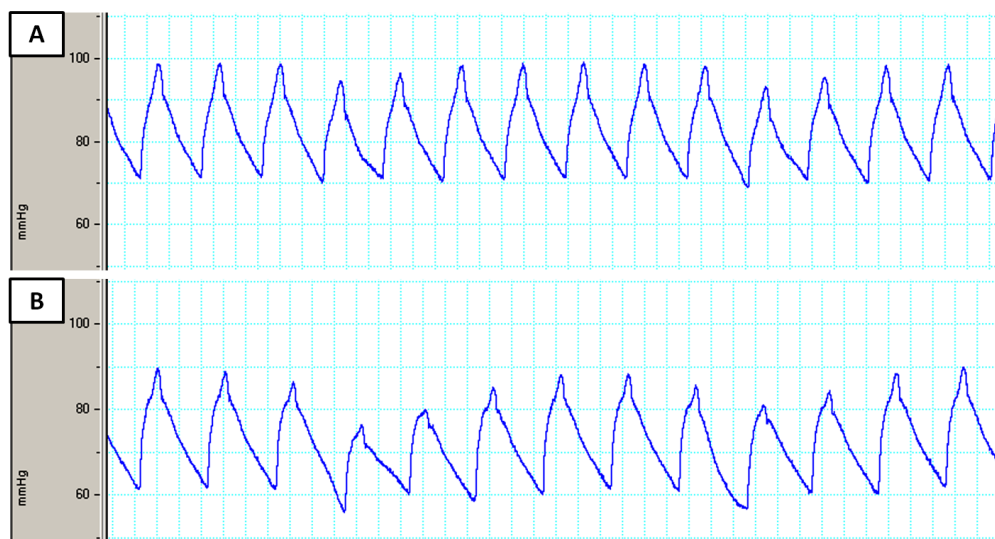
Figure 9: Myograph record of blood pressure from a control group animal (A) and from a Bu-Cy conditioned mouse (B)

during 10 minutes for later analysis. Figure 9 represents an example for blood pressure from a control animal (A) and an animal from the Bu-Cy treated group (B).

3.6.5 Measurement of the endothelial nitric oxide synthase (eNOS) expression

The mRNA expression was determined with RT-PCR. Total RNA was isolated from 3 – 5 mg of frozen mesenteric artery and aorta using RNeasy Mini Kit (Qiagen, Valencia, CA, USA) according to manufacturer's protocol. Total RNA (62 ng from mesenteric artery and 180 ng from aorta) was reverse-transcribed using iSCRIPT^{TM} cDNA synthesis kit (Bio-Rad, Hercules, CA, USA). cDNA samples were amplified using 2x SYBR Green PCR Master Mix (Bio-Rad) at optimal concentrations (10 nmol/L) of primers in a total reaction volume of 20 µl under the conditions recommended by the manufacturer. Expression levels of genes were normalized to that of ribosomal RNA S18 to control for input gene. Samples were assayed in duplicate using expression profiles generated using the comparative Ct method implemented in the Applied Biosystems 7500 Real-Time PCR System. The following primers were used (5'to3'):
eNOS F: CCTTCCGCTACCAGCCAGA,
eNOS R: CAGAGATCTTCACTGCATTGGCTA,
S18 F: CGCGGTTCTATTTTGTTGGT and
S18 R: AGTCGGCATCGTTTATGGTC.

3.6.6 Western Blot

For Western Blot analysis, $3 - 5$ mg of arterial tissues were homogenized in 100 µl ice-cold buffer containing: 50 mM Tris.HCl (pH 7.5), 150 mM NaCl, 0.5% NP-40, 5 mM EDTA, 1 mM Na_3VO_4, 20 mM NaF and a protease inhibitor cocktail (Roche GmbH, Mannheim, Germany), 1 mM dithiothreitol (DTT) and 1 mM Phenyl- methylsulfonyl fluoride (PMSF). Homogenates were cleared by centrifugation (13,000 rpm; 15 min, 4°C) and the protein contents of the supernatant were determined using Bio-Rad Protein Assay (Bio-Rad. Laboratories, ON, USA). Samples were prepared in 4x NuPAGE LDS sample buffer, 10x NuPAGE reducing agent (Invitrogen) and heated for 10 min at 80°C before electrophoresis. 5 and 30 µg of proteins from mesenteric artery and aorta, respectively, were separated on 10 % SDS-PAGE followed by transfer to a PVDF membrane. Membranes were blocked for 1 hour at room temperature in TBST containing 5% non-fat dry milk or BSA followed by incubation with eNOS or ß-Actin antibodies. Membranes were washed with TBST buffer (0.01% Tween-20) followed by incubation with HRP-IgG antibodies. Membrane blots were then exposed to ECL detection reagents (SuperSignal West Pico Chemiluminescent Substrate; Pierce) and visualized using x-ray films. Band intensities were quantified by using Quantity One software (Bio-Rad Laboratories, ON). All antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

3.6.7 Transmission electron microscopy (TEM)

Mesenteric vessels were dissected as above and pieces were fixed in 2.5% glutaraldehyde + 1% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4 at room temperature for 30 min and stored in the fixative at 4 °C. Specimens were rinsed in 0.1 M phosphate buffer, postfixed in 2% osmium tetraoxide in 0.1 M phosphate buffer, pH 7.4 at 4°C for 2 hours, dehydrated in ethanol followed by acetone and embedded in LX-112 (Ladd, Burlington, Vermont, USA). Semi-thin sections were cut, stained with toluidine blue and used for light microscopic analysis. Ultrathin sections (approximately 40-50 nm) were cut and contrasted with uranyl acetate followed by lead citrate and examined in a Tecnai 12 Spirit Bio TWIN transmission electron microscope (Fei Company, Eindhoven, The Netherlands) at 100 kV. Digital images were taken using a Veleta camera (Olympus Soft Imaging Solutions, GmbH, Münster, Germany).

3.6.8 Statistical analysis

Graph prism version 4, SPSS version 16 were both used for graph design and data analysis. Student t-test was used for the comparison between two groups. Other analysis tests were used as indicated in each study. Results consider significant when the $P < 0.05$.

4 RESULTS

4.1 STUDY I

4.1.1 BM and spleen cellularity

Bu-Cy conditioning [306] resulted in a 95% and 63% decrease in bone marrow and spleen cellularity, respectively. This conditioning significantly reduced most of the cell populations with the exception of some T subpopulations that showed resistance to the conditioning regimen. The CD11b+ myeloid cells were the most affected in BM while B cells were the most affected in spleen. We assessed the bone marrow cellularity within 21 days post HSCT in syngeneic and allogeneic settings. In syngeneic settings, the recovery of cellularity in both tissues started earlier and increased until full recovery in the BM and over 70% recovery in the spleen within three weeks. In contrast, the allogeneic HSCT showed a delayed recovery in both tissues and never reached normal levels (Figure 10).
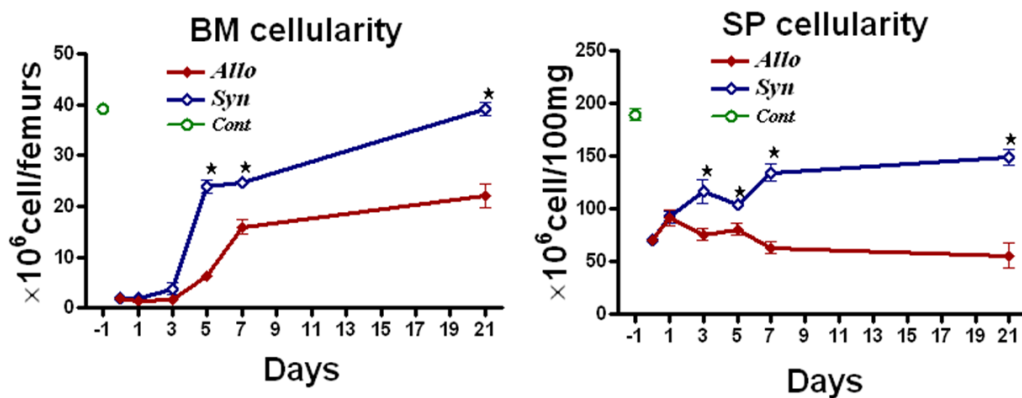
Figure 10: BM and spleen cellularity from syngeneic (connected open signs) and allogeneic (connected filled signs) transplantation

4.1.2 Expansion and phenotype of immune cells

Early expansion of natural killer (NK) cells was observed in syngeneic and allogeneic HSCT and reached an expansion peak at day +3. NK cells declined within the next days post transpation in syngeneic and allogeneic settings. In the syngeneic HSCT, NK cells expanded until full recovery while they continued to decrease in allogeneic HSCT (Figure 11A). Dendritic cells followed a pattern similar to that seen in NK cells. Interestingly, the expanding DCs were of donor origin while the host DCs decreased gradually (Figure 11B, F) . CD8+ T cells increased to reach maximal expansion at day +5 after allogeneic HSCT in BM and spleen. CD8+ T cells from BM and spleen were decreased in the syngeneic setting, in contrast to the allogeneic setting (Figure 11 D). The allogeneic setting showed that the T cells that were expanded at day +5 were mainly effector memory T cells. Hence, the number of naïve cells continued to decrease in this setting (Figure 11E). In contrast, there was no significant expansion observed at day +5 in memory T cells in the syngeneic setting. In summary, donor T cells continued their expansion in contrast to the decline in the host T cells (Figure 11).

Interestingly, the cytokine expression followed the expansion pattern of the cells. Interleukin 2 (IL-2), tumor necrosis factor alpha (TNF-α) and interferon gamma (INF-γ) in serum had all reached their highest level at day +5 in allogeneic HSCT (Figure 12).
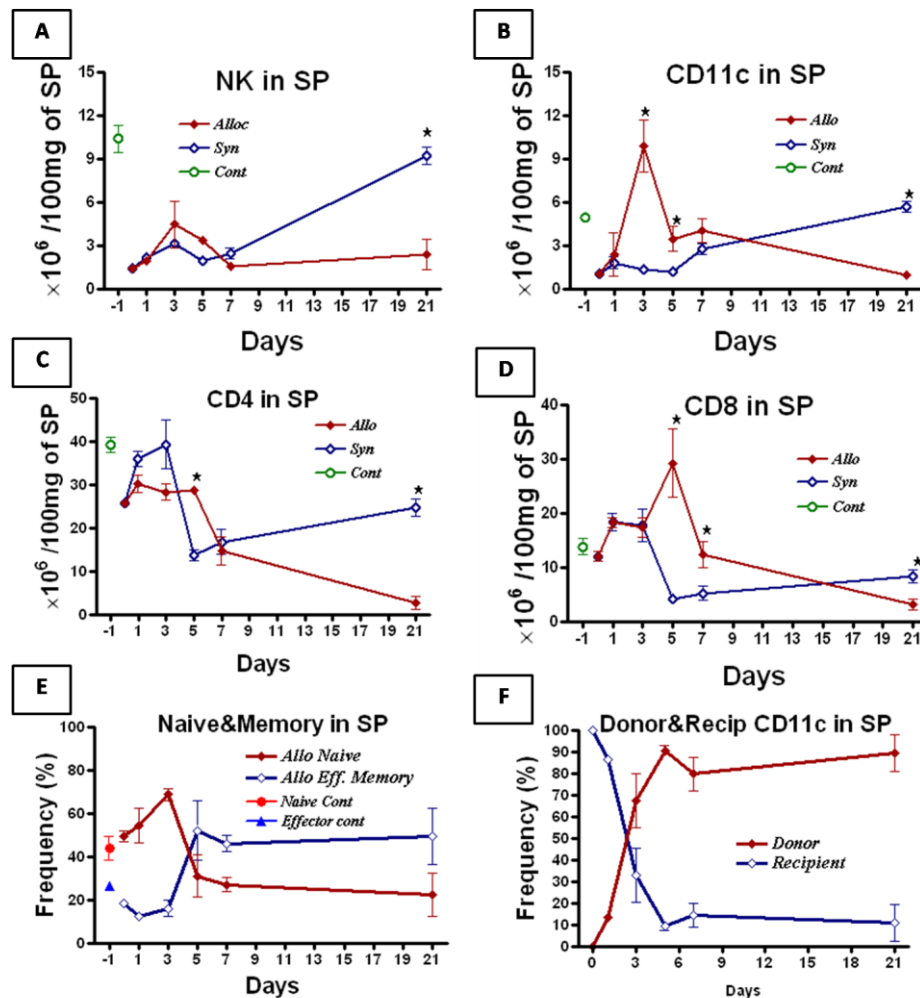
Figure 11: Shows expansion and decline of different cell population: NK, CD11c, CD4 and CD8 cells from allogeneic (connected filled signs) and syngeneic (connected open signs) HSCT. Memory cells from donor (connected filled signs) and recipient (connected open sigs). Donor (filled signs) and recipient (open signs)
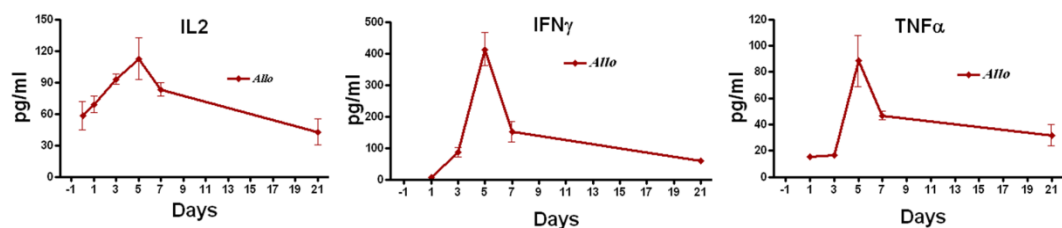
Figure 12: Serum levels of IL-2, INF-γ and TNF-α at different days after allogeneic HSCT.

4.2 STUDY II

In this study, the same established conditioning regimen was used for syngeneic and allogeneic HSCT. The mice tissues were harvested before the conditioning, after the conditioning (the same transplantation day), and then at five, seven and twenty one days post-HSCT for histology evaluation. The mice of both groups lost weight during the conditioning regimen and the weight decrement continued until day +3 and day +7 in syngeneic and in allogeneic groups, respectively. However, the mice that underwent syngeneic transplantation started to gain weight and reached normal levels within three weeks, while the weight of mice in the allogeneic group remained low. The weight loss corresponded to the intensity of the GVHD in the allogeneic group, which was not the case in the syngeneic setting.
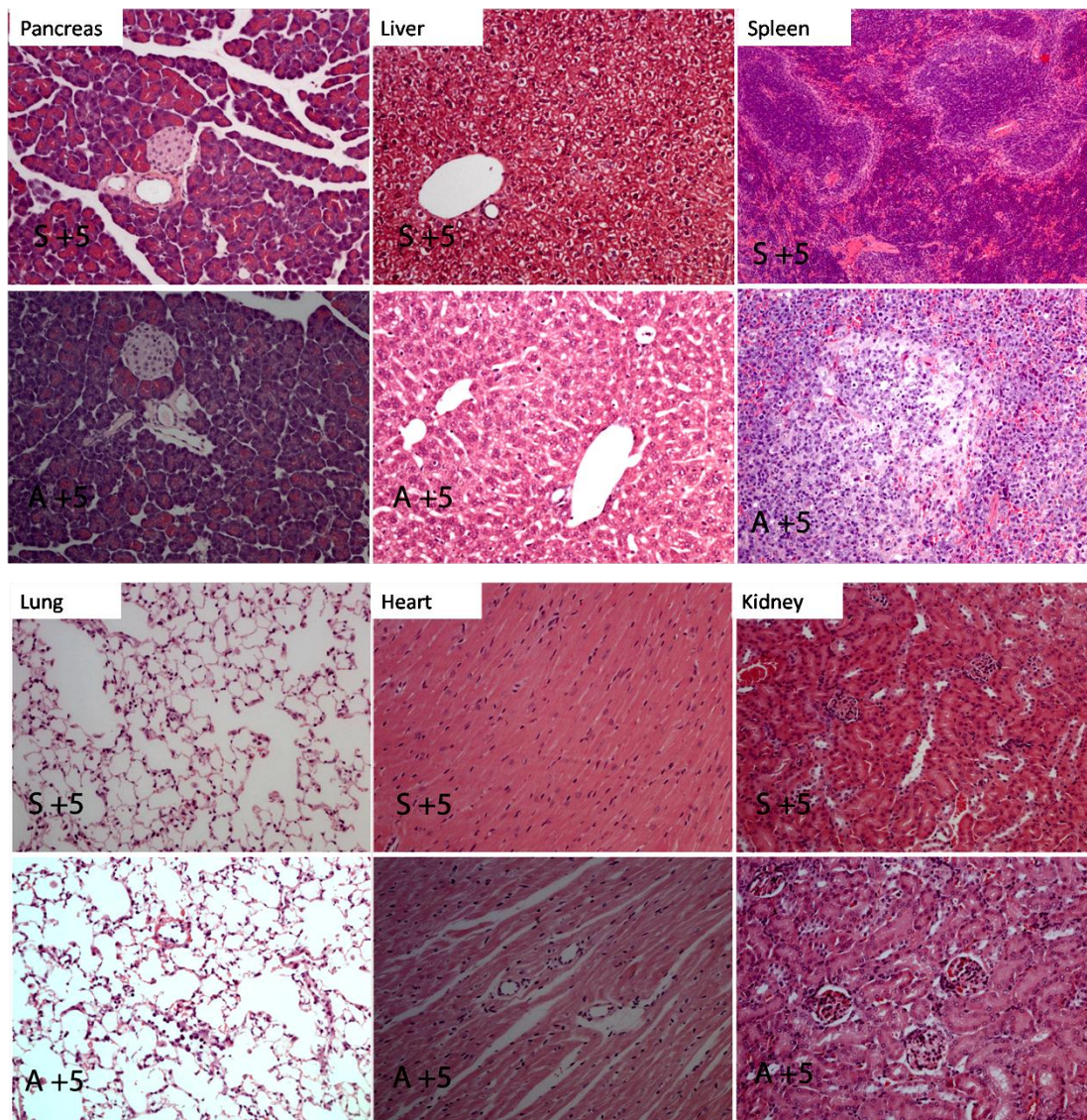
Figure 13: Histology of pancreas, liver, spleen, lung, heart and kidney from day +5 of syngeneic and allogeneic HSCT. S: syngeneic A: allogeneic

This study showed that the effect of the conditioning regimen was more obvious in some tissues compared to others. GVHD intensity also differed between different

tissues. Moreover, some tissues showed earlier effect compared to other tissues. In the pancreas, no effect of the conditioning regimen was observed and no signs of transplantation toxicity were seen in the syngeneic group. In the allogeneic group, the pancreas had some abnormalities on days 5 and 7 post HSCT, and this defect remained to a lesser extent in some mice and disappeared in other ones. The major pancreatic toxicities after allogeneic HSCT were smaller size acini, progressive loss of apical secretion granules and apoptotic cell morphology in exocrine and endocrine parts. In contrast to the pancreas, the liver was affected by the Bu-Cy conditioning. The conditioning resulted in smaller hepatocytes and decrease of cytoplasmic vacuolization. The livers from mice treated with allogeneic HSCT had an infiltration of inflammatory cells close to the central vein at day +5. On day +7, the livers from mice in the allogeneic setting had apoptotic cell forms and granulocytes adhering to the endothelium. The livers from the syngeneic HSCT mice had no significant changes. The spleen was also affected by the conditioning regimen and by allogeneic HSCT. Spleen cellularity decreased dramatically after the conditioning regimen and the allogeneic transplanted mice showed sign of change in the architecture. Hypocellularity was observed on days +5 and +7, and lymphocyte infiltration was observed within the hardly identifiable white pulp. The mice had partially recovered from the spleen damage when they were approaching three weeks post HSCT. The spleens from the syngeneic group were also hypocellular during the first week post transplantation. However, the red pulp showed extensive extramedullar hematopoiesis.

The conditioning regimen did not result in remarkable changes in lung, heart and kidney. However, lungs from the allogeneically transplanted mice had inflammatory features with adherence of granulocytes in small veins at days +5 and +7. Three weeks post transplantation granulocyte infiltration, foamy macrophages and sub-pleural fibrotic changes were still observed in some spleens from allogeneically transplanted mice. Except for alveolar foam type macrophages, there were no other abnormalities detected in the syngeneic group within three weeks post transplantation. Starting day +7, some abnormalities in the heart were found with a few apoptotic cells, minimal loss of cardiomyocytes and subendocardial accumulation of neutrophils. This observation was more pronounced by day +21. The syngeneic HSCT group showed no detectable effect within the first three weeks post transplantation. In contrast to all other tissues, syngeneic and allogeneic transplantations did not result in detectible effects on the kidney. Day +5 is very critical in GVHD development according to this established Bu-Cy model. Figure 13 shows the histopathology of different tissues in syngeneic and allogeneic HSCT on day +5.

4.3 STUDY III

4.3.1 Effect of Bu-Cy conditioning on arteries

General toxicity expressed as a weight loss and vascular toxicity were observed after Bu-Cy conditioning regimen. Significant weight loss was observed in the Bu-Cy treated animals compared to the control group injected with PBS. However, there was no effect from the Bu-Cy treatment on heart weight (both dried and wet) compared to the control. The Bu-Cy treated group had lower blood pressure compared to control group. The internal circumference (IC) of mesenteric arteries from Bu-Cy treated mice was larger compared to the IC obtained from the corresponding segment from the controls (Figure A). However, there was no difference between the groups in IC of the aorta. The micro vessels from the Bu-Cy treated group were larger and slightly stiffer than those of the control group as showed by the extrapolation between tension and IC to zero positive tension and the determination of the reference slack IC (IC_{ref}) (Figure 14 B). The relationship between passive tension (PT) and IC/IC_{ref} was non-linear with a higher stiffness (i.e. the steepness of the relationship between IC/IC_{ref} and PT) in the Bu-Cy group. The IC/IC_{ref} at the optimal length for active force was significantly ($P<0.001$) lower in the Bu-Cy group compared to the control group (Bu-Cy: 1.52 ± 0.03, n=6 and controls: 1.93 ± 0.06, n=4). The extrapolated curve is illustrated in Figure 14.
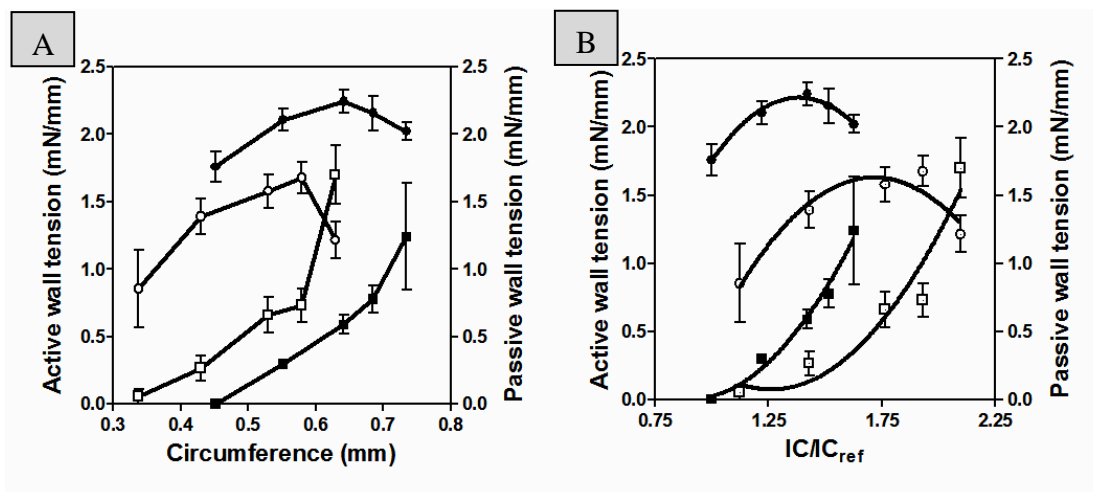
Figure 14: Force tension relationship. Control active tension (empty circle), Bu-Cy active tension (filled circle), control passive tension (empty square) and Bu-Cy passive tension (filled square).

4.3.2 Pharmacological effects

The aorta from Bu-Cy treated mice had a higher sensitivity to noradrenalin (Figure 15 B). However, there was no significant difference observed in the noradrenalin reactivity of the mesenteric arteries. This result showed that there were no major alterations in the contractile adrenoceptor signaling in the mesenteric arteries.

Acetylcholine, sodium nitroprusside and forskolin relaxant agonist effects were also investigated in mesenteric arteries and aorta from the Bu-Cy conditioned mice and the control group. Acetylcholine induces endothelium dependent relaxation and sodium nitroprusside (SNP) directly activates the relaxant nitric oxide dependent pathways

while forskolin directly activates cyclic adenosine-monophosphate (cAMP) dependent relaxation. The mesenteric arteries from Bu-Cy treated mice had a higher sensitivity to acetylcholine (Figure 15 A) while there were no significant differences between the Bu-Cy treated group and the control regarding the response to SNP and forskolin. There was no significant difference in the three relaxant agonists in the aorta between the two treated groups (Figure 16). In summary, the Bu-Cy treatment enhanced endothelium mediated relaxation in microarteries but did not change relaxation properties of the aorta.
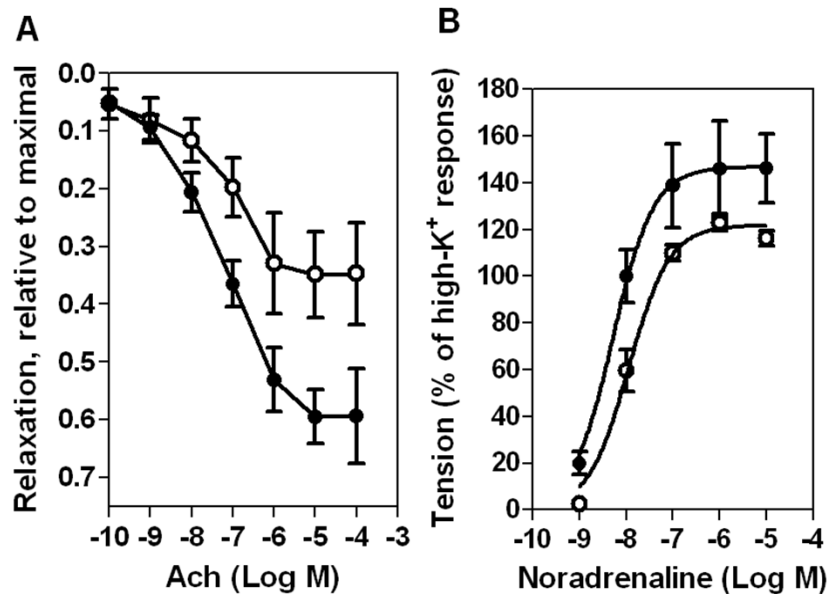
Figure 15: A; acetylcholine relaxation steps (mesenteric arteries) and B; noradrenalin contraction steps (aorta) from Bu-Cy treated mice (filled symbols) and controls (open symbols).

4.3.3 Bu-Cy Effect on endothelial cells and nitric oxide

As mentioned above we found higher relaxation response to acetylcholine in the Bu-Cy treated group compared to the control. We found that the effect was due to the increase in eNOS levels as assessed by the mRNA and the protein expression (Figure 17). There were no signs that cGMP or cAMP pathways were involved. Thus, endothelial cells, but not smooth muscles, were involved in Bu-Cy effect on arteries. Figure 16 is a schematic diagram showing the acetylcholine, cGMP and cAMP relaxation pathways.
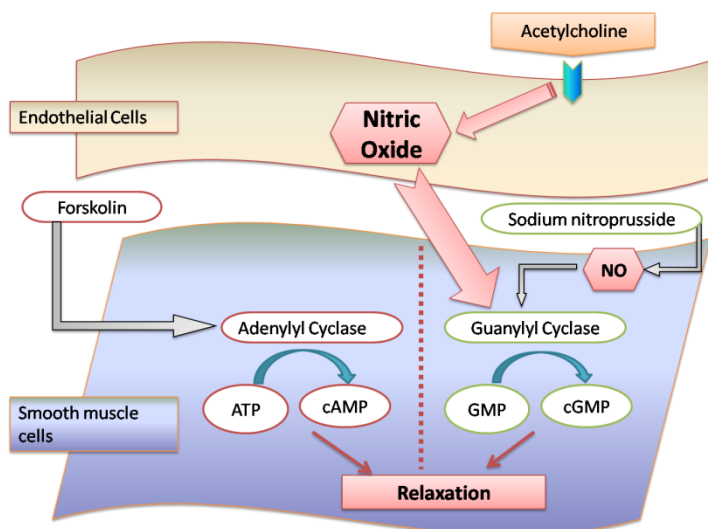
Figure 16: cAMP and cGMP relaxation pathways
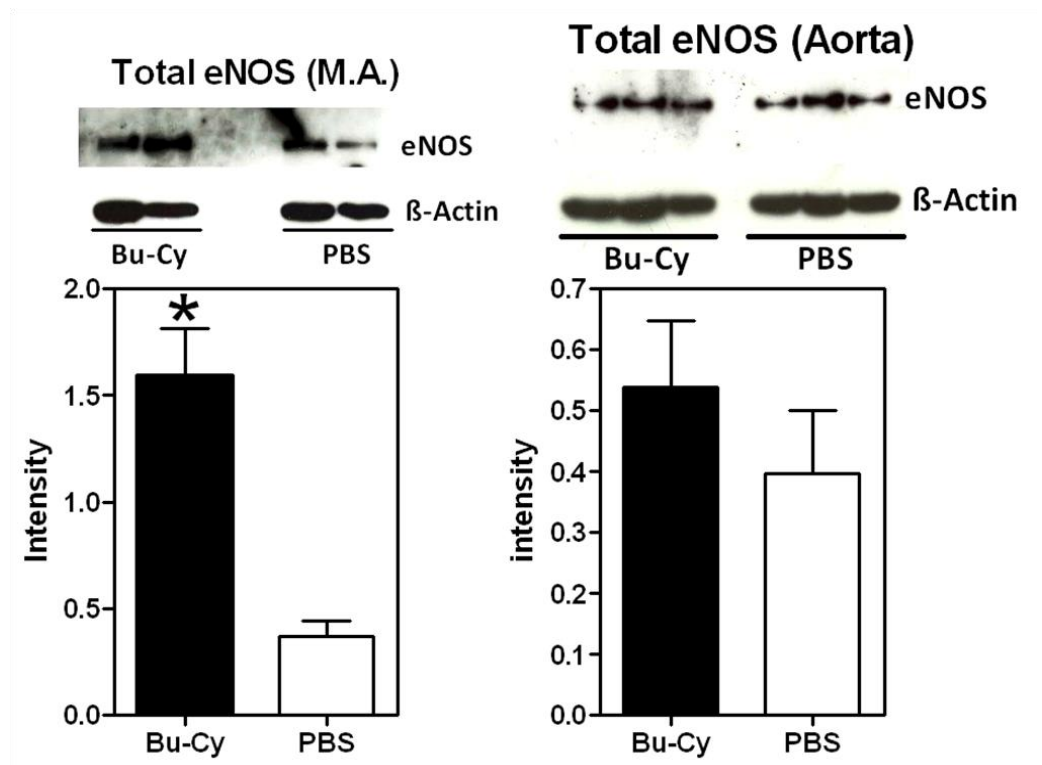
Figure 17: eNOS protein expression in microarteries (M.A.) and aorta of Bu-Cy treated (filled bars) and control (open bars) animals. Western blot analyses were normalized to β-actin.

The electron microscope confirmed the endothelial injury. The endothelial cells were detached from the extracellular matrix which disrupted the cell to cell contact (Figure 18).
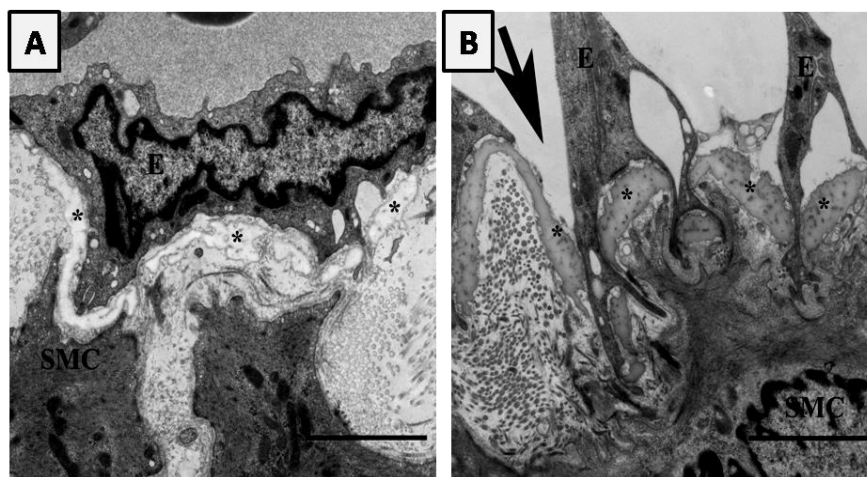
Figure 18: Electron microscope picture for control (A) and Bu-Cy treated group (B). Arrow (gaps), E (endothelial cells), SMC (smooth muscular cell), * (elastic fibers and the bars = 2 μm

4.4 STUDY IV

4.4.1 Animal weight

The mice in the omega-3 group gained more weight before the start of the conditioning regimen compared to the other two groups. The mice in the corn oil group also had a higher increase in weight compared to the mice in the standard food group. Bu-Cy conditioning regimen resulted in significant weight loss in omega-3, corn oil and control groups. However, the weight loss during the conditioning regimen was higher in the omega-3 group than in the group of mice that were fed standard food. The corn oil also presented similar results, but to a lesser extent compared to the omega-3 group. The reduction in weight was greater in the omega-3 group during the standard conditioning regimen, but there were no significant differences in weight reduction of mice treated with the reduced intensity conditioning regimen and short or long term feeding. In contrast, the eight week feeding resulted in a higher reduction of weight in the corn oil fed mice during the Bu-Cy standard conditioning.

Food supplement	(D -21)	(D -21) to (D -7) %	(D -7) to (D 0) %
Omega-3	20.06 ± 0.51 (n = 20)	9.09 ± 1.17 (n = 20)	-18.25 ± 1.29
Corn oil	20.27 ± 0.59 (n = 20)	7.05 ± 1.39 (n = 20)	-16.85 ± 1.01
Standard	20.53 ± 0.49 (n = 20)	4.59 ± 1.12 (n = 20)	-12.40 ± 1.18
T_{test} (O3 x Std)	*P* =0.52	***P* =0.01**	***P* =0.002**
T_{test} (Crn x Std)	*P* =0.74	*P* =0.18	***P* =0.01**

Table 2: Effect of omega-3 food supplement on mouse weight during Bu-Cy conditioning (O3= Omega-3; Std=Standard food; Crn= corn oil; x=versus)

4.4.2 Cellularity

4.4.2.1 *BM and spleen cellularity*

The cellularity of bone marrow did not differ significantly between the three groups in the short or long term feeding when the mice received standard Bu-Cy conditioning.

However, the group fed omega-3 had significantly lower BM cellularity when the mice had received reduced intensity conditioning with long term feeding compared to the standard food and to corn oil. The spleen cellularity was lower in both PUFA fed groups compared to the control in standard Bu-Cy conditioning. However, spleen cellularity from the omega-3 and corn oil fed groups was lower in the long term feeding and the standard intensity conditioning compared to the standard food group (Figure 19).
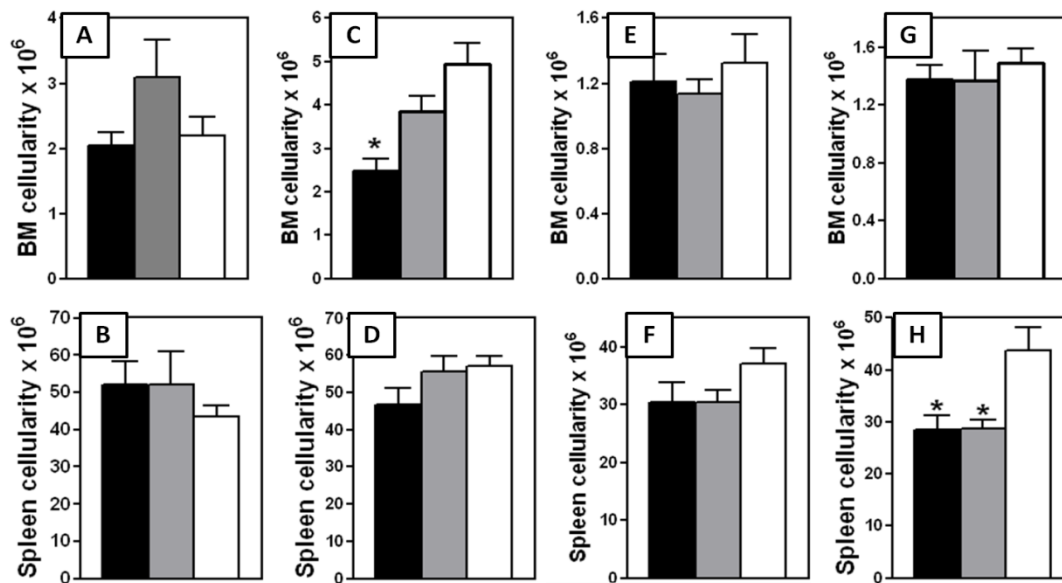
Figure 19: BM and spleen cellularity. A, B: short term feeding and RIC regimen; C, D: long term feeding and RIC; E, F: short term feeding and standard Bu-Cy conditioning and G, H: long term feeding and standard Bu-Cy conditioning

4.4.2.2 Subpopulation cellularity

CD11b+ myeloid cell population was significantly lower in the group of omega-3 fed mice compared to the other groups. The result was significant in short and long term feeding. It was also significant in reduced intensity conditioning. However, there was no significant difference in CD11b+ cells between the three supplements without Bu-Cy conditioning regimen (Figure 20). CD11c+CD86+ dendritic cells were also significantly reduced in mice fed omega-3 and given Bu-Cy conditioning. The effect of omega-3 on CD11c+CD86+ cells was significant in long and short term feeding (Figure 21).

There was no significant difference in memory and naïve T cells between the three groups in the short term feeding with the standard conditioning regimen; however, omega-3 significantly reduced both memory and naïve CD8+ T cells in long term feeding.
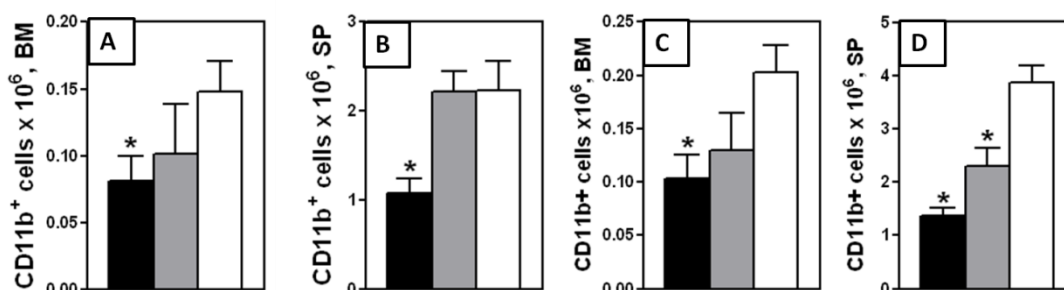
Figure 20: CD11b+ cells from standard Bu-Cy conditioning. A, B; short term feeding; C, D: long term feeding. * represents significant findings
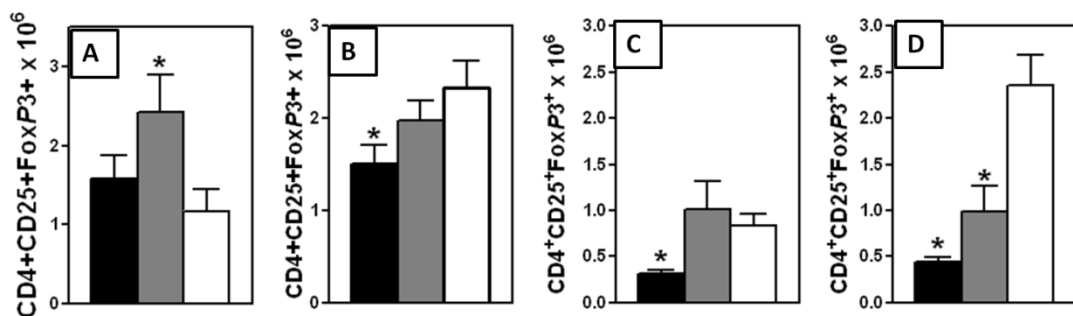
44
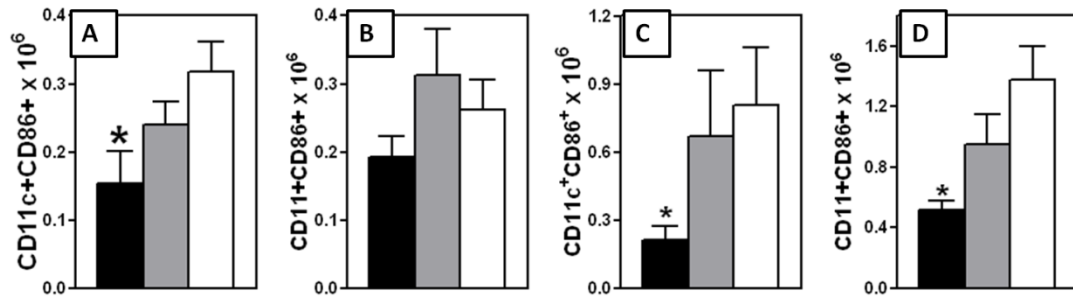
Figure 21: CD11cCD86+ cells from spleen after RIC (A, B), standard Bu-Cy conditioning (C, D). A, C; short term feeding; B, D: long term feeding.
* represents significant findings

4.4.3 Regulatory T cells and MLC

CD4+CD25+Fox$P$3+ regulatory T cells were significantly lower in the group of omega-3 fed mice which either received the Bu-Cy conditioning regimen (Figure 22) or in the group that was given the food without conditioning. To test if the effect was only on cell number or if it also affected function, we assessed this effect using mixed lymphocyte culture. The result of this experiment showed a slight effect of omega-3 on the *Treg* function. The ratio of 1:1 responder cells to *Treg* cells showed no obvious difference on *Treg* suppression between the three groups. However, as the *Treg* cells decreased, the difference between the three groups increased to the lower suppression of *Treg* cells in the omega-3 group.

Figure 22: CD4+CD25+Fox$P$3+ T cells from spleen after RIC (A, B), standard Bu-Cy conditioning (C, D). A, C; short term feeding and B, D: long term feeding.
* represents significant findings

4.4.4 Busulfan effect on CFU-GM

There was no significant difference in busulfan toxicity on CFU-GM between the omega-3, corn oil and standard food groups.

4.4.5 GVHD and animal survival

The mice fed omega-3 had a lower survival rate compared to the mice fed corn oil or standard food (Figure 23). Moreover, the mice in the omega-3 group had severe diarrhea. The mice in this group presented signs of GVHD as described in the method section. The corn oil group also had lower survival compared to the standard food. However, the mice in corn oil group did not show obvious sign of GVHD. BM and spleen chimerism from the omega-3 showed higher engraftment compared to the corn oil and the control group. The mice from the corn oil group which died had the lowest cell engraftment among the three groups.
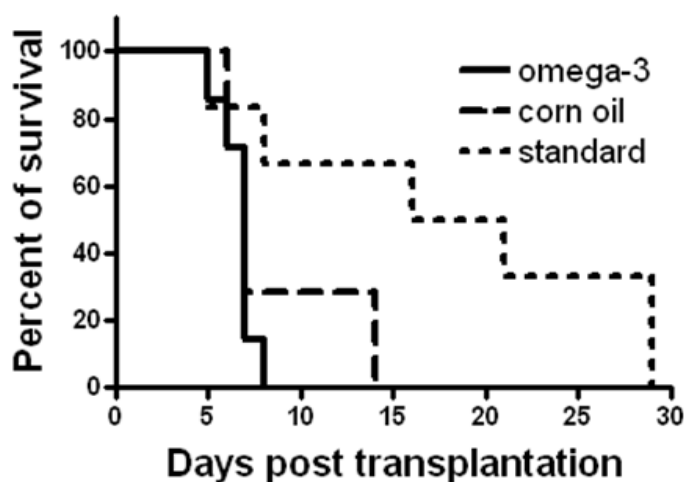
Figure 23: Survival analysis for mice fed omega-3, corn oil or standard food for three weeks before HSCT. The mice received standard Bu-Cy conditioning. Administration of the three food supplements continued after HSCT.

5 DISCUSSION

HSCT is the treatment of choice for many diseases. Despite continuous development of this procedure, HSCT is still limited by life threatening complications. Conditioning regimen is an important part of HSCT. A majority of the experimental studies are based on irradiation conditioning regimen, despite the fact that about 50% of the patients undergoing HSCT receive chemotherapy as a conditioning regimen. The studies discussed in this thesis were based on busulfan and cyclophosphamide conditioning. The rationale was the common use of this regimen in HSCT setting. As well as being used as a conditioning regimen for HSCT, these two drugs are also used for the treatment of some diseases; cyclophosphamide, for example, is also used in cancer treatment. Thus, studying their action and toxicity in HSCT will also benefit their use in other applications. Our studies focused on conditioning related toxicities and factors that may affect HSCT outcome.

In the first study we investigated the early pathophysiological events in GVHD. The Bu-Cy dose used in this study was myeloablative and resulted in over 95% elimination of BM cells. The conditioning is considered immunosuppressive with its reduction of over 60% of total splenic cells indicating that T cells might be more resistant to the chemotherapy. T cells were reduced by 30 %, while B, NK and DC cells decreased by more than 70%. Cytokine production was not high at day 0 (day of transplantation) but reached maximum levels at day +5 after HSCT. At this point, a high T cell proliferation could also be seen. This might to some extent distinguish between radiotherapy and chemotherapy conditioning; high levels of cytokines have been reported after irradiation conditioning [100, 307]. Since the levels of inflammatory cytokines were low on the day of transplantation compared to those reported in irradiation conditioning and high on day +5 in Bu-Cy conditioning, we can conclude that the cytokines are less involved in the development of GVHD when Bu-Cy conditioning has been used compared with irradiation. It might also suggest that Bu-Cy conditioning has delayed tissue injury compared to the irradiation conditioning.

As we reported in this study, the increase of NK and DC cells soon after transplantation showed the importance of these cells in the early stages of the inflammatory process, which could influence the establishment of the GVHD. These two populations reached their peak of expansion at day +3, which was also the start of T cell proliferation. The expansion of T cells reached its first maximum peak at day +5. Most of these T cells were of donor origin and the majority of them were CD8+ cells. This process was seen at the same time as two other observations were made. The first of these was the highest expression level of IL-2, TNF-α and INF-γ inflammatory cytokines. The second observation was the clinical symptoms of GVHD. GVHD is initiated by injury from the conditioning regimen. The second signal is from the expansion of the innate immune system followed by the adaptive immune system. Thus, the increase of antigen presenting cells (APC) soon after HSCT is an important signal for the production and proliferation of T cells as well as the increase of inflammatory cytokines. Although DCs on day +1 were mainly from the host, the high level at day +3 was of donor origin. Interestingly, the ratio of naïve to effector memory T cells was decreased, which means that by the time, the effector memory T cells were increasing. This indicated the

existence of GVHD. The allogeneic setting induced the inflammatory response that was absent in the syngeneic setting.

In the second study we investigated the histopathological changes in the allogeneic HSCT process at different time points from the beginning of the conditioning regimen to 21 days after stem cell transplantation. This study showed that the damage caused by the Bu-Cy conditioning regimen and GVHD are affecting different tissues by different severity grade. The study has showed that some tissues are sensitive to the Bu-Cy conditioning regimen, whereas others may be less sensitive to the treatment. GVHD has also been shown to have selective mediation to different tissues. This indicates that unaffected tissues have a protective or a fast repairing mechanism. As reported, the liver is one of the target organs [308-309]. We also observed tissue damage starting from the conditioning regimen and being enhanced by the allogeneic HSCT. A similar pattern was observed in the spleen where some chemotherapy toxicities (later enhanced by the allogeneic transplantation) were detected. We did not detect morphological changes in the pancreas and the heart after the conditioning regimen, but both organs exhibited some degree of damage during GVHD development. However, the damage in these organs was lesser than that found in the liver and the spleen. In contrast to the above four tissues, there was no morphological change detected in the kidney after Bu-Cy conditioning regimen and allogeneic HSCT.

It is possible that the Bu-Cy conditioning has no histopathological toxicity on the heart, pancreas and kidney or that the damage was minor and hence could not be detected by morphological changes. Moreover, most of the Bu-Cy toxicity reported might be caused by cylophosphamide, which might explain the obvious damage to the liver as Cy is metabolized mainly there [310-312]. However, this could not explain why the kidney was not damaged by this regimen – after all, it filters the excessive metabolites, although majority of them are inactive [313]. Although the mice were of inbred strain, there were great inter-individual differences after chemotherapy and HSCT. It is important to understand the pathophysiology of GVHD, and it is also of importance to understand the mechanism of its progression. The highest amount of damage found after HSCT developed mainly in the first week as shown previously [314].

Long-term cardiovascular toxicity after HSCT has been reported [286, 315]; however, the early effects of conditioning and HSCT on the arterial system [286] have not been yet elucidated. Hence, the third study focused specifically on Bu-Cy toxicity on the arterial system.

We selected the mesenteric arteries and the aorta to study the effect of Bu-Cy conditioning on the vascular system according to their impact in cardiovascular diseases. Micro-vessels (also called resistance vessels) have a small diameter and direct the blood to different organs. Defective micro-vessels may lead to organ dysfunction. Mesenteric arteries are micro-vessels located in the mesentery which contribute to peripheral resistance. The aorta is the large artery which passes the blood to major organs. Effects on aorta elasticity also have an impact on heart function [316]. Although the initial internal circumference (IC) of aortas from Bu-Cy treated mice was not significantly different from the control, the stretched aortas had a smaller IC compared to the control. This means that aortas from Bu-Cy conditioned mice had lower elasticity, which might have an impact on the other observation in the mesenteric arteries: the initial IC was larger in the Bu-Cy group compared to the control. This

could be the result of a compensation mechanism. The mesenteric arteries from the Bu-Cy treated mice were more sensitive to acetylcholine compared to the control. The larger IC and the increased relaxation with acetylcholine lowered the blood pressure. This result also supported the increased heart rate that was found in the Bu-Cy treated group. The increased heart rate was possibly a compensatory mechanism for the lower systemic blood pressure.

The mesenteric arteries from the Bu-Cy group had a higher production of endothelial nitric oxide (eNOS), which might explain the mechanism behind the increased sensitivity to acetylcholine dependent relaxation. The cAMP and cGMP pathways were not affected by the Bu-Cy conditioning, excluding smooth muscle defect as the source. Mesenteric arteries from Bu-Cy treated mice showed a higher level of the mRNA and the eNOS gene as well as protein expression from both genes respectively. An increased level of nitric oxide [317] and a larger internal circumference might also decrease the blood pressure, which we found in another set of experiments after Bu-Cy conditioning. Although nitric oxide deficiency is associated with some diseases [318-319], a high level of nitric oxide could also exert toxicity [320]. The electron microscope examination showed damaged endothelial layers in the mesenteric arteries and a decrease in cell to cell contact. The defects in endothelial cell to cell contact affect different features of vascular homeostasis, which in turn affects different processes such as the permeability of the vessels and the infiltration of leukocytes to the inflamed tissues [321]. The damage to the endothelial cell layers might also affect organ perfusion (and thus long term function) to some degree. The Bu-Cy conditioning had less effect on the physiological properties of the aorta compared to its effect on the mesenteric arteries. However, the higher sensitivity of the aorta to noradrenalin may also reflect damages to the endothelium [322]. The results of this study indicate an early change in the vascular system that may influence later development of cardiovascular disease after HSCT.

The fourth project investigated the effect of omega-3 on the Bu-Cy conditioning regimen and on GVHD development after allogeneic HSCT. Omega-3 effects were reported in different types of diseases, especially inflammatory and autoimmune diseases [245]. Omega-3 has been reported to modify the effect of several drugs used in cancer chemotherapy and the treatment of other diseases. Omega-3 has been reported to enhance the treatment of breast cancer [323] and to decrease tissue damage after irradiation [324]. From these studies and others, it seems that omega-3 enhances the efficacy of chemotherapy and also has beneficial effects on life quality in general [325]. Although the beneficial effects of omega-3 on cancer and chemotherapy have not been fully elucidated, they are some reported pathways. Omega-3 may increase the efficacy of chemotherapy without damaging the normal cells by reducing the oxidative stress caused by the therapeutic agents and by changing the fat composition of the cells. DHA and EPA, two major omega-3 derivatives, are reported to inhibit eicosanoid synthesis from the AA [326] and reduce the COX-2 expression [327]. The competitor properties of EPA and DHA to AA reduce the inflammatory condition through a decrease in the inflammatory mediators LTB4 and PGE2. The EPA and DHA may work as substitutes for cytochrome P450 (CYP) AA dependent metabolites and hence reduce toxicity caused by eicosanoids [328].

Despite extensive studies of omega-3 and its role in disease pathophysiology, prevention and treatment, their mechanism of action is still not well understood. The HSCT procedure is complex, involving donor and recipient immune systems,

immunosuppressive drugs to prevent and treat rejection and GVHD, drugs for treatment of other complications and frequently limited food intake. This complexity makes it hard to evaluate the effect of food supplements on chemotherapy and HSCT in clinical settings. Thus, we used the established mouse model which, to a certain extent, limits the number of variables compared to human transplantation. The food was supplemented for three weeks and two months in order to evaluate the benefits of short and long term feeding, respectively. Since the use of reduced intensity conditioning is common, we also studied the effects of omega-3 in combination with RIC. This might also reveal the effects of omega-3 which had been hidden by myeloablative conditioning. However, standard dose Bu-Cy conditioning was used with all transplantation experiments.

CD4+CD25+FoxP3+ regulatory T cells were significantly lower before the conditioning regimen in all groups and after the conditioning in the omega-3 fed group. It was reported that Cy selectively depletes *Treg* cells [329-330] and that Cy immunosuppressive properties were enhanced by EPA [239]. Hence, omega-3 is seen to suppress the *Treg* cells independent of conditioning regimen. Moreover, in the conditioning there was a dual effect resulting from the direct effect of omega-3 and the Cy enhancement effect. We then evaluated if omega-3 decreased the number of *Treg* cells and if it also diminished their suppressive function. The results showed that there was minor effect when the ratio of *Treg* to responder cells was even (i.e.1: 1). This effect was more obvious in the decreased number of *Treg* cells in omega-3 fed mice compared to those fed corn oil and standard food respectively. We conclude that omega-3 significantly deteriorates the number of *Treg* cells, but has only a slight effect on their suppressive properties. *Treg* cells are known to suppress cytotoxic T cell activity [331].

Omega-3 supplementation for three weeks, including the period of Bu-Cy conditioning, augments GVHD. The effect stemmed from the enhancement of the myeloablative effects of Bu-Cy conditioning through a decrease in the number of CD11b+ cells. The effects of omega-3 also stemmed from the reduction of activated dendritic cells, which are reported to play an important role in the initiation of GVHD [314]. Thus, omega-3 enhanced Bu-Cy conditioning and resulted in a more rapid death of the mice in the omega-3 fed group compared to the corn oil and standard food groups. The corn oil group also showed a higher death rate after HSCT as compared to the mice in the standard food group. However, the mice in the omega-3 group started to have diarrhea and display standard signs of GVHD early on, while the mice in the corn oil group showed a sign of anemia. The result was also confirmed by the full cell engraftment of the bone marrow and spleen in the omega-3 group. In contrast, the spleens from the corn oil group had a lower amount of donor cells. We also conducted another experiment where the donors were fed omega-3 before transplantation. The overall survival increased in the omega-3 recipient group, and was close to that in the standard food group. The mice in the corn oil group also had a slight improvement in survival. One of the transplanted groups that received HSCT from omega-3 fed donors and continued with omega-3 after the transplantation showed survival similar to that of the mice in the standard food group. Moreover, the mice in this group were more active compared to other groups. The results from this study showed that omega-3 augments GVHD through the enhancement of Bu-Cy conditioning and by suppressing the regulatory T cell numbers and functions. Moreover, omega-3 may help to suppress inflammatory reactions in recipients that survive the acute phase of GVHD.

6 CONCLUSIONS AND FUTURE PERSPECTIVES

- Our stem cell transplantation mouse model following busulfan-cyclophosphamide (Bu-Cy) conditioning is a reliable model for studying several factors that are involved in the development of graft versus host disease after chemotherapy. It is also a useful model for evaluating different agents in the treatment of GVHD. All studies in this thesis utilized this model.

- Naïve T cells undergo early allo-activation by host/donor APCs in the secondary lymphoid organs to produce effector/memory T cells that initiate tissue damage in GVHD.

- Decreased spleen cellularity and diminished glycogen content in the liver were observed after conditioning regimen. No morphological changes were observed in kidney in either HSCT setting.

- Histopathological changes such as vasculitis, inflammation and apoptotic cell forms in liver, spleen, pancreas, lungs and heart were observed in allogeneic transplanted mice, however, only hypocellular spleen and extramedullar hematopoiesis were detected in syngeneic transplanted animals.

- Short term consequences of Bu-Cy treatment divergently affect large and small arteries of the cardiovascular system.

- The increased noradrenaline reactivity of large elastic arteries was not associated with increased blood pressure. Instead, Bu-Cy treatment lowered blood pressure via augmented microvascular endothelial dependent relaxation, increased expression of vascular eNOS and remodeling toward a larger lumen.

- The changes in microarterial properties can be associated with direct effects of the compounds on vascular wall or possibly indirectly induced via altered translational activity associated with the reduced hematocrit and shear stress.

- Omega-3 as food supplement enhances graft versus host disease (GVHD) through a suppression of the regulatory T cells in mice receiving Bu-Cy conditioning regimen.

- Omega-3 intake caused an enhancement of Bu-Cy conditioning regimen myeloablation and toxicity.

- Corn oil as food supplement decreased the cell engraftment which led to BMT rejection or death from anemia.

1. Further evaluation of the vascular system after HSCT is warranted, including the effects of aGVHD and cGVHD. Effects of the Bu-Cy conditioning need to be studied in vessel segments from different organs.
2. More studies are needed to evaluate the effect of long term intake of omega-3 in HSCT setting. This is an important issue for patients undergoing HSCT.
3. General studies to evaluate the effects of omega-3 on the vascular system in combination with different conditioning regimens are warranted.
4. Moreover, the effects of diets in general in conjunction with HSCT might play an important role in the clinical outcome and have to be investigated.

7 ACKNOWLEDGMENTS

54

Special thanks and gratitude to **Professor Abdallah Daar,** who has supported me all the time in my graduates studies and trainings. With your extremely busy schedule, you still never delayed or hesitated to provide me with any kind of help. You are a very kind person and a great scientist whom I am very proud to know.

My Omani friends, **Fahad Al-Zadjali** and **Abdullah Al-Muniri** (the special Omani ambassadors in Sweden). Your social communication and discussion about Oman and SQU developments make me feel at home. I will never forget your support. We really had a lot of fun together (let us meet at Stockholm center). My dear friend **Said Al-Mashari**, the well known expert: thanks for introducing me to many friends.

The Egyptian friends; **Rami Genead, Mohammed Sulayem, Hamdi, Mohammed Sabri, Mohammed Yusri, Mohammed Eita, Ramy Alsayed and Mohammed Halloos**; the Sudanese friends, **Husam, Kamal Mohamad Hamzah and Alzafer**, you all are the best treatment for stress. I wish you all the best.

My treasured family; **parents, brothers, sisters and aunts**: your support is infinite and you raise my spirits when I talk with you. My wife's family; you always consider me one of you. Thanks for your enormous help, especially **Zaher**.

My beloved wife **Salma**, without you, life has no meaning.I It is difficult to express my feelings to you. You are with me all the time and you are a part of my success. As a very kind wife you are also a creative scientist; good luck in the future. My kids, **Said, Lubna, Ibrahim and Sara**; you are the flavor in my life; you bring me a smile even when I am stressed. Wish you a bright future.

8 REFERENCES

1. Kraft, A., *Manhattan transfer: lethal radiation, bone marrow transplantation, and the birth of stem cell biology, ca. 1942-1961.* Hist Stud Nat Sci, 2009. **39**(2): p. 171-218.
2. Creager, A.N., *Nuclear energy in the service of biomedicine: the U.S. Atomic Energy Commission's radioisotope program, 1946-1950.* J Hist Biol, 2006. **39**(4): p. 649-84.
3. Thomas, E.D., et al., *Intravenous infusion of bone marrow in patients receiving radiation and chemotherapy.* N Engl J Med, 1957. **257**(11): p. 491-6.
4. Thomas, E., et al., *Bone-marrow transplantation (first of two parts).* N Engl J Med, 1975. **292**(16): p. 832-43.
5. Thomas, E.D., et al., *Bone-marrow transplantation (second of two parts).* N Engl J Med, 1975. **292**(17): p. 895-902.
6. Dausset, J., *[Iso-leuko-antibodies].* Acta Haematol, 1958. **20**(1-4): p. 156-66.
7. Little, M.T. and R. Storb, *History of haematopoietic stem-cell transplantation.* Nat Rev Cancer, 2002. **2**(3): p. 231-8.
8. Thomas, E.D., et al., *One hundred patients with acute leukemia treated by chemotherapy, total body irradiation, and allogeneic marrow transplantation.* Blood, 1977. **49**(4): p. 511-33.
9. Hobbs, J.R., *Bone marrow transplantation for inborn errors.* Lancet, 1981. **2**(8249): p. 735-9.
10. Krivit, W., et al., *Prevention of deterioration in metachromatic leukodystrophy by bone marrow transplantation.* Am J Med Sci, 1987. **294**(2): p. 80-5.
11. Groth, C.G. and O. Ringden, *Transplantation in relation to the treatment of inherited disease.* Transplantation, 1984. **38**(4): p. 319-27.
12. Haynesworth, S.E., et al., *Characterization of cells with osteogenic potential from human marrow.* Bone, 1992. **13**(1): p. 81-8.
13. Prockop, D.J., *Marrow stromal cells as stem cells for nonhematopoietic tissues.* Science, 1997. **276**(5309): p. 71-4.
14. Ringden, O., et al., *Tissue repair using allogeneic mesenchymal stem cells for hemorrhagic cystitis, pneumomediastinum and perforated colon.* Leukemia, 2007. **21**(11): p. 2271-6.
15. Ringden, O., et al., *Mesenchymal stem cells for treatment of therapy-resistant graft-versus-host disease.* Transplantation, 2006. **81**(10): p. 1390-7.
16. Le Blanc, K., et al., *Treatment of severe acute graft-versus-host disease with third party haploidentical mesenchymal stem cells.* Lancet, 2004. **363**(9419): p. 1439-41.
17. Trounson, A., et al., *Clinical trials for stem cell therapies.* BMC Med, 2011. **9**: p. 52.
18. Martell, K., A. Trounson, and E. Baum, *Stem cell therapies in clinical trials: workshop on best practices and the need for harmonization.* Cell Stem Cell, 2010. **7**(4): p. 451-4.
19. Rangappa, S., R. Makkar, and J. Forrester, *Review article: current status of myocardial regeneration: new cell sources and new strategies.* J Cardiovasc Pharmacol Ther, 2010. **15**(4): p. 338-43.
20. Donovan, P.J., *The germ cell--the mother of all stem cells.* Int J Dev Biol, 1998. **42**(7): p. 1043-50.
21. Mardanpour, P., et al., *Potency of germ cells and its relevance for regenerative medicine.* J Anat, 2008. **213**(1): p. 26-9.
22. Ng, H.H. and M.A. Surani, *The transcriptional and signalling networks of pluripotency.* Nat Cell Biol, 2011. **13**(5): p. 490-6.
23. Khurana, S. and A. Mukhopadhyay, *In vitro transdifferentiation of adult hematopoietic stem cells: an alternative source of engraftable hepatocytes.* J Hepatol, 2008. **49**(6): p. 998-1007.
24. Solari, C., et al., *Induced pluripotent stem cells' self-renewal and pluripotency is maintained by a bovine granulosa cell line-conditioned medium.* Biochem Biophys Res Commun, 2011. **410**(2): p. 252-7.

25. Wu, S.M. and K. Hochedlinger, *Harnessing the potential of induced pluripotent stem cells for regenerative medicine.* Nat Cell Biol, 2011. **13**(5): p. 497-505.
26. Georgieva, B.P. and J.M. Love, *Human induced pluripotent stem cells: a review of the US patent landscape.* Regen Med, 2010. **5**(4): p. 581-91.
27. Kang, L., et al., *Induced pluripotent stem cells (iPSCs)--a new era of reprogramming.* J Genet Genomics, 2010. **37**(7): p. 415-21.
28. Li, M., et al., *How far are induced pluripotent stem cells from the clinic?* Ageing Res Rev, 2010. **9**(3): p. 257-64.
29. Yen, C.C., et al., *Stem cells in the lung parenchyma and prospects for lung injury therapy.* Eur J Clin Invest, 2006. **36**(5): p. 310-9.
30. Weissman, I.L. and J.A. Shizuru, *The origins of the identification and isolation of hematopoietic stem cells, and their capability to induce donor-specific transplantation tolerance and treat autoimmune diseases.* Blood, 2008. **112**(9): p. 3543-53.
31. Horton, R., et al., *Gene map of the extended human MHC.* Nat Rev Genet, 2004. **5**(12): p. 889-99.
32. Trowsdale, J., *The MHC, disease and selection.* Immunol Lett, 2011. **137**(1-2): p. 1-8.
33. Steinmetz, M. and L. Hood, *Genes of the major histocompatibility complex in mouse and man.* Science, 1983. **222**(4625): p. 727-33.
34. Hood, L., M. Steinmetz, and B. Malissen, *Genes of the major histocompatibility complex of the mouse.* Annu Rev Immunol, 1983. **1**: p. 529-68.
35. Gluckman, E., et al., *Hematopoietic reconstitution in a patient with Fanconi's anemia by means of umbilical-cord blood from an HLA-identical sibling.* N Engl J Med, 1989. **321**(17): p. 1174-8.
36. Shizuru, J.A., et al., *Transplantation of purified hematopoietic stem cells: requirements for overcoming the barriers of allogeneic engraftment.* Biol Blood Marrow Transplant, 1996. **2**(1): p. 3-14.
37. Zheng, J., et al., *Ex vivo expanded hematopoietic stem cells overcome the MHC barrier in allogeneic transplantation.* Cell Stem Cell, 2011. **9**(2): p. 119-30.
38. Wang, B., et al., *Purified hematopoietic stem cells without facilitating cells can repopulate fully allogeneic recipients across entire major histocompatibility complex transplantation barrier in mice.* Proc Natl Acad Sci U S A, 1997. **94**(26): p. 14632-6.
39. Moore, K.A., H. Ema, and I.R. Lemischka, *In vitro maintenance of highly purified, transplantable hematopoietic stem cells.* Blood, 1997. **89**(12): p. 4337-47.
40. Copelan, E.A., *Hematopoietic stem-cell transplantation.* N Engl J Med, 2006. **354**(17): p. 1813-26.
41. Thomas, E.D., et al., *Allogeneic marrow grafting for hematologic malignancy using HL-A matched donor-recipient sibling pairs.* Blood, 1971. **38**(3): p. 267-87.
42. Schmitz, N., et al., *Primary transplantation of allogeneic peripheral blood progenitor cells mobilized by filgrastim (granulocyte colony-stimulating factor).* Blood, 1995. **85**(6): p. 1666-72.
43. Bensinger, W.I., et al., *Transplantation of allogeneic peripheral blood stem cells mobilized by recombinant human granulocyte colony-stimulating factor.* Blood, 1995. **85**(6): p. 1655-8.
44. Korbling, M., et al., *Allogeneic blood stem cell transplantation for refractory leukemia and lymphoma: potential advantage of blood over marrow allografts.* Blood, 1995. **85**(6): p. 1659-65.
45. Rocha, V. and E. Gluckman, *Clinical use of umbilical cord blood hematopoietic stem cells.* Biol Blood Marrow Transplant, 2006. **12**(1 Suppl 1): p. 34-41.
46. Wagner, J.E., *Allogeneic umbilical cord blood transplantation.* Cancer Treat Res, 1997. **77**: p. 187-216.
47. Barker, J.N., et al., *Transplantation of 2 partially HLA-matched umbilical cord blood units to enhance engraftment in adults with hematologic malignancy.* Blood, 2005. **105**(3): p. 1343-7.

48. Takahashi, S., et al., *Single-institute comparative analysis of unrelated bone marrow transplantation and cord blood transplantation for adult patients with hematologic malignancies.* Blood, 2004. **104**(12): p. 3813-20.

49. Takahashi, S., et al., *Comparative single-institute analysis of cord blood transplantation from unrelated donors with bone marrow or peripheral blood stem-cell transplants from related donors in adult patients with hematologic malignancies after myeloablative conditioning regimen.* Blood, 2007. **109**(3): p. 1322-30.

50. Lauber, S., et al., *The Mannheim Cord Blood Bank: Experiences and Perspectives for the Future.* Transfus Med Hemother, 2010. **37**(2): p. 90-97.

51. Ringden, O., et al., *Transplantation of peripheral blood stem cells as compared with bone marrow from HLA-identical siblings in adult patients with acute myeloid leukemia and acute lymphoblastic leukemia.* J Clin Oncol, 2002. **20**(24): p. 4655-64.

52. Ringden, O., et al., *Peripheral blood stem cell transplantation from unrelated donors: a comparison with marrow transplantation.* Blood, 1999. **94**(2): p. 455-64.

53. Storek, J., et al., *Allogeneic peripheral blood stem cell transplantation may be associated with a high risk of chronic graft-versus-host disease.* Blood, 1997. **90**(12): p. 4705-9.

54. Remberger, M., et al., *Increased risk of extensive chronic graft-versus-host disease after allogeneic peripheral blood stem cell transplantation using unrelated donors.* Blood, 2005. **105**(2): p. 548-51.

55. Auberger, J., et al., *Allogeneic Bone Marrow versus Peripheral Blood Stem Cell Transplantation: A Long-Term Retrospective Single-Centre Analysis In 329 Patients.* Eur J Haematol, 2011.

56. Gallardo, D., et al., *Is mobilized peripheral blood comparable with bone marrow as a source of hematopoietic stem cells for allogeneic transplantation from HLA-identical sibling donors? A case-control study.* Haematologica, 2009. **94**(9): p. 1282-8.

57. Couban, S., et al., *A randomized multicenter comparison of bone marrow and peripheral blood in recipients of matched sibling allogeneic transplants for myeloid malignancies.* Blood, 2002. **100**(5): p. 1525-31.

58. Witkowska-Zimny, M. and K. Walenko, *Stem cells from adipose tissue.* Cell Mol Biol Lett, 2011. **16**(2): p. 236-57.

59. Yarak, S. and O.K. Okamoto, *Human adipose-derived stem cells: current challenges and clinical perspectives.* An Bras Dermatol, 2010. **85**(5): p. 647-56.

60. Tobita, M., H. Orbay, and H. Mizuno, *Adipose-derived stem cells: current findings and future perspectives.* Discov Med, 2011. **11**(57): p. 160-70.

61. Ljungman, P., et al., *Allogeneic and autologous transplantation for haematological diseases, solid tumours and immune disorders: definitions and current practice in Europe.* Bone Marrow Transplant, 2006. **37**(5): p. 439-49.

62. Ljungman, P., et al., *Allogeneic and autologous transplantation for haematological diseases, solid tumours and immune disorders: current practice in Europe 2009.* Bone Marrow Transplant, 2010. **45**(2): p. 219-34.

63. Zeher, M., G. Papp, and P. Szodoray, *Autologous haemopoietic stem cell transplantation for autoimmune diseases.* Expert Opin Biol Ther, 2011. **11**(9): p. 1193-201.

64. Sullivan, K.M., P. Muraro, and A. Tyndall, *Hematopoietic cell transplantation for autoimmune disease: updates from Europe and the United States.* Biol Blood Marrow Transplant, 2010. **16**(1 Suppl): p. S48-56.

65. Horowitz, M.M., et al., *Graft-versus-leukemia reactions after bone marrow transplantation.* Blood, 1990. **75**(3): p. 555-62.

66. Fouillard, L., et al., *Results of syngeneic hematopoietic stem cell transplantation for acute leukemia: risk factors for outcomes of adults transplanted in first complete remission.* Haematologica, 2008. **93**(6): p. 834-41.

67. Worel, N., et al., *Long-term outcome and quality of life of patients who are alive and in complete remission more than two years after allogeneic and*

syngeneic stem cell transplantation. Bone Marrow Transplant, 2002. **30**(9): p. 619-26.

68. Fefer, A., et al., *Graft versus leukemia effect in man: the relapse rate of acute leukemia is lower after allogeneic than after syngeneic marrow transplantation.* Prog Clin Biol Res, 1987. **244**: p. 401-8.

69. Gale, R.P., et al., *Identical-twin bone marrow transplants for leukemia.* Ann Intern Med, 1994. **120**(8): p. 646-52.

70. Horan, J.T., et al., *Reducing the risk for transplantation-related mortality after allogeneic hematopoietic cell transplantation: how much progress has been made?* J Clin Oncol, 2011. **29**(7): p. 805-13.

71. Little, M.T. and R. Storb, *The future of allogeneic hematopoietic stem cell transplantation: minimizing pain, maximizing gain.* J Clin Invest, 2000. **105**(12): p. 1679-81.

72. Remberger, M., et al., *Improved Survival after Allogeneic Hematopoietic Stem Cell Transplantation in Recent Years. A Single-Center Study.* Biol Blood Marrow Transplant, 2011.

73. Fuchimoto, Y., et al., *Mixed chimerism and tolerance without whole body irradiation in a large animal model.* J Clin Invest, 2000. **105**(12): p. 1779-89.

74. Giralt, S., et al., *CD8-depleted donor lymphocyte infusion as treatment for relapsed chronic myelogenous leukemia after allogeneic bone marrow transplantation.* Blood, 1995. **86**(11): p. 4337-43.

75. Slavin, S., et al., *Nonmyeloablative stem cell transplantation and cell therapy as an alternative to conventional bone marrow transplantation with lethal cytoreduction for the treatment of malignant and nonmalignant hematologic diseases.* Blood, 1998. **91**(3): p. 756-63.

76. McSweeney, P.A. and R. Storb, *Mixed chimerism: preclinical studies and clinical applications.* Biol Blood Marrow Transplant, 1999. **5**(4): p. 192-203.

77. Ildstad, S.T. and D.H. Sachs, *Reconstitution with syngeneic plus allogeneic or xenogeneic bone marrow leads to specific acceptance of allografts or xenografts.* Nature, 1984. **307**(5947): p. 168-70.

78. GroupHealth. *Clinical review criteria for stem cell and bone marrow transplantation.* 2010; Available from: http://www.ghc.org/all-sites/clinical/criteria/pdf/stem_cell.pdf

79. Services, D.o.H.H. *National Coverage Determination (NCD) for Stem Cell Transplantation (110.8.1).* 2010 April 2010 [cited 2010 23 august]; Available from: www.hhs.gov

80. Dreger, P., et al., *Indications for allogeneic stem cell transplantation in chronic lymphocytic leukemia: the EBMT transplant consensus.* Leukemia, 2007. **21**(1): p. 12-7.

81. Gratwohl, A., et al., *Hematopoietic stem cell transplantation for hematological malignancies in Europe.* Leukemia, 2003. **17**(5): p. 941-59.

82. Gratwohl, A., et al., *Results of the EBMT activity survey 2005 on haematopoietic stem cell transplantation: focus on increasing use of unrelated donors.* Bone Marrow Transplant, 2007. **39**(2): p. 71-87.

83. Tan, S.S., et al., *Stem cell transplantation in Europe: trends and prospects.* Eur J Cancer, 2007. **43**(16): p. 2359-65.

84. Ringden, O., et al., *Similar incidence of graft-versus-host disease using HLA-A, -B and -DR identical unrelated bone marrow donors as with HLA-identical siblings.* Bone Marrow Transplant, 1995. **15**(4): p. 619-25.

85. Storb, R., et al., *Second marrow transplants in patients with aplastic anemia rejecting the first graft: use of a conditioning regimen including cyclophosphamide and antithymocyte globulin.* Blood, 1987. **70**(1): p. 116-21.

86. Kondo, N., et al., *DNA damage induced by alkylating agents and repair pathways.* J Nucleic Acids, 2010. **2010**: p. 543531.

87. Wheeler, G.P., *Studies related to the mechanisms of action of cytotoxic alkylating agents: a review.* Cancer Res, 1962. **22**: p. 651-88.

88. Bignold, L.P., *Alkylating agents and DNA polymerases.* Anticancer Res, 2006. **26**(2B): p. 1327-36.

89.   Hatse, S., E. De Clercq, and J. Balzarini, *Role of antimetabolites of purine and pyrimidine nucleotide metabolism in tumor cell differentiation.* Biochem Pharmacol, 1999. **58**(4): p. 539-55.

90.   Kinsella, A.R., D. Smith, and M. Pickard, *Resistance to chemotherapeutic antimetabolites: a function of salvage pathway involvement and cellular response to DNA damage.* Br J Cancer, 1997. **75**(7): p. 935-45.

91.   Desmedt, C., et al., *Multifactorial approach to predicting resistance to anthracyclines.* J Clin Oncol, 2011. **29**(12): p. 1578-86.

92.   Galm, U., et al., *Antitumor antibiotics: bleomycin, enediynes, and mitomycin.* Chem Rev, 2005. **105**(2): p. 739-58.

93.   Ball, E.D. and H.E. Broome, *Monoclonal antibodies in the treatment of hematologic malignancy.* Best Pract Res Clin Haematol, 2010. **23**(3): p. 403-16.

94.   Kelland, L., *Broadening the clinical use of platinum drug-based chemotherapy with new analogues. Satraplatin and picoplatin.* Expert Opin Investig Drugs, 2007. **16**(7): p. 1009-21.

95.   Tutschka, P.J., E.A. Copelan, and J.P. Klein, *Bone marrow transplantation for leukemia following a new busulfan and cyclophosphamide regimen.* Blood, 1987. **70**(5): p. 1382-8.

96.   Santos, G.W., et al., *Marrow transplantation for acute nonlymphocytic leukemia after treatment with busulfan and cyclophosphamide.* N Engl J Med, 1983. **309**(22): p. 1347-53.

97.   McCune, J.S. and L.A. Holmberg, *Busulfan in hematopoietic stem cell transplant setting.* Expert Opin Drug Metab Toxicol, 2009. **5**(8): p. 957-69.

98.   Blaise, D., et al., *Prospective randomized study of GVHD prevention using an anti IL2 receptor (CD25) MoAb after allogeneic bone marrow transplantation (BMT).* Bone Marrow Transplant, 1991. **7 Suppl 2**: p. 150.

99.   Blume, K.G., et al., *A prospective randomized comparison of total body irradiation-etoposide versus busulfan-cyclophosphamide as preparatory regimens for bone marrow transplantation in patients with leukemia who were not in first remission: a Southwest Oncology Group study.* Blood, 1993. **81**(8): p. 2187-93.

100.   Clift, R.A., et al., *Marrow transplantation for chronic myeloid leukemia: a randomized study comparing cyclophosphamide and total body irradiation with busulfan and cyclophosphamide.* Blood, 1994. **84**(6): p. 2036-43.

101.   Ringden, O., et al., *A randomized trial comparing busulfan with total body irradiation as conditioning in allogeneic marrow transplant recipients with leukemia: a report from the Nordic Bone Marrow Transplantation Group.* Blood, 1994. **83**(9): p. 2723-30.

102.   Shi-Xia, X., et al., *Total body irradiation plus cyclophosphamide versus busulphan with cyclophosphamide as conditioning regimen for patients with leukemia undergoing allogeneic stem cell transplantation: a meta-analysis.* Leuk Lymphoma, 2010. **51**(1): p. 50-60.

103.   Forman, S.J., *What is the role of reduced-intensity transplantation in the treatment of older patients with AML?* Hematology Am Soc Hematol Educ Program, 2009: p. 406-13.

104.   Kiss, T.L., et al., *Blood and marrow transplantation in elderly acute myeloid leukaemia patients - older certainly is not better.* Bone Marrow Transplant, 2007. **40**(5): p. 405-16.

105.   Baron, F. and R. Storb, *Hematopoietic cell transplantation after reduced-intensity conditioning for older adults with acute myeloid leukemia in complete remission.* Curr Opin Hematol, 2007. **14**(2): p. 145-51.

106.   Verneris, M.R., et al., *Reduced-intensity conditioning regimens for allogeneic transplantation in children with acute lymphoblastic leukemia.* Biol Blood Marrow Transplant, 2010. **16**(9): p. 1237-44.

107.   Veys, P., K. Rao, and P. Amrolia, *Stem cell transplantation for congenital immunodeficiencies using reduced-intensity conditioning.* Bone Marrow Transplant, 2005. **35 Suppl 1**: p. S45-7.

108.   Satwani, P., et al., *Reduced intensity conditioning and allogeneic stem cell transplantation in childhood malignant and nonmalignant diseases.* Bone Marrow Transplant, 2008. **41**(2): p. 173-82.

109. Shigematsu, A., et al., *Increased risk of bacterial infection after engraftment in patients treated with allogeneic bone marrow transplantation following reduced-intensity conditioning regimen.* Transpl Infect Dis, 2010. **12**(5): p. 412-20.

110. Valcarcel, D., et al., *Conventional versus reduced-intensity conditioning regimen for allogeneic stem cell transplantation in patients with hematological malignancies.* Eur J Haematol, 2005. **74**(2): p. 144-51.

111. Ringden, O., et al., *Reduced intensity conditioning compared with myeloablative conditioning using unrelated donor transplants in patients with acute myeloid leukemia.* J Clin Oncol, 2009. **27**(27): p. 4570-7.

112. Aoudjhane, M., et al., *Comparative outcome of reduced intensity and myeloablative conditioning regimen in HLA identical sibling allogeneic haematopoietic stem cell transplantation for patients older than 50 years of age with acute myeloblastic leukaemia: a retrospective survey from the Acute Leukemia Working Party (ALWP) of the European group for Blood and Marrow Transplantation (EBMT).* Leukemia, 2005. **19**(12): p. 2304-12.

113. Richardson, P. and E. Guinan, *Hepatic veno-occlusive disease following hematopoietic stem cell transplantation.* Acta Haematol, 2001. **106**(1-2): p. 57-68.

114. Coppell, J.A., et al., *Hepatic veno-occlusive disease following stem cell transplantation: incidence, clinical course, and outcome.* Biol Blood Marrow Transplant, 2010. **16**(2): p. 157-68.

115. Eisenberg, S., *Hepatic sinusoidal obstruction syndrome in patients undergoing hematopoietic stem cell transplant.* Oncol Nurs Forum, 2008. **35**(3): p. 385-97.

116. Jones, R.J., et al., *Venoocclusive disease of the liver following bone marrow transplantation.* Transplantation, 1987. **44**(6): p. 778-83.

117. Meyers, J.D. and K. Atkinson, *Infection in bone marrow transplantation.* Clin Haematol, 1983. **12**(3): p. 791-811.

118. Garming-Legert, K., et al., *Long-term salivary function after conditioning with busulfan, fractionated or single-dose TBI.* Oral Dis, 2011. **17**(7): p. 670-6.

119. van der Pas-van Voskuilen, I.G., et al., *Long-term adverse effects of hematopoietic stem cell transplantation on dental development in children.* Support Care Cancer, 2009. **17**(9): p. 1169-75.

120. Dahllof, G., et al., *Disturbances in dental development after total body irradiation in bone marrow transplant recipients.* Oral Surg Oral Med Oral Pathol, 1988. **65**(1): p. 41-4.

121. Hassan, Z., et al., *Hemorrhagic cystitis: a retrospective single-center survey.* Clin Transplant, 2007. **21**(5): p. 659-67.

122. Claes, K. and P. Kestelyn, *Ocular manifestations of graft versus host disease following bone marrow transplantation.* Bull Soc Belge Ophtalmol, 2000(277): p. 21-6.

123. Riemens, A., et al., *Current insights into ocular graft-versus-host disease.* Curr Opin Ophthalmol, 2010. **21**(6): p. 485-94.

124. Westeneng, A.C., et al., *Ocular graft-versus-host disease after allogeneic stem cell transplantation.* Cornea, 2010. **29**(7): p. 758-63.

125. Weisdorf, D., *GVHD the nuts and bolts.* Hematology Am Soc Hematol Educ Program, 2007: p. 62-7.

126. Billingham, R.E. and L. Brent, *A simple method for inducing tolerance of skin homografts in mice.* Transplant Bull, 1957. **4**(2): p. 67-71.

127. Storb, R., et al., *Marrow transplantation for chronic myelocytic leukemia: a controlled trial of cyclosporine versus methotrexate for prophylaxis of graft-versus-host disease.* Blood, 1985. **66**(3): p. 698-702.

128. Storb, R., et al., *Methotrexate and cyclosporine versus cyclosporine alone for prophylaxis of graft-versus-host disease in patients given HLA-identical marrow grafts for leukemia: long-term follow-up of a controlled trial.* Blood, 1989. **73**(6): p. 1729-34.

129. Ringden, O., et al., *A randomized trial comparing use of cyclosporin and methotrexate for graft-versus-host disease prophylaxis in bone marrow transplant recipients with haematological malignancies.* Bone Marrow Transplant, 1986. **1**(1): p. 41-51.

130. Thomas, E.D., et al., *Marrow Transplants in Lethally Irradiated Dogs: The Effect of Methotrexate on Survival of the Host and the Homograft.* Transplantation, 1963. **1**: p. 571-4.

131. Latif, T., et al., *Syngeneic graft-versus-host disease: a report of two cases and literature review.* Bone Marrow Transplant, 2003. **32**(5): p. 535-9.

132. Billingham, R.E., *The biology of graft-versus-host reactions.* Harvey Lect, 1966. **62**: p. 21-78.

133. Ferrara, J.L. and H.J. Deeg, *Graft-versus-host disease.* N Engl J Med, 1991. **324**(10): p. 667-74.

134. Filipovich, A.H., et al., *National Institutes of Health consensus development project on criteria for clinical trials in chronic graft-versus-host disease: I. Diagnosis and staging working group report.* Biol Blood Marrow Transplant, 2005. **11**(12): p. 945-56.

135. Mielcarek, M., et al., *Graft-versus-host disease after nonmyeloablative versus conventional hematopoietic stem cell transplantation.* Blood, 2003. **102**(2): p. 756-62.

136. Flowers, M.E., et al., *Comparison of chronic graft-versus-host disease after transplantation of peripheral blood stem cells versus bone marrow in allogeneic recipients: long-term follow-up of a randomized trial.* Blood, 2002. **100**(2): p. 415-9.

137. Kolb, H.J., et al., *Donor leukocyte transfusions for treatment of recurrent chronic myelogenous leukemia in marrow transplant patients.* Blood, 1990. **76**(12): p. 2462-5.

138. Jacobsohn, D.A. and G.B. Vogelsang, *Acute graft versus host disease.* Orphanet J Rare Dis, 2007. **2**: p. 35.

139. Ferrara, J.L., K.R. Cooke, and T. Teshima, *The pathophysiology of acute graft-versus-host disease.* Int J Hematol, 2003. **78**(3): p. 181-7.

140. Ferrara, J.L. and P. Reddy, *Pathophysiology of graft-versus-host disease.* Semin Hematol, 2006. **43**(1): p. 3-10.

141. Travnik, R., et al., *[Graft-versus-Host Disease (GvHD) - an update : Part 1: Pathophysiology, clinical features and classification of GvHD].* Hautarzt, 2011. **62**(2): p. 139-54; quiz 155.

142. Reddy, P., *Pathophysiology of acute graft-versus-host disease.* Hematol Oncol, 2003. **21**(4): p. 149-61.

143. Vogelsang, G.B., L. Lee, and D.M. Bensen-Kennedy, *Pathogenesis and treatment of graft-versus-host disease after bone marrow transplant.* Annu Rev Med, 2003. **54**: p. 29-52.

144. Ferrara, J.L., *Pathogenesis of acute graft-versus-host disease: cytokines and cellular effectors.* J Hematother Stem Cell Res, 2000. **9**(3): p. 299-306.

145. Roncarolo, M.G. and M. Battaglia, *Regulatory T-cell immunotherapy for tolerance to self antigens and alloantigens in humans.* Nat Rev Immunol, 2007. **7**(8): p. 585-98.

146. Shono, Y., et al., *Bone marrow graft-versus-host disease: early destruction of hematopoietic niche after MHC-mismatched hematopoietic stem cell transplantation.* Blood, 2010. **115**(26): p. 5401-11.

147. Sullivan, K.M., et al., *Chronic graft-versus-host disease in 52 patients: adverse natural course and successful treatment with combination immunosuppression.* Blood, 1981. **57**(2): p. 267-76.

148. Min, C.K., *The pathophysiology of chronic graft-versus-host disease: the unveiling of an enigma.* Korean J Hematol, 2011. **46**(2): p. 80-7.

149. Prentice, H.G., et al., *Depletion of T lymphocytes in donor marrow prevents significant graft-versus-host disease in matched allogeneic leukaemic marrow transplant recipients.* Lancet, 1984. **1**(8375): p. 472-6.

150. Marmont, A.M., et al., *T-cell depletion of HLA-identical transplants in leukemia.* Blood, 1991. **78**(8): p. 2120-30.

151. Ringden, O., et al., *Long-term follow-up of a randomized trial comparing T cell depletion with a combination of methotrexate and cyclosporine in adult leukemic marrow transplant recipients.* Transplantation, 1994. **58**(8): p. 887-91.

152. Remberger, M., et al., *Long-term follow-up of a randomized trial comparing T-cell depletion with a combination of methotrexate and cyclosporine in adult*

leukemic marrow transplant recipients. Transplant Proc, 1994. **26**(3): p. 1829-30.

153. Kolb, H.J., *Graft-versus-leukemia effects of transplantation and donor lymphocytes.* Blood, 2008. **112**(12): p. 4371-83.

154. Weiden, P.L., et al., *Antileukemic effect of graft-versus-host disease in human recipients of allogeneic-marrow grafts.* N Engl J Med, 1979. **300**(19): p. 1068-73.

155. Ringden, O., et al., *The allogeneic graft-versus-cancer effect.* Br J Haematol, 2009. **147**(5): p. 614-33.

156. Bacigalupo, A., *Management of acute graft-versus-host disease.* Br J Haematol, 2007. **137**(2): p. 87-98.

157. Morris, E.S. and G.R. Hill, *Advances in the understanding of acute graft-versus-host disease.* Br J Haematol, 2007. **137**(1): p. 3-19.

158. Powles, R.L., et al., *Cyclosporin A to prevent graft-versus-host disease in man after allogeneic bone-marrow transplantation.* Lancet, 1980. **1**(8164): p. 327-9.

159. Ringden, O., *Cyclosporine in allogeneic bone marrow transplantation.* Transplantation, 1986. **42**(5): p. 445-52.

160. Ringden, O., et al., *Methotrexate, cyclosporine, or both to prevent graft-versus-host disease after HLA-identical sibling bone marrow transplants for early leukemia?* Blood, 1993. **81**(4): p. 1094-101.

161. Ferrara, J.L., *Novel strategies for the treatment and diagnosis of graft-versus-host-disease.* Best Pract Res Clin Haematol, 2007. **20**(1): p. 91-7.

162. Holler, E., *Risk assessment in haematopoietic stem cell transplantation: GvHD prevention and treatment.* Best Pract Res Clin Haematol, 2007. **20**(2): p. 281-94.

163. Georgantas, R.W., 3rd, O. Bohana-Kashtan, and C.I. Civin, *Ex vivo soluble fas ligand treatment of donor cells to selectively reduce murine acute graft versus host disease.* Transplantation, 2006. **82**(4): p. 471-8.

164. Pillai, A.B., et al., *Host NKT cells can prevent graft-versus-host disease and permit graft antitumor activity after bone marrow transplantation.* J Immunol, 2007. **178**(10): p. 6242-51.

165. Blazar, B.R., et al., *Blockade of programmed death-1 engagement accelerates graft-versus-host disease lethality by an IFN-gamma-dependent mechanism.* J Immunol, 2003. **171**(3): p. 1272-7.

166. Ho, V.T., C. Revta, and P.G. Richardson, *Hepatic veno-occlusive disease after hematopoietic stem cell transplantation: update on defibrotide and other current investigational therapies.* Bone Marrow Transplant, 2008. **41**(3): p. 229-37.

167. Cefalo, M.G., et al., *Hepatic veno-occlusive disease: a chemotherapy-related toxicity in children with malignancies.* Paediatr Drugs, 2010. **12**(5): p. 277-84.

168. Coppell, J.A., S.A. Brown, and D.J. Perry, *Veno-occlusive disease: cytokines, genetics, and haemostasis.* Blood Rev, 2003. **17**(2): p. 63-70.

169. McDonald, G.B., et al., *Venocclusive disease of the liver after bone marrow transplantation: diagnosis, incidence, and predisposing factors.* Hepatology, 1984. **4**(1): p. 116-22.

170. Carreras, E., et al., *Veno-occlusive disease of the liver after high-dose cytoreductive therapy with busulfan and melphalan for autologous blood stem cell transplantation in multiple myeloma patients.* Biol Blood Marrow Transplant, 2007. **13**(12): p. 1448-54.

171. Cesaro, S., et al., *A prospective survey on incidence, risk factors and therapy of hepatic veno-occlusive disease in children after hematopoietic stem cell transplantation.* Haematologica, 2005. **90**(10): p. 1396-404.

172. Ringden, O., et al., *Increased risk of chronic graft-versus-host disease, obstructive bronchiolitis, and alopecia with busulfan versus total body irradiation: long-term results of a randomized trial in allogeneic marrow recipients with leukemia. Nordic Bone Marrow Transplantation Group.* Blood, 1999. **93**(7): p. 2196-201.

173. Richardson, P.G., et al., *Defibrotide for the treatment of severe hepatic veno-occlusive disease and multiorgan failure after stem cell transplantation: a*

multicenter, randomized, dose-finding trial. Biol Blood Marrow Transplant, 2010. **16**(7): p. 1005-17.

174. Ramasamy, K., et al., *Incidence and management of hepatic venoocclusive disease in 237 patients undergoing reduced-intensity conditioning (RIC) haematopoietic stem cell transplantation (HSCT).* Bone Marrow Transplant, 2006. **38**(12): p. 823-4.

175. Mattsson, J., O. Ringden, and R. Storb, *Graft Failure after Allogeneic Hematopoietic Cell Transplantation.* Biol Blood Marrow Transplant, 2008. **14**(Supplement 1): p. 165-170.

176. Fleischhauer, K., et al., *Graft rejection after unrelated donor hematopoietic stem cell transplantation for thalassemia is associated with nonpermissive HLA-DPB1 disparity in host-versus-graft direction.* Blood, 2006. **107**(7): p. 2984-92.

177. Remberger, M., et al., *Major ABO blood group mismatch increases the risk for graft failure after unrelated donor hematopoietic stem cell transplantation.* Biol Blood Marrow Transplant, 2007. **13**(6): p. 675-82.

178. Marenchino, D., et al., *Consequences of ABO incompatibility in multiple myeloma patients undergoing peripheral blood stem cell transplantation after reduced intensity conditioning.* Blood Transfus, 2011. **9**(1): p. 79-85.

179. Canals, C., et al., *Impact of ABO incompatibility on allogeneic peripheral blood progenitor cell transplantation after reduced intensity conditioning.* Transfusion, 2004. **44**(11): p. 1603-11.

180. Kim, J.G., et al., *Impact of ABO incompatibility on outcome after allogeneic peripheral blood stem cell transplantation.* Bone Marrow Transplant, 2005. **35**(5): p. 489-95.

181. Dazzi, F. and C. Fozza, *Disease relapse after haematopoietic stem cell transplantation: risk factors and treatment.* Best Pract Res Clin Haematol, 2007. **20**(2): p. 311-27.

182. Miller, J.S., et al., *NCI First International Workshop on The Biology, Prevention, and Treatment of Relapse After Allogeneic Hematopoietic Stem Cell Transplantation: Report from the Committee on the Biology Underlying Recurrence of Malignant Disease following Allogeneic HSCT: Graft-versus-Tumor/Leukemia Reaction.* Biol Blood Marrow Transplant, 2010. **16**(5): p. 565-86.

183. Paulin, T., et al., *Variables predicting bacterial and fungal infections after allogeneic marrow engraftment.* Transplantation, 1987. **43**(3): p. 393-8.

184. Bow, E.J., *Invasive fungal infection in haematopoietic stem cell transplant recipients: epidemiology from the transplant physician's viewpoint.* Mycopathologia, 2009. **168**(6): p. 283-97.

185. Nucci, M., et al., *Fusarium infection in hematopoietic stem cell transplant recipients.* Clin Infect Dis, 2004. **38**(9): p. 1237-42.

186. Kontoyiannis, D.P., et al., *Prospective surveillance for invasive fungal infections in hematopoietic stem cell transplant recipients, 2001-2006: overview of the Transplant-Associated Infection Surveillance Network (TRANSNET) Database.* Clin Infect Dis, 2010. **50**(8): p. 1091-100.

187. Kriengkauykiat, J., J.I. Ito, and S.S. Dadwal, *Epidemiology and treatment approaches in management of invasive fungal infections.* Clin Epidemiol, 2011. **3**: p. 175-91.

188. Lee, S.B., et al., *Expression and characterization of antimicrobial peptides Retrocyclin-101 and Protegrin-1 in chloroplasts to control viral and bacterial infections.* Plant Biotechnol J, 2011. **9**(1): p. 100-15.

189. Strasfeld, L. and D.M. Weinstock, *Antifungal prophylaxis among allogeneic hematopoietic stem cell transplant recipients: current issues and new agents.* Expert Rev Anti Infect Ther, 2006. **4**(3): p. 457-68.

190. Therriault, B.L., et al., *Characterization of bacterial infections in allogeneic hematopoietic stem cell transplant recipients who received prophylactic levofloxacin with either penicillin or doxycycline.* Mayo Clin Proc, 2010. **85**(8): p. 711-8.

191. Cupic, M., et al., *[Conventional and molecular methods in diagnosis and monitoring of viral infection].* Srp Arh Celok Lek, 2007. **135**(9-10): p. 589-93.

192. Ljungman, P., *Molecular monitoring of viral infections after hematopoietic stem cell transplantation.* Int J Hematol, 2010. **91**(4): p. 596-601.
193. ERNA, *Polyunsaturated fatty acids (PUFA).* 2008.
194. Lands, B., *Prevent the cause, not just the symptoms.* Prostaglandins Other Lipid Mediat, 2011.
195. Arterburn, L.M., E.B. Hall, and H. Oken, *Distribution, interconversion, and dose response of n-3 fatty acids in humans.* Am J Clin Nutr, 2006. **83**(6 Suppl): p. 1467S-1476S.
196. Mattsson, J., et al., *Poor oral nutrition after allogeneic stem cell transplantation correlates significantly with severe graft-versus-host disease.* Bone Marrow Transplant, 2006. **38**(9): p. 629-33.
197. Burdge, G.C. and P.C. Calder, *Conversion of alpha-linolenic acid to longer-chain polyunsaturated fatty acids in human adults.* Reprod Nutr Dev, 2005. **45**(5): p. 581-97.
198. Berquin, I.M., I.J. Edwards, and Y.Q. Chen, *Multi-targeted therapy of cancer by omega-3 fatty acids.* Cancer Lett, 2008. **269**(2): p. 363-77.
199. Health, H.S.o.P. *Ask the Expert: Omega-3 Fatty Acids*. 2011; Available from: http://www.hsph.harvard.edu/nutritionsource/questions/omega-3/index.html.
200. Larsson, S.C., et al., *Dietary long-chain n-3 fatty acids for the prevention of cancer: a review of potential mechanisms.* Am J Clin Nutr, 2004. **79**(6): p. 935-45.
201. Zhang, P., et al., *Dietary (n-3) polyunsaturated fatty acids modulate murine Th1/Th2 balance toward the Th2 pole by suppression of Th1 development.* J Nutr, 2005. **135**(7): p. 1745-51.
202. McMurray, D.N., C.A. Jolly, and R.S. Chapkin, *Effects of dietary n-3 fatty acids on T cell activation and T cell receptor-mediated signaling in a murine model.* J Infect Dis, 2000. **182 Suppl 1**: p. S103-7.
203. Hong, M.Y., et al., *Fish oil decreases oxidative DNA damage by enhancing apoptosis in rat colon.* Nutr Cancer, 2005. **52**(2): p. 166-75.
204. Switzer, K.C., et al., *Dietary n-3 polyunsaturated fatty acids promote activation-induced cell death in Th1-polarized murine CD4+ T-cells.* J Lipid Res, 2004. **45**(8): p. 1482-92.
205. Ly, L.H., et al., *Dietary n-3 polyunsaturated fatty acids suppress splenic CD4(+) T cell function in interleukin (IL)-10(-/-) mice.* Clin Exp Immunol, 2005. **139**(2): p. 202-9.
206. Harbige, L.S., *Fatty acids, the immune response, and autoimmunity: a question of n-6 essentiality and the balance between n-6 and n-3.* Lipids, 2003. **38**(4): p. 323-41.
207. Surette, M.E., *The science behind dietary omega-3 fatty acids.* CMAJ, 2008. **178**(2): p. 177-80.
208. Calder, P.C., *Immunomodulation by omega-3 fatty acids.* Prostaglandins Leukot Essent Fatty Acids, 2007. **77**(5-6): p. 327-35.
209. Miles, E.A., et al., *Limited effect of eicosapentaenoic acid on T-lymphocyte and natural killer cell numbers and functions in healthy young males.* Nutrition, 2006. **22**(5): p. 512-9.
210. Thies, F., et al., *Dietary supplementation with gamma-linolenic acid or fish oil decreases T lymphocyte proliferation in healthy older humans.* J Nutr, 2001. **131**(7): p. 1918-27.
211. McGuinness, J., et al., *Pretreatment with omega-3 fatty acid infusion to prevent leukocyte-endothelial injury responses seen in cardiac surgery.* J Thorac Cardiovasc Surg, 2008. **136**(1): p. 135-41.
212. *Omega-3 Fatty Acids and Health*. 2011 October 2005 [cited 2011 31st of November]; Available from: http://ods.od.nih.gov/factsheets/omega3fattyacidsandhealth/.
213. Cockbain, A.J., G.J. Toogood, and M.A. Hull, *Omega-3 polyunsaturated fatty acids for the treatment and prevention of colorectal cancer.* Gut, 2011.
214. Reese, A.C., V. Fradet, and J.S. Witte, *Omega-3 fatty acids, genetic variants in COX-2 and prostate cancer.* J Nutrigenet Nutrigenomics, 2009. **2**(3): p. 149-58.

215. Fradet, V., et al., *Dietary omega-3 fatty acids, cyclooxygenase-2 genetic variation, and aggressive prostate cancer risk.* Clin Cancer Res, 2009. **15**(7): p. 2559-66.

216. Greenhough, A., et al., *The COX-2/PGE2 pathway: key roles in the hallmarks of cancer and adaptation to the tumour microenvironment.* Carcinogenesis, 2009. **30**(3): p. 377-86.

217. Center, U.o.M.M. *Omega-3 fatty acids.* 2011; Available from: http://www.umm.edu/altmed/articles/omega-3-000316.htm.

218. Bousquet, M., et al., *Transgenic conversion of omega-6 into omega-3 fatty acids in a mouse model of Parkinson's disease.* J Lipid Res, 2011. **52**(2): p. 263-71.

219. Bazan, N.G., M.F. Molina, and W.C. Gordon, *Docosahexaenoic Acid signalolipidomics in nutrition: significance in aging, neuroinflammation, macular degeneration, Alzheimer's, and other neurodegenerative diseases.* Annu Rev Nutr, 2011. **31**: p. 321-51.

220. Palacios-Pelaez, R., W.J. Lukiw, and N.G. Bazan, *Omega-3 essential fatty acids modulate initiation and progression of neurodegenerative disease.* Mol Neurobiol, 2010. **41**(2-3): p. 367-74.

221. Zhang, C. and N.G. Bazan, *Lipid-mediated cell signaling protects against injury and neurodegeneration.* J Nutr, 2010. **140**(4): p. 858-63.

222. Jicha, G.A. and W.R. Markesbery, *Omega-3 fatty acids: potential role in the management of early Alzheimer's disease.* Clin Interv Aging, 2010. **5**: p. 45-61.

223. Cederholm, T. and J. Palmblad, *Are omega-3 fatty acids options for prevention and treatment of cognitive decline and dementia?* Curr Opin Clin Nutr Metab Care, 2010. **13**(2): p. 150-5.

224. Freund-Levi, Y., et al., *Omega-3 fatty acid treatment in 174 patients with mild to moderate Alzheimer disease: OmegAD study: a randomized double-blind trial.* Arch Neurol, 2006. **63**(10): p. 1402-8.

225. Fotuhi, M., P. Mohassel, and K. Yaffe, *Fish consumption, long-chain omega-3 fatty acids and risk of cognitive decline or Alzheimer disease: a complex association.* Nat Clin Pract Neurol, 2009. **5**(3): p. 140-52.

226. Calon, F. and G. Cole, *Neuroprotective action of omega-3 polyunsaturated fatty acids against neurodegenerative diseases: evidence from animal studies.* Prostaglandins Leukot Essent Fatty Acids, 2007. **77**(5-6): p. 287-93.

227. Gleissman, H., J.I. Johnsen, and P. Kogner, *Omega-3 fatty acids in cancer, the protectors of good and the killers of evil?* Exp Cell Res, 2010. **316**(8): p. 1365-73.

228. Yee, L.D., et al., *Omega-3 fatty acid supplements in women at high risk of breast cancer have dose-dependent effects on breast adipose tissue fatty acid composition.* Am J Clin Nutr, 2010. **91**(5): p. 1185-94.

229. Bougnoux, P., V. Maillard, and V. Chajes, *Omega-6/omega-3 polyunsaturated fatty acids ratio and breast cancer.* World Rev Nutr Diet, 2005. **94**: p. 158-65.

230. Thiebaut, A.C., et al., *Dietary intakes of omega-6 and omega-3 polyunsaturated fatty acids and the risk of breast cancer.* Int J Cancer, 2009. **124**(4): p. 924-31.

231. Kelavkar, U.P., et al., *Prostate tumor growth and recurrence can be modulated by the omega-6:omega-3 ratio in diet: athymic mouse xenograft model simulating radical prostatectomy.* Neoplasia, 2006. **8**(2): p. 112-24.

232. Yamagami, T., et al., *Docosahexaenoic acid induces dose dependent cell death in an early undifferentiated subtype of acute myeloid leukemia cell line.* Cancer Biol Ther, 2009. **8**(4): p. 331-7.

233. Slagsvold, J.E., et al., *The antiproliferative effect of EPA in HL60 cells is mediated by alterations in calcium homeostasis.* Lipids, 2009. **44**(2): p. 103-13.

234. Gillis, R.C., et al., *Eicosapentaenoic acid and gamma-linolenic acid induce apoptosis in HL-60 cells.* J Surg Res, 2002. **107**(1): p. 145-53.

235. Biondo, P.D., et al., *The potential for treatment with dietary long-chain polyunsaturated n-3 fatty acids during chemotherapy.* J Nutr Biochem, 2008. **19**(12): p. 787-96.

236. Shaikh, I.A., et al., *Enhancing cytotoxic therapies for breast and prostate cancers with polyunsaturated fatty acids.* Nutr Cancer, 2010. **62**(3): p. 284-96.

237. Calviello, G., et al., *Antineoplastic effects of n-3 polyunsaturated fatty acids in combination with drugs and radiotherapy: preventive and therapeutic strategies.* Nutr Cancer, 2009. **61**(3): p. 287-301.
238. Pardini, R.S., *Nutritional intervention with omega-3 fatty acids enhances tumor response to anti-neoplastic agents.* Chem Biol Interact, 2006. **162**(2): p. 89-105.
239. Wynter, M.P., S.T. Russell, and M.J. Tisdale, *Effect of n-3 fatty acids on the antitumour effects of cytotoxic drugs.* In Vivo, 2004. **18**(5): p. 543-7.
240. Bougnoux, P., et al., *Cytotoxic drugs efficacy correlates with adipose tissue docosahexaenoic acid level in locally advanced breast carcinoma.* Br J Cancer, 1999. **79**(11-12): p. 1765-9.
241. Wall, R.L. and F.G. Conrad, *Cyclophosphamide therapy. Its use in leukemia, lymphoma and solid tumors.* Arch Intern Med, 1961. **108**: p. 456-82.
242. Fleming, R.A., *An overview of cyclophosphamide and ifosfamide pharmacology.* Pharmacotherapy, 1997. **17**(5 Pt 2): p. 146S-154S.
243. Kromann, N. and A. Green, *Epidemiological studies in the Upernavik district, Greenland. Incidence of some chronic diseases 1950-1974.* Acta Med Scand, 1980. **208**(5): p. 401-6.
244. Dyerberg, J. and H.O. Bang, *Haemostatic function and platelet polyunsaturated fatty acids in Eskimos.* Lancet, 1979. **2**(8140): p. 433-5.
245. Simopoulos, A.P., *Omega-3 fatty acids in inflammation and autoimmune diseases.* J Am Coll Nutr, 2002. **21**(6): p. 495-505.
246. Tull, S.P., et al., *Omega-3 Fatty acids and inflammation: novel interactions reveal a new step in neutrophil recruitment.* PLoS Biol, 2009. **7**(8): p. e1000177.
247. De Caterina, R., J.K. Liao, and P. Libby, *Fatty acid modulation of endothelial activation.* Am J Clin Nutr, 2000. **71**(1 Suppl): p. 213S-23S.
248. De Caterina, R., et al., *The inhibition of endothelial activation by unsaturated fatty acids.* Lipids, 1999. **34 Suppl**: p. S191-4.
249. Nowak, J.Z., *[Anti-inflammatory pro-resolving derivatives of omega-3 and omega-6 polyunsaturated fatty acids].* Postepy Hig Med Dosw (Online), 2010. **64**: p. 115-32.
250. Bonis, P.A., et al., *Effects of omega-3 fatty acids on organ transplantation.* Evid Rep Technol Assess (Summ), 2005(115): p. 1-11.
251. Takatsuka, H., et al., *Oral eicosapentaenoic acid for complications of bone marrow transplantation.* Bone Marrow Transplant, 2001. **28**(8): p. 769-74.
252. Takatsuka, H., et al., *Oral eicosapentaenoic acid for acute colonic graft-versus-host disease after bone marrow transplantation.* Drugs Exp Clin Res, 2002. **28**(4): p. 121-5.
253. Fontenot, J.D., M.A. Gavin, and A.Y. Rudensky, *Foxp3 programs the development and function of CD4+CD25+ regulatory T cells.* Nat Immunol, 2003. **4**(4): p. 330-6.
254. Waldmann, H., et al., *Regulatory T cells and organ transplantation.* Semin Immunol, 2004. **16**(2): p. 119-26.
255. Leavy, O., *Regulatory T cells: CD8+ Treg cells join the fold.* Nat Rev Immunol, 2010. **10**(10): p. 680.
256. Kim, H.J., et al., *Inhibition of follicular T-helper cells by CD8(+) regulatory T cells is essential for self tolerance.* Nature, 2010. **467**(7313): p. 328-32.
257. Kim, H.J., et al., *CD8+ T regulatory cells express the Ly49 Class I MHC receptor and are defective in autoimmune prone B6-Yaa mice.* Proc Natl Acad Sci U S A, 2011. **108**(5): p. 2010-5.
258. Varthaman, A., et al., *Physiological induction of regulatory Qa-1-restricted CD8+ T cells triggered by endogenous CD4+ T cell responses.* PLoS One, 2011. **6**(6): p. e21628.
259. Spoerl, S. and X.C. Li, *Regulatory T cells and the quest for transplant tolerance.* Discov Med, 2011. **11**(56): p. 25-34.
260. Gorantla, V.S., et al., *T regulatory cells and transplantation tolerance.* Transplant Rev (Orlando), 2010. **24**(3): p. 147-59.
261. Yu, Y., et al., *Requirement of cognate CD4+ T-cell recognition for the regulation of allospecific CTL by human CD4+ CD127- CD25+ FOXP3+ cells generated in MLR.* PLoS One, 2011. **6**(7): p. e22450.

262. Taams, L.S., et al., *Regulatory T cells in human disease and their potential for therapeutic manipulation.* Immunology, 2006. **118**(1): p. 1-9.

263. Buckner, J.H., *Mechanisms of impaired regulation by CD4(+)CD25(+)FOXP3(+) regulatory T cells in human autoimmune diseases.* Nat Rev Immunol, 2010. **10**(12): p. 849-59.

264. Viglietta, V., et al., *Loss of functional suppression by CD4+CD25+ regulatory T cells in patients with multiple sclerosis.* J Exp Med, 2004. **199**(7): p. 971-9.

265. Libera, D.D., et al., *T regulatory cells are markers of disease activity in multiple sclerosis patients.* PLoS One, 2011. **6**(6): p. e21386.

266. Costantino, C.M., C. Baecher-Allan, and D.A. Hafler, *Multiple sclerosis and regulatory T cells.* J Clin Immunol, 2008. **28**(6): p. 697-706.

267. Lindley, S., et al., *Defective suppressor function in CD4(+)CD25(+) T-cells from patients with type 1 diabetes.* Diabetes, 2005. **54**(1): p. 92-9.

268. Ehrenstein, M.R., et al., *Compromised function of regulatory T cells in rheumatoid arthritis and reversal by anti-TNFalpha therapy.* J Exp Med, 2004. **200**(3): p. 277-85.

269. Oh, S., A.L. Rankin, and A.J. Caton, *CD4+CD25+ regulatory T cells in autoimmune arthritis.* Immunol Rev, 2010. **233**(1): p. 97-111.

270. Muller, Y.D., et al., *Transplantation tolerance: Clinical potential of regulatory T cells.* Self Nonself, 2011. **2**(1): p. 26-34.

271. Wood, K.J. and S. Sakaguchi, *Regulatory T cells in transplantation tolerance.* Nat Rev Immunol, 2003. **3**(3): p. 199-210.

272. Magenau, J.M., et al., *Frequency of CD4(+)CD25(hi)FOXP3(+) regulatory T cells has diagnostic and prognostic value as a biomarker for acute graft-versus-host-disease.* Biol Blood Marrow Transplant, 2010. **16**(7): p. 907-14.

273. Edinger, M., et al., *CD4+CD25+ regulatory T cells preserve graft-versus-tumor activity while inhibiting graft-versus-host disease after bone marrow transplantation.* Nat Med, 2003. **9**(9): p. 1144-50.

274. Ukena, S.N., et al., *Acute but not chronic graft-versus-host disease is associated with a reduction of circulating CD4(+)CD25 (high)CD127 (low/-) regulatory T cells.* Ann Hematol, 2011. **90**(2): p. 213-8.

275. Iwami, D., et al., *Immunomodulatory effects of eicosapentaenoic acid through induction of regulatory T cells.* Int Immunopharmacol, 2011. **11**(3): p. 384-9.

276. Iwami, D., et al., *Purified eicosapentaenoic acid induces prolonged survival of cardiac allografts and generates regulatory T cells.* Am J Transplant, 2009. **9**(6): p. 1294-307.

277. Otto, D.A., et al., *Improved survival of heterotopic cardiac allografts in rats with dietary n-3 polyunsaturated fatty acids.* Transplantation, 1990. **50**(2): p. 193-8.

278. Grimminger, F., et al., *Omega-3 lipid infusion in a heart allotransplant model. Shift in fatty acid and lipid mediator profiles and prolongation of transplant survival.* Circulation, 1996. **93**(2): p. 365-71.

279. Gibson, S.W., et al., *Nutritional immunomodulation leads to enhanced allograft survival in combination with cyclosporine A and rapamycin, but not FK506.* Transplantation, 2000. **69**(10): p. 2034-8.

280. Kelley, V.E., et al., *Enhancement of immunosuppression by substitution of fish oil for olive oil as a vehicle for cyclosporine.* Transplantation, 1989. **48**(1): p. 98-102.

281. Hernandez, D., et al., *Dietary fish oil does not influence acute rejection rate and graft survival after renal transplantation: a randomized placebo-controlled study.* Nephrol Dial Transplant, 2002. **17**(5): p. 897-904.

282. van der Heide, J.J., et al., *Effect of dietary fish oil on renal function and rejection in cyclosporine-treated recipients of renal transplants.* N Engl J Med, 1993. **329**(11): p. 769-73.

283. Tichelli, A., et al., *Late complications after hematopoietic stem cell transplantation.* Expert Rev Hematol, 2009. **2**(5): p. 583-601.

284. Rovo, A., et al., *Late altered organ function in very long-term survivors after allogeneic hematopoietic stem cell transplantation: a paired comparison with their HLA-identical sibling donor.* Haematologica, 2011. **96**(1): p. 150-5.

285. Tichelli, A., A. Rovo, and A. Gratwohl, *Late pulmonary, cardiovascular, and renal complications after hematopoietic stem cell transplantation and recommended screening practices.* Hematology Am Soc Hematol Educ Program, 2008: p. 125-33.

286. Tichelli, A., S. Bhatia, and G. Socie, *Cardiac and cardiovascular consequences after haematopoietic stem cell transplantation.* Br J Haematol, 2008. **142**(1): p. 11-26.

287. Armenian, S.H., et al., *Predictors of late cardiovascular complications in survivors of hematopoietic cell transplantation.* Biol Blood Marrow Transplant, 2010. **16**(8): p. 1138-44.

288. Olin, J.W. and B.A. Sealove, *Peripheral artery disease: current insight into the disease and its diagnosis and management.* Mayo Clin Proc, 2010. **85**(7): p. 678-92.

289. Houtchens, J., D. Martin, and J.R. Klinger, *Diagnosis and Management of Pulmonary Arterial Hypertension.* Pulm Med, 2011. **2011**: p. 845864.

290. Plouin, P.F. and L. Bax, *Diagnosis and treatment of renal artery stenosis.* Nat Rev Nephrol, 2010. **6**(3): p. 151-9.

291. Dworkin, L.D. and C.J. Cooper, *Clinical practice. Renal-artery stenosis.* N Engl J Med, 2009. **361**(20): p. 1972-8.

292. Bian, K., M.F. Doursout, and F. Murad, *Vascular system: role of nitric oxide in cardiovascular diseases.* J Clin Hypertens (Greenwich), 2008. **10**(4): p. 304-10.

293. Furchgott, R.F. and J.V. Zawadzki, *The obligatory role of endothelial cells in the relaxation of arterial smooth muscle by acetylcholine.* Nature, 1980. **288**(5789): p. 373-6.

294. Jin, R.C. and J. Loscalzo, *Vascular Nitric Oxide: Formation and Function.* J Blood Med, 2010. **2010**(1): p. 147-162.

295. Doll, D.C. and J.W. Yarbro, *Vascular toxicity associated with chemotherapy and hormonotherapy.* Curr Opin Oncol, 1994. **6**(4): p. 345-50.

296. Doll, D.C. and J.W. Yarbro, *Vascular toxicity associated with antineoplastic agents.* Semin Oncol, 1992. **19**(5): p. 580-96.

297. Ferry, C. and G. Socie, *Busulfan-cyclophosphamide versus total body irradiation-cyclophosphamide as preparative regimen before allogeneic hematopoietic stem cell transplantation for acute myeloid leukemia: what have we learned?* Exp Hematol, 2003. **31**(12): p. 1182-6.

298. Santos, G.W., *Busulfan (Bu) and cyclophosphamide (Cy) for marrow transplantation.* Bone Marrow Transplant, 1989. **4 Suppl 1**: p. 236-9.

299. Busse, D., et al., *[Treatment of cancer with cytostatics: results with high dose therapy with cyclophosphamide and drug targeting with doxorubicin].* Pharm Unserer Zeit, 1998. **27**(5): p. 216-22.

300. Luznik, L., R.J. Jones, and E.J. Fuchs, *High-dose cyclophosphamide for graft-versus-host disease prevention.* Curr Opin Hematol, 2010. **17**(6): p. 493-9.

301. Luznik, L. and E.J. Fuchs, *High-dose, post-transplantation cyclophosphamide to promote graft-host tolerance after allogeneic hematopoietic stem cell transplantation.* Immunol Res, 2010. **47**(1-3): p. 65-77.

302. Goldberg, M.A., et al., *Cyclophosphamide cardiotoxicity: an analysis of dosing as a risk factor.* Blood, 1986. **68**(5): p. 1114-8.

303. Ayash, L.J., et al., *Cyclophosphamide pharmacokinetics: correlation with cardiac toxicity and tumor response.* J Clin Oncol, 1992. **10**(6): p. 995-1000.

304. Terpstra, W. and C.E. de Maat, *Pericardial fibrosis following busulfan treatment.* Neth J Med, 1989. **35**(5-6): p. 249-52.

305. Cooke, K.R., et al., *An experimental model of idiopathic pneumonia syndrome after bone marrow transplantation: I. The roles of minor H antigens and endotoxin.* Blood, 1996. **88**(8): p. 3230-9.

306. Sadeghi, B., et al., *GVHD after chemotherapy conditioning in allogeneic transplanted mice.* Bone Marrow Transplant, 2008. **42**(12): p. 807-18.

307. Hill, G.R. and J.L. Ferrara, *The primacy of the gastrointestinal tract as a target organ of acute graft-versus-host disease: rationale for the use of cytokine shields in allogeneic bone marrow transplantation.* Blood, 2000. **95**(9): p. 2754-9.

308. Leszczynski, D., R. Renkonen, and P. Hayry, *Bone marrow transplantation in the rat. III. Structure of the liver inflammatory lesion in acute graft-versus-host disease.* Am J Pathol, 1985. **120**(2): p. 316-22.

309. Beschorner, W.E., P.J. Tutschka, and G.W. Santos, *Sequential morphology of graft-versus-host disease in the rat radiation chimera.* Clin Immunol Immunopathol, 1982. **22**(2): p. 203-24.

310. McDonald, G.B., et al., *Cyclophosphamide metabolism, liver toxicity, and mortality following hematopoietic stem cell transplantation.* Blood, 2003. **101**(5): p. 2043-8.

311. Moore, M.J., *Clinical pharmacokinetics of cyclophosphamide.* Clin Pharmacokinet, 1991. **20**(3): p. 194-208.

312. de Jonge, M.E., et al., *Clinical pharmacokinetics of cyclophosphamide.* Clin Pharmacokinet, 2005. **44**(11): p. 1135-64.

313. Hadidi, A.H., C.E. Coulter, and J.R. Idle, *Phenotypically deficient urinary elimination of carboxyphosphamide after cyclophosphamide administration to cancer patients.* Cancer Res, 1988. **48**(18): p. 5167-71.

314. Sadeghi, B., et al., *Expansion and activation kinetics of immune cells during early phase of GVHD in mouse model based on chemotherapy conditioning.* Clin Dev Immunol, 2010. **2010**: p. 142943.

315. Chow, E.J., et al., *Cardiovascular hospitalizations and mortality among recipients of hematopoietic stem cell transplantation.* Ann Intern Med, 2011. **155**(1): p. 21-32.

316. Grotenhuis, H.B. and A. de Roos, *Structure and function of the aorta in inherited and congenital heart disease and the role of MRI.* Heart, 2011. **97**(1): p. 66-74.

317. Rajasekar, P., N. Palanisamy, and C.V. Anuradha, *Increase in nitric oxide and reductions in blood pressure, protein kinase C beta II and oxidative stress by L-carnitine: a study in the fructose-fed hypertensive rat.* Clin Exp Hypertens, 2007. **29**(8): p. 517-30.

318. Gamboa, A., et al., *Excessive nitric oxide function and blood pressure regulation in patients with autonomic failure.* Hypertension, 2008. **51**(6): p. 1531-6.

319. Dai, X. and J.E. Faber, *Endothelial nitric oxide synthase deficiency causes collateral vessel rarefaction and impairs activation of a cell cycle gene network during arteriogenesis.* Circ Res, 2010. **106**(12): p. 1870-81.

320. Boullerne, A.I. and J.A. Benjamins, *Nitric oxide synthase expression and nitric oxide toxicity in oligodendrocytes.* Antioxid Redox Signal, 2006. **8**(5-6): p. 967-80.

321. Bazzoni, G. and E. Dejana, *Endothelial cell-to-cell junctions: molecular organization and role in vascular homeostasis.* Physiol Rev, 2004. **84**(3): p. 869-901.

322. Hashimoto, M., et al., *Age-related changes in aortic sensitivity to noradrenaline and acetylcholine in rats.* Clin Exp Pharmacol Physiol, 1998. **25**(9): p. 676-81.

323. Maclennan, M. and D.W. Ma, *Role of dietary fatty acids in mammary gland development and breast cancer.* Breast Cancer Res, 2010. **12**(5): p. 211.

324. Takahashi, M., et al., *Effect of omega 3 and omega 6 fatty acids on transformation of cultured cells by irradiation and transfection.* Cancer Res, 1992. **52**(1): p. 154-62.

325. Hardman, W.E., *Omega-3 fatty acids to augment cancer therapy.* J Nutr, 2002. **132**(11 Suppl): p. 3508S-3512S.

326. Rose, D.P. and J.M. Connolly, *Omega-3 fatty acids as cancer chemopreventive agents.* Pharmacol Ther, 1999. **83**(3): p. 217-44.

327. Lee, S.A., et al., *DHA and EPA Down-regulate COX-2 Expression through Suppression of NF-kappaB Activity in LPS-treated Human Umbilical Vein Endothelial Cells.* Korean J Physiol Pharmacol, 2009. **13**(4): p. 301-7.

328. Arnold, C., et al., *Cytochrome P450-dependent metabolism of omega-6 and omega-3 long-chain polyunsaturated fatty acids.* Pharmacol Rep, 2010. **62**(3): p. 536-47.

329. Zhao, J., et al., *Selective depletion of CD4+CD25+Foxp3+ regulatory T cells by low-dose cyclophosphamide is explained by reduced intracellular ATP levels.* Cancer Res, 2010. **70**(12): p. 4850-8.
330. Greten, T.F., et al., *Low-dose cyclophosphamide treatment impairs regulatory T cells and unmasks AFP-specific CD4+ T-cell responses in patients with advanced HCC.* J Immunother, 2010. **33**(2): p. 211-8.
331. Haeryfar, S.M., et al., *Regulatory T cells suppress CD8+ T cell responses induced by direct priming and cross-priming and moderate immunodominance disparities.* J Immunol, 2005. **174**(6): p. 3344-51.

9 ABSTRAKT

Hematopoietisk stamcellstransplantation (HSCT) är en botande behandling för ett flertal maligna och icke-maligna sjukdomar. Tyvärr begränsas dess användbarhet av transplantationsrelaterade komplikationer och dödlighet. Komplikationerna kan orsakas av olika faktorer inklusive förbehandlingsrelaterad toxicitet och allogeneicitet. Trots att 50 % av transplantationspatienterna förbehandlas med cellgifter, baseras de flesta transplantationsmodeller på förbehandling med strålning. I försöken i denna avhandling använde vi en musmodell som innebär HSCT efter förbehandling med busulfan-cyklofosfamid (Bu-Cy) för att undersöka de mekanismer och faktorer som kan påverka graft versus host disease (GVHD) och/eller behandlingsrelaterad toxicitet, vilket kan påverka transplantationsresultatet.

Studie I: Denna studie formgavs för att undersöka tidig celldynamik under utvecklandet av GVHD efter allogeneisk HSCT. Vi fann en tidig expansion och aktivering av dendritiska celler (DCs) som nådde sin höjdpunkt på dag +3 post HSCT. T-cellsexpansionen startade senare och nådde sin höjdpunkt på dag +5 post HSCT. Majoriteten av dessa celler var CD8+ celler från donatorn. De inflammatoriska cytokinerna (IL-2, INF-γ och TNF-α) nådde också maximala nivåer på dag +5 efter transplantationen. Resultaten visade på den viktiga roll som donatorns DCs spelar i utvecklingen av GVHD.

Studie II: Vi studerade tidiga histopatologiska förändringar i organen vid olika tidpunkter efter förbehandling samt utvecklingen av GVHD fram till dag +21 efter transplantation. Studien visade att levern och mjälten var de organ som påverkades mest, medan inga morfologiska effekter syntes i pankreas, hjärtat, lungorna eller njurarna efter förbehandlingen. Histopatologiska förändringar såsom vaskulit, inflammation och apoptotiska cellformer i levern, mjälten, pankreas, lungorna och hjärtat observerades under utvecklingen av GVHD; hypocellulär mjälte och extramedullär hematopoies syntes däremot bara hos syngeneiskt transplanterade djur. Inga morfologiska förändringar kunde observeras i njurarna vid endera sortens HSCT. Dessa resultat kan vara till hjälp i våra försök att förstå de mekanismer som ligger till grund för utvecklingen av GVHD.

Studie III: Vi undersökte den toxiska effekt som förbehandling med Bu-Cy kan ha på artärerna. Vi fann att förbehandlingen förstärkte acetylkolinavslappning i de mesenteriska artärerna genom ett ökat uttryck av kväveoxid i endotheliet. I motsats till detta var aortans känslighet för acetylkolin ungefär densamma i den grupp som förbehandlats med Bu-Cy och hos kontrollgruppen. Aortor från cytostatikabehandlade djur visade däremot en högre känslighet för noradrenalin. De djur som behandlats med Bu-Cy hade lägre blodtryck, lägre hematokrit och större skador på endotheliet jämfört med kontrollgruppen. Dessa resultat kan vara användbara för att utveckla profylaktisk behandling för kardiovaskulära komplikationer.

Studie IV: Vi studerade effekten av omega-3 på förbehandling med Bu-Cy och på resultatet av allogeneisk HSCT med fokus på GVHD. Vi använde majsolja och standardfoder till kontrollgrupperna. De möss som fick kost som berikats med omega-3 hade den lägsta överlevnadsfrekvensen och visade tidiga tecken på GVHD. Omega-3 förstärkte effekten hos förbehandlingen genom att förhöja dess myeloablativa egenskaper, förminska uttrycket av CD4+CD25+Fox*P*3+ T celler och begränsa dessas funktion. En lägre förekomst av GVHD och en högre avstötningsfrekvens kunde ses hos kontrollgruppen som åt majsolja. Den högre mortalitetsfrekvensen hos den grupp som åt omega-3 kan förklaras av en förhöjd myeloablativ effekt och ökad förekomst av grav akut GVHD.

Sammantaget kan dessa studier förbättra vår kunskap om GVHD och förbehandlingsrelaterad toxicitet. Behandlingsstrategier kan förbättras, vilket också kan leda till bättre resultat av HSCT.