# Effects on tumor immunity, liver, gut, lung and survival after cutaneous graft-versus-host disease regulated by photochemotherapy
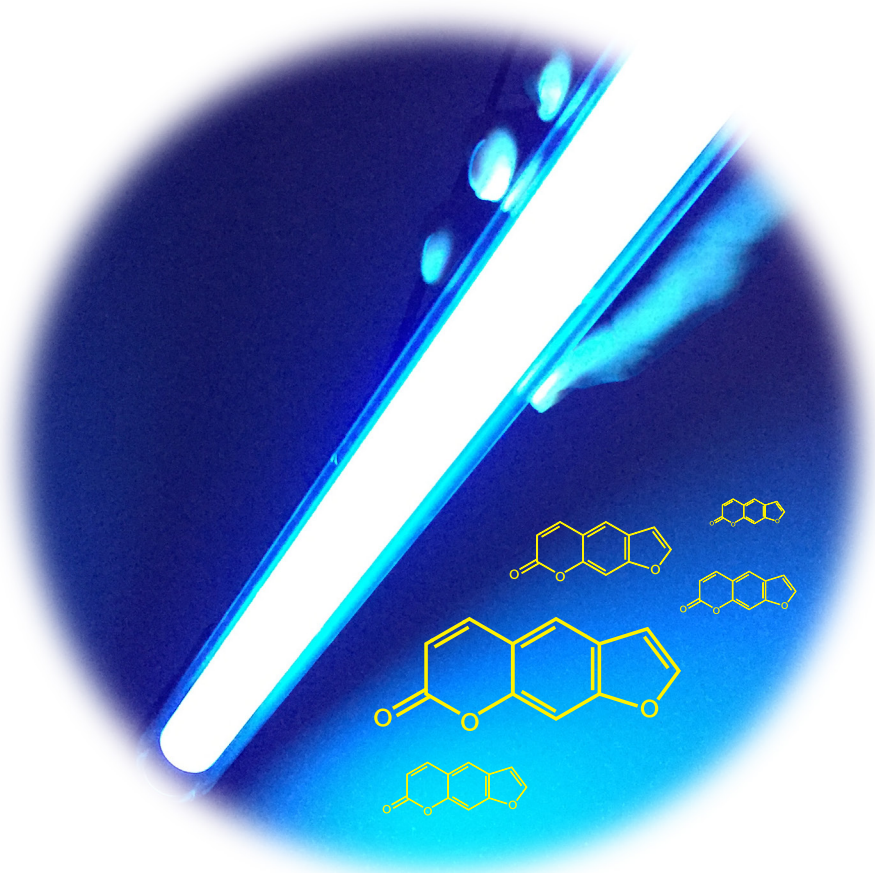
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EFFECTS ON TUMOR IMMUNITY, LIVER, GUT, LUNG, AND SURVIVAL AFTER CUTANEOUS ACUTE GRAFT-VERSUS-HOST DISEASE REGULATED BY PHOTOCHEMOTHERAPY

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To those who survived cancer, among them my *mother*
Medical Doctor Elisabeth Rimeika
and to those who died in cancer,
among them *her* sister, my *aunt*
Medical Doctor & Philosophiae Doctor Danguolé Rimeika
and my former *principal supervisor*,
Medical Doctor & Philosophiae Doctor Brigitta Omazic
but also to all of us who live a life where cancer still is a reality

Ne omittas solum ambulantem

ABSTRACT

The present thesis describes the effect and timing of photochemotherapy in relation to the onset and severity of cutaneous graft-versus-host (GvHD) in relation to the survival and cure of patients. Leukemia is the most common childhood malignancy and an entity of cancer that is increasing in elderly. Allogeneic stem cell transplantation following myeloablative treatment (conditioning) and using methotrexate-based prophylaxis still remains the best choice to cure of high-risk acute and relapsed chronic myeloid leukemia. To improve the long-term disease free survival, we have to redirect the adoptive immunity from GvHD towards graft-versus-leukemia (GvL).

The aim of the present thesis was to test the theory postulating that effects of photochemotherapy are confined to the skin. The theory was tested by whether photochemotherapy had effects on GvHD in liver and gastrointestinal tract (GI), and if it affects the anti-tumor immunity post transplantation. The complete response in GI and liver in addition to the pulmonary mortality after photochemotherapy, which intercalate with DNA, were compared in two groups of patients. One group had received myeloablative ionizing irradiation, which leaves long-lasting DNA-breaks and the second group who received myeloablative chemotherapy including busulfan, which alkylates DNA. The effect on anti-tumor immunity was determined by the effect on the cumulative GvL with regard to whether photochemotherapy was given direct after the onset of cutaneous acute-GvHD or after a week.

The main theoretical background to the present studies was the effect of photochemotherapy on cell mediated immunity evident by an inhibition of the delayed type hypersensitivity of the skin and the ability to induce circulating immunomodulatory regulatory-T cells in human. Four different patient populations were investigated; patients with cutaneous acute-GvHD; patients with cutaneous- and visceral acute-GvHD; patients with acute leukemia; and patients with chronic myeloid leukemia. Complete response, tumor immunity (GvL) and survival were considered as outcomes. Established risk factors including transplantation across the female-male barrier were assessed.

The key result was the possible synergistic-effect between ionizing irradiation and photochemotherapy, which may cure GI GvHD. Furthermore, our results indicated that cutaneous acute-GvHD may enhance the anti-tumor immunity, but also the pulmonary mortality. However, cutaneous complete response to photochemotherapy may decrease the pulmonary mortality after TBI. The studies of patients with acute-leukemia and chronic-myeloid-leukemia implied that after transplantation, the adoptive tumor immunity was modulated by the timing of photochemotherapy with regards to the onset of cutaneous acute-GvHD.

In conclusion, photochemotherapy has effects on disease in internal organs. The overall results suggest that the optimal time to start the photochemotherapy in patients with hematological malignancy is at least after the first week of cutaneous acute-GvHD.

LIST OF SCIENTIFIC PAPERS

I.   Photochemotherapy of cutaneous graft-versus-host disease may reduce concomitant visceral disease.
Feldreich N., Ringdén O., Emtestam L., Omazic B.
Dermatology 2016, Vol. 232 pp. 453-463
(DOI: 10.1159/000447058);
supplementary material www.karger.com/10.1159/000447058

II. Photochemotherapy and graft-verus-leukemia reaction in acute-leukemia: Tumor immunity and survival are dependent on timing of photochemotherapy of the skin.
Feldreich., Ringdén O., Emtestam L.
Dermatology 2017, Vol. 233 pp. 314-324
(DOI: 10.1159/000484138);
supplementary material www.karger.com/10.1159/000484138

III. Severity of graft-versus-host disease confined to the skin predicts pulmonary mortality after irradiation, photochemotherapy possibly alleviates lung injury.
Feldreich N., Ringdén O., Hassan M., Emtestam L. (Manuscript)

IV. Timing of photochemotherapy may preserve graft-versus-leukemia associated with cutaneous GvHD after transplantation for chronic myeloid leukemia
Feldreich N., Ringdén O., Hassan M., Emtestam L. (Manuscript)

CONTENTS

LIST OF ABBREVIATIONS

AAAS	American Association for the Advancement of Science
AHSCT	Allogeneic hematopoietic stem cell transplantation, the graft can be derived from bone-marrow or from peripheral blood stem cells, or from umbilical cord blood.
AIP	Acute interstitial pneumonitis
ANC	Absolute Neutrophil Count
AML	Acute Myeloid Leukemia
ALL	Acute Lymphoblastic Leukemia
APC	Antigen presenting cells
ARDS	Acute respiratory distress syndrome
ATC	American Thoracic Society
ATG	Anti-thymocyte-globuline
ATPase	Adenosintriphosphatase
B-Cells	Benmärgs-cell
BCNU	Bis-chloroethylnitrosourea (carmustine)
B7	Costimulatory protein with two subtypes B7-1 is CD80 and B7-2 is CD86 both binds to CD28
BM	Bone-Marrow
BMT	Allogeneic bone marrow transplantation, a subgroup of AHSCT
BO	Bronchiolitis Obliterans
BOOP	Bronchiolitis Obliterans Pneumonia Syndrome
BOS	Bronchiolitis Obliterans Syndrome
CAST	Center for allogeneic stem cell transplantation
CD(X)	Complex of differentiation (X)
CLA	Cutaneous lymphocyte associated antigen
CLS	Noncardiogenic Capillary Leak Syndrome
COP	Cryptogenic organizing pneumonia
CML	Chronic Myeloid Leukemia
CMV	Cytomegalovirus
CR	Complete response
CR10	Chemokine receptor 10
CT	Computer Tomography
Cy	Cyclophosphamide
DAH	Diffuse Alveolar Hemorrhage
DPTS	Delayed Pulmonary Toxicity Syndrome
DNA	Deoxyribonucleotideacid
EBMTR	European Bone Marrow Transplantation Registry
ELISA	Enzyme-linked immunosorbent assay
EP	Eosinophilic Pneumonia
FasL	Fas Ligand

FoxP3	Forkhead box 3
G1	Gap 1, Cell cycle checkpoint or cell cycle phase
Gut	Gastrointestinal tract
GI	Gastrointestinal tract
G-CSF	Granulocyte Colony Stimulating Factor
Gp120	Envelope glycoprotein 120
GvHD	Graft-versus-host disease
GvL	Graft-versus-leukemia reaction
cGy	Centi-Gray
Gy	Gray
GNC	Guanine nucleotide Cytosine
H-2(x)	Major Histocompatability Antigen (Murine)
H&E	Hemolysin & Eosin
HGF	Hepatocyte Growth Factor
HHV6	Human Herpes Virus 6
HIV	Human Immunodeficiency Virus
HLA	Histocompatability Leukocyte Antigen (Human)
HSV	Herpes simplex virus
HvGR	Host-versus-graft reaction, a rejection of the transplanted graft
HY	Human minor histocompatibility antigen Y, bound to the Y chromosome
IBMTR	International Bone Marrow Transplant Register
ICL	Interstrand cross-link
INH	Isonicotinylhydrazide
IL-(X)	Interleukin-(X)
IPS	Interstitial pneumonia syndrome
KIR	Killer cell immunoglobuline receptor
8-MOP	8-methoxysalen
MHC	Major Histocompatability Complex
MMF	Mycophenolate-mofetil
MRD	Minimal residual hematological malignant disease
MRSA	Multi Resistant Staphylococcus Aureus Type A
mTOR	Mammalian target of rapamycin
MTX	Methotrexate
MUD	Matched unrelated donor
NK-cell	Natural Killer Cell
OB	Obliterative Bronchiolitis
OP	Organizing Pneumonia
PAH	Pulmonary Alveolar Hypertension
PAP	Pulmonary alveolar proteinosis
PBSC	Peripheral Blood Stem Cell
PCT	Pulmonary Cytolitic Trombi
PERDS	Peri-engraftment respiratory distress syndrome

PTLD	Post-transplant lymphoproliferative disease
PTE	Pulmonary Tromboembolus
PRICHE	Predicted Indirectly ReCognizable Epitopes
PVOD	Pulmonary Veno-occlusive syndrome
Rad	Radiation absorbed dose (0.01 Gy)
RIC	Reduced intensity conditioning
RSV	Respiratory Syncytial Virus
STROBE	Strengthen the report of observational studies in epidemiology
SKALP	Skin-derived antileukoproteinase
SGOT	Serum glutamic-oxalacetic transaminase
SAA	Severe aplastic anemia
TBI	Total Body Irradiation
TBSA	Total Body Surface Area
T-cell	Thymocyte (immune cell that needs thymus to mature)
TCR	T-cell receptor
TGF-beta 1	Tumor growth factor beta 1
Th1	T-cells of helper type 1
Th2	T-cells of helper type 2
Th17	T-cells of helper type 17
TLR-4	Toll-like receptor 4
Tnf	Tumor necrosis factor
TRALI	Transfusion related acute lung injury
UVA	Ultraviolet light type A
UVB	Ultraviolet light type B
VOD	Veno-occlusive disease
VZV	Varicella Zoster Virus

1 INTRODUCTION TO THE FIELD

1.1   WHY SEARCH FOR THE HOLY GRAIL OF TRANSPLANTION?

To cure Leukemia has been a goal since the Greeks named the disease "White blood" - Leukemia. The noble-prize winning achievement of a stable protocol of allogenic bone-marrow-transplantation by the Noble Laurate Thomas E.D. and the Seattle Group in the seventies gave transplantation firm ground (E. Thomas et al., 1975). However, today, four decades thereafter, the separation Graft-versus-host disease (GvHD) and Graft-versus-leukemia (GvL) reactions, (anti-tumor immunity), remain as key issues to be solved. To find a well-defined strategy that separates these two events are widely acknowledged as the search for the "Holy Grail" of allogeneic stem cell transplantation.

The data supporting transplantation has improved the last decade. In 2006, Appelbaum and Pearce suggested transplantation as the premium choice in the first untreated relapse of acute myeloid leukemia (AML), and, recent data suggests that the chance for cure is better than what could be seen in the classic Figure from Appelbaum and Pearce who set the chance for cure to 25% when BMT is utilized direct as a curative treatment for relapsed AML (Figure 1). The patients with the best survival in this high risk disease were the patients with acute-GvHD of grade II or more, in whom this severe disease must be associated with GvL (Appelbaum & Pearce, 2006; Gyurkocza, Lazarus, & Giralt, 2017).

The number of transplantations can today be estimated to exceed 500.000 cases worldwide (Gratwohl et al., 2015). Pediatric patients who survive cancer have higher chances to live a long and healthy life (Asdahl et al., 2017). Retrospective data have pointed out that relapse is the major cause of treatment failure after transplantation. Studies have shown that myeloablative conditioning including higher dose of total-body irradiation is associated with an increased tumor eradication at the stake of a higher toxicity of liver, gut, and lung and an increased rate of severe acute-GvHD. Although acute-GvHD grade II or more is associated with a better relapse free survival in high-risk AML, GvHD is a major cause of death after transplantation (Gyurkocza et al., 2017; Nassereddine, Rafei, Elbahesh, & Tabbara, 2017). Allogeneic stem cell transplantation of acute-lymphoblastic leukemia (ALL) in first complete remission GvHD induces the most potent anti-tumor immunity through the induction of GvL (Inbar, Rowe, & Horowitz, 2017). Together, it is conceivable that acute-GvHD and the GvL are at least partly correlated/associated and/or connected. Therefore, it is a matter of uttermost importance for the treatment of leukemia to separate acute-GvHD and GVL i.e. to find the Holy-Grail of transplantation.

Figure 1. The classic Figure from Appelbaum F.R. et al. who set the chance for cure to 25% in a theoretical calculation when transplantation is utilized direct as a curative treatment for first untreated relapse of acute-myeloid leukemia
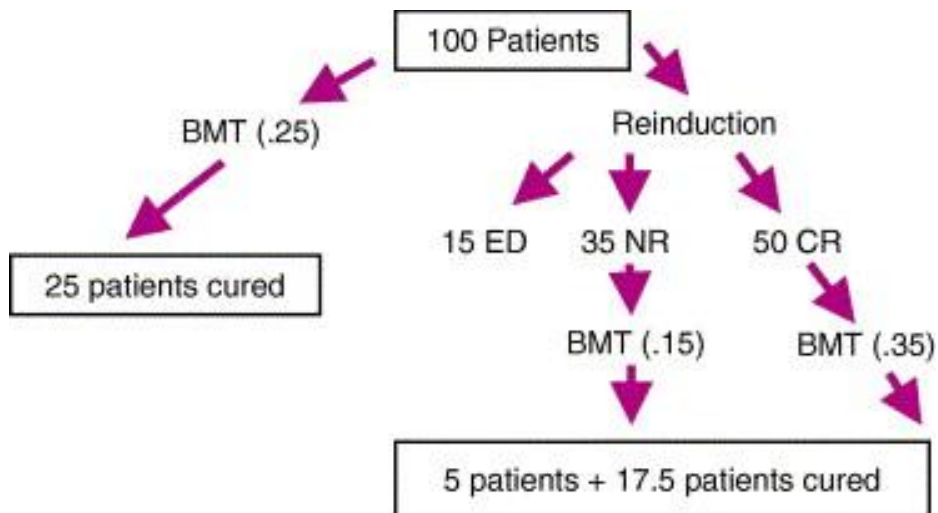
1.2 THE SYNGENEIC TRANSPLANTATIONS

The syngeneic kidney transplantation was a breakthrough in the transplantation field (HarrisonM.J.P. & MurrayJ.H., 1955). Following in the footsteps of the successful syngeneic kidney transplantation, and in an attempt to find a sanctuary from the "secondary disease" (GvHD – and the more common Host-versus-graft disease (HvGD), when the immune defense of the patient rejects the graft); Thomas E.D. and colleagues proceeded on the path to cure acute-leukemia when they offered two children with leukemia and their families a possible cure with lethal irradiation aimed to destroy the leukemia and bone-marrow transplantation from the patients respective twin. Thomas and colleagues at Mary Imogene Bassett transplanted the first human with isologous marrow, referred from the Johns Hopkins Hospital in Baltimore, MD. This patient and the following patient referred from the Eglin Air Force Base Hospital, FL. survived their transplantation, but the leukemia relapsed (E. D. Thomas, Lochte, Cannon, Sahler, & Ferrebee, 1959). The conclusions were that there was additional means needed to cure leukemia beyond irradiation and that the isologous marrow did not prevent the leukemia from relapse. The authors discussed two sources of increased anti-leukemic treatment; first the addition of chemotherapy to the lethal irradiation to reduce the burden of leukemia further before rescuing the patient with a transplantation of bone marrow and then the potential anti-tumor effect seen in animal models of homologous transplantation. This effect had been discussed during the 1950's, when an effect of the graft against the tumor was noted in experimental animal models, even more so when the number of leukemic cells in the recipient was small (Barnes, Corp, Loutit, & Neal, 1956; E. D. Thomas et al., 1959).

1.3 SKIN GRAFT HISTOCOMPATABILITY AND GVL IN HUMAN

Cutaneous immunization by skin grafts determined the tissue-antigen or the histocompatability between the donors and the recipient in the first transplantation where GvLwas experienced in human. In 1963, Mathé G. et al. searched for donors for a recipient in need of cure for acute-lymphoblastic-leukemia who had several relapses following chemotherapy treatments during several years. The Paris group determined histocompatibility by first transplanting a skin graft from the patient to the six donors who were in fact, the father, mother, sister, and the three brothers of the patient. The donors immunized and rejected the skin-graft in between $12 - 22$ days. Thereafter, the donors received a skin-graft from all of the potential donors with the hypothesis, that the graft that was rejected the fastest, would be the one closest to the recipient since immunization against the recipient already was in place in that donor. One thing was certain after this procedure, that the donors where immunized against antigens in the patient's skin.

The patient received 800 rads from two opposing Co^{60} and was successfully transfused with 330 mL of bone-marrow from each of the six donors. The transplantation of the patient was a five-hour transfusion procedure, during which he received a total of 2000 mL of blood with 5.8×10^{10} mononucleated cells. During the transfusion, 1700 mL blood was withdrawn from

the patient. He was kept in aseptic conditions in a room with ultraviolet light from the 23rd April – 4th July. During this time period, the patient experienced a severe GvHD with cutaneous, liver and gastrointestinal involvement that were treated with steroids. Furthermore, he had suspected lung-infiltrates that were successfully treated with isoniazid and isonicotinylhydrazide (INH) (900 mg/daily) and streptomycin (1 gram/day) in a similar treatment protocol as tuberculosis.

On June 6th that corresponds to day 44 after SCT, the patient again received skin allograft from all donors. One of them remained perfectly intact for seven months; these others remained for 15 – 60 days. The brother who had the skin graft that remained intact was chosen for donor-lymphocyte-infusions, and during October the patient received weekly infusions of 29 - 45 x 10^6 nucleated cells, which revoked the graft-versus-host disease. The patient survived 12 months without signs of recurrent leukemia (Mathe et al., 1965).

1.4   PHARMACOLOGICAL IMMUNOSUPPRESSION

1.4.1   The barrier of graft-versus-host disease

The GvHD and the HvGD remains a barrier within transplantation. However, patients who had received kidney transplants before the syngeneic transplants did not experience the immense type of rejection seen in the animal models, something the surgeons believed was mediated by immunosuppression, since the transplants were cadaver kidneys from patients with congestive heart failure and the recipients were in end-stage chronic uremia. From the moment this thought was evoked, and on, throughout the transplant field, the search for suitable immunosuppression to allow optimal post-transplant graft and patient survival, has been ongoing. In a canine model, especially methotrexate was found to be a promising prophylaxis against GvHD after transplantation. Thomas E.D. expressed his astonishment when he concluded that the anti-metabolite methotrexate that suppresses the bone marrow does not suppress the engraftment of bone marrow in dogs when the treatment started three – five days after transplantation (E. D. Thomas, Collins, Herman, & Ferrebee, 1962).

1.4.2   The final candidates

The development was not easy and a wide range of pharmaceutics was tried in several small and large animal models. After studies on several pharmaceutics in rodents, primates and canines, antithymocyte globuline (ATG), cyclophosphamide (Cy) and methotrexate (MTX) were identified as potential candidates for suppression of GvHD, and among these, methotrexate turned out to be an outstanding GvHD prophylaxis in the HLA-matched setting (Storb, Epstein, Graham, & Thomas, 1970; E. D. Thomas et al., 1971). The construction of a protocol with iterative doses of methotrexate to protect from the development of acute-GvHD opened up the possibilities to start transplants in human with siblings and unrelated matched donors - with the aim to cure hematological malignancies including leukemia.

1.5 THE CURE OF LEUKEMIA

The stable Kaplan-Meier curve presented by the Seattle group in 1975 after addition of cyclophosphamide in to TBI in the conditioning regimen and methotrexate as a GvHD-prophylaxis made it possible to start to identify the clinical symptoms and signs necessary for a graft-versus-leukemia effect (E. Thomas et al., 1975). During the improvement of their protocol and their care, the survival increased each year towards a cure of Leukemia (Figure 2). Research has subsequently tried to identify the time for the development of GvL, and if possible to separate this phenomenon from GvHD to offer a future cure for cancer with minimal adverse effects.

1.5.1 Tumor immunity and clinical GvHD

Initially, both acute-GvHD and chronic-GvHD were reported to be associated with GvL (Weiden et al., 1979; Weiden et al., 1981). In 1990, the International Bone Marrow Transplantation Register (IBMTR) could gather 439 patients transplanted for ALL, 1046 patients transplanted for AML, and 769 patients transplanted for CML. The study had three key results. First, it was confirmed that GvL was mediated by T-cells in human i.e., the T-cell depleted recipients had a higher relapse rate. Second, GvL can result from major mistocompatibility complex (MHC) differences between the donor and the recipient as the syngeneic twins had the highest relapse rate. Third, the GVL effect was associated with GvHD (Horowitz et al., 1990). The major shortcoming of this study was that no landmark was used in the analysis which overrated the importance of chronic GvHD. The chronic-GvHD is an event that comes when the hazard-ratio for treatment-failure, including early relapse, already has decreased. Thus, a landmark needs to be set at a time-point at least 100 days after transplantation so that not all censored events e.g. treatment failure and relapse before the time-window when chronic-GvHD starts, not falsely indicate an increased modulation of the adoptive immunity during chronic-GvHD. This Landmark can be set at different time-points, one year has been used and six months have been used, dependent on what the outcome of the **Study I**s (Ringden et al., 2017; D. J. Weisdorf et al., 2009). In an attempt to determine if there can be a GvL effect in the absence of GvHD in patients transplanted for AML and ALL, 5200 autologous transplantations, where reinfusion of leukemic cells is a possibility, with 44% TBI as the conditioning regimen, were compared to1039 transplantations, without any GvHD and with 68% TBI in the conditioning. The two year relapse incidence was significantly lower in the allograft group in patients 27% ±2 vs. 43% ±1 in autografts. This study cannot exclude a GVL effect in the absence of clinical acute-GvHD and point towards the possibility to separate GvHD and GVL (Ringden et al., 2000).

Figure 2. Survival curves in patients (n=70) transplanted by a Human Leukocyte Antigen matched graft for acute leukemia during the development of the Seattle protocol.
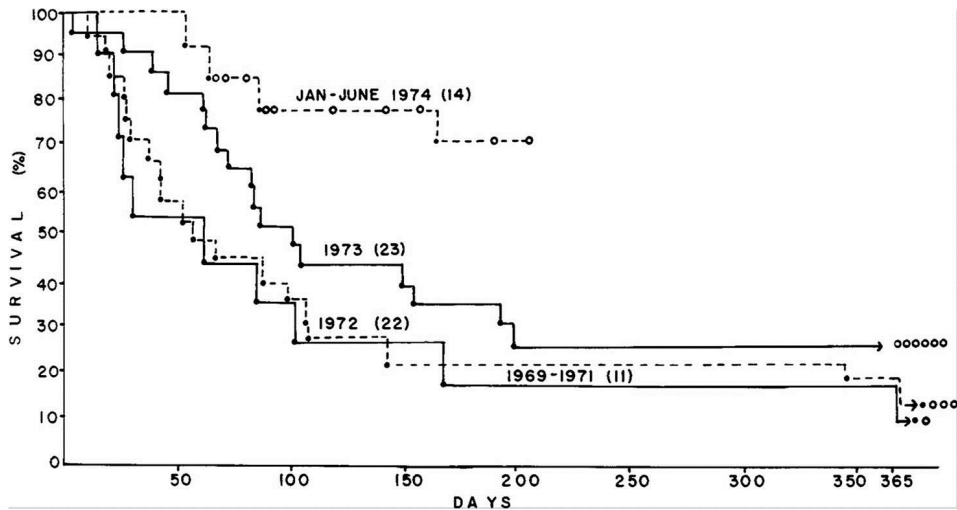
Open circles denote survivors.

1.5.2 Cutaneous acute-GvHD and anti-tumor immunity

Cutaneous acute-GvHD was found to have a good GvL effect and was associated with chronic leukemia in the first chronic phase when the European group for Blood and Marrow Transplantation Registry (EBMTR) presented a study of more than1000 patients with a five year follow-up. The patients were transplanted between 1979 and 1990. The study postulated an anti-leukemic effect of cutaneous acute-GvHD in chronic leukemia (Gratwohl et al., 1995). The important GvL effect caused by cutaneous acute-GvHD was confirmed in a study of 2122 patients with acute leukemia in first remission and 780 patients with chronic myeloid leukemia in first remission that received their first transplant from an HLA-identical donor. In the study, it was found that grade I acute GvHD, (acute GvHD covering 50% or less of the TBSA), was associated with a superior leukemia free survival (Ringden et al., 1996).

1.5.3 The best donor

In a re-classification of the degree of HLA-match from the study presented in 2002 by Weisdorf D.J. et al. of 2464 patients who received their transplant from unrelated donor, and 450-HLA identical matched sibling transplantations, the findings of the previous study could be confirmed, HLA-mismatch do not increase the GvL effect in chronic leukemia significantly, the best matched donor is the best donor (D. J. Weisdorf et al., 2002; D. J. Weisdorf et al., 2009).

1.5.4 Methotrexate and graft versus leukemia (GvL)

Methotrexate confirmed the position in the graft-versus-host prophylaxis in 2012 when the IBMTR gathered 4022 patients who were transplanted in the period1997-2005 and received myeloablative conditioning without ex vivo T-cell depletion. No significant difference in relapse rate was observed between patients with or without GvHD. However, in this study compared to the previous study of Horowitz et al. combined calcineurin and methotrexate or mycophenolate mofetil (MMF) where more common, and a graft-versus-host prophylaxis with less GvL promoting effect than methotrexate alone and cyclosporine and methotrexate together may have had an impact on the results. Consequently, Chronic-GvHD and acute-GvHD followed by chronic-GvHD had a decreased leukemia free 5 year survival (70%)/(64%) and a decreased overall survival (73%)/(65%) (D. Weisdorf et al., 2012).

1.6 IMMUNOLOGY OF CUTANEOUS ACUTE-GVHD

1.6.1 Unrecognizable T-cells

GvHD was first encountered after cardiac surgery and then termed postoperative erythrodermia (POE). In a fatal adverse event, six out of twelve recipients of sibling blood transfusion died in a transfusion associated GvHD (ta-GvHD), that started with a postoperative erythrodermia, i.e. a cutaneous acute-GvHD and spread to the liver and the gastrointestinal channel (Vriesendorp & Heidt, 2016). The risk for GvHD is increased in immunosuppressed patients, who cannot identify or defend themselves against the exogenous T-cells. Neonates are especially sensitive to ta-GvHD (Funkhouser et al., 1991). In blood transfusions of unirradiated blood between close relatives, there is a risk that the blood donor is homozygotous for a HLA-antigen where the recipient is heterozygotous. The result is that the recipient cannot identify and reject the donor T-cells in a host-versus-graft reaction; the recipient will instead die in a graft-versus-host reaction unleashed by the unrecognizable T-cells without any hope of engraftment, - a blood transfusion does not contain enough stem-cells. A donor homozygotous for HLA to a recipient heterozygotous for the same HLA, which can occur within the same family, is also a risk in solid organ transplantation, especially when recipients receive parts of a liver from a living donor within their own family. After transplantation, GvHD not seldom affects the skin, the whole gastrointestinal tract and the liver (Figure 3.), and may develop into multiorgan failure, why GvHD prophylaxis is used when donor T-cells encounter the antigens of the conditioned recipient after a transplantation (Ferrara, Levine, Reddy, & Holler, 2009; Rappeport, 1990; Vriesendorp & Heidt, 2016); (Zeiser et al., 2006).

Figure 3. GvHD mainly affect the skin, the whole gastrointestinal tract and the liver
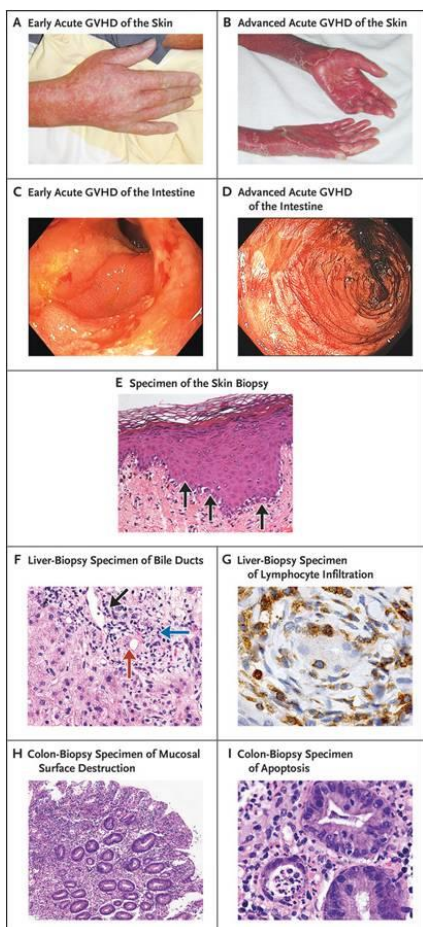
1.6.2 Tissue damage

During the development of GvHD, the first step is an increased antigen presentation through an upregulation of human leukocyte antigens (HLA) on the cell surfaces of the patient. The increased density of antigens is triggered by the inflammation caused by the tissue damage inflicted by the conditioning regimen. The conditioning regimen increases the release of cytokines, the expression of adhesion molecules and HLA, as well as the damaged cells and cell components, in the skin, the liver, the lung and the gut. Furthermore, the tissues that are involved in the GvHD after transplantation may have been damaged by the previous medical history of the patient, i.e. by the underlying malignancy and the previous treatment thereof, and from the infections that are common in patients with hematological disease. These chronic- and acute injuries provide targets for the GvHD. The skin is more commonly affected by acute-GvHD compared to liver or gut (Antin & Ferrara, 1992; Ferrara et al., 2009). The acute tissue damage can heal, and the inflammation may resolve, but several factors in the allogeneic setting change the immunomodulatory signals in the allogeneic setting, such as IL-10 polymorphism and vitamin D polymorphism, and increase or decrease the likelihood for GvHD (Lin et al., 2003; Middleton et al., 2002).

1.6.3 T-cell activation and proliferation

In addition to the antigen upregulation of step one, donor T-cells from the graft have to meet the antigen and be stimulated to proliferate. After transplantation, the grafted T-cells are recruited to the tissue by the chemokines and cytokines that are released from the tissue secondary to the damage caused by the conditioning regimen. The damaged tissue presents adhesion factors which enables the T-cells to enter the skin. By tissue inflammation, the T-cells and the professional antigen presented cells (APC) are activated and drained to the local lymph nodes where they have a higher probability to meet. The secondary step in the development of acute-GvHD, the clonal expansion of donor naïve T-cells, does not necessary occur, but if the factors favoring a continued immune response are present, GvHD pathogenesis may progess. One such factor is the number of possible immunological synapses where the antigens are presented through HLA to the memory T-cells in a costimulatory environment (Curtsinger, Johnson, & Mescher, 2003; Isakov & Altman, 2012). HLA-class I is presented on almost all cells in the skin, but in an inflammatory situation HLA-class II is upregulated in the tissue too and presented on cells outside the hematopoietic lineage e.g. (B-cells, macrophages and dendritic cells). In contact with the HLA-molecule which presents an antigen epitope, the T-cell will recognize if it is foreign or not. In addition to the immunological synapse, the "second signal" decides if the T-cell will be activated, partially activated or go into anergy (shut down of response). In close proximity to the T-cell-receptor (TCR) and the HLA is costimulatory molecules and ligands such as CD28 on the T-cell and B7 on the professional antigen presenting cell. If both the stimulation through HLA:TCR and the co-stimulation, i.e. B7:CD28, is in place, the T-cell starts to accumulate and secrete IL-2 which binds to the IL-2 receptor in an autocrine mode and activates the T-cell (Guinan E. 1995). The activated donor T-cells start to proliferate in the local lymph node. The release of IL-2 can also act to stimulate the presence of regulatory T-cells which can

inhibit the conventional T-cell response, this has been suggested to be an adverse effect of the calcineurin inhibitors (Zeiser et al., 2006). For CD8+ T-cells to proliferate, they need prolonged contact with the antigen in the immunological synapse, they need costimulation, but they also need a third cytokine signal. This signal can be either IL-12 which is produced by macrophages and dendritic cells secondary to the autocrine interferon-gamma from the activated donor T-cells. The third signal can also be IL-7 together with IL-18. Both IL-7 and IL-18 has been associated with the development of acute-GvHD (Dean et al., 2008; Shaiegan, Iravani, Babaee, & Ghavamzadeh, 2006). The combination is shown to induce proliferation of CD8+ T-cells together with costimulation and stimulation of the T-cell receptor (Walsh M.C. 2014). The possibility for activation of donor memory T-cells is increased with a more intense conditioning regimen, an increased number of APCs in the patient, an increased number of infused donor cells or a concomitant infection (Antin & Ferrara, 1992). The regulatory T-cells have been suggested to leave the skin in number after an immune response, where after they pass the local lymph node and recirculate to the skin to dampen the immunity (Tomura et al., 2010).

1.6.3.1 Clinical application

T-cell depletion of the graft (ex-vivo) or anti-thymocyte-globulin given during the conditioning regimen, (in-vivo-T-cell depletion), which decreases the number of T-cells in the graft was developed to decrease the second step in GvHD (Busca & Aversa, 2017; Marmont et al., 1991).

1.6.4 Systemic inflammation

Finally, the proinflammatory IL-2 activates the mononuclear cells graft to start to produce inflammatory cytokines, e.g., IL-1, tnf-alpha, and interferon-gamma. The T-cells continue to develop and traffic to the tissue where they induce cytotoxic cell death through Fas-ligand, Granzymes and Perforin. This lead to an increased level of tissue destruction and T-cell proliferation and lead to the severe cytokine storm of acute-GvHD (Antin & Ferrara, 1992; Ferrara et al., 2009).

GvHD is a paradigm of systemic inflammation and in its more severe form threatens the life of the patient that enters multi-organ failure with symptoms coupled to the dysepitheliation caused by the tissue infiltrated cytotoxic T-cells. The organ failure becomes evident with massive diarrhea, icteric liver failure secondary to destruction of the biliary ducts and skin desquamation with increased fluid loss. The dysregulation of the immune-defense, the tissue-damage and the immunosuppressive treatment all increase the risk for infections. The mortality is high. To diagnose GvHD early and correct, several biomarkers such as Elafin, interleukein-8, TNF-receptor 1, IL-2 receptor alpha, and Hepatocytic Growth Factor have been identified (Paczesny et al., 2010).

Cutaneous acute-GvHD is indicated by an increase of Elafin/SKALP which is produced by the Keratinocytes in response to inflammation (Figure 4). Elafin has also been found to be an early marker in acute-respiratory distress syndrome (Paczesny et al., 2010; Wang et al.,

2017). The Cytokines released during cutaneous acute-GvHD further include interleukin 8 (Chang T.P. 2015), a proinflammatory cytokine, that is inhibited in response to TGF-beta 1. TGF-beta 1, in turn, is produced by CD4+ regulatory T-cells which are distinguished by the IL-2 receptor CD25. Tissues and cells that are activated or inflamed shed cytokine receptors, and some of these receptors have become markers of acute-GvHD e.g. TNF-receptor 1 and IL-2-receptor alpha. Shedding is suggested to protect against severe innate immune hyper reactivity (Xanthoulea et al., 2004). When the inflammation including the cell mediated immunity starts to damage the tissue, cell size in tissues decreases, which also increases the expression of factors such as Hepatocytic Growth Factor (HGF) which is released secondary to a decrease of the cell size of fibroblasts, a cell in the skin (Qin, Worthen, & Quan, 2017). The cytokines released also regulate the T-cell response, whereas IL-2 alpha stimulates a regulatory-T cell response, which balances the response to local antigens in the skin, and the contact between conventional T-cells and regulatory T-cells in the lymph node (Billroth-MacLurg, Ford, Rosenberg, Miller, & Fowell, 2016). A dysregulation of the regulatory T-cells are suggested to be present in acute-GvHD (Rieger et al., 2006), which may explain the elevated IL-8 levels. In this connection, therapies that increase the amount of regulatory-T-cells in patients with GvHD are warranted.

Figure 4. Cutaneous acute-GvHD is indicated by an increase of Elafin / SKALP which is produced by the Keratinocytes in response to inflammation
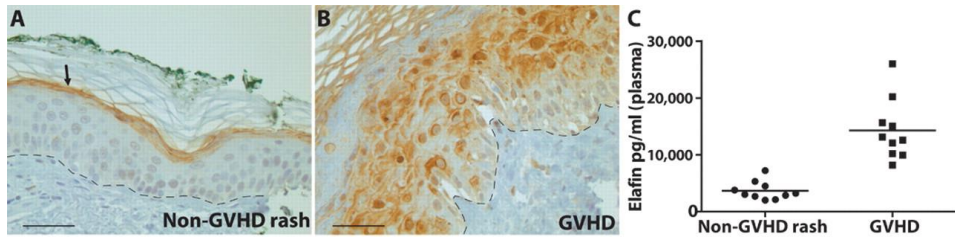
From Sophie Paczesny, Thomas M. Braun, John E. Levine, Jason Hogan, Jeffrey Crawford, Bryan Coffing, Stephen Olsen, Sung W. Choi, Hong Wang, Vitor Faca, Sharon Pitteri, Qing Zhang, Alice Chin, Carrie Kitko, Shin Mineishi, Gregory Yanik, Edward Peres, David Hanauer, Ying Wang, Pavan Reddy, Samir Hanash, James L. M. Ferrara, 2010: Elafin Is a Biomarker of Graft-Versus-Host Disease of the Skin; Science Translational Medicine, Vol. 2 pp. 1-13, Reprinted with permission from AAAS.

1.6.5 Clinical Cutaneous acute-GvHD from Th1 paradigm to Th17 presence

T-helper cells are CD4+ cells that direct the immune response by the secretion of cytokines and by cell-to-cell contact were primarily categorized to two cell phenotypes. The Th1 CD4+ cells are distinguished by the production on IL2 and IFN-gamma and the Th2 by IL-4 and Il-10. For twenty years Th1 and Th2 were the only two known types of T-helper cells, where CD4+Th1 cells promoted cell-mediated-immunity and CD4+Th2 cells supported a development of B-Cells, antibodies and the defense against parasites. Today, the CD4+ subpopulations have expanded and in addition toTh1 and Th2 cell types, the CD4+CD25+ regulatory T-cells and CD4+Th17 cells have been identified by the production of interleukin-17 and interkeukin-22, which are is important for the immunity in the parenchyma. The increased knowledge has triggered a re-evaluation of the Th1 paradigm that dominated cutaneous acute-GvHD. The conditioning regimen does impact the development of CD4+ cells as well as the CD8+ cells why it is important to relate the clinical research of the cytokine pattern and the T-helper balance to the clinical protocol used. Preferably, the T-helper response and the cytotoxic T-cell response should be studied during cutaneous acute-GvHD after different conditioning regimen whereafter the results should be compared between the clinical protocols e.g. the response after RIC compared to the response after myeloablative regimen, this is not the case in the reports found.

The cytokine profile and the cytotoxic involvement has been evaluated in three studies with predominantly myeloablative conditioning. In a study of HLA-identical sibling transplants (n=22), given after a Cyclophosphamide and a TBI based myeloablative conditioning with cyclosporine and methotrexate as GvHD prophylaxis, IFN-gamma, IL-1, and IL-6 were expressed by the circulating lymphocytes. In the cutaneous acute GvHD, the skin was infiltrated by lymphocytes, which expressed low levels of genes encoding for IL-1 and IL-6, but neither a Th1 (IL-2/IFN-gamma) nor a Th2 (IL-4/IL-10) cytokine phenotype was present in this organ (Carayol et al., 1997). In unrelated donors, TNF-alpha, IFN-gamma, and IL-10 were elevated in serum in patients with acute GvHD II-IV compared to patients with mild cutaneous acute GvHD. The theory that cytotoxic T-cell killing is a major mediator of acute-GvHD is strengthened by that the Fas Ligand was increased on the CD8+ cells in patients with acute GvHD in the related setting and in the serum of unrelated recipient (Lee et al., 1997; Remberger, Jaksch, Uzunel, & Mattsson, 2003). However, the FasL increased also in patients who converted to donor-chimerism after unrelated transplantation (Remberger et al., 2003), why Fas L may be a major pathway to eradicate the remaining recipient cells.

The possible Th17 involvement in cutaneous acute-GvHD was evaluated in a study with an even mix of related (18/36) and unrelated transplants (18/36), the majority with an myeloablative conditioning regimen, cyclophosphamide and busulfan in (16/36) and cyclophosphamide and TBI in (9/36), with (35/36) on methotrexate and cyclosporine GvHD prophylaxis. The prospective nature of the study made it possible to sample skin biopsies before prednisolone was started. Immunohistochemistry reviled low levels on IL-17 and IL-22 producing CD4+Th17 cells in the skin. Instead, a three weeks cell cultures on 3D matrix after skin punch biopsies showed a CD4+ IFN-gamma positive profile (Broady et al., 2010).

On the contrary, there are two studies with mixed myeloablative and reduced-intensity conditioning that report an IL-22 phenotype in acute-GvHD (Bruggen et al., 2014; Reinhardt et al., 2014). In the latter, a study designed to evaluate a phagocyte dependent induction of the Th17 response in acute-GvHD, the cutaneous and gastrointestinal GvHD was followed and sampled during fourteen days. In the study of 47 patients; 35/37 children and 12/47 adults, a majority with related matched or unrelated donors, and a minority with haploidentical and mismatched unrelated donors - with a reduced intensity conditioning (Bacigalupo, 2004); the CD4+ peripheral blood monocytes released IL17 and IFN-gamma upon toll-like-receptor type-4 (TLR4) stimulation by S100 which is produced by phagocytes. This finding suggested a link between the development of Th17 response, bacterial infection and the emergence of GvHD (Reinhardt et al., 2014).

Viral proteins have also been used to modulate the CD4+ population. In a murine in-vivo model the human immunodeficiency virus (HIV) protein gp-120 was used to stimulate the potency of regulatory T-cells which attenuteted GvHD (Schloder, Berges, Tuettenberg, & Jonuleit, 2017).

1.6.6 The time dependent changes of cell populations and cytokines in cutaneous acute-GVHD

It is important to know the time pattern in the immunological changes in the skin during cutaneous acute-GvHD in order to be able to choose the right clinical response to the disease. The normal healthy epidermis constitutes a distinct T-cell compartment with a predominant memory effector T-cell phenotype (Spetz, Strominger, & Groh-Spies, 1996). After transplantation, the immune reconstitution of the lesion and non-lesion skin in patients have been followed in sex-mismatched recipients utilizing fluorescent in situ hybridization (FISH) to discriminate between donors and recipients - while immunohistochemistry was used to identify the changes in the cell population. Already at day fourteen after a myeloablative TBI transplant, the intradermal CD1a+ Langerhans cells were depleted. Donor derived cells approached the dermis as early as day 23+. Lesions of acute GvHD was mediated primarily by CD8+ T-cells, and in non-lesion skin, the CD1a+ seem do dominate in this minor-HY mismatched setting (Stewart R.L. 2017).

The acute-GVHD incidence is dependent on the degree of HLA-A, -B, (-C) and HLA-DRB1 match between the donor and the recipient. The important HLA-DRB1 is expressed on the APCs, and while the HLA-class II molecules are constitutively present on the hematopoietic cells (monocytes, dendritic cells, and B-cells) they can be upregulated on a range of tissues after inflammation. The Class I HLA-A, -B, and -C function as the presentation module of antigens for the nucleated cells. In acute cutaneous GvHD, skin infiltrating T-cells seem to accumulate with an increase in recipient-specific epitopes present on donor-T-cells. In clinical cutaneous acute-GvHD, a multitude of epitopes from an antigen mismatch elicit the concomitant response from CD4+ and CD8+ T-cells (Thus et al., 2015). Immunohistochemistry revealed that CD4+ cells appeared more frequently in the dermis and CD8+ cells in the epidermis. Furthermore, a mismatch in the class II HLA and HY-

presenting infiltrate was associated with a more pronounced CD4+ infiltration, while a class I HLA and HY-presenting infiltrate was associated with CD8+ infiltration. This was revealed by Predicted Indirectly ReCognizable Epitopes (PRICHE), which is a term for peptides presented at HLA that differs from the donor-self derived epitope (Thus et al., 2015). Weeks after the start of clinical cutaneous acute-GvHD, in a group of 15 patients, the majority on methylprednisolone treatment after an acute-GvHD of the skin, (11/15) with a TBI based conditioning, compared to (2/8) in the control group with Bu-Cy in their conditioning, (p=0.039, Fishers two-tailed test), the CD8+ population in the skin did not have any chemokine ligands on them. The chemokine receptors were more common on the CD4+ population (CD4+ CR10+ and CLA+ cells) (Faaij et al., 2006). This suggest later arrival or a more transient presence of the different CD4+ cells in the dermis where they can enter and release their cytokines to steer the CD8+ cells in the epidermis.

In a prospective study where six out of seven patients with myeloablative conditioning developed cutaneous acute-GvHD, acute-GvHD initiated release of interferon-gamma and interleukin-10 into the bloodstream. The patients have received grafts from HLA-identical donors and received a methotrexate and cyclosporine GvHD prophylaxis. However, interleukin-12 or interleukin-4 could not be detected at any level of disease which may depend on the detection threshold (ELISA after -80 oC freezing &thawing). Both Th1 (CD4+Interferon-gamma+ cells) and Th2 (CD4+Interleukin-4+ cell) activity was increased. Specifically, interleukin 10 –levels increased with hepatic disease severity (Yeh S.P 2012). This suggest that different organ involvement can be coupled to different cytokine prophiles, which may switch during the time-course of the acute-GvHD when the organ involvement changes.

1.6.7 The antigen presenting cells and the regulation of the immune response

Knockout animal models suggest that highly efficient antigen presenting cells (APCs); the dendritic cells from the recipient; initiates the acute-GvHD through cell to cell contacts with the T-cells in the graft. The effect may however not be totally dominant and may possibly be overruled by the total amount of antigen and receptor contacts, and thus may be revoked e.g., by a large number of T-cells in the graft (Shlomchik et al., 1999). In human, several types of dendritic cell have been identified. In the skin, the Langerhans cell population which is a part of the CD1a+ population, and the CD14+ APCs are dominated populations (Angel et al., 2007). In human, the dendritic cell populations, including the Langerhans cell population express the CD1a, CD1b and CD1c lipid antigen presenting molecules which may be associated to the HLA-1 and T-cell interplay. Interestingly, while the CD1c is more efficient in cross presentation, the CD1a may be involved in the induction of tolerance, since the loss of CD1a cell surface expression is not associated with conventional immune defects (Kashem, Haniffa, & Kaplan, 2017). The conditioning regimen damages the skin and heavily damages the cells within the lymphocytoid cell populations, of which a large part does not survive but enters necrosis and/or apoptosis. That certainly occurs due to the soluble factors

and the direct contact between activated apoptotic cells and antigen-presenting-cells upregulate molecules on the APC that are costimulatory to the T-cells e.g. CD80 and CD86 (S. K. Pathak et al., 2012). The fact that the recipient dendritic cells at large have left the skin at day 14 after transplantation, points out that the antigen specific priming of the donor T-cells that venture to the skin to elicit acute-GvHD possibly is done elsewhere. It is well known that the dendritic cells of the skin travel to the local lymph node where they mediate and appropriate immune response which may include the facilitation of a clonal expansion of T-cells (Angel et al., 2009). The damaged host dendritic cells may also be necessary for the suppressive action of donor-derived regulatory T-cells (Kushwah, Oliver, Zhang, Siminovitch, & Hu, 2009). A plausible model is that early in the process, that leads to lesion cutaneous acute-GvHD, the patient is at the mercy of the interplay between the donor T-cell population which favors an aggressive and an suppressive function, - and the host-antigen presenting cells. This theory suggests that the aggressive or suppressive outcome of this interplay determines if an acute-GvHD will be elicited or not. One way tolerance towards and antigen response can be mediated is by the work of regulatory T-cells. A meta-analysis on the graft composition finds that a higher ratio of regulatory T-cells in the infused graft is associated with an increased overall survival, specifically decreased non-relapse mortality, probably to a decreased acute GvHD (Fisher et al., 2017). The clinical studies of infusions of regulatory T-cells before- and after transplantation support a role where regulatory T-cells may balance the ability of T-cells to induce acute-GvHD (Brunstein et al., 2011; Di Ianni et al., 2011).

1.6.8 The keratinocyte and it´s key role in cutaneous immunity

The keratinocyte, the epithelial cell of the epidermis, has a key role in cutaneous immunity. At the damaged epithelial surface, these cells produce cationic antimicrobial peptides (AMPs) and precursors of interleukin-1 alpha and interleukin-1 beta. A study of delayed hypersensitivity (DTH) after topical application of skin sensitizers, like poison ivy, found that the keratinocytes were already activated when the T-cells entered the skin (Griffiths & Nickoloff, 1989). When activated, the keratinocytes release cytokines from the inflammosome and chemokines which attract T-cells. With increasing inflammation, and after particularly after stimulation by The Th17 T-cell produced cytokines IL-17A and IL-22, the keratinocytes increase their AMP production (Liang et al., 2006). Acute-GvHD models have revealed that the keratinocytes function as non-professional APCs. Keratinocytes may produce IL-1, IL-6, IL-10, IL-18 and tumor-necrosis-factor alpha and upregulate MHC class-II (HLA-class II). These cells cannot themselves launch a T cell-mediated immune response, but they seem to be able to process and present peptides derived from the outside of the cell membrane to CD8+ cells and guide them to target cell lysis. The keratinocytes of the skin are also able to present peptide antigens to CD4+ cells which in turn may induce Th1 and Th2 type cytokines (Nestle, Di Meglio, Qin, & Nickoloff, 2009).

1.6.9   The histopathology of acute-GvHD

1.6.9.1   Elafin a biomarker of GvHD

The GvHD biomarker, Elafin is predictive of non-relapse-mortality. Elafin is an AMP which also is known as proteinase 3 inhibitor (PI3) or Skin-derived antileukoproteinase (SKALP), and is expressed by polymorphnuclear leukocytes and squamous epithelia. The proteinase inhibiting domain protects against detachment mediated by polymorphonuclear leukocyte (PMN) derived enzymes such proteinase 3 and elastase (Levine et al., 2012; Paczesny et al., 2010). The serine protease inhibitor was discovered in keratinocytes of the human hyperproliferative epidermis. The proteinase is linked to multiple domains which bind to extracellular and cell envelope proteins through transglutaminase substrates at the NH2 terminal. Elafin is expressed in epithelia that are exposed to inflammatory stimuli such as the hair follicular of the skin, the vagina and the oral cavity (Pfundt et al., 1996). Elafin is also a biomarker for ARDS (Sallenave et al., 1999; Wang et al., 2017). It is expressed by squamous epithelia and is present in many suprabasal layers in the upper gastrointestinal tract (Esofagus). The esophageal lining has multiple positive cell layers and the superficial layers stain polarized and intense. In the suprabasal layer, Elafin colocalizes with type 1 transglutaminase (Pfundt et al., 1996). The esophagus has a high constitute expression of elafin (Hosaka et al., 2008), in the lower levels of the gastrointestinal channel may induce elafin in response to antimicrobial invasion (Wehkamp, Schmid, & Stange, 2007).

1.6.10 The gastrointestinal disease and its relation to the cutaneous disease

In transplantation patients there is a concomitant development of cutaneous acute-GvHD and gastrointestinal acute-GvHD to a high degree >50% (Martin et al., 1990). Acute gastrointestinal GvHD is the most common cause of gastrointestinal persistent nausea and anorexia after transplantation (Wu et al., 1998). If it is possible to do an endoscopic assessment, gastrointestinal disease are confirmed by histological grading. A routine histopathology protocol is compromised by fixation of tissue by 10% of buffered formalin. The specimens are assessed on three sections where inflammation, necrosis of epithelial cells (single cell necrosis), epithelial atypia, loss of glands or crypts, dilatation of glands or crypts, crypt obstruction and abscesses are rated. In the small intestine, the status of the villous architecture is described additionally. Cytoreductive therapy before allogeneic transplantation may leave histological changes which are similar to the pathology of GI acute-GvHD, therefore histological studies have included patients after day 20, generally or in subgroups (Roy et al., 1991; Snover et al., 1985).

Single cell necrosis is the established positive biopsy finding of gastrointestinal acute-GvHD (Snover et al., 1985). At autopsy, rectal biopsies present single cell necrosis, crypt degeneration, crypt obliteration and crypt abcesses (Sale G. 1979). If cytolomegalovirus (CMV) inclusions can be identified by peroxidase-antiperoxidase techniques the GvHD of the gastrointestinal tract cannot be diagnosed histologically, with reliability (Snover et al., 1985). The confounding by CMV can be the cause why the early study of Snover et al. with

patient inclusion between 1981-1983 where cytolomegalovirus was diagnosed on clinical suspicion show a positive predictivity (0.55) for gastrointestinal acute-GvHD by cutaneous acute-GvHD in patients with biopsies made between 20 – 100 days after transplantation i.e. cutaneous acute-GvHD may predispose for gastrointestinal acute-GvHD, but not for gastrointestinal CMV infection. This study shows a correlation between diarrhea and gastrointestinal disease of the small intestine and correlation between positive biopsies of the small intestine and the rectum. The patients with upper gastrointestinal acute-GvHD more often had symptoms with nausea, vomiting, and anorexia (Snover et al., 1985). Thus gastrointestinal disease can be present in a part or the whole of the gastrointestinal tract.

The association between cutaneous acute-GvHD beyond stage one and upper GI acute-GvHD was presented by Roy et al. in 1991 in a retrospective study of allogeneic stem cell transplantation recipients. The combined measure of a significant gastrointestinal biopsy from the upper and the lower intestine acquired at day 38 and day 40 after transplantation resulted in 16/26 patients with cutaneous acute GvHD grade II or more with a positive gastrointestinal acute-GvHD histology, and 10/26 with a negative - compared to the 51 patients with no- or stage 1 cutaneous acute-GvHD of whom 18/51 patients had a positive biopsy and 33/51 patients had a negative biopsy (Roy et al., 1991). These results in a specificity of 0.77 and a predictive positive value of 0.62 of acute cutaneous GvHD grade II or more for patients with a positive GI biopsy. In specific, there was a significant association between a cutaneous acute-GvHD with a rash that covered more than 25% of the TBSA and GvHD was stronger than the association between upper and lower acute-GvHD GI biopsies with positive histology.

The relation between cutaneous acute-GvHD and the GI acute-GvHD can also be found in a study where the median time to intestinal biopsy by endoscopy was 54 days (range 21-98). In this retrospective study where 15/40 (38 %) of the patients with a biopsy proven GI acute GvHD also had cutaneous rash, while 2/40 (5%) with skin rash did not have GI acute-GvHD, the endoscopic evaluation of gastrointestinal acute-GvHD was prompted by diarrhea (70%), 27/40 or nausea and vomiting in 28/40, (67%). All patients with ulceration had GI GvHD, while a normal endoscopy could not exclude GI acute GvHD. The biopsies were examined immunohistochemically, and viral cultures were made, in addition to the hematoxylin and eosin staining (Sultan, Ramprasad, Jensen, Margolis, & Werlin, 2012). This reveals a specificity rate of 0.75 and a positive predictive value of 0.88 for cutaneous acute-GvHD.

The high incidence of concomittant cutaneous- and GI acute-GvHD is supported prospectively. In total 76 patients entered a study after two weeks of symptoms of nausea, vomiting, and anorexia (15.4 +- 11.4 days), at a mean of 57 days after transplantation (+- 31 days). The symptoms were evoked during a taper of prednisone in 23 of the patients who already had acute-GvHD if the skin and/or liver or skin, liver and intestine. The majority had T-cell depleted grafts and received a myeloablative conditioning. Acute-GvHD was the single cause of symptoms in 83%, 63/76. Intestinal upper endoscopy biopsies were performed in 52 out of these 63 patients. At this time point, the majority, 51/52 patients had edema and patchy

erythema of the gastric antral mucosa, with a pathology that ranged from, "scattered, isolated crypt cells", to "lymphocytic infiltrate in the lamina propria associated with crypt drop-out and apoptotic cells" (Wu et al., 1998).

In a study with patients of 273 patients included between 1996 and 2001, Schulenburg et al. presented the main disease panorama in patients with symptoms of the GI tract after allogeneic transplantation as GvHD as; (62%), gastritis/esophagitis; (19%), CMV; enteritis (11%); bacterial enteritis (6%); toxic mucosal damage (2%) (Schulenburg et al., 2004). CMV enteritis is seen during 100 days after transplantation and may be prevented with acyclovir (Meyers et al., 1988), and treatment start early after transplantation or at engraftment with ganciclovir may be monitored and guided by pp65 antigen and quantitative PCR (Boeckh et al., 1996; Schulenburg et al., 2001). An association between liver acute-GvHD and gastrointestinal acute-GvHD was not seen in the histological studies by Roy et al. and Fischer et al. (Roy et al., 1991); (Fischer et al., 2015). Treatment of GI disease of a disease severity beyond II has since 1997 included 2 mg/ body weight and a topical addition of budenoside (9 mg/day orally) (Schulenburg et al., 2004).

1.6.11 Regulatory T-cells is scarce and CD8+ T-cells common in the gastrointestinal GvHD

The hypothesis of a dysregulation of the regulatory response in acute-GvHD was supported in a retrospective study of 95 paraffin-embedded tissue samples from duodenum and colon biopsies of 49 patients. In this study where the majority (46/49) had matched related or unrelated transplant with an cyclosporine and methotrexate or melphalan GvHD-prophylaxis with the addition of anti-thymocyte globuline in case of an unrelated transplant, (25/49) had myeloablative conditioning divided on TBI (n=17/25) and chemotherapeutics (n=8/25); the ratio of CD8+ and regulatory T-cells revealed by double immunoenzymatic staining in mucosal biopsies was highly significant (Figure 5) for gastrointestinal acute-GVHD (Rieger et al., 2006).

Figure 5. The ratio of CD8+ and regulatory T-cells as revealed by double immunoenzymatic staining in mucosal biopsies was highly significant for gastrointestinal acute-GVHD
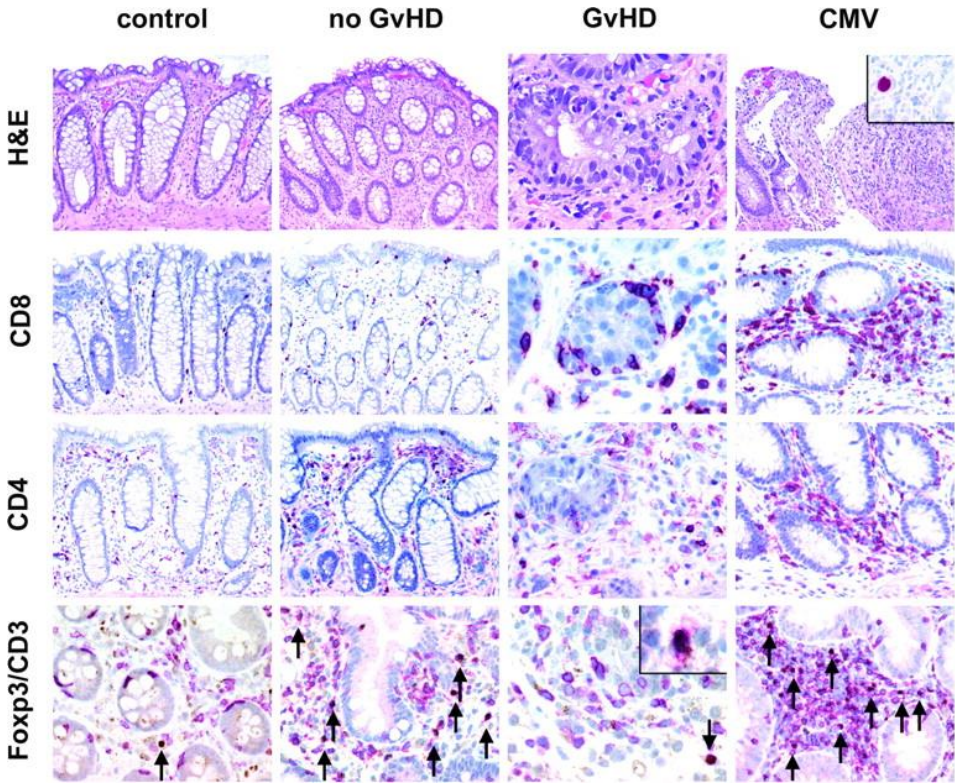
1.6.12 The liver disease and its relation to the cutaneous disase

The liver is perceived as the organ with a relatively low incidence of acute-GvHD compared to the skin and the gut. In an analysis of 1986 patients after myeloablative conditioning and a GvHD prophylaxis with methotrexate or methotrexate and cyclosporine in a majority of the patients, (37%), (740/1986) had acute-GvHD and the incidence of acute-GvHD of the liver was the lowest (19%), (369/1986). Among the patients with acute-GvHD in any organ did 50% did have liver involvement, compared 54% with acute-GvHD of the gut, and 81% had GvHD of the skin (Martin et al., 1990). In later studies of secondary treatment with monoclonal antibodies, high liver involvement in severe acute-GvHD has been suggested as a reason to inferior results, e.g. 52% liver involvement and 17% six months survival (Horse derived Anti-thymocyteglobulin) (Khoury et al., 2001), 50% liver involvement and 0% six months survival (Alemtuzumab) (Martinez et al., 2009), 32% liver involvement and 28% six months survival (Dacluzimab) (Perales et al., 2007). Consensus of histopathological pathognomous findings for liver acute GvHD is missing, but a recent report suggests that within the portal field, bile duct damage and bile duct intraepithelial lymphocytes may be indicative of liver GvHD in general (Eskandari et al., 2017).

1.6.13 The lung disease and its relation to the cutaneous disease

The lung function detoriates after transplantation and in both the population surviving acute-GvHD. In a post-mortem **Study I**n patients who received myeloablative conditioning, a correlation between onset of pulmonary disease and onset of acute GvHD within 150 days was found, together with a post-mortem association of bronchopneumonia or extensive acute broncitis and acute GvHD grade II-IV, has been reported (Figure 6.), (Beschorner, Saral, Hutchins, Tutschka, & Santos, 1978). Recovery of lung-function comes late, among 180 days survivors of acute-GvHD grade I-IV, an improvement in lung-function (FVC and TLC) could be seen first 1.5 years after Transplantation (Gore, Lawton, Ash, & Lipchik, 1996). Today, the majority of the lung diseases after transplantation are grouped in a syndrome called interstitial pneumonia syndrome (IPS) (Table 1). IPS has an incidence of 3-15% after myeloablative conditioning (Panoskaltsis-Mortari et al., 2011).

Figure 6. In patients who received myeloablative conditioning, a correlation between onset of pulmonary disease and onset of acute GvHD within 150 days was found, together with a post-mortem association of bronchopneumonia or extensive acute broncitis and acute GvHD grade II-IV.

$$y = 1.06X - 2.11$$
$$r = 0.962$$
$$p < 0.001$$
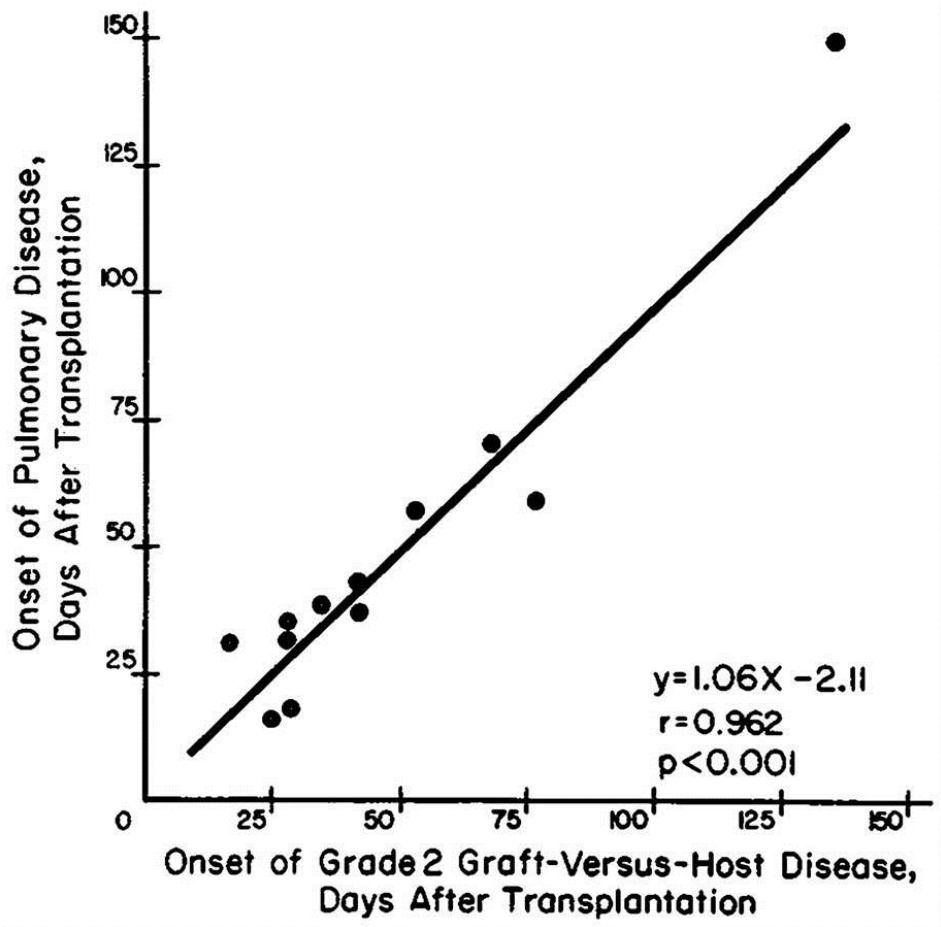
TABLE 1.

DEFINITION OF IDIOPATHIC PNEUMONIA SYNDROME

I: Evidence of widespread alveolar injury:
    a. Multilobar infiltrates on routine chest radiographs or computed tomography
    b. Symptoms and signs of pneumonia (cough, dyspnea, tachypnea, rales)
    c. Evidence of abnormal pulmonary physiology
        1. Increased alveolar to arterial oxygen difference
        2. New or increased restrictive pulmonary function test abnormality

II: Absence of active lower respiratory tract infection based upon:
    a. Bronchoalveolar lavage negative for significant bacterial pathogens including acid-fast bacilli, *Nocardia*, and *Legionella* species
    b. Bronchoalveolar lavage negative for pathogenic nonbacterial microorganisms:
        1. Routine culture for viruses and fungi
        2. Shell vial culture for CMV and respiratory RSV
        3. Cytology for CMV inclusions, fungi, and *Pneumocystis jirovecii* (*carinii*)
        4. Direct fluorescence staining with antibodies against CMV, RSV, HSV, VZV, influenza virus, parainfluenza virus, adenovirus, and other organisms
    c. Other organisms/tests to also consider:
        1. Polymerase chain reaction for human metapneumovirus, rhinovirus, coronavirus, and HHV6
        2. Polymerase chain reaction for *Chlamydia*, *Mycoplasma*, and *Aspergillus* species
        3. Serum galactomannan ELISA for *Aspergillus* species
    d. Transbronchial biopsy if condition of the patient permits

III: Absence of cardiac dysfunction, acute renal failure, or iatrogenic fluid overload as etiology for pulmonary dysfunction

Definition of abbreviations: CMV = cytomegalovirus; HSV = herpes simplex virus; RSV = respiratory syncytial virus; VZV = varicella zoster virus.

There is no controversy over the immunological impact transplantation has on the lung, but the presence or not of a lung cute-GvHD is discussed, and the histopathological definition IPS encompasses what may be lung GvHD. TBI is one major cause of pulmonary toxicity and decrease in function. Infections are another cause, and it is very important to diagnose pulmonary disease early to initiate proper treatment of infections in the immunosuppressed individual (Hoglund, Sandin, & Simonsson, 2015; Svensson, Lundstrom, Hoglund, & Cherif, 2017).

Due to the lack of histopathological studies of lung GvHD within the first hundred days, the pattern of lung pathology within the first five months of transplantation in two studies is described (Xu, Drachenberg, Tavora, & Burke, 2013; Yousem, 1995).

There are few reports where the relation of cutaneous acute-GvHD can be related to what is described as a concomitant GvHD in the lung. The clinical spectrum of disease is categorized by the presumed site of tissue injury (Table 2) (Panoskaltsis-Mortari et al., 2011). In a histopathological study of transbronchial or open lung biopsy specimens, out of 17 described patients, four specimens were from <5 months after GvHD. The remaining specimens were from median 12 months after transplantation (range 6-110). Special microbial stains (Grocott, acid fast, Gram) and bacterial, fungal and viral cultures were negative. The two patients with GvHD of the skin both were on steroid treatment and had specimens from the fourth month, and thus possible had originated together with a cutaneous acute-GvHD. These patients were both females and were transplanted for CML. The biopsies revealed BO and Bronchiolitis obliterans organizing pneumonia (BOOP), both were alive at follow-up, one with no sign of pulmonary disease at six months, and one with pulmonary disease at 39 months. The patient with BOOP presented with dyspnea on exertion, cough, and malaise. The chest X-ray show "patchy bilateral alveolar and interstitial infiltrates". The morphology of the small airways in showed "epithelial apoptosis" and "epithelial transmigration of lymphocyte". Granulation that filled the airways was seen in all specimens, which was accompanied by perivascular and mononuclear infiltrates. There was an "up-and-down" pattern of epithelial regeneration, with "stretches of cuboidal cells alternating with hyperplastic tall columnar epithelium". The patient with cicatrial bronchiolitis obliterans (BO), were grouped with five others, in a group who had "airways obliterated by dense fibrous scar tissue". Two of the patients with bronchiolitis obliterans had post pulmonary transplantation biopsies. Three had perivascular and interstitial mononuclear cells. All had the "up-and-down" pattern of the epithelia and the "peribronchial mononuclear infiltrate percolated into the overlying epithelium, where apoptosis was readily noted" (Yousem, 1995).

TABLE 2.

CATEGORIZATION OF THE CLINICAL SPECTRUM OF LUNG INJURY
FOLLOWING HEMATOPOIETIC STEM CELL TRANSPLANTATION

Clinical Spectrum of Disease as Categorized by Presumed Site of Primary Tissue Injury

Pulmonary Parenchyma	Vascular Endothelium	Airway Epithelium
Acute interstitial pneumonitis (AIP)[*]	Peri-engraftment respiratory distress syndrome (PERDS)[*]	Cryptogenic organizing pneumonia (COP)/Bronchiolitis obliterans organizing pneumonia (BOOP)[*]
Acute respiratory distress syndrome (ARDS)[*]	Noncardiogenic capillary leak syndrome (CLS)[*]	Bronchiolitis obliterans syndrome (BOS)[*]
BCNU pneumonitis	Diffuse alveolar hemorrhage (DAH)[*]	
Radiation pneumonitis	Pulmonary veno-occlusive disease (PVOD)	
Delayed pulmonary toxicity syndrome (DPTS)[*]	Transfusion-related acute lung Injury (TRALI)	
Post-transplant lymphoproliferative disease (PTLD)	Pulmonary cytolytic thrombi (PCT)	
Eosinophilic pneumonia (EP)	Pulmonary arterial hypertension (PAH)	
Pulmonary alveolar proteinosis (PAP)	Pulmonary thromboembolus (PTE)	

[*]Conditions routinely included under the classification of idiopathic pneumonia syndrome (IPS).

A recent study of another 17 specimens had four specimens from <5 months after transplantation, three of these had concomitant cutaneous GvHD, the rest were from median 17 months (range 6-82). In this study, viral and bacterial etiologies were excluded by culture, diagnostic findings and special stain (Gomori methenamine silver, acid fast bacillus, cytomegalovirus, herpes simplex virus). These were distributed in different groups based on bronchiolar histology. The first categorized in the "acute-lung-injury pattern", was a 60 y.o. male who died within 6.5 months after transplantation, The second was categorized as a histological obliterative bronchiolitis "OB-pattern", the 40 y.o. female with ground glass densities with bilateral nodulus on CT, who complained of shortness of breath, had a GvHD that included the skin and the oral cavity, thereafter the pulmonary function were reported stable at three months but the GvHD recurred and she died of systemic GvHD at 1 y. The third, a 49 y.o. female was categorized as a chronic interstitial pneumonia (CIP-pattern), and had shortness of breath with nodules and organizing pneumonia (OP), (CT). She had concomitant GvHD of the stomach, duodenum the liver and the skin. She stabilized, but died in multi-resistant staphylococcus aureus (MRSA) infection after having developed bronchiolitis obliterans syndrome (BOS). The histological pattern is diverse, but the mean bronchiolar CD3 cells/50 respiratory cells were 30 +-10 (SD) and all but one patient (16/17) in the study had apoptotic bodies, (one were autologous), (Xu et al., 2013).

The clinical improvement of reduced-intensity conditioning (RIC) leads to a decreased tissue damage (Giralt et al., 1997) and thus less acute-GvHD and IPS, was developed secondary to the high incidence of GvHD and IPS in patients over 40 yrs old (Ringden et al., 1993). RIC has made transplantation possible with low toxicity (Ringden et al., 2013).

1.6.14 The balance between GvHD and GvL

In GvHD, the tissue damage from T-cell mediated cell lysis recruits additional immune cells. With increasing inflammation, the tissue destructive process is spreading, and the cross-presentation between activated immune cells and the antigen presenting cells are increased. The oligoclonal response of activated T-cells in cutaneous acute-GvHD are evident and a multitude of clonal T-cells lines from a single skin biopsy can be visual already at five days in a T-cell cloning assay (Gaschet et al., 1996). Without proper treatment, this process of oligoclonal expansion of recipient antigen specific T-cells may become fatal to the patient. However, if the cutaneous tissue lesions of GvHD do not progress, or can be controlled by anti-GvHD therapy, the presence of GvHD is associated with a decreased incidence of recurrent malignant disease (Ringden et al., 2000). There are indications that the GvL effect is coupled antigen mismatches between the donor cells and the malignancy of recipient origin, predominantly in the related donor-recipient pair (Hobo et al., 2013; Spierings et al., 2013). Thus, the anti-tumor immunity associated with cutaneous acute-GvHD in human may be mediated by adoptive immunity towards minor histocompatibility antigens. A plausible model which is congruent with long term cancer free survival is that after the priming of an antigen specific memory T-cell response, antigen specific memory T-cells with tissue specificity patrol the patient and eradicate remnant tumor (Woodland & Kohlmeier, 2009).

1.7 DEFINITION OF ACUTE-GVHD

The organ damage is staged and categorized by the Glucksberg Criteria and the IBMTR classification (Glucksberg et al., 1974; Rowlings et al., 1997).

1.7.1 Stage of organ involvement

The clinical manifestation and the histopathological changes in organ involvement as staged by Glucksberg:

"Skin: +1, A maculopapular eruption involving less than 25% of the body surface; +2, a maculopapular eruption involving 25-50% of the body surface; +3, generalized erythroderma with bullous formation and often with desquamation.

Liver: +1, Moderate increase of SGOT (150-750 IU) and bilirubin (2.0-3.0 mg/100 ml); +2, bilirubin rise (3-5.9 mg/100 ml) with or without an increase in SGOT; +3 bilirubin rise (6-14.9mg/100 ml) with or without an increase in SGOT; +4, bilirubin rise to >15 mg/100 ml with or without an increase in SGOT. Increases in SGOT were temporarily related to the onset or worsening for skin rash.

Gut: Diarrhea, nausea, and vomiting were graded +1 to +4 in severities, and the severity of gut involvement was assigned to the most severe involvement noted. It was difficult to quantitate most of these manifestations except for diarrhea.

Diarrhea: +1, >500 ml of stool/day; +2, >1,000 ml of stool/day; +3, >1,500 ml of stool/day; +4, >2,000 mL of stool/day."(Glucksberg et al., 1974).

The Total Body Surface Areas (TBSA) affected by cutaneous acute-GvHD can be determined by the "rule of nines". The area of surface in the body parts in the adult is 9% for the head, 9% for each arm, chest & back 18% each, 1% for genitalia and 18% for each leg. In the children, the surface area of the head is 19% at birth, but 1% shall be derived each year until ten year of age, and instead, be added to the legs. In children, each arm is 9% and the thorax is 36%, while the legs start at 13% each (Waslen, 1986).

1.7.2 Severity of GvHD

"Based on severity and number of involved organs, patients were divided into four categories:

Grade 1: +1 to +2 skin rash without gut involvement with no more than +1 liver involvement; no decrease in performance status or fever.

Grade 2: +1 to +3 skin rash with either +1 to +2 gastrointestinal involvement or +1 to +2 liver involvement, or both. All exhibited a mild decrease in performance status and some had fever.

Grade 3: +2 to +4 skin rash with +2 to +4 gastrointestinal involvement with or without +2 to +4 involvement. All exhibited a marked decrease in performance status and many experienced fever.

Grade 4: Pattern and severity of GvHD similar to grade 3 with extreme constitutional symptoms."

1.7.3 The histopathological grading

Skin;

Grade 1: the mildest change in the skin was focal or diffuse vacuolar degeneration of epidermal basal cells and acanthocytes.

Grade 2: in addition to basal vacuolar degeneration, focal or diffuse spongiosis (separation of basal cells and acanthocytes by intercellular edema) and dyskeratosis or eosinophilic degeneration of epidermal cells which tended to occur in scattered individual cells.

Grade 3: clefts and spaces after necrosis of basal cells and acanthocytes in the basal and more superficial layers often resulting in separation of the dermal-epidermal junction.

Grade 4: frank loss of epidermis.

Gut;

Grade 1. The mildest changes in the gastrointestinal tract were focal dilatation and degeneration of the mucosal glands.

Grade 2: the bowel, in addition to the changes described under grade 1, showed focal to diffuse loss of intestinal glands.

Grade 3: focal mucosal denudation.

Grade 4: diffuse mucosal denudation.

Liver;

Although hepatic GvHD causes variable degeneration and eosinophilic necrosis of hepatocytes, the most constant finding was that of degeneration and necrosis of small bile ducts.

Grade 1: less than 25% pathological changes in small bile ducts.

grade 2: 25-49%;

grade 3: 50-74%;

grade 4: more than 75%.

Above are quotations from page 297, from the first and second paragraph in Glucksberg H. et al. (Glucksberg et al., 1974). The corresponding levels of bilirubin in micromole are: liver. +1 < 34: +2, 34-102; +3: 103-255; +4: > 255. The CIBMTR revised the scoring and suggested following groups based on the staging by Glucksberg: A: stage +1 skin; B: stage +2 skin or stage 0-2 skin and stage 1-2 visceral acute GvHD (liver and/or gut); C: stage +3 of any skin/liver or gut; D: stage 4 of any skin/liver or gut (Rowlings et al., 1997).

1.7.4 Immune-mediated and cytotoxic assault of the lung

The view of non-infectious lung damage as an idiopathic syndrome has changed into a view where the lung is considered to be under a complex immune-mediated and cytotoxic assault after transplantation. The mortality remains as high as 60-80% and above 95% in mechanically ventilated patients (Panoskaltsis-Mortari et al., 2011); (Fukuda et al., 2003). The gut-liver-lung axis is predominant in the experimental animal models referred to in the official statement, and treatment directed against Tnf-alpha is suggested. The American thoracic society (ATS) now defines Idiopathic Pneumonia Syndrome (IPS) accordingly:

"An idiopathic syndrome of pneumopathy in which infectious etiologies and cardiac dysfunction, acute renal failure, or iatrogenic fluid overload has been excluded. Moreover, IPS has been further classified into specific entities" … " based in large part on the primary anatomical sites of inflammation and dysfunction". It is defined clinically by widespread alveolar injury, absence of infection and absence of cardiac, renal or iatrogenic ethiology (Panoskaltsis-Mortari et al., 2011).

IPS should be differentiated by bronchoalveolar lavage (BAL), Computer tomography (CT), echocardiography and lung biopsy into specific entities mainly related to: Interstitial tissue, vascular tissue, airway tissue and IPS, unclassifiable (Table 1&2) (Panoskaltsis-Mortari et al., 2011).

1.8 IS THERE A TIME WINDOW TO SEPARATE GVL FROM GVHD

The time window for the elicitation of cell-mediated immunity evoked against leukemia was studied by Saltztein and Bortin in the beginning of the seventies. They suggested that critical time to catch the cells and to transfer the allogeneic GvL response was during the first week (6 days) (Bortin, Rimm, & Saltzstein, 1973; Saltzstein, Glasspiegel, Rimm, Giller, & Bortin, 1972). The experimental setup was accordingly, in murine model experiments, the 70 days survival of secondary recipients was measured and related to the day of transplantation of splenic or lymph-node derived cells from primary recipients (normal AKR (H-2k) mice). The primary mice had been previously seeded with spleen cells harvested from AKR mice dying from long-passage lymphocytic leukemia, thereafter treated with TBI and cyclophosphamide and selected to an intervention by adoptive immunotherapy by inoculations by allogeneic BM cells or lymph-node cells from C57BL/6 (H-2b) or not. The 70 days survival of the secondary recipient peaked on day 6 compared to day 4 and 8. This suggests that in non-immunosuppressed mice, the optimal day for cell harvesting from spleens and lymph-nodes to catch cells vital to transfer the allogeneic graft-versus-leukemia effect after the initiation of an immune response into a secondary host is at day 6 (Saltzstein et al., 1972).

In a follow up experiment where adoptive immunotherapy was the exclusive anti-leukemic treatment; leukemic cells were transferred to primary hosts of different strains; at day six their spleens were harvested and transferred to a secondary recipient and the 60 day survival was tested. A significant GvL effect was found when the secondary recipient differed from the primary recipient in the H-2 locus (Bortin et al., 1973). This confirms that at day six, the anti-leukemic priming of an unprimed immune response has taken place and further suggests that an antigen mismatch on one locus increases the GvL effect possibly by cross-presentation or increased danger signaling.

In another murine model, Sadeghi et al. studied the kinetics of the elicitation phase of GvHD. In these studies reciepient female BALB/c mice received busulfan (80 mg/kg) for 4 days followed by cyclophosphamide (200 mg/kg) for 2 days as myeloablative chemotherapy, followed by syngeneic transplantation and allogeneic transplantation of male C57BL/6 mice. These experiments show an early peak of CD11c+ dendritic cells in the spleen followed by a major peak of CD8+ cells at day seven (Sadeghi et al., 2010). The time windows similar to that presented in the studies of GvL and GvHD by Saltztein et al. and Bortin et al. and suggest that GvHD and the GvL may be parallel processes in murine model.

1.9 THE ROLE OF CONDITIONING REGIMEN

1.9.1 Three reasons for conditioning before transplantation

Transplantation has been considered an optional treatment, for several hematological malignancies, after a clinical risk-benefit decision and informed consent of the procedure has been acquired by the patient and in the cases of children, the whole family of the patient. In order to facilitate the transplantation procedure, patients are treated with total body irradiation with or without high doses of chemotherapy (conditioning regimen) prior to allogeneic stem

cell transplantation. The conditioning regimen is, in general, a combination of myeloablative and immune supervise treatment that serves several aims; First to decrease the risk for host-versus-graft reaction which rejects the graft and prevents the engraftment in the primary and secondary hematopoietic organs of the recipient; The second purpose of the conditioning regimen may be to eradicate a malignant residual cells; The third purpose may be immunomodulatory, e.g. to decrease the risk for graft-versus-host disease while preserving the graft-versus-leukemia reaction, or to decrease the risk for host-versus-graft reaction while preserving the innate immunity. One immunomodulatory option of the graft is to give in-vivo T-cell depletion by using anti-thymocyte globulins.

1.9.2 Myeloablative conditioning

A myeloablative conditioning can be reached through several established clinical protocols. Total-body-irradiation (TBI) is a mainstay and may at higher dose ranges alone make the recipient susceptible for engraftment of the transplanted graft. The use of TBI in patients may induce adverse effects within the acute-irradiation-syndrome (ARS) and irreversible chronic damage to the lung. The TBI may be given as a single (920 – 1000 cGy) or by fractionated doses with repeated lower doses which limits the secondary tissue injuries but increases the accumulated irradiation e.g. dose 1320 cGy (Brochstein et al., 1987; E. D. Thomas et al., 1977; Vriesendorp & Heidt, 2016). Irradiation of dual Co^{60} source gives a homogenous radiation exposure e.g. 5.5 rads/min – 8.0 rads/min, in a field where the patient has more marginal to move (E. D. Thomas et al., 1977). Linear accelerators can provide higher dose rates and linear irradiation is easier to shield (protect) the lungs, but where the patient is immobilized (Giebel et al., 2014). To cure leukemia chemotherapeutic agents are added to TBI as the doses needed to eradicate leukemia by TBI alone would induce lethal damage in the patient. Reduced intensity conditioning (RIC) may use DNA-synthesis blocking agents such as fludarabine and may be necessary in the older population or in patients with a previous medical history who do not sustain the toxicity of the myeloablative conditioning (Aschan, 2007; Slavin et al., 1998).

1.9.3 Cyclophosphamide

Cyclophosphamide is an effective immunosuppressive and anti-neoplastic agent. The maximum tolerated dose is 200 mg/kg limited by the side effect of hemorrhagic myocarditis. The protocol used in the majority of the patients in this thesis was cyclophosphamide for two days before TBI at a dose of 60 mg/kg/day in a central venous line (Ringden et al., 1996). Protocols used were developed and verified by several centers, but the protocols vary, and it is unlikely that to centers use the exact same protocol.

1.9.4 Busulfan

Busulfan is a myeloablative alkylating agent, which may substitute total-body-irradiation in myeloablative protocols. The nonmyeloablative cyclophosphamide are then used with the myeloablative Busulfan (a metabolite of treosulfan), in accumulated doses of 120 mg/kg and 16 mg/kg (Ringden et al., 1999). The treatment at Center for Allogeneic Stem Cell

transplantation (CAST) was optimized and intravenous and monitored by plasma concentration measurements to reach the targeted steady-state to optimize the effect versus the adverse effects of the treatment (Hassan et al., 2002). Cyclophosphamide and TBI has a potentially better overall tumor effect but may lead to secondary malignancies and irradiation injuries of the lung. The cyclophosphamide and busulfan protocol lead to permanent alopecia and an increase risk for obstructive bronchiolitis, veno-occlusive-disease in the liver and hemorrhagic cystitis (Aschan, 2007; Ringden et al., 1999).

1.9.5 T-cell depletion

The in-vivo T-cell depletion used was by different anti-thymocyte globulins. The use of anti-thymocyte globulins increases the number of patients that can be offered a transplantation as the in-vivo T-cell depletion lowers the risk for GvHD after transplantations with grafts from matched unrelated donors (MUD). The use of anti-thymocyte globulins also decreases the risk for graft-rejection in heavily transfused recipients, which may be a remnant to the era of non-filtered blood-products (Aschan, 2007). In-vitro and in-vivo T-cell depletion has made it possible to choose grafts from donors in registers worldwide which has increased the possibility to locate suitable donor fast.

1.10 THE HISTOCOMPATABILITY

1.10.1 The barrier

The allogeneic hematopoietic stem-cell transplantation (AHSCT) is transplantation within the human species between genetically disparate individuals. The transplantation faces the bidirectional barrier of host-versus-graft disease and graft-versus-host disease. The barrier can be estimated by comparing the degree of human-leukocyte-antigene (HLA)-match after tissue typing and genotyping of the HLA-genes for both recipient and donor (Olerup & Zetterquist, 1992; E. Thomas et al., 1975). The functional magnitude of the barrier may be assessed by the mixed-lymphocyte-reaction, intracellular ATPase activity of selected immuncells which are established for CD4+ T-cells, Limited Dilution tests, Tetramer analysis which is prevalent for CD8+ T-cells or enzyme-linked immunospot which is a common measure of interferon-gamma (Jeras, 2002; Mehrotra, Leventhal, Purroy, & Cravedi, 2015). The HLA-bind antigens and present them on the cell surface so that the antigen can be recognized by an antigen-specific T-cell receptor on T-cells. There are a high degree of polymorphism among the HLA-genes within the 7.6 Mb region where the HLA-A, -B, -C, -DR, -DQ and -DR are transcribed. The HLA-barrier is minimum in syngeneic twins, among siblings there is a 48% chance of a HLA-match (Petersdorf, 2007; E. Thomas et al., 1975). NK-cells can be mediate cytotoxicity in addition to T-cells if the patient does not have killer cells immunoglobulin receptors (KIRs) which match HLA class I donor alleles (Farag, Fehniger, Ruggeri, Velardi, & Caligiuri, 2002).

1.10.2 Related transplantation

Recipients not related to the donor, have a higher risk for graft-failure with an increased donor-recipient mismatching why the least mismatched donor should be selected in related transplantation (Anasetti et al., 1989). Further, there must not be a mismatch in the homozygotous locus of a related recipient homozygotous for HLA, as this is associated with a high risk of host-versus-graft disease. Further, pre-transplant cytotoxic cross-matching should be performed, as HLA-antibodies increase the risk for graft-failure (Petersdorf, 2007; E. Thomas et al., 1975).

1.10.3 Unrelated transplantation

In unrelated transplantation the risk for graft-failure and GvHD is higher with antigen mismatch than with allele mismatch. NK-cell alloreactivity give lower GvHD and lower relapse after specific conditioning and immunosuppression regimens, KIR-ligand incompatability can be used to select the donor of a graft with the beneficiary NK-cell effect (Petersdorf, 2007). In unrelated donors, the chance for a match is low and international cooperation is needed to find a donor in time from the international registries. Since 1987 the NIH has demanded a $5 - 6$ match in the HLA-A, HLA-B and HLA-DRB1 loci in unrelated donors (Hurley et al., 2003).

1.11 THE TRANSPLANTATION

1.11.1 Trilinear engraftment by bone-marrow-transplantation

The transplantation must provide trilinear engraftment of erythrocytes, immune-cells, and platelets. The graft is given to the conditioned patient as a transfusion during several hours under the direct supervision of a specially trained team comprising physicians and nurses. The day and time-point for the transplantation in relation to the conditioning regimen given is crucial for patient survival. The number of nucleated cells in the graft is a surrogate of hematopoietic stem cells and lymphocytes which are decisive to reach a successful engraftment. The Bone-Marrow is harvested in multiple aspirations under general anesthesia (E. D. Thomas et al., 1971).

1.11.2 The nucleated cell dose

A nucleated cell dose of above $3x10^8$ cells/kg marrow has been identified as a key number to achieve a stable engraftment with a minimum degree of graft-versus-host disease in sibling and in syngeneic donor-recipients of bone-marrow-grafts (BM) (Barrett et al., 2000; Ringden & Nilsson, 1985). The crucial number in unrelated matched transplants has been suggested to $3.65x10^8$/kg (Sierra et al., 1997). Immunohistochemistry has revealed that in allogeneic stem cell transplantation not only the progenitor cells are important for engraftment. The increased use of peripheral-blood-stem-cells (PBSC) mobilized to the peripheral blood of the donor by using G-CSF provides grafts not only rich for in CD34+ progenitor cells but also CD3+ and CD56+ cells. The CD34+ count in BM is usually about 50% of the count found in PBSC, and

the CD3+ and the CD56+ is about 10% of the PBSC dose, yet transplantation outcome remain similar regardless of stem cell source (Remberger et al., 2001).

1.11.3 The CD34+ cell dose

Retrospective analysis of HLA-identical transplantations where positive selection of hematopoietic progenitor CD34+ cells have caused to depletion of CD3+ cells (T-cells) showed that the CD3+ T-cells are needed in a minimum of 0.2×10^6 cells/kg to prevent from graft-failure (Urbano-Ispizua et al., 2001). In a comparative study by Professor Remberger M. et al. from CAST and two additional European centers, which included 107 patients who received BM and 107 patients who received PBSC, - the CD34+ threshold (time in days) to achieve three consecutive days with an absolute neutrophil count (ANC) of 0.5×10^9 (neutrophil engraftment) was lower than the platelet engraftment. The neutrophil engraftment was reported to be at day 19 (range 10 -– 31 days), in the BM-recipients, compared to the PBSC-recipients where ANC-engraftment was reached at day 15 (range 7-27 days), four days earlier, and the platelet engraftment (A week with platelet counts $> 30 \times 10^9$/L without transfusion), was 27 days (range 18-210 days) in BM-recipients, compared to 20 days (range 8-395 days) in PBSC recipients (Remberger et al., 2001). The optimal dose range of CD34+ in the PBSC graft was suggested to be $(4 – 8 \times 10^6$ cells/kg) but may be wider $(2.5 – 11 \times 10^6$ cells/kg) where a higher number may decrease the overall survival secondary to GvHD (Sierra et al., 1997); (Remberger et al., 2015).

1.12 THE ROLE OF GRAFT-VERSUS-HOST PROPHYLAXIS IN TRANSPLANTATION OUTCOME

1.12.1 The methotrexate protocol

To overcome the histocompatibility barrier remaining after the HLA-matching it is necessary to treat the recipient with an immunosuppressive prophylaxis in order to prevent from GvHD. In HLA-matched siblings, a methotrexate protocol elaborated in canine models preserves engraftment in human while decreasing GvHD (Storb et al., 1970; E. D. Thomas et al., 1971). The methotrexate GvHD-prophylaxis protocol was shown to preserve a substantial graft-versus-leukemia effect that is needed to cure leukemia (E. Thomas et al., 1975; Weiden et al., 1979). The introduction of cyclosporin A in connection with therapeutic drug monitoring provided an alternative to methotrexate. However, while the lung-toxicity decreased, an increased relapse incidence was observed with cyclosporine A instead of methotrexate (Atkinson et al., 1988; Backman, Appelkvist, Ringden, & Dallner, 1988).

1.12.2 Cyclophosphamide postransplantation

The action of drugs on tolerance is not the same before and after allogeneic transplantation. The use of post-transplant cyclophosphamide which has made Haploidentical-transplantation possible in a large scale is an example of that. There are two theories to why posttransplant cyclophosphamide induces tolerance. Initially, the theory was that cyclophosphamide given in the allogeneic setting deleted the proliferating donor-T-cells after transplantation (Mayumi

et al., 1987). Recently, however, it has been suggested that the donor T-regulatory cell are more resistant to cyclophosphamide given after transplantation than the conventional T-cells (Ganguly et al., 2014). In analogue, methotrexate was found by Thomas E.D. to have an effect as GvHD-prophylaxis while preserving GvL (E. D. Thomas et al., 1971). Recently, however, methotrexate has been suggested to work as a pharmaceutic substitute for a dysfunctional regulatory function (Talme, Bergdahl, & Sundqvist, 2016). Therapies and drugs may have regulatory effects after allogeneic transplantation with outcomes on GvL and GvHD than expected, why the timing of the use of therapies after transplantation is important. Haploidentical transplantation with a three day post-transplant cyclophosphamide graft-versus-host prophylaxis is now an established way of transplantation, with the advantage that donors can be found to a much larger extent within the family (Luznik et al., 2008).

1.12.3 Different prophylaxis in different leukemias

The hard outcome measure of graft-versus-host prophylaxis must be treatment-failure. The combined treatment with a short-term methotrexate added to cyclosporin A weakens the barrier between the graft and the host at the cost with less acute-GvHD at the cost of a somewhat higher rate of relapses compared to methotrexate alone. Different graft-versus-host prophylaxes have different toxicity, different effects on acute-GvHD and chronic GvHD and effect on GVL. In children with acute or chronic leukemia who received a HLA-matched sibling graft after a Cyclophosphamide and TBI conditioning, none of these GvHD prophylaxes seem superior. In adults, generally, methotrexate has a pulmonary toxicity and is associated to the interstitial pneumonia syndrome, but preserves the graft-versus-leukemia effect best in ALL, when compared to cyclosporin A and MTX added to cyclosporine A, while cyclosporine A is superior in AML, while methotrexate and cyclosporine is superior in CML (Ringden et al., 1993).

1.12.4 The Calcineurin inhibitors

The calcineurin inhibitor cyclosporin and methotrexate is superior to tacrolimus and methotrexate and is the established alternative for GvHD prophylaxis. The criticism against the calcineurin inhibitors is that cyclosporin is nephrotoxic while tacrolimus is neurotoxic and that they inihibit IL-2 which leads to a decrease in CD4+CD25+FoxP3 regulatory cells, which theroretically may increase chronic GvHD that is a great problem for quality of life after transplantation (Storb, Antin, & Cutler, 2010).

1.12.5 Myelotoxic suppressors

To potentially myelotoxic drugs; sirolimus and mycophenolate has been tried in combination instead of cyclosporine and mycophenolate. Sirolimus that bind to the FK-binding protein 12 and form a complex with mammalian target for rapamycin (mTOR) arrests the cell cycle in G1 through inhibition of DNA-transcription. Mycophenolate mofetil (MMF) inhibits inosine monophosphate dehydrogenase. The combination has been observed in a study with reduced intensity conditioning (RIC) in high-risk leukemia without inferior results beyond a later

ANC take. In one study with myeloablative busulfan conditioning which resulted in a pre-study arrest secondary to adverse events coupled to busulfan and sirolimus e.g. sever sinusoidal obstructive syndrome, portal vein thrombosis altered mental status and risk for poor wound healing, the degree of acute-GvHD, however was not inferior to calcineurin inhibitors (Johnston et al., 2012; Schleuning et al., 2009).

1.12.6 Unrelated transplantations and T-cell depletion

The increased relapse in recipients of T-cell depleted grafts made before 1988 are a major evidence of the graft-versus-leukemia effect mediated by the depleted cells in the graft (Marmont et al., 1991). The GvHD-prophylaxis need to be more intense with a stronger barrier in unrelated donor-recipient pairs, -and with donor-recipient HLA-mismatches. The use of T-cell depletion weakens the barrier further in unrelated transplants where a high risk of fatal acute-GvHD is present, which may be especially important in adults (Remberger et al., 1998).

T-cell depletion may be made ex-vivo by positive selection of the graft by CD34+ antibodies, or by negative selection by CD3+ cell antibodies. Anti-thymocyte globulines (ATG) from animals sensitized to the human leukocytoid cells given during the preparative regimen, but avoiding the transplantation day have become the established method of in-vivo T-cell depletion (Busca & Aversa, 2017). The ATG stays in the recipient and elicit in-vivo T-cell depletion on the graft. These polyclonal anti-thymocyte globulines have an affinity to a range of different molecules on cell surfaces, among them CD4+ and CD11a+ depending on how specific the selection of the leukocytoid cells that are infused into the animal which are to develop adoptive immunity against the human cells are. All of the anti-thymocyte globulines have a strong anti-CD3 effect (Bourdage & Hamlin, 1995; Fang, Fehse, Engel, Zander, & Kroger, 2005).

1.12.7 Donor lymphocyte infusions

If the graft fails to engraft, or the leukemia is coming back, the patient has to be saved. Donor cells can be obtained in the same way as when obtaining a graft for transplantation, by marrow aspiration or by mobilization from the peripheral blood by G-CSF and harvesting by leukapheresis (Remberger et al., 1998). Donor Lymphocyte Infusion is an adoptive immunotherapy to increase the GvL effect (Kolb et al., 1990), which can be used to stop a fulminant relapse when molecular- or cytogenic residual disease is detected (Sairafi et al., 2010). The GvL after DLI has been monitored by cytogenic markers in CML and when the leukemia can be detected in the blood the GvL has been found to come two weeks earlier than GvHD (Hari, Logan, & Drobyski, 2004).

1.13 SUPPORTIVE THERAPY AND HOME CARE

The supportive therapy managed by the staff at the transplant center is the key of transplant success. In addition to the transplant protocol described, there is a need of professional and experienced care during the pancytopenic phase. In addition to the frequent blood sampling

and the administration of the graft-versus-host prophylaxis and the prophylactic antibiotics used during the pancytopenic phase, the need for discussion with and advice from experienced staff is imminent. The place for care may have an impact on the outcome of transplantation, in a single center study and a long-term follow up, home-care has been superior to care at the ward with protected environment, with less incidence of acute-GvHD (Svahn et al., 2002).

1.14 THE FIRST AND SECOND LINE TREATMENTS OF ACUTE-GVHD

1.14.1 Corticosteroids in the treatment of acute GvHD

The most up-to date recommendations of acute-GvHD treatment are the first and second-line treatment recommendations of the American society for Blood and Bone Marrow Transplantation from 2012. There is a broad consensus on a first line treatment for acute-GvHD grade$\geq$II with prednisolone 2 mg/kg/day or methylprednisolone 2.0 – 2.5 mg/kg/day, with a complete response rate of 40-50% (Deeg, 2007; Martin et al., 2012).

1.14.2 Second line therapy

Photochemotherapy has so far been offered for patients suffering predominantly acute-GvHD of the skin. There is a lack of prospective randomized studies on second line treatment. However, most second line treatments rely on retrospective studies, and there is no consensus on the best second line treatment. The results obtained from these retrospective studies of second-line treatments for acute GvHD suggest several treatment strategies including methotrexate, MMF, photochemotherapy, narrowband ultraviolet B, Extracorporeal photochemotherapy, daclizumab, alemtuzumab, horse ATG, etanercept, infliximab, horse ATG+etanercept, daclizamab+infliximab, daclizamab+infliximab+horse ATG and sirolimus (Deeg, 2007; Feldstein, Bolanos-Meade, Anders, & Abuav, 2011; Furlong et al., 2002; Martin et al., 2012; Wiesmann et al., 1999).

1.14.3 Phase II & III studies

Phase II studies has been carried out based on several treatments including basiliximab, daclizumab, inolimomab, denileukin diftitox, alemtuzumab, horse ATG, mesenchymal cells and the combination of daclizumab and etanercept. Phase III studies have been carried out using horse ATG. At this time-point, no treatment can be determined as superior or inferior to any other. The response rate and the complete response rate at day 28 of treatment, as well as the 6-months survival, are identified as important end-points for future research design of treatment studies of acute-GvHD (Martin et al., 2012).

1.14.4 Etanercept combinations

Two prospective studies of severe steroid resistant acute-GvHD with combinations with etanercept have been carried out and were reported 2015 and 2017.

In the first study, the majority had gastrointestinal acute-GvHD stage 3-4 (n=19) after ASCT and a few (n=5) after donor-lymphocyte-infusion. These patients were treated with a combination of etanercept (anti-tumor necrosis factor-alpha) and inolimab (anti-IL-2 receptor alpha) with a complete response rate of 4/21 at day 28 and a two years survival of 10% (van Groningen et al., 2016).

In the second study etanrecept and basiliximab (anti-IL-2 receptor alpha) were given to a patient group where the majority experienced acute-GvHD of the skin and a minority had visceral acute-GvHD. In this multicenter study of 65 patients, the majority had acute leukemia and was treated with myeloablative conditioning. These patients developed grade III-IV severe acute-GvHD. Treatment using a combination of basiliximab and etanercept showed a higher complete response (75.4% vs. 29.6%, p<0.001), compared to a retrospective cohort identified from the database of the first affiliated hospital of Zhejiang University School of Medicine using all patients (n=27) with severe SR-acute-GvHD before 2009. The patients in the control group received some other second-line salvage treatment including high-dose steroid (2-5 mg/kg) (n=2), FK506 (n=7), CD3 antibody (OKT3)(n=5), CD25 antibody (daclizumab) alone (n=9), or plasmapheresis (n=6) at the discretion of the physicians. The combination etanercept and basiliximab resulted in a 2-year relapse incidence of 19% and a non-relapse mortality of 24.9%, and a one year survival of 62.1% and a two year survival of 54.7%, compared to the previously best reported CR (69%) and 6-month survival of 56% of a retrospective report of predominantly steroid resistant visceral acute-GvHD of the combination of horse ATG and etanercept (Kennedy et al., 2006; Tan et al., 2017).

1.15 THE DEVELOPMENT OF PHOTOCHEMOTHERAPY

1.15.1 First generation:

Phototherapy and photochemotherapy was firstly used 3400 years ago and is the oldest therapy described in human history. Psoralens are tricyclic furanocoumarins found in the common fig, celery, parsley, west Indian satinwood and in citrus fruits (M. A. Pathak, Daniels, & Fitzpatrick, 1962). The compounds are known to intercalate and form crosslinking with the pyrimidines in the DNA strands after they have been photosensitized by sunlight (Figure 7). This concept provides an opportunity for a group of therapies that is the oldest known therapies still in use. Psoralens were administered topically, in the absence of light, and when sufficient amount of photosensitizer were taken up by tissue, exposing the patient to sunlight will then activates the photosensitizer. This therapy has been recommended to cure of the T-cell mediated cutaneous leprosy and the T-cell mediated Vitiligo and can be traced to the sacred book of Atharva Veda (Bloomfield, M.: Sacred Books of the East; Hymns of Atharva-Veda, Vol. XLII, Claredon Press, Oxford, 1897).

"Born by night art thou,
O plant, dark, black, sable do thou,
that art rich in color, stain
this leprosy and the gray spots."

1.15.2 The second generation:

Noble Laurate Niels Finsen (1903) introduced the second generation of phototherapy when he created a lamp that could eradicate cutaneous tuberculosis far more efficient than any other previous therapy. This therapy was the basis for the eradication of cutaneous Tuberculosis in Scandinavia.

"In recognition of his contribution to the treatment of diseases,
especially lupus vulgaris, with concentrated light radiation, whereby he has opened
a new avenue for medical science", "The Nobel prize in Physiology or Medicine 1903"

1.15.3 The third generation:

The construction of fluorescent lamp systems that can provide an even exposure of the total body surface e.g. provide an even exposure over 213 by 122 centimeter where it emits high-intensity radiation between 320 and 390 nm with a peak emission of 365 nm, together with the oral pharmaceutic formulation of 8-methoxysalen opened up the next generation of photochemotherapy, which was a revolutionary cure for psoriasis (Parrish, Fitzpatrick, Tanenbaum, & Pathak, 1974).

Figure 7. DNA Crosslinking agents
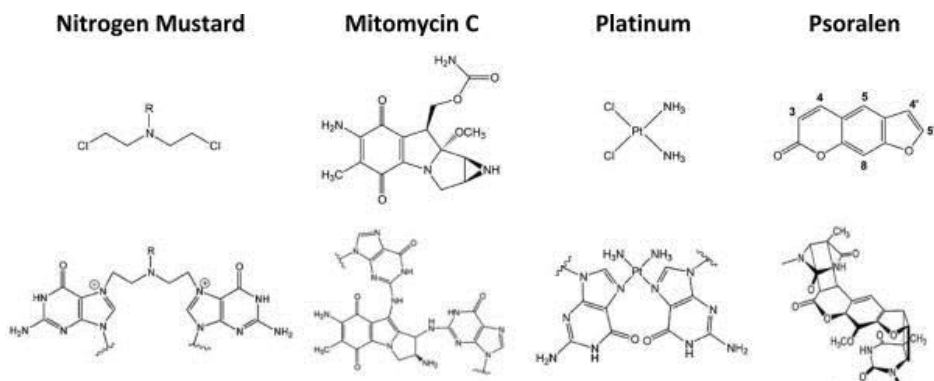
Examples of DNA Crosslinking Agents. The chemical structure of the indicated agent is shown in the top row. The bottom row depicts the nature of the ICL. (i) Nitrogen mustards form a 1,3 ICL between the N7-positions of two G residues on opposite strands within a 5'-d(GNC) sequence. Mitomycin C (MMC; ii) and cisplatin (iii) generate ICLs between juxtapose Gs of 5'-d(GC). (iv) [SC1]Psoralen can produce ICLs between the T residues of 5'-d(TA) complementary sequences upon exposure to UVA. The frequency of ICLs for cisplatin is around 5–10% of the total adducts, about 5% for nitrogen mustards, less than 10% for MMC, and 20–30% for psoralen. Reprinted from Trends in Biochemical Sciences, Vol. 35, David M. Wilson, Michael M. Seidman, A novel link to base excision repair? pp. 247-252. Copyright (2010), with permission from Elsevier

1.16 PHOTOCHEMOTHERAPY IN THE TREATMENT OF CUTANEOUS ACUTE-GVHD

1.16.1 The clinical experience of photochemotherapy on cutaneous acute-GvHD

The clinical experience of the effects of treatment of cutaneous acute-GvHD by photochemotherapy, has been based on measuring the effects in the skin. In 1986, Atkinson K. reported the results of the treatment of three patients, who received HLA-identical sibling transplantation for severe-aplastic anemia (SAA), CML and ALL respectively, with photochemotherapy, at a dose of 0.75-1.25 J daily for four days per week after the administration of 0.6 mg/kg (oral 8-methoxypsoralen) orally. The conditioning regimen was cyclophosphamide 50 mg/kg for x 4 four times for SAA, and cyclophosphamide 60 mg/kg x2 + fractionated TBI 12 gray for the malignancies. Graft-versus-host prophylaxis was cyclosporine and in the last case addition of T-cell depletion, subsequently, the last patient was treated for an acute-cutaneous GvHD stage IV. The patient with ALL started at day 73 post-transplant after a treatment with methylprednisolone since day 11, and antithymocyte globulines repeatedly since day 40, followed by cyclophosphamide 200 mg/day during two weeks and during the period of ten treatments of photochemotherapy, the skin scaling, edema, erythema and petechial decreased in intensity. The treatment had however to stop, since the patient deteriorated in septicemia and died within 100 days post-transplantation of staphylococcal infection.

In 1990, Eppinger T. reported the results of the treatment of 11 patients with cutaneous acute GvHD. The 8-methoxypsoralen photosensitized the skin after oral administration, two hours before irradiation with ultraviolet type A. Four patients with cutaneous acute-GvHD were treated, two with HLA-identical donors who received cyclosporine as GvHD-prophylaxis, and two one antigen mismatched donors who received methotrexate and campath-1 antibody in addition. The conditioning was cyclophosphamide 4x50 mg/kg and etoposide (50 mg/kg/bw) or busulfan (4x4 mg/kg/bw) respectively for the matched recipients, and cyclophosphamide 2x60mg/kg/bw, TBI 12 gray for the recipients who received antigen mismatched grafts, where the last one also received etoposide (30mg/kg/bw). The matched recipients started photochemotherapy at 70 days and 72 days post-transplant respectively and received 23 and 14 treatments with an accumulated dose of 67 and 31.5 J/cm^2, after which they went into complete remission. The patients who received antigen-mismatch marrow and was treated with Campath-1 (an antibody against CD52 on the mature lymphocytes, the monocytes and the dendritic cells) after transplantation showed only had a partial remission and survived only 45 and 250 days, respectively.

In a study of acute- and chronic GvHD, from 1995, Aubin F. et al. reported the results of photochemotherapy of four patients with cutaneous acute-GvHD after HLA-identical sibling grafts. The first three patients with ALL (12 y.o.), AML (6 y.o.) and CML (54 y.o.) received a conditioning regimen of TBI in combination with either cytarabin/ and melphalan for the children, or cyclophosphamide for the adult, before they started a graft-versus-host

prophylaxis of cyclosporine and methotrexate. The cutaneous acute-GvHD was stage III-IV and had an onset at day 18 (range 15-30). The children received photochemotherapy after a line of treatments beginning with corticosteroids, followed by cyclosporine and anti-CD5 & anti-CD8; corticosteroids and additional cyclosporine and thalidomide or anti-CD25 and finally anti-CD25 or anti-TNF. The adult received photochemotherapy as a second-line treatment in addition to initiated corticosteroids and cyclosporine as first line treatment. The start of photochemotherapy was at day median 54 (range 25-66) of acute cutaneous GvHD. These three patients had to a complete response. At seven months the 6 y.o. and the 54 y.o. developed chronic GvHD which responded to therapy. The survival was at least median 42 months (52 – 73) after transplantation. The fourth patient, a 20 y.o. female, who was treated for cutaneous acute-GvHD which started at day 13 after a GvHD-prophylaxis of methotrexate and cyclosporine, had received busulfan, cytarabin and melphalan for ALL. She developed a stage III cutaneous acute-GvHD and was treated with corticosteroids and +cyclosporine and anti-CD25, thalidomide and finally addition of photochemotherapy at day 98. The cutaneous GvHD did not repond to the therapy and the patient was only able to receive three treatments of photochemotherapy before she died in interstitial pneumonia four months after transplantation. The treatments were given two hours after photosensitization with 8-methozysalen with a starting dose of 0.5 J/cm^2 three times a week. The three survivors accumulated median 23 (11-25) treatments with an accumulated dose of 85 (30-90) J/cm^2 (Aubin et al., 1995).

A larger other study came was published in 1999 by Wiessman A. et al. who reported their treatment of twenty patients with cutaneous acute-GvHD after transplantion for different hematological malignancies, i.e. CML, AML, ALL, MDS, CLL and chronic myeloproliferative syndrome. The conditioning regimen was cyclophosphamide (120 mg/kg) in combination with the myeloablative addition of busulfan (16 mg/kg; n=13) and fractionated TBI (12 Gy; n=7). Thirteen were transplanted from a related donor and seven were transplanted with a graft from an unrelated donor. The graft-versus-host prophylaxis was cyclosporine with a conventional basic treatment of methotrexate. Five patients received anti-thymocyte globuline. The lowest degree of acute-cutaneous GvHD included in the study was stage II, i.e. >25% of the TBSA covered by erythema, why it is difficult to determine four how long the skin symptoms had been present until the prednisolone therapy was initiated. The initial therapy of acute-GvHD was prednisolone 2mg/kg and if the cutaneous acute GvHD progressed after three days photochemotherapy was started. The authors used a unique scoring system for skin-disease severity, intensity of erythema x % body surface area + size of bullae x % body surface area affected. The combined prednisolone and early photochemotherapy led to a progression of cutaneous acute-GvHD in four cases, four had relapse of malignancy, and none of these patients had concomitant visceral disease. Only two patients with visceral disease resolved the disease. Overall 55% (n=11) survived, and the overall 50% reduction of prednisolone was 35 days (5-133) and the response rate for acute-GvHD of the skin was 92% (Wiesmann et al., 1999).

In 2002, the by far most numerous experience of cutaneous acute-GvHD treated by photochemotherapy was published by the Seattle group at Sloan Kettering. The study photochemotherapy was initiated at median day 30 (range 11 – 79), and a high ratio of the patients, 92% of the evaluable 95 developed chronic GvHD, but no relapse data was reported. The Kaplan and Meier estimated survival at five years from the start of photochemotherapy as 43%. This study had a majority of unrelated donors 74/103 vs. 29/103 related donors, with a majority who received a cyclosporine plus methotrexate as acute GvHD prophylaxis. The mean number of treatments was 16 (range, 1-78), with a mean cumulative exposure of 41 J/cm^2. The majority had stage III skin disease, with a cutaneous rash that covered > 50% of the body surface area. In this study only patients with hepatic or gastrointestinal disease were treated if the symptoms seemed mild or were resolving (Furlong T. 2002). In both these studies the majority tolerated photochemotherapy well, and in the Furlong study a majority went through a six week therapy (Furlong et al., 2002; Wiesmann et al., 1999).

1.17 CUTANEOUS EFFECTS OF PHOTOCHEMOTHERAPY

1.17.1 Suppression of DTH and carcinogenesis

The delayed type hypersensitivity reaction in the skin of patients treated by photochemotherapy is known to decrease (Moss, Friedmann, & Shuster, 1980). This effect differs from the effect of ultraviolet light type B, as photochemotherapy suppresses the delayed type hypersensitivity reaction independently of the erythematous reaction of the skin in allergic patients (Kalimo, Koulu, & Jansen, 1983).

The modern photochemotherapy was established as a treatment to induce remission in psoriasis before the era of monoclonal antibodies (Parrish et al., 1974). Extensive use (1000 J/cm^2) can however cause skin cancer, a reason for why the therapy should be used in moderate doses. Photochemotherapy has three important co-carcinogens, two where cases of cancer have been described in patients with accumulated doses between (51 – 895 J/cm^2), e.g. ionizing radiation therapy and arsenic exposure, while the third risk factor is skin cancer. The lowest reported accumulated dose is from a single patient who had four years of superficial X-ray therapy between 1942 - 1946 and developed squamous cell carcinoma in 1982 after a treatment of 51 J/cm^2 of photochemotherapy during 1.5 years (Studniberg & Weller, 1993; Torinuki & Tagami, 1988).

1.17.2 Downregulation of costimulation with HLA-class II at the surface

Photochemotherapy further stands out from UVB by the histopathological response in psoriatic patients. In a comparative study of punch biopsies of 4 mm skin, the majority from the glutea, before and after response to cyclosporine, UVB or photochemotherapy, photochemotherapy was reported to more significantly decrease the numbers of CD1a+ cells in the epidermis and in the dermis in psoriatic patient responding to treatment. In the epidermis however, the photochemotherapy does not downregulate the HLA class-II molecules, but do downregulate the co-stimulatory molecule CD86. Cyclosporine alone keeps the CD1a+ cells in the epidermis and the CD4+ and the CD1a+ cells in the dermis.

UVB keeps the CD1a+ cells in the epidermis together with CD68+ expression. All treatments downregulate the CD8+ population in psoriatic skin (Erkin G. 2007) Photochemotherapy has effects on cell-surface molecules associated with adhesion and costimulation on several cell-types in the psoriatic skin (Figure 8) (Stern, 2007).

Figure 8. The effects of photochemotherapy on the psoriatic skin
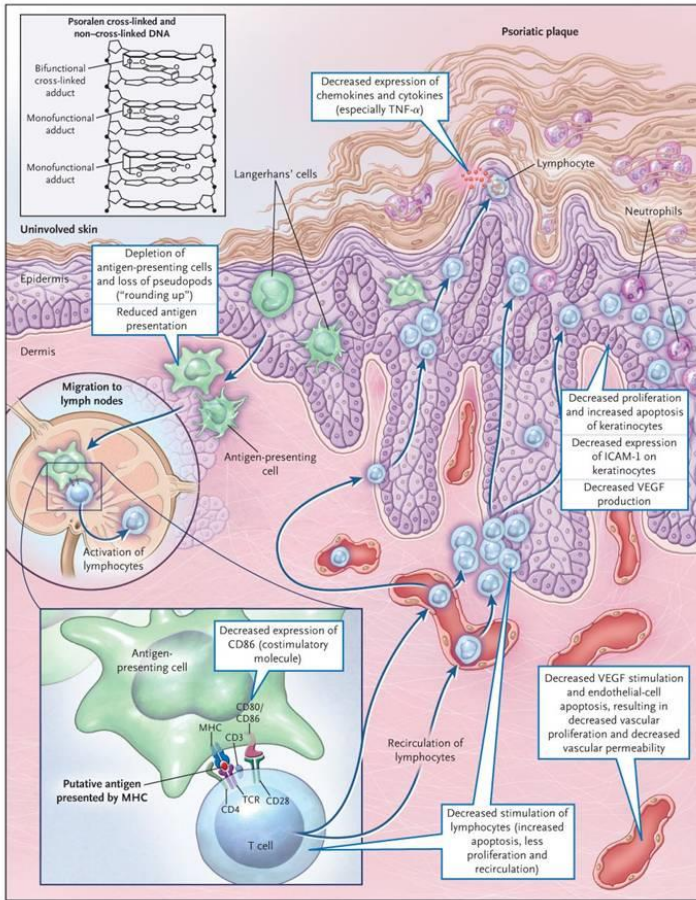
Reproduced with permission from Stern R. 2007: Psoralen and Ultraviolet A Light therapy for psoriasis; New England Journal of Medicine, Vol. 357 pp. 682-90 Copyright Massachusetts Medical Society

1.17.3 Photochemotherapy and tissue-antigen presenting capacity

The in-vitro antigen stimulatory capacity of the epidermal skin seems to decrease after treatment of 8-MOP and UVA. In study of predominantly patients with psoriasis who underwent routine photochemotherapy, but where the lower left arm was shielded from UVA, the difference in cell populations by cell surface markers were compared within the same individual after harvesting of epidermal sheets from both arms by the suction-blister method. The harvested cells were suspended in an epidermal solution and their examined by fluorescence activated cell sorter (FACS) and their allo-stimulatory capacity was evaluated. The overall number of HLA DR+ CD1a+ Langerhan cells in the epidermis decreased dependent on the dose of photochemotherapy received, and at higher doses of a median of 40 J/cm^2 (range 22.8 – 59.7) the decreased number remained down regulated up to ten days, while at a low dose 10.7 J/cm^2 the repopulation was almost total at eight days (89%). The autologous antigen presenting capacity was diminished after concavalin and tetanus toxin provocation, while the capacity to induce an allogeneic response was decreased in epidermal-lymphocyte reactions, and in unsorted epidermal cell mixed lymphocyte reactions, but not after the aliquots of epidermal sheets from the exposed and UVA-unexposed forearms had been enriched by FACS to contain the same number of HLA-DR+ CD1a+ cells (Ashworth, Kahan, & Breathnach, 1989).

1.17.4 The effect on regulatory T-cells

The regulatory T-cells within the CD4+CD25+ cell population were found by Sakaguchi S. et al. (Sakaguchi, Sakaguchi, Asano, Itoh, & Toda, 1995) who transplanted CD25 depleted and CD25 repleted CD4 cells to the athymic nu/nu mice, which lacks their own production of T-cells. After transplantation of CD25- depleted cells, all athymic mice developed autoimmune diseases, including GvHD. Furthermore, the nu/nu repleted CD4CD25+ had a better tolerance towards allogeneic skin grafts, than the CD4CD25- or the CD8+ cell transplants (Sakaguchi et al., 1995). The CD4+CD25+ population has been further investigated, and the identification of the transcription factor FoxP3 more precise defined a cell population within the CD4+CD25+ with a high cytokine production, but with an ability to suppress other T-cells upon cell contact (Hori, Nomura, & Sakaguchi, 2003). The human FoxP3+ population can be separated in at least three populations, among these resting cells (CD4+CD25+RA+Foxp3low) and activated T-regulatory cells (CD4+CD25+RA-FoxP3high), however the FoxP3 population is dynamic and may be diverted into a Th17 population which aggravates an immune response (Miyara et al., 2009).

In classic murine studies, high-dose topical photochemotherapy and subsequent provocation with haptens known to induce DTH has been found to induce suppressor cells in the spleen and in the local lymphnodes, which attenuate the DTH with antigen specificity in syngeneic recipients (Kripke, Morison, & Parrish, 1983; Wolf et al., 2006). Singh et al. could in repeated studies of the K5.hTGF-beta 1 transgenic mice, which display a poriatic skin type, show an increase of the regulatory T-cells after repeated low dose photochemotherapy (0.25 J-0.50 J/Cm^2 twice a week four weeks in a row with a good treatment response (Singh et al.,

2010). In human, the regulatory cells in the CD4+ cell population, (CD25+FoxP3+/CD4+), in peripheral blood increase with bath-PUVA treatment and are associated with with a treatment response of psoriasis after an accumulated dose of 31.2 J/Cm^2 (range 6-54) (Saito C. 2009). The regulatory T-cell compartment can be divided into different subgroups which are suggested to be correlated to their suppressive function. Within the regulatory T-cell group (CD4+CD25+), the cell surface markers CD45RA and FoxP3 can be used to identify cells that are activated (CD4+CD25+CD45RA-FoxP3high) and are suggested to have the most suppressive function (Miyara et al., 2009). The Bath-PUVA has been studied extensively with regards to the induction of regulatory-T-cells and their coupling to the remission of psoriasis, where a shift from circulating Th17 cells to circulating CD4+CD25+Foxp3+ cells have been seen by FACS (Furuhashi T. 2013). In a study of whole blood on psoriatic patients (n=15) with controls (n=11), Bath-PUVA changed the balance between resting T-regs and activated T-regs in patients, while the opposite happens in the healthy controls, which turn from activated to resting T-regs. Furthermore, Bath-PUVA has a maximum induction dose of 15 treatments whereafter the balance in peripheral blood is partly reversed. The subgroup of regulatory T-cells were reported to be evoked most easily in psortiatic patients that are juvenile to Bath-PUVA (Kubo et al., 2017).

In Bath-PUVA, Bath-water containing 8-methoxypsoralen which photosensitizes the skin after which ultraviolet type A irradiation is administered to the skin of the patient, which is considered an atoxic and inexpensive treatment. The remission period of psoriasis has been found to be longer with bath-PUVA (Photochemotherapy after topical administration of psoralen), than with Narrowband UVB (Brazzelli et al., 2008), the same is known to be true for photochemotherapy of the skin (Stern, 2007). Photochemotherapy reduces the IL-6, IL-8. IL-2, Interferon-gamma, and Tnf-alpha in skin biopsies from psoriatic patients, whereas IL-10 was not affected (Olaniran et al., 1996).

1.17.5 Photochemotherapy and Vitamin D

Photochemotherapy elevates the levels of 25-OH vitamin D (Shuster, Chadwick, Moss, & Marks, 1981). Ultraviolet light initially produces 1,25-Dihydroxyvitamin D3 that in murine models induce retinoic acid production in professional antigen presenting cells and the induction of FoxP3 (Kang et al., 2012; Sato et al., 2013). The retinoic acid producing APCs in the skin draining lymph nodes are derived from the dermis (Guilliams et al., 2010) and stimulate a gut-homing, IL-10 producing population of regulatory T-cells (Bakdash, Vogelpoel, van Capel, Kapsenberg, & de Jong, 2015; Iwata et al., 2004).

Hypothethically, the effects of photochemotherapy through the induction of Vitamin D may attenuate lung disease, as increased levels of 1,25-Dihydroxyvitamin D3 in the skin induces regulatory cells in the local lymph nodes with suppressive effects on lung responsiveness in a hypersensitivity murine model (Gorman, Judge, Burchell, Turner, & Hart, 2010). Low levels of vitamin D have also been reported in children and adults with airway hypersensitivity and impaired lung function (Beyhan-Sagmen, Baykan, Balcan, & Ceyhan, 2017; Maatta et al., 2017).

1.17.6 Photochemotherapy in immunocompromised conditions

The immunity against viral infections seems not to be inhibited by photochemotherapy. In patients with advance stage HIV and psoriasis (n=2), prurigo (n=1) or folliculitis (n=3), cutaneous photochemotherapy does not seem to increase the viral load or have any effect on the CD4+ count in the doses of 0.5-1 J/cm^2 (Horn, Morison, Farzadegan, Zmudzka, & Beer, 1994).

1.17.7 T-regulatory cells and remaining leukemia

In addition to the research on photochemotherapy, two aspects have to be taken into consideration when post-transplantation setting is discussed. Firstly, the regulatory T-cell compartment is modified by immunosuppressive drugs (Daniel, Trojan, & Opelz, 2016), secondly, T-regs seem to mediate a part of their regulatory function through cytotoxicity, since their function in an experimental model is coupled to granzyme A (Velaga et al., 2015). The regulatory capacity thus may eradicate cells that elicit the acute-GvHD, but they may also possibly target cells within the leukemia, and thus exercise GvL.

1.18 TRANSPLANTATION OF LEUKEMIA AND THE CLINICAL OUTCOME

1.18.1 Acute Lymphoblastic Leukemia (ALL)

Patients with acute Lymphoblastic Leukemia (ALL) in first remission have a potent graft-versus-leukemia effect if they are transplanted with a related donor. Relapsed leukemia, as well as leukemia with remaining minimal residual disease positivity as well as Philadelphia chromosome positivity or Philadelphia like positivity and T-cell, are at high-risk and should be offered allogeneic stem cell transplantation (Inbar et al., 2017).

1.18.2 Acute Myeloid leukemia (AML)

In Acute Myeloid leukemia (AML) allogeneic stem cell transplantation with HLA-identical sibling donor can offer a curative long-term leukemia free survival in patients in primary complete remission, and in some patients in relapse. The increased availability of unrelated donors can offer the opportunity to find a KIR-mismatched donor if no sibling donors is at hand (Gyurkocza et al., 2017).

1.18.3 Chronic myeloid leukemia (CML)

The incidence of chronic myeloid leukemia is slightly higher for females (1.2-1.7 times), and with an onset at 57-60 years of age, the prevalence is increasing from 10-12/100.000 due to improved therapeutic options (Höglund M 2015). Allogeneic stem cell transplantation remains a curative alternative forCML, as a primary alternative or when chimeric-antigen T-cell therapy fails and tyrosine inhibitor resistance develops (Patel, Wilds, & Deininger, 2017).

1.18.4 The diagnosis of haematological Remission & Relapse

In morphological examination, clinical remission is considered when less than 5% blast cells were found among 200 nucleated cells. Relapse is diagnosed with bone-marrow (BM) aspirates from the iliac crest in local anesthesia and comfort sedation (Diazepam or Midazolam). A BM aspirate is acquired at clinical signs of relapse or at 3, 6 or 12 months after transplantation and then yearly (Remberger et al., 2001).

Early relapse is diagnosed at 5-30% of blasts in the BM-aspirate. More than 30% of blasts in BM aspirates were considered a relapse. Further subtyping is made according to the subtyping of the blasts within each disease, and classification within diagnostic criterias e.g. French-American-British criteria for AML with assorted genetic abnormalities (FAB) (Figure 9). The classification of the malignancy that made the transplantation necessary is at this stage already known. The presence of extramedullary leukemic cells was considered as an extramedullary relapse.

Minimal residual disease & Molecular relapse are diagnosed by reverse transcripts of BCR/ABL in diseases with malignant cells positive for this marker, e.g., ALL and CLL (Mattsson et al., 2000; Provan et al., 1996). Chimerism analysis with PCR amplification of variable tandem repeats (VNTRs) can be used in patients where translocation breakpoints are not present (Mattsson, Uzunel, Tammik, Aschan, & Ringden, 2001).

Figure 9. Cytologic Findings in Bone Marrow Specimens and Peripheral-Blood Smears from a Patient with Subtype M2 AML and the t(8;21)(q22;q22) translocation (FAB); Leukemic blasts.
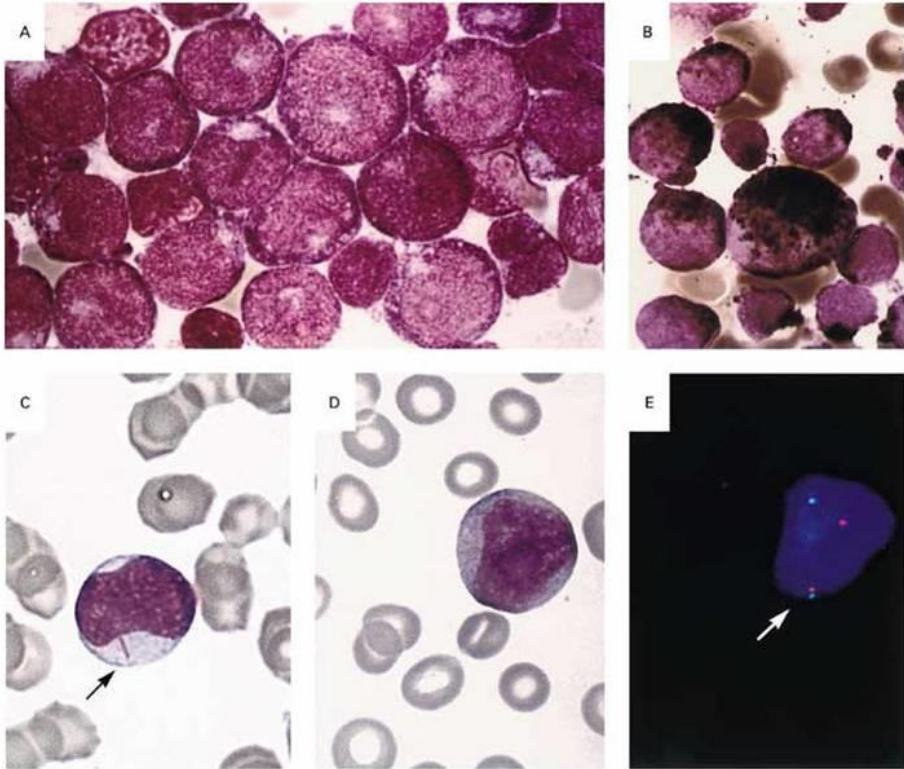
In Panel A, a bone marrow specimen contains medium-sized blasts, cytoplasm with no granulation, and nucleoli that are sometimes clearly visible (May–Grünwald–Giemsa, ¬1600). Panel B shows a bone marrow specimen with myeloperoxidase-stained blasts (¬1600). Panel C shows a leukemic blast with an Auer body (arrow) (May–Grünwald–Giemsa, ¬1600). Panel D shows a blast stained with May–Grünwald–Giemsa in a peripheral-blood smear (¬1000). Panel E shows the results of fluorescence in situ hybridization of the cell shown in Panel D with probes specific for the breakpoint regions of chromosome 8(q22) (isolated green spot) and chromosome 21(q22) (isolated magenta spot) (¬1000). The arrow indicates the chromosomal fusion (clustered green and magenta spots).

2 DISCUSSION OF THE LITERATURE

Leukemia has been a burden for mankind throughout history, and although the field of allogeneic hematopoietic transplantation has branched with haploidentical transplantations (Luznik et al., 2008) and reduced intensity conditioning (Giralt et al., 1997). Still the myeloablative transplantation with matched unrelated donors remains an established therapy with competitive results within ALL, AML and CML (Gyurkocza et al., 2017; Inbar et al., 2017; Patel et al., 2017).

The key question to increase long-term-disease free survival is to decrease the treatment-failure and the two main reasons are recurrence of cancer and GvHD (Gyurkocza et al., 2017; Nassereddine et al., 2017). These two outcomes are found to be associated in several large register studies (Gratwohl et al., 1995; Horowitz et al., 1990; Ringden et al., 1996). The time-point for the initiation of the GvL in relation to the GvHD reaction after transplantation has however not been clarified. Despite that animal studies have suggested that the cell mediated immunity that leads to GvL and GvHD takes a week to be ready to be promoted further (Bortin et al., 1973; Saltzstein et al., 1972) and GvL has been shown to be present two weeks before acute-GvHD after DLI against CML, the chronic GvHD is still by many regarded as the crucial event for disease free survival (Horowitz et al., 1990). This view is however contested by the landmark analysis of the IBMTR patients transplanted in the period 1997 – 2005 (D. Weisdorf et al., 2012). However, two studies identified early cutaneous acute-GvHD as important for the GvL in CML (Gratwohl et al., 1995; Ringden et al., 1996). In addition to this comes the experience from AML in first untreated relapse, where acute GvHD grade II-III is identified as a key factor for a successful eradication of cancer and survival (Appelbaum & Pearce, 2006; Gyurkocza et al., 2017), The temporality of the GvL effect thus may be shifted from chronic GvHD to be evoked by an acute-GvHD which extends into or is followed by a chronic GvHD.

The organ staging of the GvHD organ involvement of the skin, the liver and the gut established by Glucksberg et al. has been stable for over four decades (Glucksberg et al., 1974). When the IBMTR adjusted the overall grading to the outcome data after the first two decades of transplantation, the staging of gut, liver and cutaneous acute-GvHD was not changed (Rowlings et al., 1997). The postmortem report of Beschorner et al. postulated a direct correlation between acute-GvHD and onset of deadly pulmonary disease. The autopsy findings showed an association between bronchopneumonia or extensive acute bronchitis and acute-GvHD grade II-IV (Beschorner et al., 1978). Subsequently, the high incidence of GvHD and IPS in myeloablative transplantation leads to the development of RIC (Giralt et al., 1997; Ringden et al., 1993). There is a shift within the view on lung disease after transplantation, from being regarded as idiopathic, towards being viewed partly as secondary to the immune response evoked by the graft. Lung acute-GvHD is only established within the experimental animal models, where a gut-liver-lung paradigm is predominant. The early lung disease after transplantation remains fatal with a reported mortality of 96% in patients needing mechanical ventilation (Fukuda et al., 2003; Panoskaltsis-Mortari et al., 2011).

Specific reports on the histopathological pattern of lung biopsies in patients with cutaneous acute-GvHD are missing, but after the first 100 days, the fourth and fifth month after transplantation lymphocyte infiltration is general, but beside that, the histological picture in the lung is somewhat diverse, and cathegorized as BOOP, BO, ALI, OB, OP, which however all fits in within the IPS (Panoskaltsis-Mortari et al., 2011; Xu et al., 2013; Yousem, 1995).These reports do not have the numbers to support or dismiss a skin-lung connection after transplantation and the albeit minor histopathological diversity the picture in the lung reported is a picture of lymphocyte infiltration which is similar to what is seen in the skin during cutaneous acute-GvHD. Cutaneous acute-GvHD and biopsy proved upper gastrointestinal GvHD are associated, and both the upper GI and the skin, as well as the lung, may produce elafin, thus if they share the same constitutive expression and the same response to inflammation, they may hypothethically by analogy also be the shared target area for acute-GvHD.

The transplantation demands a conditioning regimen where either TBI or Bu can be used. Ionizing irradiation and photochemotherapy are known cocarcinogens (Studniberg & Weller, 1993; Torinuki & Tagami, 1988). Thus, a possibly stronger effect on cells can be expected by photochemotherapy in the myeloablative TBI group. These groups are therefore analyzed separately. The T-cell depletion in the majority of patients are given as the established in-vivo depletion by ATG ought also to be analysed either as a group or as a predictor, as it is well known that ATG decreases both DTH, but also cell-mediated immunity (Busca & Aversa, 2017; Katsura, Inaba, Izumi, & Uesaka, 1977). The T-cell depletion was coupled to relapse in early reports but with the established protocol of *in vivo* ATG, the relapse risk has decreased (Busca & Aversa, 2017; Marmont et al., 1991). The finding that T-cell depletion not only abolished the GvHD, but also the GvL, established the theory that T-cells mediated both GvHD, but also a significant part of the GvL in human. The definition of GvL is however not stringent. If it is to be the effect of the graft against the leukemia, the GvL should not extend when the graft-failed, or monitoring with cytogenic – or molecular sampling gives the opportunity to use a DLI to prevent a fulminant relapse. To be stringent, GvL should be only the effect of the transplanted graft. Relapse incidence should instead measure the total quality of the care given at the center, including the protective effects of the DLIs that are given. This separation of GvL from relapse incidence does make the effects of the anti-cancer effect of the conditioning regimen clearer and it further allows for transparent evaluation of the graft-versus-host prophylaxis and theGvHD.

There is no recommended second line treatment after the first line of corticosteroids. The combination with etanercept and basiliximab in mostly severe cutaneous acute-GvHD is promising; with 75.4% CR but holds a two year survival of 54.7% and a relapse incidence of 19%. These results should be compared to the studies of severe cutaneous acute-GvHD by photochemotherapy, where Wiesmann et al. had grade II-III of cutaneous acute-GvHD with a response of 92% and a 55% survival with 20% relapse (absolute numbers), and Furlong et al. had the majority grade III of cutaneous acute-GvHD with 57% response and 51% survival at 129-1883 days. It is, however, difficult to compare these numbers, as it is at least fifteen years

between the study of etanercept and basiliximab and the studies of photochemotherapy, and the results of transplantation has improved world-wide due to improved protocols and supportive therapy.

Photochemotherapy has a high potential to induce interstrand links when compared to other DNA crosslinking agents as Nitrogen Mustard, Mitomycin C and Platinum (Wilson & Seidman, 2010). This comparison underscores the biological effects of photochemotherapy. The photochemotherapy is described to decrease the antigen presenting cell load and the costimulatory capacity of the skin which are essential for the T-cell maturation and proliferation that causes GvHD. Photochemotherapy promotes apoptosis of cells in the skin, and after phagocytosis of apoptotic dendritic cells, the phagocytizing dendritic cell aquires the ability to induce antigen specific regulatory T-cells, to the antigen, that the apoptotic dendritic cell was presenting (Kushwah et al., 2009). Recently focus on the mediation of the effects of photochemotherapy have shifted to lower doses of topical photochemotherapy and bath-photochemotherapy where an induction of regulatory T-cells has been found both in animal and in human (Furuhashi et al., 2013; Kubo et al., 2017). Regulatory T-cells are missing in gastrointestinal biopsies from patients with intestinal GvHD after UVB (Rieger et al., 2006), and an experimental murine model of acute-GvHD of the gut has shown a curative effect of UVB coupled to the induction of regulatory T-cells in the gastrointestinal tract. The mechanism behind suberythemal ultraviolet light induced effects on experimental murine acute-GvHD after allogeneic bone-marrow-transplantation, (C57BL/6 to BALB/c), was studied in a routine TBI protocol without control group. The results showed an increased survival and a gastrointestinal acute-GvHD that resolved, associated with an increased infiltration of regulatory T-cells in the viscera. In addition, a decreased incidence of interstitial pneumonitis syndrome (IPS) was noted, together with an increase presence of regulatory (FoxP3+) cells in the lungs of the hosts (Hashimoto et al., 2016). An analogy would be that photochemotherapy mediate cure in the same way, by inducing regulatory T-cells, which home to the gut. This would be intriguing since both bath-PUVA and photochemotherapy have longer lasting effects compared to UVB in psoriasis (Brazzelli et al., 2008; Stern, 2007). One mechanism behind this could be that photochemotherapy induces Vitamin D3 (Shuster et al., 1981), which stabilizes the inhibitory T-regulatory function and increases dendritic cell production of retinoic acid which in turn increases the gut homing of regulatory T-cells (Bakdash et al., 2015; Iwata et al., 2004; Kang et al., 2012; Sato et al., 2013).

There is a controversy of whether there is a predominant Th1 or a predominant Th17 pattern within the cutaneous acute-GvHD (Broady et al., 2010; Bruggen et al., 2014; Reinhardt et al., 2014). It is not clear whether this is secondary to different modalities of conditioning regimens, with more RIC therapies in the Th17 studies, whether it depends on the laboratory methods used, or if it depends on the time-point for the skin biopsy compared to the onset of the cutaneous acute-GvHD, were the Th17 biopsies may have been later in the disease. Photochemotherapy, however, has been associated both with a downregulation of Th1 cytokines in the skin (Olaniran et al., 1996), and a shift from the th17 towards the regulatory

T-cell population (Furuhashi et al., 2013), why this controversy over the cytokine profile fits well with photochemotherapy, regardless of the outcome. There are indications between an association between the skin and the gastrointestinal tract, indicated by clinical retrospective report (Martin et al., 1990) and from histopathology studies where concomittant cutaneous acute-GvHD has a slightly elevated specificity and sensitivity for GvHD confirmed by a biopsy from the upper gastrointestinal channel (Roy et al., 1991; Wu et al., 1998). In the experimental transplant model with TBI conditioning regimen, the regulatory T-cells induced by phototherapy (UVB) was found in the gut but also in the lung (Hashimoto et al., 2016). This suggests that photochemotherapy may have an effect on the adverse immunity in the lung too. In addition, the Vitamin D3 induced by photochemotherapy (Shuster et al., 1981), has been suggested to be deficient in lung disease (Beyhan-Sagmen et al., 2017; Maatta et al., 2017). To conclude, when treating patients with cutaneous acute-GvHD, the efficacy of photochemotherapy is at the same level as other therapies. The relapse incidence or survival does seem to be in comparison to the latest antibody therapy of acute-GvHD. Systemic effects of photochemotherapy, beyond the indications of circulating regulatory cells, are not generally acknowledged.

3 THEORY AND AIM OF THE THESIS

3.1 THE THEORY IN USE

Treatment with photochemotherapy of the skin can be made without any effects on internal disease; i.e., without effects on pathological process in any organs beside the skin. Photochemotherapy is used to avoid a general immunosuppressive effect by steroids or other immunosuppressive pharmaceutic therapy.

Cutaneous acute-GvHD is known as the most common manifestation of GvHD (Martin et al., 1990). The clear important logic interpretation of a theory of a selective effect on cutaneous acute GvHD by photochemotherapy would be: when cutaneous acute-GvHD erupts, photochemotherapy is the "Holy Grail", the treatment that may separate the systemic graft-versus-leukemia from the graft-versus-host disease in the skin.

The prevalence of a paradigm of a selective immunosuppressive effect on the skin by photochemotherapy was supported by the previous studies of photochemotherapy where no measurement on relapse or visceral disease was assessed (Atkinson, Weller, Ryman, & Biggs, 1986; Aubin et al., 1995; Furlong et al., 2002; Wiesmann et al., 1999).

The current praxis is that photochemotherapy is used for acute-GvHD predominantly in the skin (Deeg, 2007).

3.2 THE STRENGTH OF THIS THEORY:

The theory of an effect selective to the skin is simple, "what you treat is what you see". It focuses on the easily understandable view, that if you treat a disease in the skin, you should treat the skin. It relays on the tradition of that the photochemotherapy is used to treat diseases of the skin since ancient time. With a predominant perception that GvL is a systemic process and that GvL is most dominant in chronic GvHD or at least a chronic GvHD that follows an acute GvHD, photochemotherapy of the skin early after GvHD would not be an issue for the adoptive tumor immunity.

3.3 THE WEAKNESS OF THIS THEORY:

The theory does not consider the skin as the biggest organ in the body. It does not acknowledge the systemic effect which antigen provocation through the skin has been proven to have, evident by the effects of intradermal vaccinations (Bacille Calmette-Guérin by Mantoux technique). In addition, the theory of a selective effect on the skin is in conflict with animal studies where systemic suppressor cells were found (Kripke M.L. 1983; Wolf P. 2006) Further, these studies pointed out that to suppress a delayed type hypersensitivity and induce cell that could be transferred to a syngeneic animal, you would have to treat unaffected skin, this is also in conflict with the theory as it is an acute-GvHD, predominantly in the skin that is the indication for treatment (Deeg, 2007).

3.4 THE QUESTION

The question to be elucidated was if the theory was true, did photochemotherapy not have any systemic effects after transplantation? Was photochemotherapy the "Holy Grail" of transplantation? Could photochemotherapy separate GvL from GvHD? These question is of high relevance for the field of transplantation as recurrence of disease are the most common cause of treatment failure, and GvHD is the second most common cause of death after transplantation, and cutaneous GvHD is an important predictor of GvHD treatment failure when concomitant visceral GvHD disease is percent (McDonald et al., 2017; D. Weisdorf et al., 1990).

3.5 GENERAL AIM

To test if the theory that the effect of photochemotherapy is confined to the skin could be falsified.

3.5.1 Specific aims

1. To evaluate the effect of photochemotherapy on acute-GvHD in relation to the stage of cutaneous acute-GvHD, the dose of photochemotherapy, and the previous therapies.

The efficacy of photochemotherapy on disease beyond the skin would be tested on:

- The GvHD of the liver and the gastrointestinal tract
- The effect on pulmonary mortality

2. To investigate the safety of photochemotherapy in cutaneous acute-GvHD with regards to GvL in relation to temporality between the onset of cutaneous acute-GvHD and start of photochemotherapy:

- No effect would be seen according to theory?
- An early start of treatment would be longer from chronic GvHD and safer?
- An early start would abrogate a cell mediated response that started together with acute-GvHD?

4 METHOD

4.1 FORMATION OF A COHORT

The patients transplanted at Karolinska University Hospital Huddinge between 1980 – 2005 who had signed written consent for prospective data collection (425/97) were assessed for eligibility by two researchers who screened the CAST-register, and by two researchers who screened the archives at the Dermatology department. The results were double checked with the records at Haematology and the prospective charts at CAST by one researcher and one physician, and one physician and one researcher reviewed the acute-GvHD diagnosis and the steroid resistancy (Le Blanc et al., 2008). A cohort of 116 transplants in 115 patients was presented in Firenze at the 34 th EBMT meeting 2008. The primary cohort data was reported to and retrieved from the CAST-database based by written consent to ethical permission 425/97. The studies followed the declaration of Helsinki and were approved by the local ethical committee by ethical permission 425/97 and 2012/969-31/3, with addendum 2014/1569-32.

4.2 DESIGN OF DISEASE SPECIFIC FOLLOW-UP

The ten year follow-up was organized by disease, and clinical uncertainties concerning the follow-up data was resolved after control with the patient records together with Omazic B., Ringdén O and Emtestam L. The following diseases were investigated in patients with cutaneous acute-GvHD. The studies were designed to falsify the theory of photochemotherapy as inducing effects isolated to the skin.

Study I: Patients with GvHD of the liver and the gut (n=33), a flowchart of the study design is shown in **Figure 1., Study I.**

Study II: Patients with acute-leukemia (n=47) assessed for effects on tumor immunity (GvL), the study design is shown in **Figure 1., Study II.**

Study III: Patients with disease only in the skin (n=79), assessed for effects on the lung, the **Figure 1., Study III.,** provides a flowchart of the study design.

Study IV: Patients with chronic-leukemia with HLA-ID related donor, (n=22) assessed for effects tumor immunity (GvL). A reference group was formed with comprising patients (n=22), who had received T-cell depletion, either ex-vivo, or in-vivo (ATG). The study design for **Study IV** is shown in **Figure 1., Study IV.**

The follow-up data was provided from the CAST-database (co/ Uhlin M. Remberger M. and Mattsson J). The reports were based on the STROBE protocol and ClincalTrials.gov was used for protocol registration for **Study** 2 and 3, identifier NCT02631993 and NCT03320928 (NLM at the NIH).

4.3 TRANSPLANT CHARACTERISTICS

The disease risk was stratified into high-risk and low-risk (Alyea et al., 2010; Ringden et al., 1988). The donor and the recipient were matched for HLA-A, HLA-B, and HLA-DR. The predominant conditioning regimens were based on cyclophosphamide together with myeloablative total-body-irradiation or myeloablative busulfan (Hassan, 1999; Ringden et al., 1996). The majority of the unrelated donors received anti-thymocyte globulin (ATG) (Busca & Aversa, 2017; Ringden et al., 2000). The graft-versus-host prophylaxis was mainly based on methotrexate with cyclosporine; a minority of the patients had single therapy of either methotrexate or cyclosporine (Ringden et al., 1993; Storb et al., 1986).

4.4 ACUTE CUTANEOUS GVHD OF THE SKIN

The skin disease was stratified at the start of photochemotherapy, at the end of photochemotherapy and up-until fourteen days after photochemotherapy. The skin disease was diagnosed based on the clinical presentation together with a skin biopsy when possible and required. The skin diagnose was stratified by the total body surface area (TBSA) covered with rash according to Glucksberg et al. and the IBMTR severity index (Glucksberg et al., 1974; Rowlings et al., 1997).

4.5 TUMOR IMMUNITY

Adoptive immunity with preserved anti-tumor immunity was considered present if the patient did not have relapse of malignant disease after photochemotherapy, or minimal residual hematological malignant disease (MRD) that demanded a donor-lymphocyte infusion (DLI) (Sairafi et al., 2010). Relapse or MRD were diagnosed and followed-up as mentioned in 1.18.4, and was reported to the CAST database. Death or graft-failure, including retransplantation or booster not triggered by relapse or MRD (Remberger et al., 1998), was considered as a competing event.

4.6 DISEASE OF THE LIVER, GUT, AND LUNG

The disease of liver and gut was staged on the same time-points as cutaneous acute-GvHD. The lung disease was considered significant if the patient died from an infectious or non-infectious cause with presumed pulmonary tissue involvement and comprised pneumonia, IPS with or without pulmonary infection, interstitial fibrosis and undefined respiratory insufficiency.

4.7 PHOTOCHEMOTHERAPY

The skin was photosensitized with 8-methoxysalen (0.4-0.8 mg/kg) orally two hours before irradiation with Long-wave UVA. Long-wave UVA (320-400 nm) was administered with the full-body UV1000 supine unit (Waldmann, Villingen-Schwenningen, Germany) with 26 Waldmann F85 100-W fluorescence photochemotherapy lamps or Waldmann UV3003K half-body unit with 15 Waldamann F85 100-W photochemotherapy lamps. The total dose of photochemotherapy was measured as the total number of treatments, the dose J/cm^2 per

treatment and the total accumulated dose J/cm^2. The genital area of males was shielded during irradiation, and eye protection was used 24 hours after the oral administration of 8-methoxysalen (Parrish J.A. 1974; Henseler T. 1981).

4.8 ADDITIONAL GVHD TREATMENT

Photochemotherapy was primarily started when the GvHD was steroid resistant after persistent GvHD after one week of prednisone (2mg/kg i.v.) or on the recurrency of a steroid taper, but was also started as single therapy, or concomittant with steroids. Methotrexate was given concomitantly with photochemotherapy as a part of the GvHD-prophylaxis protocol or as a GvHD treatment. Additional GvHD treatment was recorded when present.

4.9 STATISTICS

Descriptive statistics were used to describe the patient- and transplant characteristics. Non-parametric statistics were used to compare confounding associations between groups.

Cox-F-test was used to compare difference between groups, Cox-Proportional Hazards Ratio was used for multivariate analysis, Log-rank was used where no complete events were present in either group to base the cox-analysis on, and Kaplan-Meier was used to depict the groups and to estimate the cumulative survival (Cox D.R. 1964; Kaplan E.L. 1958). The events per variable was controlled to ensure the stability in the multivariate analysis (Peduzzi P. 1995). Statistica 64, 1985-2015, Dell Inc. (2015). Dell Statistica (data analysis software system), version 13. Software dell.com was used for the statistics.

5 THE RESULTS

5.1 STUDY I.

The majority of the patients, 94% (31/33), were transplanted for hematological malignancies, with a homogenous myeloablative conditioning regimen in 97% (32/33), which was based on TBI in the majority of the cases, 85% (16/28), and busulfan in 18% (5/28). All but one received cyclophosphamide, and one patient received fludara (RIC). The GvHD prophylaxis was mainly, 76% (25/33), methotrexate and cyclosporine, with six patients who had cyclosporine in monoprophylaxis and three patients who received T-cell depletion. There was no significant difference with regards to the characteristics of the patients, (n=33), in relation to skin disease stage (1-3), at start of photochemotherapy, which can be seen in **table 1, Study I.** The patients, had acute GvHD encompassing one of the visceral organs, liver or the GI in addition to the skin. Liver acute-GvHD was present in 69% (24/33) and GI acute-GvHD was present in 56% (17/33). The patients with most cutaneous acute-GvHD, (stage 3), had least involvement of disease in both the liver and the GI (0/11), compared to (4/11), (stage 2) and (4/10) for the patients with acute cutaneous-GvHD stage 1.

The acute-GvHD was resistant to steroid treatment in (93%) when photochemotherapy started. The patients with the middle severity of acute-GvHD, (stage 2), had a slightly earlier onset of acute-GvHD and photochemotherapy after transplantation compared to the group with stage 1 and the group with stage 3 cutaneous acute-GvHD. The distribution of gastrointestinal tract acute-GvHD in relation to the severity of the cutaneous acute-GvHD can be seen in **Figure 2a, Study I**, and the distribution of liver acute-GvHD in relation to the cutaneous acute-GvHD can be seen in **Figure 2b, Study I**. The characteristics of the acute-GvHD in relation to the skin disease stage (1-3) at start of photochemotherapy can be seen in detail in **table 2, Study I.**

The photochemotherapy dose was higher in the patients that responded completely in their visceral disease, median 11 treatments (range 5-33), (p = 0.03), compared to 6 treatments, (range 1-26) in the patients who did not reach a CR in visceral acute-GvHD. The photochemotherapy in the CR group was given during median 25 days (range 10-94), (p=0.06), compared to median 19 days (range 4-48). The average dose was median 1.55 (range 0.79 – 3.10), vs. 1.25 (range 0.57 – 2.33) and the accumulated dose was median 19.2 J/cm^2 (interquartile range 8 – 40; range 5.79 – 65.0), vs. 11 J/cm^2 (range 4-46) in the patients who did not reach a response, (p = 0.05). The majority of the patients with a complete response in the visceral and the cutaneous acute-GvHD, 81% (13/16), could have a steroid taper (>50% decrease in (mg/kg bodyweight), compared to 29% (5/17) of the patients who

did not respond completely in their visceral disease (p = 0.003). The degree of cutaneous acute-GvHD at start of treatment did not correlate to a steroid taper.

The complete response to cutaneous acute-GvHD in the skin and viscera was 39% (13/33) and the responding patients, (CR + partial response (PR)), was 64%. No response could be seen in 24% (8/33) and 12% (4/33), progressed (CIBMTR classification). Beside the GvHD-prophylaxis, methotrexate was added in two patients who did respond completely, and anti-thymoglobulin or mycophenolate was added to two patients who did not respond. The liver and/or gastrointestinal response in relation to the cutaneous acute-GvHD stage can be seen in **Figure 2b & 2d, Study I.**

The patients who responded in their visceral acute-GvHD, did respond after a lower dose of photochemotherapy (p = 0.04), (n=15), and a lower dose of UVA per treatment, (p = 0.04), (n=16) when they had a less severe cutaneous acute-GvHD, **Figure 3. a & b, Study I,** and a slightly lower accumulated UVA dose (n=13), **Figure 3c. Study I.** The chance of a response in the viscera tended to be higher in the patients with cutaneous acute-GvHD stage 1, (p = 0.07) at day 28, day 56 and day 100, (Figure 4a) **Study I**. The cumulative proportion of GI acute-GvHD with anti-thymocyte treatment as GvHD-prophylaxis during the conditioning (in-vivo T-cell depletion), and liver acute-GvHD, are plotted against the CR in GI acute-GvHD in patients without ATG during the conditioning, p = 0.01)., **Figure 4b. Study I.**

The patients who received total body irradiation in the myeloablative conditioning regimen responded completely in their visceral disease after photochemotherapy at a higher rate (16/28) compared to none of the five patients with myeloablation by busulfan (0/5), (p = 0.045). A post-hoc analysis of pediatric patients is limited to two cases among the patients with TBI and photochemotherapy with (100%) 2/2 cutaneous response, 100% (1/1) in liver acute GvHD and 100% (1/1) in GI acute GvHD. The adult & pediatric experience show a cumulative response among the surviving at 67% (p = 0.034, Log-Rank-Test), when comparing the patients with TBI to those without, and a better 10-year relapse free survival and overall survival (32%), (p= 0.039) and (32%), (p = 0.039), respectively.

The 6-months overall survival was 64%. To start photochemotherapy at cutaneous acute-GvHD stage 1, showed a trend to a better 10-year survival compared to the a more severe cuteanous acute-GvHD (p = 0.096), **Figure 5a. Study I.** The group with CR in visceral disease started photochemotherapy at median 21 days (range 12 – 28) after the onset of acute cutaneous-GvHD. A complete response after photochemotherapy predicted a better 10-year overall survival compared to if visceral acute-GvHD remained after photochemotherapy (p = 0.003), **Figure 5b. Study I.**

5.2   STUDY II.

The outcome of the group with the time-point for start of photochemotherapy within the first week after the onset of cutaneous acute-GvHD and the outcome of the group with photochemohterapy start after the first week of cutaneous acute-GvHD were comparable, since the demographics and transplantation characteristics of the patients at the start of photochemotherapy with regards to the time-to-photochemotherapy after the onset of acute GvHD did not differ, **table 1. Study II**.; The GvHD prophylaxis and treatments, did differ, with regards to corticosteroid treatment **table 2. Study II**.; The GvHD organ disease stage at the start of photochemotherapy and characteristics of photochemotherapy, was similar at the time-point when photochemotherapy was initiated **table 3. Study II.** The time from transplantation to cutaneous acute-GvHD and the time from transplantation to photochemotherapy was lower in the patients who started photochemotherapy early, the median time for start in the early group was day 1 (range 0-7) compared to day 19.5 (8-52), in the group with a more delayed treatment, **table 4. Study II.**

The GvHD answered similarly to treatment, regardless if the photochemotherapy was started early, where 77% (10/13) had CR; 85% (11/13) responded (CR+PR), and there was one non-responder, and one patient progressed, compared to start of photochemotherapy after the first week of cutaneous acute-GvHD, when the CR was 76% (26/34), and 80% responded (CR+PR), whereas three did not respond, and three progressed.

The GvL was predicted by a start after the first week of cutaneous acute-GvHD (p = 0.0018). This more delayed start of photochemotherapy was at day median 19.5 (range 8-52), and the relapse risk was predicted by an early start of photochemotherapy and a lower stage of cutaneous acute-GvHD at start of photochemotherapy (p = 0.004) in the cox-proportional hazards ratio (HR) univariate and multivariate analysis with 5,000 days follow-up after transplantation, for details see **table 5. Study II.** The Kaplan-Meier Curves of the cumulative proportion of GvL coupled to photochemotherapy, and the cumulative proportion of leukemia relapse risk can be seen in **Figure 2 and Figure 3 a& b, Study II.**

The survival with preserved GvL and the leukemia-free-survival were both predicted by a delayed start of photochemotherapy, but not the overall 5,000 day survival, **table 6, Study II.** The Kaplan-Meier curves of the cumulative proportion of survival with preserved GvL, the cumulative proportion of leukemia-free survival and the cumulative proportion of survival are shown in Figure **4 a,b & c. Study II.**

The time-to photochemotherapy after the onset of cutaneous GvHD counted in days, a continous variable, was a predictor for GvL (p = 0.017), in a model which was as valid as

when time-to photochemotherapy was handled a binary variable, with early vs. delayed start (start during the first week vs. after the first week of onset of cutaneous acute-GvHD), (p = 0.003). The multivariate analysis of the time-to photochemotherapy as a binary and a continuous predictor for GvL can be seen in **table 7. Study II.**

5.3 STUDY III.

The majority of the patients, 77% (61/79), received myeloablation by total body irradiation (TBI), – fractionated irradiation was given (3 Gy/day) on four consecutive days to an accumulated dose of 12 grays, and non-fractionated TBI was given as a one day single dose of 10 Gy. Myeloablative busulfan (16 mg/kg), was given in 20% (16/79). In one patient reduced conditioning was given with reduced busulfan (8mg/kg) and in one patient reduced TBI (2 Gy) was given, both in combination with Fludara.

Bone-marrow-grafts were given to the majority of the patients 86% (68/79), whereas peripheral blood stem cells were given to 14% (11/79). Anti-thymocyte globulin was given in 39% (31/79). The graft-versus-host prophylaxis in 91% (72/79) was based on methotrexate, with or without cyclosporine, T-cell depleted bone marrows were given to four patients, and three patients received cyclosporine without methotrexate, two of them together with prednisolone.

At start of photochemotherapy, the acute-GvHD was confined to the skin where acute-GvHD covered was stage 1 and covered up to 25% of the TBSA in 43% (34/79); stage 2 in 41% (32/79) and stage 3 where the acute-cutaneous GvHD covered more than 50% of the TBSA in 16% (13/79) of the patients. The majority of the patients 75% (59/79) had CR of their cutaneous acute-GvHD, and the response, (CR+PR), was 93% (73/79). The CR in cutaneous acute-GvHD after 30 days or less of photochemotherapy was 54% (41/76) and the CR in acute GvHD was 49% (37/76); (MD data for exact finish day of photochemotherapy (n=3)); The CR after 56 days or less was 68% (53/78) for skin and 62% (48/78) in total; (MD for exact day of finish of photochemotherpy (n=1); and the CR after 100 days or less was 75% (59/79) and 68% (54/79) in total (all patients with onset of visceral disease during photochemotherapy were counted as a non-responders). The cutaneous acute-GvHD severity, acute-GvHD prophylaxis and treatment characteristics are shown in **table 2. Study III.** In a non-parametric fisher-exact test, a stage 3 cutaneous acute-GvHD at start of photochemotherapy was associated with a CR in cutaneous acute-GvHD; (p = 0.016); but not with CR in acute-GvHD, (p = 0.10), as gastrointestinal disease started in one patient with cutaneous acute-GvHD stage 1, and three patients with cutaneous acute-GvHD stage 2, of whom one in addition had onset of liver acute-GvHD, whereas one patient with cutaneous

acute-GvHD stage 3 had onset of GI acute-GvHD during the course of photochemotherapy. Methotrexate was associated with an increase in creatinine, ALAT and an increased risk for chronic GvHD. Pulmonary mortality was 19% (15/79) and occurred at median day 217 (range 63-1297). Mortality after relapse occurred at median day 499 (range 97-3396), (p<0.05). The pulmonary mortality associated with opportunistic infections came at median day 122 (range 104 − 217) and pulmonary mortality of non-opportunistic cause came at median 390 days [358 − 892]. A more severe cutaneous acute-GvHD of the skin was the multivariate predictor for pulmonary mortality; HR 4.49, 95% CI [2.05 − 9.82], (p < 0.001). The pulmonary mortality was present in the group treated with TBI, methotrexate and hematological malignancy. In the subgroup treated with TBI (n=61), CR of cutaneous acute GvHD after photochemotherapy protected from pulmonary mortality in a univariate analysis HR 0.34 [0.12 − 0.96], (p = 0.04), but a more severe cutaneous acute-GvHD remained the multivariate predictor HR 4.06 [1.91 − 8.64]; (p < 0.001). The results of the univariate- and multivariate analysis are presented in **table 3, Study III.**

The cutaneous acute-GvHD severity (The TBSA covered by rash), protected from relapse, as it was the inverse predictor for relapse risk; HR 0.51 95% CI [0.26 − 0.99], (p > 0.05); **Figure 3. Study III.** The cumulative proportion of survival and relapse free survival are shown in **Figure 4&5, Study III.**

5.4 STUDY IV.

The majority received myeloablative TBI (10 Gy), (7/8) in the group that started photochemotherapy within a week after onset of cutaneous acute-GvHD, whereas in the group who delayed the start of photochemotherapy after the first week, (10/14) received 10 Gy and one patient received 7.5 Gy. One patient in the group with early treatment start and three in the group with delayed treatment start received busulfan. All patients received cyclophosphamide. The demographics and clinical characteristics of the patients at start of photochemotherapy grouped by the time to photochemotherapy after onset of acute-GvHD did not differ between the groups in the study group (n=22), or in the T-cell depleted reference group (n=22), **table 1A & 2A Study IV.**

The graft-versus-host prophylaxis was mainly Cyclosporine and Methotrexate; one in the group which started photochemotherapy more than one week after the onset of cutaneous acute-GvHD received single methotrexate prophylaxis and one received cyclosporine. There was one female to male donor-recipient relationship in the early group. The group who started photochemotherapy within one week had only one steroid resistant acute-GvHD (1/8), whereas the majority (13/14) in the delayed group were steroid resistant, (p < 0.001).

The dose of photochemotherapy (the number of treatments), the dose per treatment (J/cm^2) and the accumulated dose (J/cm2), were similar regardless of photochemotherapy started within a week from onset of acute-GvHD or whether it started after one week of unrelenting cutaneous acute-GvHD, **table 2A, Study IV.**

The cutaneous acute-GvHD responded completely (CR) in the majority, 88% (7/8), in the group who started photochemotherapy within a week, at median 3.5 days (range 0 – 7), from the onset of cutaneous acute-GvHD. Two patients who resolved the cutaneous acute-GvHD had onset of liver acute-GvHD and in one patient the cutaneous acute-GvHD was persistent and in addition a GI acute-GvHD had developed. In the group where photochemotherapy was started after one week of cutaneous acute-GvHD at median 21.5 days (range 8 – 40) after the onset of cutaneous acute-GvHD, the GvHD responded in 93% (13/14), (CR+PR) and responded completely (CR) in 86%, 12/14. The remaining patient progressed into a fulminant stage IV cutaneous acute-GvHD. There was an onset of liver acute-GvHD in two patients who had resolved their GvHD in the group who started photochemotherapy after the first week of cutaneous acute-GvHD. In the ex-vivo or in-vivo T-cell depleted reference group, who received unrelated transplants, there was a higher degree of antigen-mismatched transplants and the patients who started photochemotherapy after the first week of cutaneous acute-GvHD, at median 15.5 days (range 8 – 43), had a lower degree of CR of cutaneous acute-GvHD. The doses of photochemotherapy was comparable, with a tendency to a lower dose per treatment in the T-cell depleted patients who had received photochemotherapy within a week of onset of cutaneous acute-GvHD, at median 3.5 days (range 0 – 6) (p = 0.06), **table 2B. Study IV.**

The group who started photochemotherapy after the first week of cutaneous acute-GvHD (n=14) had a stronger GvL effect compared to the patients who started photochemotherapy within a week from the onset of cutaneous acute-GvHD (p = 0.038 cox F-test), **Figure 3A Study IV.**

The relapse incidence did not differ with regard to the time-to treatment between onset of acute-cutaneous GvHD and start of photochemotherapy in the depleted control cohort, (p = 0.49 log rank), **Figure 3B, Study IV**

Three patients in the non-depleted group had received anti-thymocyteglobulin after transplantion, the GvL remained strong in these patients, who all were in the HLA-identical group, who started treatment at more than one week after the onset of cutaneous acute-GvHD, (n=11) vs. (n=8), (p = 0.022), **Figure 3C. Study IV.**

The ten-year survival estimate was 50% (Kaplan-Meier) when photochemotherapy started more than one week after the onset of cutaneous acute-GvHD, this trended to be superior to

start of photochemotherapy during first week of cutaneous acute-GvHD (p = 0.09) overall survival, (p = 0.07) leukemia free survival, **Figure 4A and 4B, Study IV**. Likewise, the survival and the leukemia free survival was slightly better in the patients that received ex-vivo or in-vivo T-cell depletion concomitant with the transplantation and started photochemotherapy after the first week of cutaneous acute GvHD, **Figure 7A and 7B, Study IV**.

Chronic GvHD had a cumulative incidence of 80%, in the patients who were transplanted without a depletion procdure, i.e. (5/8) and (10/14), whereas the incidence of chronic GvHD in the group with T-cell depletion during the transplantation was 70%. In a landmark analysis, where the median day of chronic GvHD onset was used (day 171), GvL was not improved by acute- and chronic-GvHD Figure **5A., and Figure 6A & 6B, Study IV.** In the T-cell depleted transplantations, which includes the unrelated transplantations where anti-thymocyteglobulin was used, chronic-GvHD did not improve the GvL, **(Figure 5B. and 6C-D), Study IV.**

6 THE DISCUSSION OF THE THESIS

The key result of **Study I** is the association between total body irradiation (TBI) in the conditioning regimen and the complete response (CR) in visceral acute-GvHD by photochemotherapy in patients after clinical transplantation. This was further supported in a post-hoc analysis, where TBI predicts both visceral GvHD response and long-term overall and relapse free survival. An explorative analysis indicates that ATG may inhibit the effects in the viscerawhich suggest that the T-cells depleted by ATG is present in the mediation of the systemic effect after photochemotherapy. The results support a biological gradient where photochemotherapy has a stronger effect when a lower degree of the skin is affected by cutaneous acute-GvHD. The response of the acute-GvHD is well within the range of established systemic immunotherapies used as secondary therapies against acute-GvHD (chapter 1.14). The results suggest a systemic effect of photochemotherapy after transplantation.

The relation between total-body-irradiation (TBI) and complete response (CR) in visceral disease after photochemotherapy is an intriguing result. The mechanism of an increased response when the DNA-damaging photochemotherapy follows after TBI is suggested to be secondary to the additional DNA-damage (**Study I**), but there may be a synergistic effect. At 30-days after TBI DNA double strand lesions remain, which theoretically would increase the density of underwound DNA in the recipient cells in the skin. The remaining double stranded lesions not only add to the load of the total-DNA-damage, in addition, in the repair process the supercoiling of the DNA-molecule is changed into an under wound state which makes DNA repair possible (Cech & Pardue, 1977; Corless & Gilbert, 2017), and furanocoumarins, like psoralen, are more likely to bind to the DNA in the underwound state (Cech & Pardue, 1977). This repair process may hypothetically still be ongoing to repair lesions that are not yet cured thirty days after TBI when photochemotherapy started. Thus there may be a synergy between ionizing irradiation and furanocoumarin-based therapies like photochemotherapy. During the peer-review process of **Study I**, Hashimoto et al. submitted and published a paper on the induction of regulatory T-cells by UVB which heal gastrointestinal acute-GvHD caused by transplantation after a conditioning regimen by TBI in an experimental murine model. This finding has been discussed in chapter 2. In comparison to **Study I**, Hashimoto et al. has no other conditioning regimen, and one cannot say that the results presented are dependent on the effects remaining after the TBI; however the mechanistic presentation of the induction of regulatory T-cells after phototherapy and the

effects on acute-GvHD in the liver, lung, and gut is substantial (Chapter 2). An increased level of regulatory (FoxP3+) T-cells is well documented in human after Bath-photochemotherapy (Furuhashi et al., 2013; Kubo et al., 2017). In clinical transplantation, a dysregulation of the conventional T-cell ratio (CD8+) and the regulatory T-cell ratio is described in the small- and the large intestine (Rieger et al., 2006). In **Study III** we find that a CR of cutaneous acute-GvHD after photochemotherapy possibly is associated with a decreased incidence of pulmonary mortality in patients who has received a conditioning regimen with myeloablative total-body-irradiation (TBI). The first time an increased response on acute-GvHD after phototherapy (UVB or UVA+psoralen) has been associated to TBI is in the **Study I** comparison with chemotherapy (busulfan) myeloablative conditioning. The results from **Study I**, i.e. that photochemotherapy of the skin after a conditioning with TBI may resolve acute systemic GvHD, e.g. liver and gut, is strengthened by the findings in **Study III**, where a decreased pulmonary mortality is present after CR in the cutaneous acute GvHD after treatment with photochemoterhapy. These results from a study of patients with cutaneous acute-GvHD and liver and/or gut acute GvHD (**Study I**), and from a study where CR in cutaneous acute-GvHD by photochemotherapy decreases pulmonary mortality in patients treated by TBI (**Study III**), support a theory where photochemotherapy has effects on acute-GvHD during the first 100 days after a conditioning with myeloablative TBI.

Study III suggests that cutaneous acute-GvHD predisposes for pulmonary mortality. The perspective in this **Study I**s from the clinical presence of a well-defined skin disease towards the cause of death secondary to a decreased organ function in the lung. It has long been well-known that a patient after allogeneic stem cell transplantation that is in need of mechanical ventilation has a fatal prognosis (Fukuda et al., 2003). In addition it is well known the lung function during the first year after transplantation is decreased, especially in patients with acute GvHD. But from the opposite point of view, from a histopathological perspective the association between cutaneous acute-GvHD and a particular pulmonary histopathology seems unlikely. In two reports of the early histopathological pattern after cutaneous disease five out of five patients are placed into different groups secondary to the histopathological pattern found in lung biopsies, e.g. cicatrial bronchiolitis obliterans (BO), bronchiolitis obliterans organizing pneumonia (BOOP), acute lung injury (ALI) and bronchiolitis obliterans syndrome (BO), (Xu et al., 2013; Yousem, 1995). An evaluation from the histopathological perspective thus only may reveal cutaneous acute-GvHD as a part of the causes of each different pattern of pulmonary acute-GvHD. However, in all different patterns, there is however an infiltration of lymphocytes typical for acute GvHD (Xu et al., 2013; Yousem,

1995). The histopathology and the search for causes of pulmonary mortality in human gets even more diverse when infections are taken into account, which were thoroughly excluded from these retrospective histopathological studies discussed here and in Chapter 1.6.13. However, infections are common in the lung of the patients with immunosuppression secondary to acute-GvHD and acute-GvHD high dose steroid treatment. A comparison to smoking, which damages the lung, reveals from the histopathological viewpoint different disease patterns which each may lead to death, e.g. chronic obstructive pulmonary disease (COPD) with severe pulmonary emphysema or with an acute excacerbation by infectious ethiology and as a final point different lung cancers. From each of these predictors, it is in a minor set of patients not as easy to identify the tissue damage of smoking as the common cause. However, if the outcome is set to "death in pulmonary mortality", in the same way as it was set secondary to evaluate the tissue damage the patient were exposed to associated with cutaneous acute-GvHD in **Study III**, a biological gradient (a dose-response correlation) may be evident also in a limited group of patients. The results of **Study III** are straightforward, the association between cutaneous acute-GvHD severity and pulmonary mortality is highly significant, highly prevalent and death comes early. Furthermore, in temporality, the higher danger of an opportunistic infection is evident, as viral and fungoid causes of death comes significantly earlier than non-opportunistic causes of death. This emphasizes that severe cutaneous acute-GvHD ought to be regarded as a systemic disease with effects on internal organs. There is a high risk of concomitant systemic involvement, were the histopathological pattern of the disease in the tissue in specific can be diverse. The patients that are transplanted are a population of patients with severe disease, often with a long an compromising previous medical history, and a long period of immunosuppression, where the lung is an organ that is at high risk for viral, bacterial and mucosal infections in the time period before transplantation and as well as after transplantation e.g. before, during and after cutaneous acute-GvHD. Therefore it is suggested that when searching for the organ failure underlying different causes of death with systemic impact after transplantation, infections in the organ and what seems like non-infectious causes shall include a stage where infectious and non-infections histopathological disease patterns, or presumed primary tissue injury, shall be pooled together. The infiltration of allogeneic cytotoxic T-cells in the organ with specificity for the patient tissue, and not for infections, both destroys the organ, but also may allow infections to thrive in the damaged tissue. In addition these patients often are on heavy pharmaceutic systemic immunosuppression, and are for a long time not fully reconstituted in their immune defence which further increases the risk for fatal mold and viral disease. To conclude, this study reveals a high incidence of pulmonary mortality coupled to cutaneous

acute-GvHD, which cannot be revealed if the cause of each infection or each histopathology is analyzed separately. The possible protection against pulmonary death if a complete response in cutaneous acute-GvHD can be reached by photochemotherapy must be investigated further. Furthermore, the **Study III** suggests a biological gradient of systemic damage evoked by cutaneous acute-GvHD. The Chapter 1.6 introduces the systemic inflammation of acute-GvHD, and the upregulation of Elafin that is a marker of cutaneous acute-GvHD, but also of the acute-respiratory-distress syndrome (Chapter 1.6.9). Finally, Chapter 1.7.4 presents the correlation between severe acute-GvHD and lung disease that Beschorner et al. described (Beschorner et al., 1978)). It was known that acute-GvHD grade II or more was associated with severe lung disease after transplantation, but that the pulmonary mortality was directly depended on the TBSA covered by cutaneous acute-GvHD was not acknowledged. The pattern of presumed lung tissue injury after cutaneous acute-GvHD indicated by **Study III** can can be specific to the lung or general to the internal organs i.e. either is this systemic damage of the activated T-cells in cutaneous acute GvHD more fatal specifically to in the lung, which is a site of brisk immune responses (Strieter, Belperio, & Keane, 2002), or either does the cutaneous acute-GvHD exaggerate acute-GvHD generally, including the lung. T-cell driven mediation of early pulmonary damage is supported by the finding showing that T-cell depletion reduces early severe complications in the lungs (Ho et al., 2001).The impact of the increased dose-response effect of photochemotherapy related to a lower degree of cutaneous acute-GvHD found in **Study I**, subsequently must be regarded as confounded by the concomitant increased impact on the internal organs by the severe cutaneous acute-GvHD. The increase in the systemic inflammation may increase the risk for increased organ involvement of acute-GvHD systemically, which includes the visceral acute-GvHD, and thus may make it more difficult to bring the visceral disease into CR. This impose that the visceral disease may be harder to treat by the systemic inflammatory response secondary to an increased stage of cutaneous acute-GvHD and thus need an increased dose of photochemotherapy. The results of **Study I** stands however well out when comparing to other secondary therapies of mainly steroid resistant acute-GvHD (Martin et al., 2012), and with the indications of the effects of cutaneous acute-GvHD on the lung indicated in **Study III**, as well as the well-known increased risk for treatment failure for visceral GvHD with concomitant cutaneous acute-GvHD (McDonald et al., 2017; D. Weisdorf et al., 1990), the results indicate that photochemotherapy induces a systemic immunosuppressive effect with effects on the disease in internal organs,

The good of the treatment of cutaneous acute-GvHD by photochemotherapy is underscored by both **Study I** and **Study III**, especially after a conditioning regimen of TBI. In general, all studies and times encounter, there was a good complete response of cutaneous acute-GvHD treated by photochemotherapy 83% (range 75%-88%), a response which is in similarity to the previous clinical experience of photochemotherapy of cutaneous acute-GvHD (Atkinson et al., 1986; Aubin et al., 1995; Eppinger, Ehninger, Steinert, Niethammer, & Dopfer, 1990; Furlong et al., 2002; Wiesmann et al., 1999);(Chapter 1.16.1). The pooled results of extracorporeal photochemotherapy from eight studies of pediatric acute GvHD has a pediatric cutaneous response of median 80% (50 – 100%), liver median 67% (range 0 – 100%) and gut median 74% (range 57% - 83%) (Alfred et al., 2017). In **Study I**, the cumulative adult- and pediatrics results in the TBI group were a CR of 67% on acute-GvHD, and although the number of patients in **Study I** was limited, the results in the two pediatric cases it was 100%, why the results for photochemotherapy after TBI may be in the same magnitude as extracorporeal photochemotherapy.

The **Study II** is a reports of a regulation of the GvL reaction by timing of a secondary GvHD therapy. The GvL seem to be regulated by the time between photochemotherapy and cutaneous acute-GvHD, in general, but predominantly in the patients without T-cell depletion and/or ATG. This effect is present both in patients with Acute- and chronic leukemia (**Study II & IV**). The window when the tumor immunity (GvL) is elicited is what we know, not defined for any other secondary GvHD therapy. The weak GvL secondary to photochemotherapy during the first week after the onset of cutaneous acute-GvHD as well as the identification of photochemotherapy as a continuous predictor of GvL suggests that photochemotherapy touches important mechanisms within the elicitation of GvL. The time window for the mechanism behind GvL in early animal studies has been identified to be during the first week (Chapter 1.8). This was not perceived as a problem when photochemotherapy was given with the goal to minimize the immunosuppression to the skin, in benefit of the patient and the GvL more than ten years ago. The mechanism behind a possible decrease in the GvL during the first week after the onset of cutanenous acute-GvHD can be caused by the decreased antigen-presentation of the skin, and the change in costimulation caused by photochemotherapy. If we allow ourselves to see GvHD and GvL as adaptive immunity against different antigens on different cell surfaces, GvL could be understood by the same mechanisms that govern GvHD. The first step is tissue damage (we know that the conditioning regimen is important to eradicate cancer (Chapter 1.9, but we do also know that the tissue damage by the conditioning regimen upregulates the antigen

presentation on common tissues, and more so on tissues of hematopoietic lineage, and the leukemia is of hematopoietic lineage). The second step is T-cell activation and proliferation, during this stage a crosspresentation and oligoclonal expansion also may take place. It is during this stage photochemotherapy was administered when it was administered early. Photochemotherapy does downregulate adhesion markers, which makes it more difficult for the T-cells to enter the skin, further photochemotherapy decreases the antigen presentation of the skin as a tissue and does downregulate the costimulatory capacity of the antigen presenting cells of the skin (Chapter 1.17). These mechanisms is suggested to limit the expansion of activated T-cells towards effector T-cells against the antigen presented. Hypothethically, this early photochemotherapy may also decrease the possibility of the adaptive immunity against the cancer, the tumor immunity.

A later start of photochemotherapy was given to a majority of patients with steroid-resistant disease. These patients have at a higher degree been exposed to the systemic inflammatory stage of GvHD (Chapter 1.6.4). It cannot be ruled out that the steroid treatment may have had an effect on the GvL, but the identification of photochemotherapy as a continuous predictor indicates that the role of corticosteroids may be limited. There may be GvL without GvHD in patients that are not treated with steroids (Ringden et al., 2000), why the effect of steroids on GvL in general and in this study may not be critical, why the time-dependent-difference with regard to photochemotherapy ought to be considered as the main result. This said it cannot be clearly delineated if the photochemotherapy weakens the GvL if given early, or strengthens the GvL if it is given late. Besides the more developed systemic immune response with a later start of photochemotherapy, which now may have launched the GvL safely in analogy with what we know about the mechanisms in GvHD, we have the reports of the induction of regulatory T-cells by photochemoterapy (Chapter 1.17). These regulatory T-cells are reported to have a cytotoxic effect against cells of hematopoietic lineage (Chapter 1.17.7), and the leukemia is of hematopoietic linage. Hypothetically, the effects of photochemotherapy may both be an inhibition of adaptive immunity if given early, but also a healer of GvHD by restoration of the regulatory T-cell balance in the tissue, and possibly an eradicator of tumor-cells by the induction of regulatory-T-cells.

The incidence of chronic GvHD were evaluated in **Study IV**, and was found to be (80%), and (70%) in patients with ATG. In general ATG seem to inhibit the effects of photochemotherapy, suggested by the possibly decreased effect on gastrointestinal acute-GvHD in patients treated by photochemotherapy after total-body-irradiation (**Study I**), but also by the indications of a preserved GvL in patients where photochemotherapy was given

during the first week after onset of acute GvHD, in patients given ATG or T-cell depletion and/or ATG during the conditioning regimen (**Study II** or **Study IV**). The use of ATG diminished the dendritic cells and the T-cells in the graft, as an in-vivo T-cell depletion. This suggests that it is cell mediated immunity modulated in photochemotherapy, and is compatible with the hypothesis of regulatory-T-cells as the population that is mediating the effect of decreased tissue damage in the lung and the gut after photochemotherapy of cutaneous acute-GvHD.

There is a controversy over the cytokine pattern in cutaneous acute-GvHD, where recent findings of a Th17 (Bruggen et al., 2014; Reinhardt et al., 2014) pattern is questioning persistent reports of a Th1 cytokine pattern (Broady et al., 2010; Remberger et al., 2003); (Chapter 1.6.5). Whether this controversy is secondary to novel conditioning regimens in the Th17 studies, sampling later on in the time course of GvHD, where also more steroids have been used, or whether it is secondary to differences in laboratory methods is not clear. However, photochemotherapy is reported to both decrease the Th1 and the Th17 pattern in cutaneous disease, why this controversial may be of lesser importance to the clinical utility of photochemotherapy on cutaneous acute-GvHD.

The internal validity of the results has been safe-guarded by the use of register data from the established database at CAST which is an internationally highly regarded transplant center. In addition, objective outcomes were used e.g. complete response (CR) in acute GvHD and time-points for start of treatment, as well as survival and relapse. The major weaknesses of these studies are the limited number of patients which increases the risk of a statistical error, and the retrospective nature which induces a risk for selection bias regarding the basis for the clinical decision on when and on what clinical signs photochemotherapy were to be started. The single center setting do reduce the impact of this bias as the longitudinal presence of a stable cadre of attending physicians were present during the time-interval of the study. The relative strength of the studies is that they comprise highly homogenous treatment regimens of photochemotherapy and highly homogenous transplant protocols with the overwhelming majority of patients myeloablative conditioning regimens (cyclophosphamide and myeloablation with TBI or busulfan) and graft-versus-host prophylaxis (cyclosporine and/or methotrexate) with addition of anti-thymocyte globulin in the unrelated transplants.

The external validity regarding the efficacy of photochemotherapy is limited to acute GvHD within which photochemotherapy most possible has a variable effect depending on the type

of previous treatment, the type of conditioning regimen, regarding which antibodies that may have been used during the conditioning regimen and as previous GvHD therapy, and what cell populations they deplete and for long, e.g. are the cell populations that takes part in the mechanism secondary to photochemotherapy still present or have they been depleted? Photochemotherapy after ATG seem to have less systemic effect as shown in **Study I-IV**. It is not guaranteed that the same effects can be seen in GvhD after transfusion or after liver transplantation.. However, with the findings of the stronger effect after TBI and the temporality of photochemotherapy after transplantation, the present research indicate that photochemotherapy has effects on disease in internal organs after transplantation. Based on the present research I recommend that to optimize GvL photochemotherapy should be withheld the first week of onset of cutaneous acute-GvHD, but if ATG has been used treatment may start earlier if the need is urgent. The present research is however made on a transplant protocol that is in relatively close proximity to the myeloablative protocol of Thomas E.D. which on the otherhand has been close to an established international standard within allogeneic transplantation of acute-leukemia's for over forty years. Patients who received donor-lymphocyte infusions or booster doses of T-cells were not eligible and the use of donor-lymphocyte infusions were considered as a loss of the graft-versus-leukemia effect from the adoptive immunity transplanted. This strict study design limits the validity of the studies of photochemotherapy to treatment after a myeloablative conditioning, after a predominantly HLA-matched transplantation before DLI. Haploidentical transplantation with posttransplantation cyclophosphamide which may have an effect on the T-regulatory compartment early after transplantation (Chapter 1.12.2), has not been studied. There is a need for additional studies of the effects of photochemotherapy after DLI, where GvL has been reported to be elicited before GvHD (Chapter 1.12.7). The majority of the studies on secondary therapies against GvHD are retrospective, and the transparency of the tumor immunity of the **Study I-IV** is of good standard. The present research is done on the immunity adopted from the original graft both with regards to GvHD and GvL, which increases the reproducibility of the results.

To conclude, **Study I & III** suggest that photochemotherapy of cutaneous acute-GvHD has beneficiary effects on the acute-GvHD of the GI and that it may decrease the risk for pulmonary mortality after transplantation, to which cutaneous acute-GvHD is identified as a risk factor. **Study II & IV** support two issues, first, that the first week of initial development of cutaneous acute-GvHD is critical for GvL, second, that photochemotherapy has systemic effects after transplantation. Thus, the theory of an effect of photochemotherapy confined to

the skin is suggested to be replaced by a theory where photochemotherapy has systemic effects after myeloablative allogeneic stem cell transplantation. The time-point for the clinical decision to use photochemotherapy of the skin may improve the disease of the gut, the lung, and the long-term cancer free survival. By comparing the four separate studies of this thesis, I suggest that the optimal time point for start of photochemotherapy in patients with hematological malignancy is at least after the first week of cutaneous acute GvHD.

7 FUTURE PERSPECTIVES

7.1 TRANSPARANT REPORT OF GVL

When it comes to the use of donor-lymphocyte-infusion (DLI), to hinder relapse and treat MRD in allogeneic transplantation, term "window of opportunity" has been established. However, when it comes to studies of secondary therapy, the term of relapse incidence is mostly undefined and it is not outspoken whether or not the use of DLI determines a loss of the GvL that the patient adopted from the primary transplantation. If the DLI succeds and the relapsing cells are eradicated, it is not clear that it is counted as a loss of GvL which is possibly induced by the therapies in place when the MRD became significant in the blood of the patient, or previous by previous therapies. Thus the modulation of the transplanted adoptive immunity by pharmacuetic therapies used to treat acute-GvHD may be underreported. The elaboration of a more transparent separation of GvL and relapse incidence defined as in the present thesis may improve the quality of the reports of clinical trials of GvHD therapies.

7.2 THE TIMING OF PHOTOCHEMOTHERAPY

The timing of photochemotherapy in relation to the onset of cutaneous acute-GvHD with regard to the effects on tumor immunity can be precised further. In the experimental murine studies, the GvL increased until day 6 (Chapter 1.8). In the present study, the lower limit of the group with optimal GvL is from day eight but the median day is during the third week after onset of cutaneous acute-GvHD. With regard to the detrimental effects on pulmonary disease correlated to cutaneous acute-GvHD, a closure of this time-gap with the hope of a better effect on GvHD could deliver an advantage for the patient. Prospective research coupled to photochemotherapy could also increase the knowledge of cutaneous acute-GvHD, with regards to the Th1 and the Th17 profile. The research also would give an opportunity to learn more of the regulatory cell population in regards to the optimal induction dose by photochemotherapy, as well as the effects within different tissues and with regard to tumor immunity. Foremost prospective randomized controlled research of photochemotherapy treatment of cutaneous acute-GvHD is needed for its promising effects on gastrointestinal- and lung GvHD which are great threats against the cure of leukemia. Importantly, a possible attenuation of the adverse effects of irradiation injury of the lung by photochemotherapy would be of great benefit to mankind as an established therapy to prevent from respiratory failure secondary to the acute-irradiation-syndrome is missing today.

7.3 THE COMBINATION OF IONIZING IRRADIATION AND PHOTOCHEMOTHERAPY

The effect of the combination of ionizing irradiation and ultraviolet light type holds promises of a new field of DNA modulation with secondary effects on immunology and cancer therapies. The combination may further have advantages within disinfection of surfaces and cutaneous infections as well as for systemic immunosuppression. The known risk for cancer

in patients with higher doses ionizing irradiation followed by photochemotherapy, where the lowest reported case is the patient who had four years of superficial X-ray therapy between 1942-46 and developed squamous cell carcinoma in 1982 after a treatment of 51 J/cm^2 during 1.5 years (Torinuki W. 1988), sets the limit for exposure below an accumulated does of 50 J/cm^2. The median response dose associated to visceral complete response in **Study I**. was 19.2 J/cm^2, but 50 J/cm^2 was in the fourth quadrant in the patient group with most severe cutaneous acute-GvHD. The vast majority in the present studies responded to lower doses, and the dynamics of the regulatory cell population in psoriatic patients suggest that the appropriate effect can be reached well before an accumulated dose of 50 J/cm^2 (Kubo R. 2017). If a combined apparatus for ionizing irradiation and ultraviolet light type A is further developed, it can be used both extra corporeally and directly towards the skin and tumors. It is not known to what levels the ionizing irradiation may be minimized to provide and additive- or synergistic effect to UVB or photochemotherapy, but since DNA-damage which is sub-lethal to the cell is repaired at a high speed, there is a need to provide the ionizing irradiation and the ultraviolet light if possible as a part of the same procedure to minimize the adverse effect of each source of irradiation. The DNA-damage in living cells are dependent on the dose of ionizing irradiation (Banath, Fushiki, & Olive, 1998). DNA repair processes are present 7.5 at mSv (Nguyen et al., 2015), which is a dose that may be present during computer tomography, why therapeutic effects for severe disease may be within reach using the doses that diagnostic imaging use today. In addition, to the plausible induction of regulatory T-cells of a combined therapy, the field holds promise to give novel knowledge of the coupling between DNA-damage, DNA-repair and the link between DNA-damage and immunosuppression. A dysregulation of regulatory T-cells has been suggested in psoriasis, multiple sclerosis, necrotizing enterocolitis and myasthenia gravis (Danikowski, Jayaraman, & Prabhakar, 2017; Dingle et al., 2013). Estimated from the response of photochemotherapy on acute-GvHD a limited combined dose to treat severe diseases may provide competitive effects compared to the present therapies. A primary goal will be to evaluate the effect in animal models to see if regulatory T-cells can be induced by a combined therapy and to see if these regulatory T-cells in turn may cure acute-GvHD, and possible also provide a granzyme dependent anti-tumor effect within the hematologic cell population.

7.4   STRESSED DNA, A NOVEL TARGET

A fourth field of future research is the possibility to increase the stress of DNA by modest levels of ionizing irradiation or ultraviolet light, to easier insert molecules in underwound

DNA secondary to DNA damage. The DNA can be human or prokaryote or viral, and besides to attach molecules to the DNA, the stress of the DNA-damage can also make it easier to target the process of DNA-repair. Furthermore, a combined therapy can have unexplored antibacterial properties secondary to DNA-damage. Novel ways of eradicating bacteria are needed with regard to the increased resistance against the therapies of today. The process of DNA repair is different between eukaryotes and prokaryotes, why this could lead to the invention of new antibiotics.

7.5 PHARMACEUTIC MODULATION OF ACUTE-GVHD TO ERADICATE CANCER

There is a growing awareness that acute-GvHD may crucial to succeed to cure the high-risk cancers which may be the mainstay indication for future myeloablative transplantation when pharmacuetic- and cellular immunetherapies e.g. tyrosinase kinase -inhibitors or chimeric antigen receptor (T-CAR) therapies, have failed or does not match with the patient diagnosis (Chapter 1.1 & 1.18). I propose that the importance of acute-GvHD will continue to grow in importance, and with that will come the need of a detailed knowledge on how GvHD therapies will regulate the associated anti-tumor immunity that is the key to patient survival. In the post-transplantresearch we can rely on the knowledge from the development of graft-versus-host prophylaxis, (Chapter 1.4 and Chapter 1.12.1 & 1.12.2), we do not know upfront, what effects a certain therapy will have on a certain type of GvL, against a certain type of cancer, with a certain type of GvHD. We can only continue to measure, continue to do our physical assessment, sample our biomarkers to improve the information upon which we make our clinical decision making and continue to assess the clinical outcome. Thus, future studies on the regulatory effect on GvL of acute-GvHD therapies with regard to the timing after the onset of acute-GvHD, with regard to the systemic biomarker pattern, the changing cell populations during the different phases of acute-GvHD and secondary to the impact of the previous GvHD-prophylaxis and GvHD-therapy as well as the underlying malignancy are warranted.

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