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Graft-versus-Host Disease | Julia Cromvik

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Eosinophils, Chimerism and Clinical Features in Patients Undergoing Allogeneic Hematopoietic Stem Cell or Multivisceral Transplantation

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Till min familj

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ABSTRACT

Graft-versus-host disease (GVHD) is a potentially severe complication that may develop after allogeneic hematopoietic stem cell transplantation (HSCT). It can also occur after transplantation with isolated intestinal grafts or after multivisceral transplantation (MVTX). GVHD is difficult to diagnose. The aims of this thesis were to 1) investigate the potential of the eosinophilic granulocyte as an immunoregulatory cell and biomarker in GVHD, 2) determine the incidence, risk factors and clinical features of GVHD in MVTX, 3) evaluate the utility of lymphocyte chimerism analyses to predict overall survival and risk of GVHD after HSCT. In paper I, we used an *in vitro* model of GVHD to see if eosinophils could inhibit allogeneic T cell proliferation. In paper II, flow cytometry was used to examine patterns of surface receptors on blood eosinophils from transplanted patients +/- GVHD and +/- systemic glucocorticoids. Paper III is a retrospective epidemiological study of patients with acute GVHD after MVTX. In paper IV, the predictive capacity of chimerism analyses and impact of chimerism status on the duration of immunosuppression was evaluated. It was found that eosinophils can inhibit allogeneic T cell proliferation *in vitro* and that eosinophils in patients with acute and chronic GVHD have an activated phenotype, which is altered by systemic steroid therapy. Our conclusion is that the blood eosinophils are activated and have immunoregulatory capacity in GVHD, and might serve as a biomarker of GVHD. In MVTX, it was seen that a tumor diagnosis or neoadjuvant chemotherapy were possible risk factors for GVHD. Finally, chimerism analyses could not predict relapse, survival or GVHD after HSCT. However, patients with mixed chimerism or chronic GVHD had longer treatment time with cyclosporine A.

Keywords: Graft-versus-host disease, eosinophilic granulocyte, intestinal transplantation, multivisceral transplantation, chimerism analysis **ISBN**: 978-91-628-9824-3 (print), 978-91-628-9825-0 (PDF) http://hdl.handle.net/2077/42336

SAMMANFATTNING PÅ SVENSKA

Den så kallade graft-versus-host sjukdomen (GVH) eller "transplantat mot värd-sjukdomen" är en av de vanligaste komplikationerna efter transplantation med benmärg eller blodstamceller. Dessa transplantationer ges med syfte att bota svårbehandlade benmärgssjukdomar som akuta leukemier. Cirka hälften av de transplanterade får en akut eller kronisk form av GVH. Även vid transplantationer av andra organ som tunntarmstransplantation eller då tunntarmen transplanteras tillsammans med magsäck, tolvfingertarm, lever och bukspottskörtel (en så kallad multivisceral transplantation), finns en risk för GVH.

GVH uppkommer då de transplanterade immuncellerna reagerar mot vävnad hos den transplanterade. Tidigare forskning har föreslagit att en vit blodkropp, den eosinofila granulocyten skulle kunna användas som en laboratoriemarkör för GVH. En laboratorieanalys som tas regelbundet efter stamcellstransplantationer är så kallade chimerismanalyser, vilket visar hur stor andel av patientens immunceller som kommer från den som donerat blodstamcellceller (givaren) respektive från patient själv.

I denna avhandling undersöker vi GVH ur flera perspektiv. I delstudie I, används en laboratoriemodell av akut GVH för att se om eosinofiler kan hämma aktiverade T-celler. I delstudie II, har vi undersökt om eosinofiler i blodet hos transplanterade patienter har ett speciellt molekylmönster vilket skulle kunna utnyttjas vid diagnostik. Vi har studerat patienter med akut och kronisk GVH, samt med och utan kortisonbehandling. I delstudie III, har vi genom att studera patientjournaler undersökt förekomst, prognos och klinisk bild av patienter som drabbats av akut GVH efter multivisceral transplantation. I delstudie IV, studerade vi om läkaren genom chimerismanalys vid tre månaderskontrollen efter transplantation kunde förutsäga överlevnad, återfallsrisk, och utveckling av GVH, och om dessa faktorer påverkade hur länge patienten stod på immunhämmande behandling med cyklosporin A.

Våra resultat visar att eosinofiler kan hämma aktiverade immunceller (T-celler) i en laboratoriemodell av GVH, samt att eosinofilerna uttrycker olika molekylmönster på sin yta vid akut och kronisk GVH. Detta kan indikera att blodeosinofilerna aktiveras vid GVH och möjligen kan hämma GVH-reaktionen. Vi såg också att en cancerdiagnos eller cytostatikabehandling före tarm- eller multivisceraltransplantation är en möjlig riskfaktor för att patienten ska drabbas av akut GVH. Chimerismstatus kunde inte förutsäga återfall, överlevnad eller förekomst av GVH efter blodstamcellstransplantation. Däremot hade patienter

med blandad chimerism och kronisk GVH längre behandlingstid med cyklosporin A.

LIST OF PAPERS

This thesis is based on the following studies, referred to in the text by their Roman numerals.

I. **Eosinophils from hematopoietic stem cell recipients suppress allogeneic T cell proliferation.**
Andersson J*, Cromvik J*, Ingelsten M, Lingblom C, Andersson K, Johansson JE, Wennerås C. *shared first authorship
Biol Blood Marrow Transplant. 2014 Dec;20(12):1891-8.

II. **Eosinophils in the blood of hematopoietic stem cell transplanted patients are activated and have different molecular marker profiles in acute and chronic graft-versus-host disease.**
Cromvik J, Johnsson M, Vaht K, Johansson JE, Wennerås C.
Immun Inflamm Dis. 2014;2(2):99-113

III. **Graft-versus-host disease after intestinal or multivisceral transplantation: A Scandinavian single-center experience.**
Cromvik J*, Varkey J*, Herlenius G, Johansson JE, Wennerås C. *shared first authorship.
Transplant Proc. 2016;48(1):185-90.

IV. **T cell chimerism after allogeneic hematopoietic stem cell transplantation: impact on graft-versus-host disease and tapering of immunosuppression.**
Cromvik J, Wennerås C, Johansson JE.
In manuscript.

TABLE OF CONTENT

ABBREVIATIONS

ALL	acute lymphoblastic leukemia
AML	acute myeloid leukemia
APC	antigen-presenting cell
ATG	anti-thymocyte globulin
BMT	bone marrow transplantation
CCR3	C-C chemokine receptor type 3
CCL	chemokine (C-C motif) ligand
CD	cluster of differentiation
CLC	Charcot Leyden crystal
CMV	cytomegalovirus
CRTH2	chemo attractant receptor-homologous molecule expressed on Th2 cells
CTL	cytotoxic T lymphocyte
CsA	cyclosporine A
CXCL	chemokine (C-X-C motif) ligand
DC	dendritic dell
DFS	disease-free survival
DLI	donor lymphocyte infusion
EBMT	European Group for Blood and Marrow Transplantation
ECP	eosinophilic cationic protein
EDN	eosionophil-derived neurotoxin

EDTA	ethylenediaminetetraacetic acid
EPX/EDO	eosinophilic peroxidase
FACS	fluorescence activated cell sorter
FBS	fetal bovine serum
FITC	fluorescein isothiocyanate
FMO	fluorescence minus one
FOXP3	forkhead box P3
FPR	formyl peptide receptor
G-CSF	granulocyte colony-stimulating factor
GI tract	gastrointestinal tract
GM-CSF	granulocyte-macrophage colony-stimulating factor
GVHD	graft-versus-host disease
GVL	graft-versus-leukemia
HLA	human leukocyte antigen
HSCT	hematopoietic stem cell transplantation (refers to allogeneic HSCT when not otherwise specified)
ICAM-1	intercellular adhesion molecule-1
IDO	indoleamine 2, 3-dioxygenase
IFN	interferon
Ig	immunoglobulin
IL	interleukin
ITX	intestinal transplantation
LTX	liver transplantation

KRG	Kreb's ringer glucose
mAb	monoclonal antibody
MAC	myeloablative conditioning
MBP	major basic protein
mFI	median fluorescence intensity
MHC	major histocompatibility complex
MLR	mixed leukocyte reaction or mixed lymphocyte reaction
MVTX	multivisceral transplantation
NIH	National Institute of Health
NRM	non-relapse mortality
OPLS-DA	orthogonal partial least squares-discriminant analysis
PBMC	peripheral blood mononuclear cell
PBS	phosphate-buffered saline
PCA	principal component analysis
PCR	polymerase chain reaction
PDGF	platelet-derived growth factor
PerCP	peridinin chlorophyll protein complex
PGE2	prostaglandin E2
PI	propidium iodide
RI	relapse incidence
RIC	reduced intensity conditioning
RICT	reduced intensity conditioning transplantation

RNA	ribonucleic acid
ROS	reactive oxygen species
TNF	tumor necrosis factor
TGF-β	transforming growth factor-β
Th cell	T helper cell
Treg	regulatory T cell
VCAM-1	vascular adhesion molecule 1
VIP-value	variable importance in the projection value

1 INTRODUCTION

1.1 ALLOGENEIC HEMATOPOIETIC STEM CELL (HSCT) OR BONE MARROW TRANSPLANTATION (BMT)

Transplantation of allogeneic hematopoietic stem cells or bone marrow is a treatment option for patients with malignant diseases or bone marrow failure syndromes. The technique of allogeneic HSCT was first developed for patients with severe bone marrow failure syndromes, like aplastic anemia, and by help of cells from related donors.[1] The discovery of the immunosuppressive drug cyclosporine A[2] made it possible to use bone marrow transplantations with unrelated donors. The transplantations became an established treatment during the 1970s.[3] According to the World Health Organization,[1] altogether more than one million bone marrow or stem cell transplantations have been done and approximately 50 000 are done per year worldwide.

"Allogeneic" means that the patient receives cells from another individual, as opposed to "autologous" stem cell transplantation, when the patient receives their own cells. The allogeneic transplant can consist of bone marrow cells harvested from the crista iliaca (hip bone) of the donor. Another option is peripheral blood stem cell transplantation.[1] In this case, the donor has taken injections with granulocyte-colony-stimulating factor (G-CSF) to stimulate the hematopoietic stem cells ($CD34^+$ cells) in the bone marrow, so they leave the marrow and enter the bloodstream. These cells are harvested by help of apheresis. In both cases, the patient then receives the harvested stem cells as an intravenous infusion.

There are two mechanisms behind the use of allogeneic HSCT in malignant diseases. 1) firstly to give the patient an increased dose of chemotherapy in the so called conditioning therapy which can be either myeloablative (MAC), which means that it depletes the bone marrow irreversibly of hematopoietic stem cells, or reduced (RIC), where there is residual hematopoiesis. The use of transplantations with RIC (RICT) has made it possible to transplant older patients, up to 70 years of age, owing to reduced toxicity. 2) secondly to induce a so called graft-versus-leukemia (GVL) effect meaning that the new cells will recognize the tumor cells as foreign and suppress them in an immunological way. The GVL effect is a desirable effect, but it cannot be separated from the undesirable graft-versus-host reaction that may result in graft-versus-host-disease (GVHD).

1.2 INTESTINAL (ITX) AND MULTIVISCERAL TRANSPLANTATION (MVTX)

Transplantation of the isolated small bowel is used as a treatment option in patients with intestinal failure combined with life-threatening complications of total parental nutrition. Intestinal failure occurs when there is a reduced absorptive surface area in the small intestine. Among adults, it can occur after extensive surgical resections and in children as a consequence of congenital diseases.[4] The definition of intestinal failure is "the reduction of gut functions below the minimum necessary for the absorption of macronutrients and/or water and electrolytes, such that intravenous supplementation is required to maintain health and/or growth".[4] Unfortunately, long-term total parental nutrition is associated with risk of central venous catheter-related infections, occlusions, thrombosis and also increased risk of intestinal failure associated liver failure.[5]

In patients with intestinal failure-associated liver disease or tumors/desmoids/neuroendocrine pancreatic tumors spreading to the mesenteric root, a multivisceral transplantation can be performed. In the classical multivisceral transplantation, all abdominal organs are completely eviscerated and then transplanted “en bloc”: stomach, duodenum, pancreas, small intestine and liver. These operations are performed with some variations, for example the modified multivisceral, when the liver is not transplanted. Another alternative is that the spleen or kidney can be included in the transplant. The first intestinal transplantations were performed in the 1980s. The Intestinal Transplant Registry started to report data in 1985. According to this registry, 2887 intestinal transplants had been performed worldwide until February 2013.[6] The registry includes both small intestine transplants, liver and intestinal transplants, modified multivisceral and multivisceral transplants. An illustration of the different grafts can be seen on the next page.

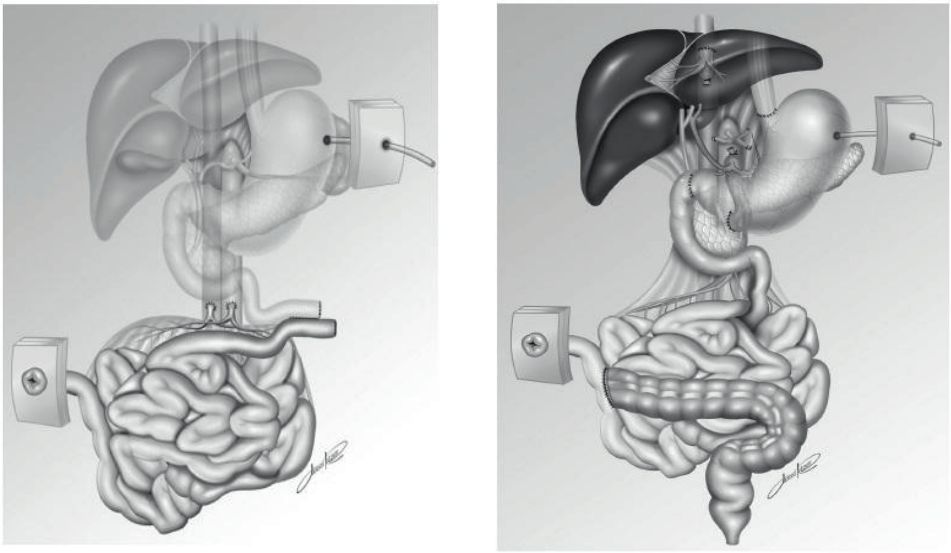

Figure 1. *Isolated small bowel transplantation to the left and multivisceral transplantation including stomach, duodenum, liver, small intestine to the right. The illustration is adapted and used after permission from Pécora RA, et al: Arq Bras Cir Dig.2013 Jul-Sep; 26(3):223-9.* [7]

1.3 GRAFT-VERSUS-HOST DISEASE (GVHD) AFTER TRANSPLANTATION

1.3.1 Immunopathogenesis

GVHD occurs when T cells from the donor react against the patient's tissues. The GVHD reaction depends on three components: 1. a graft containing immunologically competent cells, 2. a recipient, whose tissues express tissue antigens, not present in the donor 3. an immunosuppressed recipient, not capable to immediately eliminate the response of the transplanted cells. This was proposed 50 years ago by Billingham.[8] Further research has confirmed that GVHD reactions occur when tissue (blood products, bone marrow or solid organs) containing T cells are transferred allogeneically, which means from one individual to another. Later studies in mice have shown that the immunological cells are T cells[9] and that an increase in T lymphocytes in cell-depleted bone marrow transplant correlates with development of acute GVHD in humans.[10] The donor-derived T cells do not attack the graft as they are HLA-identical. Accordingly, intestinal GVHD cannot occur after intestinal transplantation.

The most common clinical situation with GVHD reaction is after allogeneic BMT or HSCT. According to the Ferrara model,[11] the immunopathogenesis of acute GVHD consists of three phases. In phase 1, there is a tissue damage induced by the conditioning regimen. Especially the gastrointestinal tract is affected and intestinal permeability increases, causing endotoxins like lipopolysaccharide (LPS) to translocate the bloodstream, where it stimulates secretion of cytokines through Toll-like receptors. This leads to an activation of antigen presenting cells. This facilitates phase 2, in which donor T cells become activated and start to proliferate, stimulated by interleukin 2 (IL-2). In the final phase 3, the effector phase, both cellular effector cells like natural killer (NK) cells and cytotoxic T cells together with soluble inflammatory mediators like TNF-α, IFN-γ, IL-1 and NO all amplify each other. This "cytokine storm" may cause transient or permanent tissue damage, especially in the skin, gastrointestinal tract (GI tract) and liver, which are the most common affected organs in acute GVHD after HSCT.

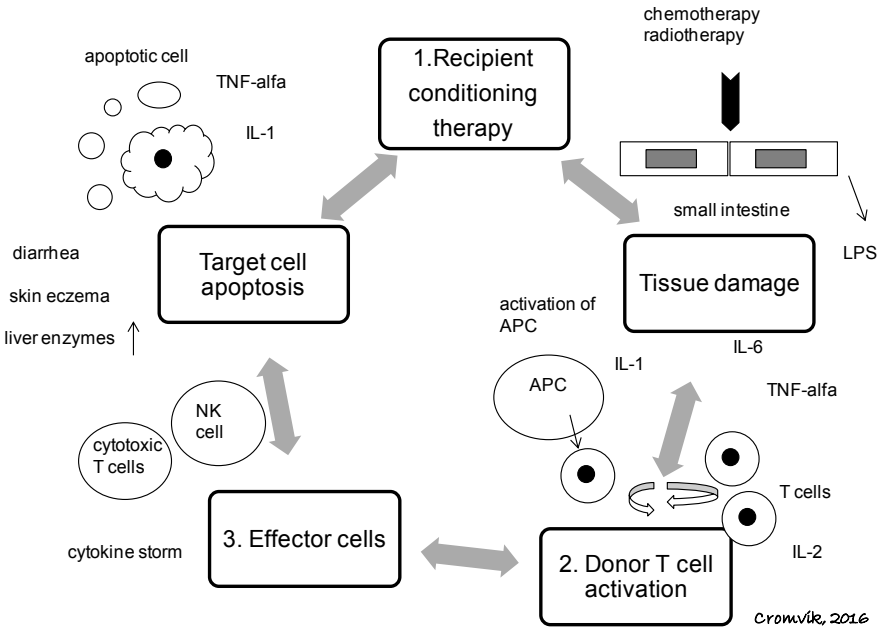

Figure 2. Immunopathogenesis of acute GVHD. *Abbreviations: LPS= lipopolysaccharide, APC=antigen presenting cell.*

However, these allogeneic T cells cannot alone explain why suppression of allogeneic T cells does not always cure GVHD. Another question is why only skin, GI tract and liver are affected in acute GVHD. Here some authors[12]

emphasize the role of the recipient tissue and propose that an impaired three-way crosstalk between immune cells, tissue homeostasis and the insults (resident microflora, toxins) lead to decreased immune responses and increased tissue damage, resulting in development of GVHD in these tissues.

The pathogenesis of chronic GVHD reactions is less known. Chronic GVHD is a heterogeneous multiorgan systemic disease, with similarities with rheumatological diseases like systemic sclerosis. There are indications that there are disturbances among donor B cells, donor T cells and probably also among other cells, like the eosinophilic granulocyte. One sign of a disturbed B-cell development is the occurrence of alloreactive autoantibodies, which are reactive with recipient cells. For example, male recipients with stem cell grafts from a female donor can develop antibodies against the Y-chromosome-encoded HY-protein. These antibodies can precede chronic GVHD.[13] In addition, autoantibodies directed against the PDGF receptor have been seen in extensive chronic GVHD and in patients with scleroderma. These autoantibodies have been proposed to be biologically active and stimulatory of collagen expression and reactive oxygen signaling.[14] Perhaps they can increase fibrosis development in patients with chronic GVHD. However, the precise role of autoantibodies in chronic GVHD needs to be further specified.

Donor T cells are thought to play a key role in chronic GVHD, since *in vivo* T cell depletion is a prophylactic measure that effectively prevents the development of acute and chronic GVHD.[15] Thymic damage is also proposed to be a mechanism, as patients with extensive chronic GVHD display delayed immune recovery and production of naïve thymus-derived T cells, as assessed by measurement of T-cell receptor excision circle (TREC) contents.[16]

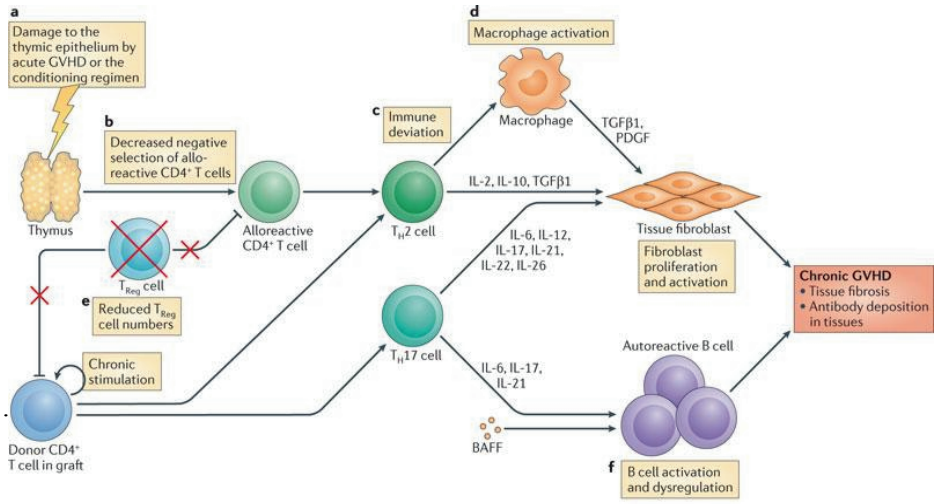

Figure 3. The pathophysiology of chronic graft-versus-host disease. *Six features of chronic GVHD. a) thymic dysfunction due to conditioning regimen and in some cases also prior acute GVHD. b) decreased negative selection of T cells in thymus. c) immune deviation to Th2-type cytokine response, d) release of Th2 cytokines and release of fibrogenic cytokines like IL-2, IL-10 and TGF-β and platelet-derived growth factor (PDGF) released by macrophages. e) decreased numbers of regulatory T cells and f) B cell dysregulation and production of autoantibodies. Reproduced with permission from Nature Publishing Group.* [17]

In conclusion, the pathogenesis of chronic GVHD is still poorly understood, but most probably it is a combination of defective immunological tolerance concerning both B and T cells, which results in autoantibody production, pro-fibrotic pathways and a defective central tolerance due to thymic dysfunction.[17]

1.3.2 Prevalence and outcome

GVHD has been well-known as a serious complication after HSCT since the first published studies from the Seattle group.[18] Acute GVHD occurs in approximately 50%[17] of the patients, after an allogeneic HSCT and is responsible for the majority of of transplant-related mortality in hematopoietic stem cell recipients.[17] Chronic GVHD occurs in approximately 50% of the patients surviving one year.[19] A diagnosis of GVHD can cause a prolonged use of immunosuppression, permanent organ dysfunction, and increased risk of infections.

GVHD is more uncommon in solid organ transplant recipients compared with allogeneic HSCT recipients However, the highest rates are seen among MVTX

recipients, where 12% incur acute GVHD compared to 4.6% among those with an isolated small bowel transplant.[20] In contrast, the mortality due to GVHD after solid organ transplantation is very high, with mortality rates of 80-100%.[21, 22]

1.3.3 Diagnosis and clinical criteria

GVHD is traditionally divided into two variants, acute and chronic. According to the original Glucksberg-Seattle classification[23] all types of GVHD that occurred within 100 days after transplantation were classified as acute GVHD. Chronic GVHD was diagnosed as GVHD occurring after 100 days. Later research has revealed a more complex picture and the NIH consensus group[24] have proposed the following categories:

- Classic acute GVHD occurring within 100 days after transplantation. No diagnostic or distinctive features of chronic GVHD.
- Persistent, recurrent and late onset of acute GVHD. Debut of symptoms > 100 days after transplantation. Can occur after withdrawal of immunosuppression or after donor lymphocyte infusions (DLI: s).
- Classic chronic GVHD: Can occur at any time after transplantation.
- Overlap syndrome: Can occur at any time after transplantation. Has a feature of both chronic GVHD and acute GVHD. Sometimes referred to as "acute on chronic" GVHD. New category in the NIH 2005 classification.[25]

According to the existing classification from the NIH[24], it is the clinical features of GVHD that determine whether it is an acute or chronic form. Thus, after 2005, diagnosis of GVHD is primarily based on clinical picture rather than on the temporal relationship to the transplantation, as originally defined in the Glucksberg-Seattle classification.[23] The NIH consensus groups for diagnosis[24] and for histopathological disease[26] recommend biopsies to confirm the diagnosis of GVHD in situations, where there are no diagnostic features and only distinctive clinical features of GVHD, for example nail loss or depigmentation. It is not compulsory if the patient has at least one diagnostic manifestation, for example lichen-planus in mouth or genitalia. Unfortunately, a biopsy can be hard to obtain (in some organs, especially if the patients have low platelet levels). Another problem is that the histopathological biopsies can be inconclusive due to inadequate tissue sampling or that immunosuppressive treatment was started before the biopsy was taken.

The NIH pathology working group has defined minimal histopathological criteria to classify active acute or chronic GVHD. They propose that organ-specific criteria should be used, for example ductopenia and portal fibrosis in liver biopsies.[26] Ductopenia means reduction in the number of intrahepatic bile ducts in the liver. There are also minimal criteria for acute/active GVHD like apoptosis in epidermal skin layer or variable apoptotic crypt in the GI tract.

1.3.4 Acute GVHD- classification and grading

The acute forms of GVHD usually affect the skin, the liver or the intestine. In skin-GVHD the patient has an exanthema. In acute liver GVHD the patient has elevated liver enzymes, which in the worst case scenario can lead to manifest liver failure. The intestinal GVHD manifests as voluminous diarrheas and in severe cases also as melena. Here an intestinal biopsy by help of a colonoscopy or sigmoideoscopy[27] is necessary to differentiate acute GVHD from intestinal CMV infection or other infectious causes of diarrheas. There also exists a form of acute GVHD in the upper gastrointestinal tract with symptoms of anorexia, dyspepsia, food intolerance, nausea and vomiting.[28]

Several grading systems of acute GVHD are in use. The first grading of acute GVHD was the Glucksberg-Seattle classification[23] from 1974 in which grading (grades I-IV) of skin, liver and GI tract were used together with performance status. This was revised some years later by Thomas et al,.[29] A more recent grading classification is the International Bone Marrow Transplant Registry (IBMTR)[30] grading system (grades A-D), in which the patient's performance status is not included. In this grading, there is first an organ grading of skin, liver and GI tract depending on rash size, bilirubin levels and diarrhea volumes. These values together constitute the overall grade:

- Grade A: there is only a stage 1 skin involvement (maculopapular rash over <25% of the body).
- Grade B: stage 2 skin involvements together with stage 1 or 2 gut or liver involvement. Stage 1-2 liver involvement occurs when total bilirubin is 34-102 μmol/l and stage 1-2 gastrointestinal involvement is when volume of diarrhea is 550-1500 ml/day.
- Grade C: Stage 3 involvement of any organ system: (generalized erythroderma >50% of body area, elevated bilirubin 103-255 μmol/l or diarrhea >1500mL/day).
- Grade D: Stage 4 involvement of any organ (generalized erythroderma with bullous formation, total bilirubin>255 μmol /l, and severe pain and ileus.

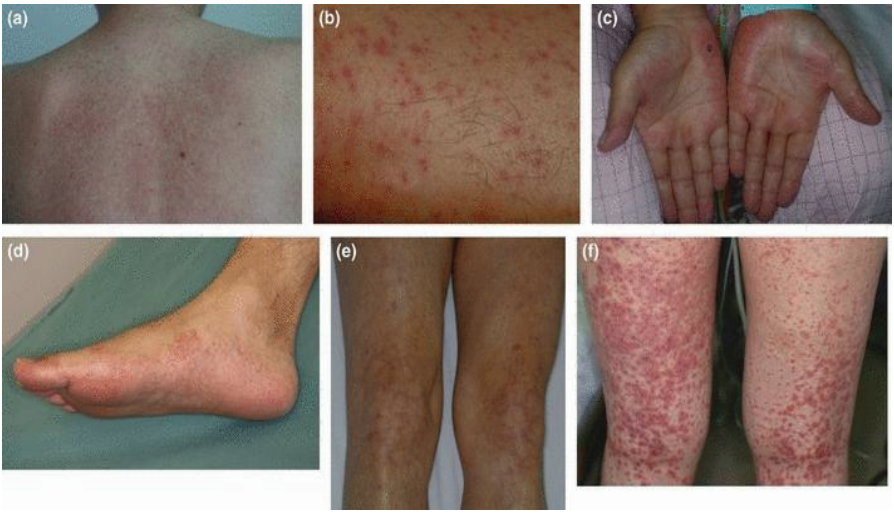

Figure 4. *Different manifestations of acute skin GVHD. A) disseminated maculopapular exanthema; b) perifollicular papular lesions; c, d) erythematousus rash of the c) palms and d) soles; e) reticular erythema and f) purpura. Used with permission from John Wiley and Son.*[31]

1.3.5 Chronic GVHD- classification and grading

The picture of chronic GVHD is complex as characterized by several clinical manifestations of varying severity and clinical course. The disease can involve diverse organs including skin, nails, hair mouth and oral cavity, eyes, genital organs, GI tract and liver, lungs and the hematopoietic, neurologic and musculoskeletal systems. The clinical features are often similar to systemic rheumatological diseases like systemic sclerosis or Sjögren's syndrome, as many patients suffer from dryness of the eyes and mouth and sclerodermatous skin.

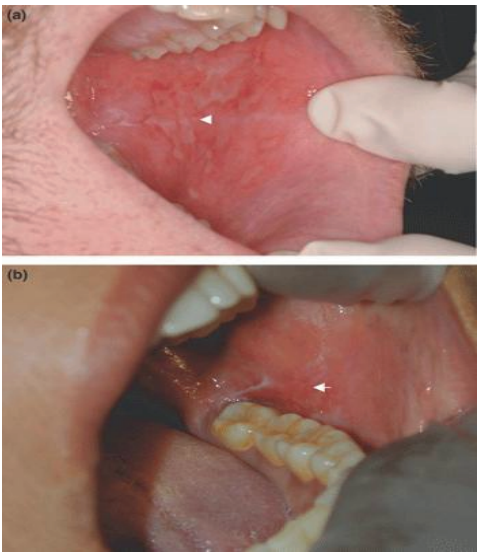

Figure 5. *Similarities in clinical picture of oral chronic GVHD and oral lichen planus. Ulcerative and erythematosus lesions of the buccal mucosa in oral chronic GVHD (a) upper picture and in oral lichen planus (b) lower picture. Used with permission from John Wiley and Son.* [32]

According to the latest classification of chronic GVHD, the NIH consensus group[24] recommends that the diagnosis of chronic GVHD requires at least one diagnostic manifestation of chronic GVHD or at least one distinctive manifestation of chronic GVHD plus a pertinent biopsy, laboratory, or other test (e.g., pulmonary function test), evaluation by a specialist (ophthalmologist, gynecologist), or radiographic imaging showing chronic GVHD. In the same way as in acute GVHD, alternative diagnoses, e.g. nail dystrophy due to onychomycosis, Candida albicans infections of the mouth and drug reactions need to be excluded. An overview of diagnostic, distinctive and other features of chronic GVHD is given in Table I. The table is a simplified and adapted version from Table 1 in the NIH Consensus development on criteria for clinical trials in chronic GVHD, Jagasia et al, Biology of Blood and Marrow Transplantation 21 (2015) 389-401.[24]

Table I. Signs and symptoms of chronic GVHD

Organ or Site	Diagnostic	Distinctive[1]	Other features [2]	Common [3]
Skin	Poikiloderma Lichen-planus Sclerotic featues Morphea	Depigmentation Papulosquamous lesions	Sweat impairment Hypopigmentation Hyperpigmentation	Erythema Maculo-papular rash
Nails		Onycholysis, nail loss		
Scalp and body hair		Loss of body hair	Thinning scalp	
Mouth	Lichen-planus	Ulcers		Erythema Gingivitis
Eyes		New onset dry, gritty or painful eyes	Photophobia Periorbital hyperpigmentation	
Genitalia	Lichen-planus Lichen-sclerosis			
GI tract	Esophageal web		Exocrine pancreas insufficiency	Nausea Vomiting Diarrhea Weight loss
Liver				Bilirubin, ALP> 2 x upper limit of normal
Lung	Bronchiolotis obliterans (BOS)[4]	Air trapping and bronchiectasis on chest CT	Cryptogenic organizing pneumonia	
Mucles, fascia **joints**	Fasciitis or joint stifness or contrac-tures due to fasciitis or sclerosis	Myositis or polymyositis[5]	Edema Muscle cramps	
Hematopoietic and immune			Thrombocytopenia Eosinophilia Autoantibodies	
Other			Pericardial and pleural effusions	

[1] In all cases, infection, drug effect, malignancy, or other causes must be excluded

[2] Can be acknowledged as part of the chronic GVHD manifestations if diagnosis is confirmed

[3] Common refers to shared features by both acute and chronic GVHD

[4] BOS can be diagnostic for lung chronic GVHD only if distinctive sign or symptoms are present also in another organ

[5] Diagnosis of chronic GVHD requires biopsy

Traditionally, chronic GVHD has been graded as "limited" or "extensive" on the basis of a retrospective study from Seattle of twenty patients.[18] In the present grading from the NIH consensus group[24]: the grading of chronic GVHD consists of a performance score according to Eastern Cooperative Oncology Group (ECOG) in addition to an organ scoring of eight organs: skin, mouth, eyes, GI tract, liver, lung, joints and fascias, and genital tract. Every organ is scored with respect to the presence of GVHD as either 0 (none) to 1 (mild), 2 (moderate) or score 3 (severe). In addition, other indicators should be mentioned like eosinophilia, thrombocytopenia, and ascites. It is recommended that eyes and female genitalia are scored by an ophthalmologist and gynecologist, respectively. The score from these eight organs are then used in order to calculate a global score of mild, moderate or severe chronic GVHD.

1.3.6 Overlap GVHD

The category of overlap GVHD was introduced by the NIH consensus group[25] in 2005 as a sub-category of chronic GVHD. According to that classification, patients with simultaneous features of both chronic and acute GVHD should be classified as overlap GVHD. Sometimes overlap GVHD is called "acute on chronic GVHD" in clinical practice. Some studies have indicated that these patients have a worse prognosis compared to those with classical chronic GVHD.[33,34] Several authors report it to be difficult to use the overlap classification in clinical practice[35] and therefore the definitions have been clarified in the new NIH consensus document from 2014.[24] In this document they recommend documentation of specific manifestations (acute and chronic), when scoring organ severity at debut and at any time of chronic GVHD with the purpose to achieve a more complete documentation of chronic GVHD syndrome and to facilitate retrospective confirmation of the "overlap" diagnosis in patient with clinical chronic GVHD.

1.3.7 Possible biomarkers for GVHD

There are no generally accepted diagnostic or therapeutic biomarkers in GVHD. Therefore NIH has established a biomarker working group. They have proposed three groups of biomarkers in chronic GVHD in their latest consensus document from 2015: a) diagnostic biomarkers, b) prognostic biomarkers, used to foretell the risk of developing chronic GVHD and c) predictive biomarkers to forecast the response to therapy.[36] Most biomarkers hitherto identified belong to the group of diagnostic biomarkers. Eosinophilic counts were proposed as diagnostic biomarker in the first consensus document from 2006[37], but are no longer mentioned in the update from 2015[36]. This could be due to a recent study from a Danish group. Their study could not find any association between eosinophilic counts and long-term outcome in patients with chronic GVHD.[38] The NIH consensus group updated its consensus report in 2015[36], and concludes that much remains to be done before we

have validated consensus biomarkers for GVHD.

During the last years, several research groups have launched candidate biomarkers, for example Levine et al.,[39] who proposed an algorithm composed of the cytokine receptors: tumor necrosis factor receptor 1 (TNFR1) and ST2 together with regenerating isled-derived protein 3α (Reg3α), which is lectin secreted by Paneth cells in the intestinal mucosa. This algorithm could ameliorate prediction of non-relapse mortality at six months and adjustment of immunosuppressive therapy. Others[40] have proposed the cytokines: B cell activating factor, and the chemokine C-X-C motif chemokine ligand 10 (CXCL10) as predictive biomarkers in acute GVHD and BAFF, CXCL10 and C-X-C motif chemokine ligand 11 (CXCL 11) as diagnostic biomarkers in chronic GVHD.

1.3.8 Prophylaxis and first and second line therapy of GVHD

The recommended standard prophylaxis is the immunosuppressive calcineurin inhibitor, cyclosporine A (CsA) and a short course of the cytotoxic drug methotrexate. After transplantation CsA is recommended during six months in the absence of GVHD. The dose should not be tapered if there are signs of acute GVHD or signs of chronic GVHD exceeding mild skin disease.[41] Another strategy is so called T cell depletion using polyclonal anti-thymocyte globulins. A recent meta-analysis has shown that ATG, significantly reduces the risk of severe acute GVHD (grade II-IV), but do not improve overall survival. The prevalence of chronic GVHD was not possible to evaluate in the meta-analysis.[42] The doses and timing of ATG differ at different centers. At Sahlgrenska University Hospital, it is often used before transplantation with unrelated donors. During the last years, new protocols with high-dose of cyclophosphamide during one of the first days after transplantation have been developed.[43] Cyclophosphamide can inhibit alloreactive T cells generated after a HLA-mismatched transplantation, for example after haploidentical HSCT,[44] and leave the non-dividing hematopoietic stem cells unaffected.

The first line therapy of acute GVHD is systemic glucocorticoids, such as prednisone given at 1-2 mg/kg. This regimen has been used during the last decades. Glucocorticoids are steroid hormones secreted from the adrenal cortex. They have physiological effects in normal life, as they control metabolism by stimulating gluconeogenesis in the liver and mobilize amino acids from extra-hepatic tissues. Glucocorticoids also inhibit glucose uptake in muscles and adipose tissue and mediate the stress response.[45] In clinical practice glucocorticoids are used as anti-inflammatory and immunosuppressive drugs. Most of the actions of the glucocorticoids are mediated through the intracellular glucocorticoid receptor (GR) in the cytoplasm. After activation, the receptor-steroid complex translocates

to the nucleus, where it binds to DNA and induces and represses the expression of target genes.[46] This activation is called the genomic mechanism of the glucocorticoid receptor. The process is time-consuming and therefore accounts for a delayed onset of the clinical effect (6-8 hours) in clinical practice. Glucocorticoids also express more immediate reactions (within minutes), like cerebral effects as euphoria, apathy and depression. This action is mediated through non-genomic mechanisms of membrane-coupled receptors in the plasma membrane.[47]

In eosinophilic diseases, glucocorticoids are an effective anti-inflammatory drug. This effect is due to an inhibition of the synthesis of the cytokines that prolong eosinophilic survival, like interleukin 3 (IL-3), IL-5 and granulocyte-macrophage colony stimulating factor (GM-CSF)[48] and also an induction of programmed cell death, so called apoptosis.[49] There is also an inhibition of the release of mature eosinophils from the bone marrow and an increased destruction of eosinophils in the reticuloendothelial system after intake of glucocorticoids.[50] Among physicians it is well known that glucocorticoids result in a rapid decrease in eosinophilic blood counts. There are two different pathways of apoptosis[51]: the extrinsic (receptor-mediated pathway) in which there is an activation of the "death"-receptor, FAS/CD95 followed by formation of the death-inducing complex and caspase-8 activation. The other pathway is the intrinsic pathway (mitochondrion-centered) pathway. This can be induced by DNA damage, oxidative stress or cytosolic $Ca2^{+}$ overload. Apoptosis of human eosinophils can be induced by one of the pathways, for example Fas-mediated apoptosis[52] or by help of both the intrinsic and extrinsic pathways.[53]

For patients with limited chronic GVHD in the eyes, skin or genital areas, an alternative is local treatment for example topical glucocorticoids for skin and genital involvement and eye drops. Systemic immunosuppression should be considered in patients with chronic GVHD and a score of 2 or more in any single organ.[24] An unanswered question is what treatment to choose when the glucocorticoids do not work, so called steroid resistant GVHD. In one study only 55% of the patients with acute GVHD responded to systemic steroid therapy and 35% achieved a durable complete remission.[54] This is in congruence with earlier studies in which 44% obtained an overall complete or partial response.[55] In 2012, the American Society of Blood and Marrow Transplantation (ASBMT) reviewed 29 studies concerning GVHD therapy for steroid-refractory cases[56] and concluded they could neither recommend any therapy before another, nor dissuade the use of any substance out of the following immunosuppressive therapies: mycophenolate mofetil, methotrexate, extra-corporeal photopheresis, daclizumab, alemtuzumab, horse ATG, and sirolimus.

There is a lack of therapeutic guidelines, but there exist a multitude of therapies which have been tested at different centers. For example, extra-corporeal photopheresis is supposed to eradicate T cells but the exact mechanism is unknown, and there is a lack of randomized clinical trials evaluating this method.[57] Another new alternative is ruxolitinib (Jakavi®), a JAK2-inhibitor supposed to inhibit inflammation in steroid resistant acute and chronic GVHD.[58,59] Also cell therapies with regulatory cell types have been tried and are examined in clinical studies. The prospect is that these cells would down-regulate the allogeneic T cell activity in GVHD. Here bone-marrow derived mesenchymal stromal cells have been used in order to treat steroid-resistant acute GVHD, since a case-report in 2004.[60] In one phase I study of 40 patients, no acute toxicity was reported.[61] A Swedish phase II study has also shown promising results.[62] As the human placenta contains cells with similar capacities, decidual stromal cells have been analyzed in GVHD. They cells have shown to highly suppress allogeneic T cell proliferation in vitro[63] and to induce an overall response rate of 75% in eight patients with acute steroid resistant GVHD.[64] Studies have also been performed in the preventive setting using regulatory T cells from umbilical cord blood, which have been tolerated in eleven patients and resulted in both lower incidence of acute GVHD on 100 days and absence of chronic GVHD.[65]

1.3.9 GVHD after ITX or MVTX

Among solid organ transplanted patients, GVHD is a very rare disease, but among solid organ grafts it is most prevalent after multivisceral (MVTX:s) and isolated intestinal transplantations (ITX:s). An American study of 241 patients, showed an incidence of 12% after MVTX and 5% after ITX[20], which is in congruence with 6% of GVHD in another study of 250 ITX patients[66] and 13% among MVTX patients.[67] The incidence of GVHD after an isolated liver transplantation is 1%.[68]

Patients with GVHD after solid-organ transplantation typically develop fever, skin rash or pancytopenia two to six weeks after transplantation.[68] The most prevalent clinical symptom is skin rash, but also diarrheas can occur unless the patient do not have a small bowel graft, as GVHD can not occur in the graft.[69]

Another immunological complication, which can occur after AB0 or Rhesus (Rh) mismatched solid-organ transplantation is passenger lymphocyte syndrome (PLS). Here donor B cells react with the recipient's erythrocytes and start producing antibodies, causing alloimmune hemolysis. This phenomenon can be severe and occurs in 18% of transplantations witch AB0 incompatibility.[70] However, the diagnostic process of GVHD and PLS is complicated as these symptoms also can occur due to hemophagocytosis or as drug reactions. The clinical picture is also different depending on different grafts, as GVHD by definition can not occur in transplanted organs, for example can a recipient of intestinal grafts not incur

intestinal GVHD. The prevalence of chronic GVHD seems to be very rare and there exist only few studies, most of them are single center studies or case reports about chronic GVHD in this population.[71, 72]

Traditionally the same diagnostic criteria have been used for GVHD among solid organ transplanted patients as for patients after HSCT. However, the situation is different compared to after allogeneic HSCT, as the recipient after MVTX or ITX still have their own bone marrow and immune system. The GVHD after these transplantations is caused by T cells in the graft. Therefore grafts with extensive lymphoid tissue, for example MVTX are a known risk factor of GVHD compared to ITX grafts.[20] A situation with persistence of donor cells (less than 1% of total leukocytes), so called microchimerism often occurs after solid-organ transplantation.[73] Normally, these donor cells are eliminated by the recipient by help of their immune system and the patient recover chimerism with 100% recipient cells. This is the opposite compared to after allogeneic HSCT, in which the desirable state is 100% donor cells. Macrochimerism (more than 1% donor cells of total leukocytes) has been proposed to be useful in the diagnosis of GVHD after liver transplantation.[74] However, a recent article reports that macrochimerism may occur without GVHD and that macrochimerism was more common after MVTX than after ITX.[75] The diagnostic of GVHD after MVTX and ITX is therefore still complex and challenging.

The mortality has been reported to be very high after solid organ transplantation with mortality rates of 80%[21] up to 100%[22] after liver transplantation. There is a lack of consensus guidelines about treatment and both increased immunosuppression with corticosteroids have been described[21] no treatment when mild disease,[76] and a complete withdrawal of immunosuppression.[77]

1.3.10 Other complications after allogeneic HSCT

Rejection. A rejection means that the remaining immune system of the transplanted patient recognizes the transplanted organ as foreign and rejects it, leading to graft failure. Chimerism analyses are often used for early diagnosis of rejection. (See chimerism analysis section).

Relapse. Even if the purpose of allogeneic stem cell transplantation is to achieve cure or a long remission, there is a risk of relapse of the underlying hematological disease. The relapse risk is dependent on the original diagnosis and the risk of relapse is more significant in patients with a malignant disorder than in those with a benign diagnosis.

1.4 THE IMMUNE SYSTEM IN TRANSPLANTED PATIENTS

1.4.1 The reconstitution of the immune system after allogeneic HSCT

Before the stem cell infusion, the patient receives a conditioning treatment consisting of chemotherapy, sometimes with the addition of radiotherapy. The main purposes are to eradicate the malignant cells and to prevent rejection, so the new allogeneic stem cells can establish themselves in the bone marrow.

After infusion of the allogeneic hematopoietic stem cells, the cells actively cross the blood/bone marrow endothelium barrier and stay in the bone marrow by activation of adhesion interactions.[78] This process is called homing and starts within hours after the transplantation. Other cells like human neutrophils and specialized T cells can also home to the bone marrow, but only $CD34^{+/}CD38^{-}$ cells can establish in the endo-osteal niches and reach long-term repopulation.[78]

After homing, the engraftment starts, i.e., the transplanted stem cells start to proliferate and to repopulate the patient's blood system. Some stem cells remain as quiescent stem cells, other self-renew or become committed to progenitor cells in the myeloid or lymphoid lineage.[79] In clinical practice, the peripheral blood cell levels are very low, so called cytopenia, during the engraftment process. After allogeneic HSCT, the time until neutrophil engraftment (to $0.5 * 10^9$ cells/L on two consecutive days) usually ranges from 14 to 28 days.[80] During this time of severe cytopenia, the patient is at high risk of infections.

After engraftment, the establishment of the donor immune system in the recipient continues, a process that takes months to years to complete.[81] The innate immune system recovers within weeks, for example the epithelial barriers, which often have been damaged by the conditioning regimens. The barrier function recovers quickly, but the protective secretions such as IgA and lysozyme can be chronically subnormal, especially in chronic GVHD.[82] Although the neutrophil counts recover within weeks, their functional properties such as chemotaxis, phagocytosis, production of superoxide, and killing of bacteria takes months to recover.[81] Eosinophil engraftment appears to follow the same kinetics as neutrophils, re-appearing in the blood within 1 month post-transplantation.[83]

The reconstitution of the adaptive immune system takes longer time. During the first months, the dominating cells are post-thymic T cells expanding from transplanted T cells.[84] These T cells originating from grafted T cells, can have both

antimicrobial and antileukemic effects and dangerous pro-GVHD effects. The number of T cells co infused with the marrow illustrates that the majority of T cells after BMT is regenerated through peripheral expansion and not through thymus.[85, 86]

The B cells only exist in very low number during the first months after transplantation. Antibodies of recipient origin can be found for years after the transplantation, as long-lived plasma cells resident in the bone marrow can be resistant to chemotherapy and radiotherapy.[87] Both the engraftment and the immune reconstitution processes can be delayed due to infections, GVHD or systemic treatment with glucocorticoids.

1.4.2 The eosinophils

The eosinophilic granulocyte belongs to the granulocytes in the innate immune system. The production of blood cells (hematopoiesis) starts in the red bone marrow in adults. All cells of hematopoietic origin derive from the pluripotent hematopoietic stem cell, which is recognized by the surface marker combination $CD34^{+}CD38^{-}$.[88] These cells are harvested and used in allogeneic HSCT. There are two parts of the hematopoiesis, the lymphoid progenitors, which develop into the lymphocytes (B, T and NK cells). There are also myeloid progenitors which develop into platelets, erythrocytes (red blood cells), monocytes, dendritic cells, and the granulocytes. The granulocytes are cells with granules in the cytoplasm. The most common granulocyte is the neutrophilic granulocyte, essential for the defense against bacterial and fungal infections.

The eosinophilic granulocytes are recognized by their coarse granules that stain red with eosin and their lobulated nucleus. Therefore, the research on eosinophils was limited by methodological difficulties until the 1990s, when the development of immunomagnetic depletion based on the neutrophil's expression of CD16 and the eosinophil's lack thereof made it possible to obtain eosinophils.[89, 90]

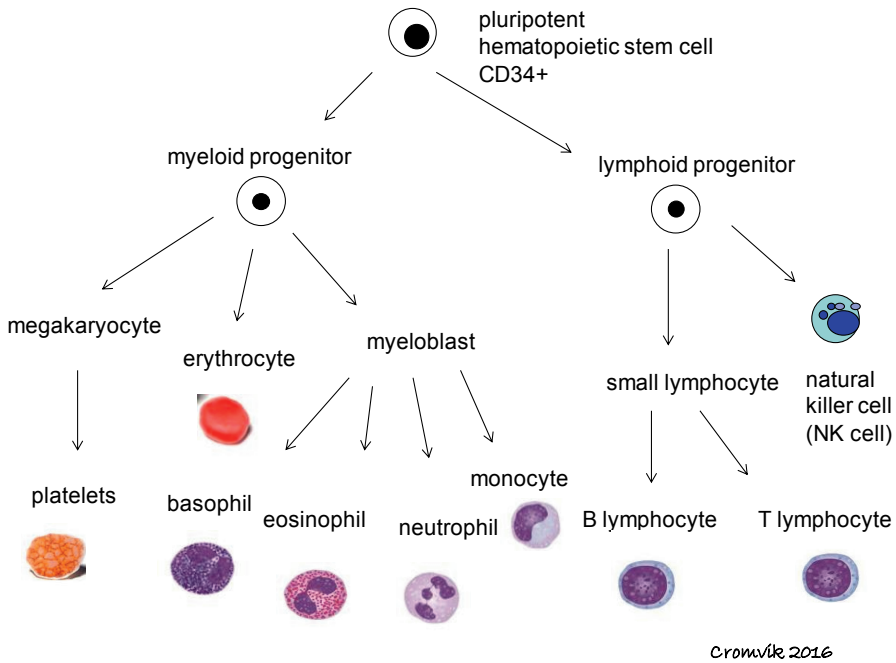

Figure 6. The normal hematopoiesis in adults*. The cells in color are the cells, which can be found in peripheral blood. The other cells are normally only found in the bone marrow, thymus or in the lymph nodes. The illustration of the cells is used with permission from DocCheck pictures.*

1.4.3 The eosinophils in healthy individuals

In healthy individuals, the eosinophil levels are normally 0,04 - 0,4 x 10^9/L.[91] Approximately 4% of the population have eosinophilia.[92] The cells are generated continuously from the bone marrow. They only remain in the bloodstream for hours until they enter the tissue. Their intravascular life span is thought to be 25 hours compared to 10 hours for neutrophils.[93] They are primary tissue-dwelling cells and can survive for several days in tissues[94] and up to three months if activated.[95] In healthy individuals in non-inflammatory conditions, eosinophil infiltration have been found in spleen, lymph nodes and thymus and both infiltration and degranulation have been found in the gastrointestinal tract.[96]

Earlier studies have shown that eosinophils of varying similarity exist in both vertebrates and non-vertebrates like insects. They seem to have an evolutionary conserved role in the immune system, although this role still is unclear.[97] However, unexpected eosinopenia in patients with normal leukocyte concentration is very rare. A study showed that less than one of 1000 patients had eosinopenia, and the

majority of these patients were under treatment with glucocorticoids.[98] There also exist observations indicating that true eosinophil deficiency is often associated with conditions of dysregulated immunity (thymoma or hypogammaglobulinemia), or in combination with deficiency of basophils or in association with allergic diseases like asthma or urticaria.[99]

Human eosinophils can produce and secrete over 30 cytokines, including both Th2-associated cytokines like IL-4, IL-13, and the Th1-associated cytokines IL-12 and IFN-γ, but also immunoregulatory cytokines such as the proinflammatory TNF-α and the immunosuppressive IL-10.[100] Eosinophils from humans can degranulate through several mechanisms. The most common mechanism is so called "piecemeal degranulation", which means that fractions of preformed cytokines and/or granule proteins are selectively secreted through a regular vesicular-based process "piece by piece".[101] Classical "exocytic degranulation" with fusions of granules to the plasma membrane and release of all granule contents is rare *in vivo,* except when eosinophils are on the surface of a helminthic parasite. Also "cytolysis", which means deposition of clusters of free extracellular granules upon lysis of the cell can occur as a "degranulation" process.[102] However, the piecemeal degranulation seems to be the most relevant mechanism of storage and secretion of granules contents during physiological conditions.[103, 104] The presence of preformed granule proteins prior to secretion makes the eosinophils different from most other immune cells, such as lymphocytes. The secretion of the preformed eosinophil cytokines is a rapid and stimulus-specific process, and different stimuli results in differential secretion of cytokines.[100] Together with the cytokines, eosinophils also contain four cationic proteins stored within their cytoplasmic granules, eosinophil peroxidase (EPO), major basic protein (MBP), eosinophil cationic protein (ECP) and eosinophil-derived neurotoxin (EDN), which all under certain conditions can induce tissue damage and dysfunction.[105]

The eosinophils also express several receptors. Resting human eosinophils highly express cysteine-cysteine chemokine receptor-3 (CCR3), which is the main receptor of eotaxins 1-3.[106] The eotaxins are chemoattractants inducing eosinophil recruitment into tissue sites.[107] In addition, the chemoattractant receptor-homologous molecule expressed on T-helper type 2 (CRTH2) is commonly expressed on human eosinophils.[108] Prostaglandin D2 selectively induces chemotaxis in eosinophils via CRTH2.[109] Further, sialic acid immunoglobulin-like lectin 8 (Siglec-8) is primarily expressed on eosinophils.[110] Several surface receptor have been reported to be up-regulated on activated eosinophils, like CD69, intercellular adhesion molecule-1 (ICAM-1, CD54) and the IL-5 receptor and Fc receptors in asthma[111] and CD25 in acute GVHD.[112] Also purified human blood eosinophils can express several integrin heterodimers: CD49d[113], CD11b, CD11a, CD11c, CD18[114]. Integrin receptors like CD11b can be up-regulated on

human eosinophils and then bind to vascular cell adhesion molecule-1 (VCAM) after culture with IL-5.[114] The eosinophils with expression of CD18 can bind to ICAM-1, ICAM-2 and to endothelial cells in inflammatory disorders like, eosinophilic esophagitis.[115]

1.4.4 Functional properties of the eosinophils

Traditionally, the eosinophil has been connected to helminthic infections[116] and allergic diseases as an effector cell, which can effect tissue destruction mediated by cytotoxic cationic granule proteins. However, more recent studies have challenged the perception of the eosinophils as tissue-destructive cells. For example, IL-5 blocking monoclonal antibodies (mepolizumab) decrease eosinophil levels in patients with allergic asthma, but could not ameliorate the late asthmatic response or airway hyper responsiveness to histamine in patients.[117] A similar result has been seen in patients' sufferings from eosinophilic esophagitis. In these patients, the IL-5 blocking antibody did decrease the number of tissue eosinophils in the esophagus, but without any convincing symptom relief.[118]

Human eosinophils can also recognize and become activated by both Gram-negative and Gram-positive bacteria. *Escherichia coli* is a strong eosinophilic activator and can induce chemotaxis, degranulation and respiratory burst in human eosinophils.[119] Bacterial lipopolysaccharides (LPS) from Gram-negative bacteria can induce IL-5 or IFN-γ primed eosinophils to release mitochondrial DNA in a catapult-like fashion.[120]

The last decades of research have also given a more complex picture, proposing that the eosinophil may have immunoregulatory properties in health and disease.[121] For example studies have shown that eosinophils can regulate T cell subset selection by indoleamine 2,3-dioxygenase (IDO).[122] These authors continued these studies and found eosinophils which expressed IDO in thymus from children. Both IL-5 and IL-15 were found in thymus supernatant. Also thymic IDO immunoreactivity was highest in the youngest children. This can be an indication that eosinophils might regulate Th2 responses in young children.[123] Animal studies from mice have proposed that activated eosinophils in the thymus are active in negative selection of T cells.[124]

Inspired by these results, in 2010, mouse eosinophil researchers proposed a new paradigm and hypothesized that eosinophils are actually regulators of *L*ocal *I*mmunity *A*nd/or *R*emodeling/*R*epair in both health and disease- the *LIAR* hypothesis.[125] For example, they propose that eosinophils gather in tissues, where there is a co-existence of dying cells and a significant pool of proliferating cells and/or stem cell activation, for example in the bone marrow, in the gastrointestinal tract, in the thymus and in the uterine endometrium. They can also suppress

immune responses, for example in a Th1/Th17 environment by help of immunosuppression.[122] This is in congruence with Swedish studies from the 1980s, which have shown that purified eosinophilic granule proteins can inhibit lymphocyte proliferation[126] *in vitro* and that eosinophils can alter T cell responses toward T helper 2[122] or amplify Th 1 and Th 2 types of cytokine secretion *in vitro.*[127] In allergic inflammation, eosinophils also seem to be active in repair and tissue modeling. For example, co-culture of primary human dermal fibroblasts together with human eosinophils of atopic subjects resulted in increased transcript of procollagen.[128]

Newer studies of mice have shown that eosinophils localize together with plasma cells in the bone marrow of mice. The role of the plasma cells is to produce antigen-specific antibodies. *In vivo* studies in mice indicate that eosinophils secrete cytokines, like the proliferating-inducing ligand and IL-6, which support the survival of the plasma cells in the bone marrow. If the eosinophils were depleted, the plasma cells died by apoptosis.[129] Similar results have also been shown the gut of mice. Here eosinophils also co-localized with eosinophils in the intestinal lamina propria and were essential for the maintenance of IgA+ plasma cells. Eosinophil-deficient mice had a defective intestinal mucous shield and altered intestinal microbiota.[130] Also malignant plasma cells seem to be supported by eosinophils. A study of human bone marrow biopsies from patients with multiple myeloma showed co-localization of eosinophils and plasma cells and confirmed that eosinophils promoted the growth of malignant plasma cells *in vitro.*[131] Eosinophils have also been connected to other malignancies, as they have been found to induce proliferation of Reed-Sternberg cells in Hodgkin's lymphoma.[132] Later studies have proposed that tissue eosinophilia is a strong unfavorable prognostic factor in patients with the subgroup nodular sclerosis in Hodgkin's lymphoma.[133]

1.4.5 Galectin -10 also called Charcot Leyden Crystal protein (CLC)

The Charcot Leyden Crystal protein (CLC) was discovered in 1853 by J.M Charcot[134], who identified it in the spleen of a patient with leukemia and later also Leyden[135] studied this protein. It is found extracellularly in a variety of tissues, body fluids and secretions with inflammatory infiltrates including eosinophils and basophils. The CLC-protein accounts for 10% of the cellular protein in mature human eosinophils.[136] In undamaged mature eosinophils, CLC-protein is located in minor primary granule. In reactive or damaged eosinophils it is located in nuclei, cytoplasmic areas and in the extracellular matrix.[137] The details about the secretion of the CLC protein are not known. The CLC protein has unique auto crystallizing properties and can form distinctive hexagonal bipyramidal crystals. It was

originally proposed that CLC-protein was synonymous with eosinophil lysophospholipase[138], but this has now been questioned[139]. Further studies of the CLC-protein structure have found that its x-ray crystallographic structure[140] and the gene structure[141] are similar to the members of the galectin family. CLC-protein is therefore designated galectin-10. Also $CD4^+CD25^+$ Tregs express galectin-10, which seems to be responsible for their suppressive capacity.[142] Therefore the eosinophil researcher Helene Rosenberg[143] proposed in an editorial that human eosinophils, which are the cell in the body with the highest expression of galectin-10/CLC perhaps also could inhibit the function and proliferation of $CD4^+$ T cells.

The role of galectin-10/CLC in diseases is still enigmatic. It has been proposed to be a diagnostic marker to measure esophageal inflammation in eosinophil esophagitis together with other eosinophil-derived proteins like MBP, EPX[144] and elevated galectin-10/CLC on patients with asthma or bronchopulmonary infection.[145]

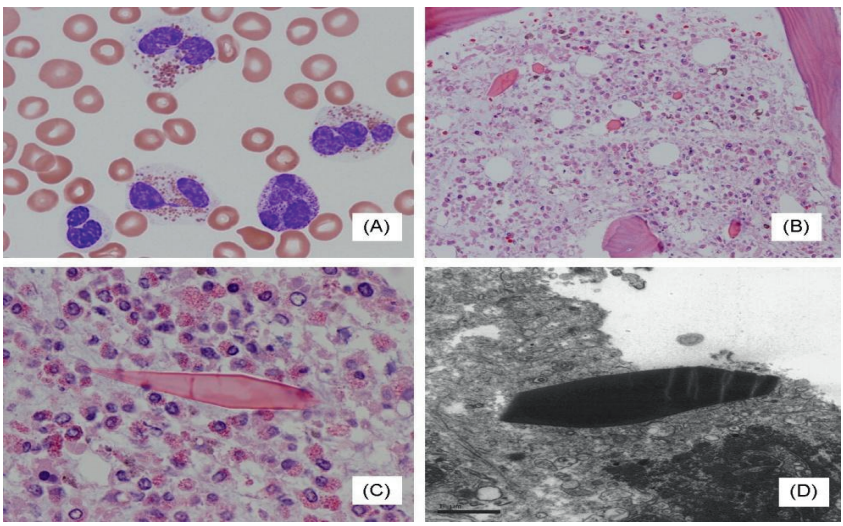

Figure 7. Pictures of abnormal eosinophils and Charcot-Leyden Crystals (CLCs) associated with acute myeloid leukemia. *A) Abnormal eosinophils, (B) extensive bone marrow necrosis with CLCs, (C) bipyramidal shaped CLC with abnormal eosinophils in the background, and (D) electron photomicrograph of CLC. Reproduced with permission from Elsevier.*[146]

1.4.6 The eosinophils in disease

Many diseases are associated with elevated levels of blood eosinophils (eosinophilia). There are two different groups:

- The hematological diseases (primary, clonal disorders)
- The secondary or reactive diseases

According to the World Health Organization (WHO)[147], the first group consists of: myeloid and lymphoid neoplasm with eosinophilia and abnormalities of the platelet-derived growth factor receptor (PDGFRA, PDGFRB or PDGFR1), chronic eosinophilic leukemia not other specified (CEL, NOS) or WHO-defined myeloid neoplasm with associated eosinophilia, and idiopathic hypereosinophilic syndrome and lymphocyte-variant hypereosinophilia and idiopathic hypereosinophilic syndrome (HES), which is a diagnosis of exclusion. The recommended therapy according to WHO[147] is glucocorticoids in HES and lymphocyte-variant hypereosinophilia and hydroxyurea or IFN-α in CEL, NOS and tyrosinkinase inhibitor, imatinib in PDGFRA/B rearranged disease.

Before the diagnosis of a primary hematological disease, it is important to exclude a secondary (reactive) cause of eosinophilia. In developing countries, the most common reactive causes of eosinophilia are infections, especially tissue-invasive parasites.[148] In the developed countries, the majority of eosinophilia cases in outpatient care are due to allergic diseases, like allergic asthma.[149] Drug reactions may also give rise to secondary eosinophilia. Other possible causes of eosinophilia can be eosinophil-associated cutaneus and fibrotic diseases e.g. eosinophilic fasciitis[150] or eosinophil-associated respiratory diseases e.g. idiopathic chronic eosinophilic pneumonia[151] or allergic granulomatosis and angiitis [Churg-Strauss syndrome].[152] Another disease with increasing incidence is eosinophilic esophagitis[153] an antigen-driven chronic inflammatory disorder characterized by infiltration of eosinophilic granulocytes into the esophagus, which normally is totally devoid of eosinophils.

1.4.7 The eosinophils in GVHD

Some other diseases have been associated with the eosinophil, for example: GVHD since the first report of in patients from the Seattle group[18] in 1980. They observed that blood eosinophilia preceded GVHD which also was reported later among eleven of 39 patients after busulfan and cyclophosphamide conditioning.[154] Early studies in 25 patients in 1980 showed an elevation of immunoglobulin E (IgE) in transplanted patients simultaneously with the development of acute GVHD.[155]

Several case-reports have associated eosinophils with GVHD: for example

activated tissue eosinophils in a conjunctival biopsy in a patient with chronic GVHD[156], and acute eosinophilic pneumonia after a debut of acute GVHD[157,158] and in association with chronic GVHD[159] or severe eosinophilia concomitant with acute GVHD after donor lymphocyte infusion.[160] Also eosinophilic fasciitis has been connected to chronic GVHD.[161] A French case-report describes hypereosinophilia in two patients before debut of acute GVHD.[162] There also exists a case-report indicating a possible association between GVHD and eosinophils after solid organ transplantation.[163] One case-report describes a cutaneous eosinophilic infiltrate in a patient with lichenoid skin GVHD after liver transplantation.[163]

There are also more extensive studies than case-reports about eosinophils and GVHD. In an American study, eight of ten children with hypereosinophilia had or developed chronic GVHD.[164] Other report that eosinophilia is connected with a special features of chronic GVHD like sclerotic GVHD.[165] Also levels of eosinophil cationic protein (ECP) on day +28 after transplantation have been connected with concurrent acute GVHD in children.[166] However, there also exist other eosinophilic disorders after allogeneic HSCT, without occurrence of GVHD. For example in one case-report of eosinophilic folliculitis.[167] Some authors also report that the presence of tissue eosinophil is not a reliable indicator of histological differential diagnosis of GVHD, as tissue eosinophils often exist also in drug-induced dermatitis.[168]

Later studies have given contradictory results concerning the meaning of elevated eosinophil counts: The NIH biomarker consensus group proposed the eosinophil as a cellular marker of chronic GVHD in their article from 2005[37], but the eosinophil is not included in their newer report from 2015.[36] Studies have indicated that blood eosinophilia could be used as a negative prognostic factor[169] or as a marker for a less-severe course of acute GVHD or chronic GVHD and even indicate improved overall survival.[170-173] Another study found a high prevalence of eosinophilia (44%) at diagnosis of chronic GVHD, but no association with overall survival, relapse-incidence or non-relapse mortality.[174]

The most detailed study ever about eosinophilic counts in blood samples in patients with chronic GVHD is written by Mortensen[38] in 2014 and their retrospective analysis of blood eosinophilia in 142 allogeneic stem cell transplanted patients. Their analysis showed no connection between concomitant blood eosinophilia and the appearance of chronic GVHD. They reported a significant decrease of blood eosinophil counts after start of systemic glucocorticoid treatment. There was no difference in prognosis between patients with or without eosinophilia with regard to GVHD.

There are very few studies about the phenotypic or functional properties of the eosinophil in GVHD. One study of eosinophils from peripheral blood from patients with acute GVHD, detected the IL-2 receptor subunit CD25 receptor on the surface of the eosinophils in almost all patients with acute GVHD[112]. An extensive prospective study from France did histopathological analyses of intestinal biopsies in all patients with suspected upper gastrointestinal GVHD.[175] All 36 patients with confirmed histological digestive GVHD had tissue eosinophilia, in other words, eosinophils were only present when there were histological signs of GVHD, and eosinophil counts correlated with GVHD severity. In addition, immunohistochemical studies showed the presence of IL-5 and eotaxin in the tissue and electron micrographs showed many degranulated eosinophils in the tissue. This can be an indication that the tissue eosinophils were activated in the patients with GVHD in the upper gastrointestinal tract.

1.5 CLINICAL FOLLOW-UP AFTER TRANSPLANTATION

1.5.1 Chimerism analysis- a sign of engraftment

The definition of "chimerism" is a state in an organism, when there are cells (not germ cells) from two or more lineages at the same time. Chimera is a word from the Greek mythology, describing a creature with the strength and body parts of many animals. Chimerism analysis is used to determine the genotypic origin of the hematopoiesis after allogeneic transplantations and determines the proportion of lymphocytes (or other subsets of cells) that derive from the donor and recipient respectively. It is necessary to obtain genetic markers from the donor and the recipient before the transplantation. Chimerism analyses are used to monitor engraftment kinetics after allogeneic HSCT. The analyses determine the proportions of lymphocytes (or other cells) that derive from the donor and recipient, respectively. There are different states of chimerism:[176]

After allogeneic HSCT:

- Complete chimerism: no recipient cells can be detected the in patient (the exact definition depends on the chimerism analysis method). Suggesting complete hematopoietic replacement after allogeneic HSCT.
- Mixed chimerism: recipient T cells are detected, in particular cells like lymphocytes. 5-90% donor cells set the criteria.
- Split chimerism: one or more lineages are of recipient-origin and one or more lineages are of donor-origin, e.g. e myeloid cells are 100% from the recipient and 100% of T cells are from the donor .

After solid organ transplantation:

- Macrochimerism: more than 1% donor cells are detected.
- Microchimerism: less than 1% donor cells are detected. Normally found after solid organ transplantation.

Traditionally patients who receive a MAC reach complete donor chimerism, as the engraftment process is faster. Repeated chimerism analyses are optional after standard MAC using conventional GVHD prophylaxis, as the majority will have a complete donor chimerism, according to the guidelines of the International Bone Marrow Transplant Registry[177] Newer studies[178,179] have verified that chimerism surveillance after MAC is of very limited clinical value as only 5% had <95% donor derived T cells in bone marrow at two to six months after transplantation.[178] On the other hand, among patients who received RICT, it is very common with mixed chimerism status. For example 43% in one study had a mixed chimerism <90% donor T cells on day 30 after transplantation.[180] Therefore, chimerism analyses at 1,3,6 and 12 months are recommended after RICT during the first year in order to detect GVHD, graft loss or relapse early and because interventions such as donor lymphocyte infusions may depend on chimerism status.[177]

If the chimerism analyses show mixed chimerism, it can signal threatening relapse or rejection. Proposed interventions are to decrease the immune suppression or to perform donor lymphocyte infusions (DLI). At the moment, there are no clear guidelines about these interventions and no national guidelines regarding the care about allogeneic HSCT recipients in Sweden.

After solid organ transplantation, the interpretation of the chimerism analyses is different. Microchimerism <1% donor cells is known to occur in the immediate post-operative period and it can perhaps facilitate graft acceptance[181], but the the existence of macrochimerism >1% donor cells in peripheral blood, bone marrow or other organs may precede threatening rejection.[75] Macrochimerism >1% donor T cells can also occur after intestinal or multivisceral transplantation without signs of GVHD.[75]

There are no international guidelines about the frequency of chimerism sampling after neither ITX or MVTX nor clear recommendations how to proceed when macrochimerism and/or GVHD is suspected. The recommendations concerning GVHD treatment are diverging and both increased immunosuppression[21] or a complete withdrawal of immunosuppression[77] have been proposed.

1.5.2 Immunosuppression after allogeneic HSCT

Immunosuppression is essential due to two reasons after allogeneic HSCT. The first reason is to prevent activation of the immune cells in the graft and consequently from attacking the recipient's tissue and thereby induce a GVHD reaction. The other reason is to decrease the recipient's immune system, so it will not recognize the hematopoietic stem cells as foreign and start a rejection process followed by graft failure. The standard immunosuppression after transplantation consists of an induction phase directly after transplantation and a consolidation phase. Most often methotrexate (MTX) is given 3-4 times during the first week after transplantation together with cyclosporine A, CsA, a calcineurin inhibitor, which diminishes the capacity of T cells to divide. The concentration of CsA is measured weekly during follow up during the first months post transplant. Alternative treatments to CsA are tacrolimus (Prograf®), mycophenolate mofetil (Cellcept®), sirolimus (Rapamune®) or cyclophosphamide.[182] No routine glucocorticoid treatment is given unless the patient develops GVHD or other complications. The immune suppressive therapy is normally tapered after the transplantation and if no complications occur, it can be discontinued after six months if the patient has achieved complete donor engraftment.

The situation after solid organ transplantation is different, as the largest threat is graft rejection (acute cellular rejection or chronic rejection) and not GVHD. Another difference is that the recipient maintains their own immune systems, as they do not change hematopoietic stem cells. Therefore the immune cells, will constantly recognize the graft as foreign and the patients will therefore need lifelong immunosuppression. Otherwise, threatening graft rejection can occur at any time after transplantation. Among solid organ transplants, recipients of intestinal or multivisceral grafts need the most intensive immune suppression therapy, probably due to the volume of immune cells in the grafts. At Sahlgrenska University hospital, the standard protocol currently used is the protocol from Pittsburg.[183] It consists of two major principles, 1) recipient preconditioning with a single-dose of antithymocyte globulin induction therapy in combination with minimization of post transplant maintenance immunosuppression, 2) elimination of glucocorticoids that earlier had been a part of the maintenance immunosuppression. Later studies in mice have proposed that high doses of glucocorticoids and calcineurin inhibitors (like tacrolimus or CsA can cause potent Th1 suppression and thereby promote a Th2 deviation, which can increase the risk of chronic rejection.[184]

2 HYPOTHESES AND AIMS

The main focus of this thesis was to better understand the incidence, prevalence, risk factors and pathogenesis of GVHD in patients after an allogeneic HSCT or intestinal transplantation (ITX) or multivisceral transplantation (MVTX). Concerning the pathogenesis, we have been especially interested in the role of the eosinophilic granulocyte and its interaction with T cells.

The thesis is based on the following hypotheses:

- Eosinophilic granulocytes from healthy individuals can decrease allogeneic T cell proliferation in an *in vitro-model* of GVHD
- Eosinophils from allogeneic transplanted patients have an increased capability to inhibit T cell proliferation compared to eosinophils from healthy persons
- The eosinophils of HSCT patients with GVHD have a different phenotype compared to eosinophils from transplanted patients without GVHD
- The surface receptors of the eosinophils are different in patients with acute and chronic GVHD
- Eosinophils are activated in patients with GVHD
- Treatment with systemic glucocorticoid treatment changes the phenotype of the eosinophil
- Patients receiving MVTX have a higher risk of acquiring GVHD compared to patients with intestinal transplants
- A tumor diagnosis and neoadjuvant chemotherapy are risk factor of GVHD after multivisceral transplantation
- Chimerism status on day 100 after allogeneic HSCT can predict overall survival, disease-free survival and development of chronic GVHD
- Patients with mixed chimerism status have shorter duration of cyclosporine A therapy because clinicians will be prone to withdraw immunosuppression

To test this, the thesis has the following specific aims:

- By using functional tests to see whether eosinophilic granulocytes can decrease allogeneic T cell proliferation in an in vitro-model of GVHD (a so called mixed-lymphocyte reaction). Further studies of possible T cell suppressive mechanisms used by eosinophils, such as the requirement for cell-cell contact, IDO, galectin-10,

and IL-10.

- To see if eosinophils from patients with chronic GVHD can inhibit T cell proliferation
- Perform flow cytometry analysis of eosinophilic surface receptor in eosinophils from HSCT recipients with and without GVHD.
- To use multivariate analysis for comparison of the patterns of surface receptors in HSCT recipients with and without acute GVHD, chronic GVHD and with and without systemic glucocorticoid treatment, respectively.
- To do a retrospective clinical study and determine the incidence of GVHD, as well as clinical features, risk factors, response and choice of therapy in patients after ITX and MVTX.
- By using survival analyses to determine whether chimerism status in allogeneic HSCT patients after RICT could predict overall survival, disease-free survival, non-relapse mortality and relapse incidence and using Cox regression to determine if the time with CsA was different among subgroups and Fischer's exact test to see if incidence of acute GVHD was different among T cell chimerism groups (complete versus mixed).

3 METHODOLOGICAL CONSIDERATIONS

In this chapter, more general considerations are discussed. Detailed descriptions of the methods used are found in the published studies and manuscript.

3.1.1 Patients and study persons

In study I, 50 healthy study persons were recruited to donate peripheral blood for *in vitro*-assays of eosinophilic T cell regulatory function. Persons with ongoing glucocorticoid treatment were not included. The majority of these persons were recruited from students and employees from the research laboratories. As a consequence of the recruitment, there were more women among the healthy study persons and they also had a younger age, than the patients. The study was complemented with blood from eleven allogeneic HSCT recipients. Among these eleven patients, five had chronic GVHD and six did not have chronic GVHD. All patients were recruited from the Bone Marrow transplantation Unit, Sahlgrenska University Hospital. Unfortunately, we could not include patients with suspected acute GVHD, because of practical reasons and difficulties to include them before systemic glucocorticoid treatment had begun. All participants gave written informed consent.

In study II, altogether 35 adult allogeneic HSCT patients were recruited from the Bone Marrow Transplantation Unit, at Sahlgrenska University Hospital after written informed consent. These patients were followed clinically and donated altogether 78 blood samples on different occasions, when they visited the out-patient clinic. They were divided into groups depending on GVHD status and whether they were under systemic glucocorticoid treatment or not.

In study III, consecutive intestinal or multivisceral patients, of all ages, who were transplanted at the Transplant Unit, Sahlgrenska University Hospital from January 1, 1998 to December 31, 2014 were included retrospectively. This cohort included 26 patients. No exclusion criteria were used.

In study IV, all adult patients who received an allogeneic hematopoietic stem cell transplant with reduced intensity conditioning at Sahlgrenska University Hospital between January 1, 2005 and December 31, 2014 were investigated in retrospect. A total of 105 patients with a hematologic malignancy and who had undergone RICT, and with available blood chimerism data on day 100, and whom had not suffered from a relapse before day 100 were included. Those with a benign diagnosis were excluded as the risk of relapse is lower in this population, and they

usually receive other protocols of immunosuppression. Patients who received myeloablative conditioning were not included, as they normally do not develop a mixed chimerism. Other exclusion criteria were cord blood, haploidentical, or syngeneic transplantation as the chimerism is impossible or more difficult to interpret in such cases. Patients not in remission before transplantation were also excluded.

3.1.2 Purification of eosinophils

The eosinophils were purified from heparinized blood within three hours after venipuncture. The erythrocytes were removed by help of 20 minutes of dextran sedimentation (dextran: blood=1:1) at room temperature. After the dextran sedimentation, differential gradient centrifugation at 400 *g* for 20 minutes at 4°C on a Ficoll gradient was used to separate mononuclear from polynuclear cells. The Buffy coat with mononuclear cells was removed. The granulocytes and remaining erythrocytes were found in pellet. Hypotonic lysis was used to remove the erythrocytes from the granulocytes. The hypotonic lysis was done in 3-4 cycles; by help of 6 ml distilled water for 35-40 seconds, followed by addition of 2 ml of 3.4% NaCl in order to stop the reaction and achieve isotonicity. The granulocytes was washed in 15 ml Ca^{2+}-free Kreb's ringer glucose (KRG) (120 mM NaCl, 5 mM KCL, 1.7 mM KH_2PO_4, 8.3 mM Na_2HPO_4, 10 mM glucose, 1.5 mM $MgCl_2$; pH 7.3). Next, eosinophils were separated from neutrophils by help of negative immune depletion as follows: All granulocytes were incubated together with magnetic beads coated with mAbs (MACS; Miltenyi Biotec Inc., Bergisch Gladbach, Germany) directed against CD3 (T cells), CD14 (monocytes), CD16 (neutrophils), and CD19 (B cells). As eosinophils lack these receptors, they could pass the magnetic cell sorter VarioMACS, CS-column (MACS; Miltenyi Biotec), while the other cells were stuck in the magnetic column. Finally the eosinophils were washed twice in KRG. As a control of purity of the eosinophils, 200 cells were counted in a light microscope after cytospin (Cytospin; Shandon Scientific Co. Ltd., London, UK) and staining with Diff-Quick (Dade Behring AG, Deerfield, IL, USA). The purity was routinely ≥98%.

3.1.3 Mixed lymphocyte reaction (MLR)

Healthy volunteers donated blood from which the PBMC responder cells were isolated. Pooled PBMC from 11 healthy volunteers were gamma-irradiated (25Gy; ^{60}Co source) and used as trigger cells. The concept is that the non-irradiated responder cells (T cells) respond to foreign tissue antigens (HLA) expressed by the mixture of PBMC from 11 non-related donors. This method has been adapted from the method used at the Clinical Immunology Laboratory, Sahlgrenska University Hospital. The purpose of the gamma-irradiation is to inhibit proliferation, so only the responder cells are able to proliferate, a so called one-sided MLR. Freshly

purified eosinophils were added either on the same day as the start of the MLR or two days later. After incubation for 6 days at 37°C, a low-dose beta-emitter ^{3}H-thymidine (1μ Curie/well: Perkin Elmer, Waltham, MA, USA) was added for 6 hours and the cells were harvested using a cell harvester. A beta-scintillation counter (1450 Micro Beta TriLux; Perkin Elmer) was used to measure the incorporation of radioactive thymidine as a measure of cellular proliferation, which was expressed as counts per minute (cpm). To examine if cell-cell contact was necessary for the inhibiting effect, transwell-96 culture plates were used. In these, eosinophils were either cultured in permissive transwell inserts (3μm) or in non-permissive (0.4 μm) ones. The MLR culture was cultured in the receiver plate. The cell medium used in all MLR: s was RPMI 1640 supplemented with 10% human AB serum, 2 mM L-glutamine, 50 μg/ml gentamicin and 0.05 mM β-mercaptoethanol.

3.1.4 Chimerism analyses

The purpose of the chimerism analyses is to detect the percentage of donor CD3+ cells (T lymphocytes), $CD19^{+}$ (B lymphocytes) or hematopoietic stem cells ($CD34^{+}$) or other hematopoietic cells in peripheral blood or bone marrow of the recipient. The chimerism analysis reveals if a transplanted patient's blood cells are strictly donor-derived (complete chimerism) or a mixture of donor-derived and the patient's own remaining cells (mixed chimerism). Quantitative chimerism is part of the routine surveillance after bone marrow transplantation, but it is only done on demand after solid-organ transplantation. A prerequisite for the method to be performed is that pre-transplantation blood samples from the recipient and donor are available as references. These assays have been performed at the Department of Clinical Genetics, Sahlgrenska University Hospital as follows: Separation of mononuclear cells in to two fractions: non T cells and T cells by help of immunomagnetic beads (Dynabeads CD3 from Thermo Fisher Scientific). In some cases, $CD34^{+}$ cells from the bone marrow were analyzed in addition using Dynabeads CD34. For fragment analysis, DNA was extracted from the isolated cells, followed by PCR amplification of short tandem repeats (STRs) with fluorescent tagging. STRs (sometimes called microsatellites) are short highly polymorphic DNA sequences repeated in tandem multiple times. After PCR amplification, the fluorescently labeled PCR-products were separated and detected by capillary electrophoresis on a Genetic analyzer 3500 or 3500xL (Applied Biosystems, CA, USA). The relative amounts of donor and recipient cells were calculated after genotyping, using the GeneMapper software (Applied Biosystems, CA, USA). This method was used when the donor and recipient have the same sex. In case of sex mismatch, a FISH-based method was used to score hybridization signals using chromosome X and Y specific DNA probes on interphase nuclei. At least 500 nuclei were included in the latter analysis. The FISH assay can occasionally be more sensitive than STRs, in sex mismatched cases. The reason is

that it directly measures the frequency of the minor component marker.[185]

3.1.5 Flow cytometry (FACS)

In studies I and II, flow cytometry of surface receptors on eosinophils was done using 3-color or 4-color flow cytometry. A FACSCanto II™ Flow Cytometer (BD Biosciences, Franklin lakes, NJ, USA) with Diva 6 software was used in all analyzes of the expression of surface markers. Flow Jo software (Tree Star Inc., Ashland, OR, USA) was used to analyze the results.

In study I, T cells present in the eosinophil: MLR co-cultures were stained to determine if eosinophils preferentially suppressed the proliferation of CD4+ T cells, CD8+ T cells or regulatory T cells. The cells were incubated for 30 minutes at 4°C with CD4-APC-H7, CD8-FITC or CD8-PerCP-Cy5.5, CD25-APC or CD25PE, and CD127-Alexa Fluor 647 or CD127-FITC. Intracellular staining of the T cells was done after fixation and permeabilization with a Foxp3 Fixation/Permeabilization kit (eBioscience, San Diego, CA, USA). Proliferation was monitored by intracellular staining for the proliferation marker Ki-67. Regulatory T cells were gated as $CD4^{+}$, $CD25^{+}$ and $CD127^{low/neg}$. Fluorescence Minus One (FMO)[186] technique was used as a control of background staining. All data about surface receptors are expressed as median fluorescence intensity (median-FI).

In study II, the analyses were done on whole blood (non-fractionated leukocytes) to avoid non-intentional activation of the eosinophils by the purification process. All flow cytometry analyses were done within 24 hours after venipuncture as we have shown that eosinophils alter their expression of surface markers if the cells are delays in the analyses. EDTA-anticoagulated blood was used and the erythrocytes were removed by repeated hypotonic lysis. Unfractioned leukocytes were incubated at 4°C for 15 minutes in the dark with a panel of fluorochrome-conjugated mouse monoclonal antibodies directed against surface receptors. The gating strategy was to first set a granulocyte gate and then to gate for eosinophils based on high side scatter and low/no expression of CD16, to differentiate them from neutrophils.

3.1.6 RNA extraction, cDNA synthesis and real-time quantitative PCR (qPCR)

In study I, real-time quantitative PCR was used. First eosinophils were isolated as described under purification of eosinophils. Then RNA was extracted using the Rneasy Mini Kit (Qiagen, Valencia, CA, USA) and RNA quality and concentration were evaluated in the Experion Automated Electrophoresis System (Bio-Rad Laboratories, Berkeley, CA, USA). Total RNA was used to synthesize cDNA by

AMV reverse transcriptase, PCR-dNTP mix, RNAse inhibitor and random primer pd(N)6, (all from Roche Applied Bioscience, Mannheim, Germany). The protocol for cDNA synthesis was; 5 minutes at 20°C, 50 minutes at 42°C, 5 minutes at 70°C and a final step at 4°C.

Eosinophilic expression of the genes for galectin-10 and hypoxanthine phosphoribosyltransferease were analyzed by Taqman gene expression assays (Applied Biosystems, Foster City, CA, USA) using 7500 real-time PCR system and 7500 System SDS software (Applied Biosystems). Eosinophils from healthy individuals were used as controls. We used the Pfaffl method algorithm[187] to calculate the transcript levels of galectin-10 relative to those of the housekeeping gene hypoxanthine phosphoriboyltransferase.

3.1.7 Restrospective clinical studies

Study III and study IV included studies of the medical records. In study III, we created a table for each transplanted patient and collected clinical data such as protocol of immunosuppression, histopathological biopsy results and the clinician's description of GVHD features. This study is a part of a larger research project about the follow-up of patients after ITX or MVTX. Study IV about chimerism analyses also included studies of the medical records. We used our local part of the EBMT registry to identify patients who fulfilled the inclusion criteria. Both studies have been approved by the Regional Ethics Committee in Gothenburg.

3.2 STATISTICAL ANALYSES

3.2.1 Univariate statistics

In study I, paired Wilcoxon signed-rank test and Mann-Whitney U-test were used to test for statistical significance between groups. In study II, the Mann-Whitney U-test was used to analyze variables derived from the multivariate analyses with VIP-values >1.0. In studies III and IV, Fischer's exact test of independence was used to detect differences between groups. All these analysis were done using GraphPad prism software (GraphPad, San Diego, CA, USA). In study IV, logistic regression was used to evaluate the impact of chimerism status on development of chronic GVHD. Cox's proportional hazard modeling techniques were used to see whether duration of cyclosporine A therapy differed among groups. Logistic regression and Cox's proportional hazard were done in SPSS Statistics 21 (SAS Institute, Cary, NC, USA. A p-value <0.05 was considered to be statistically significant in all studies.

3.2.2 Multivariate statistics

In study II, we used multivariate analysis of pattern recognition "Orthogonal Partial Least Squares-Discriminant Analysis" (OPLS-DA) and "Orthogonal-Projection to Latent Structures" (OPLS) using the software SIMCA (version 13.03) statistical package (Umetrics, Umeå, Sweden). OPLS-DA is a form of "scatter analysis" that is used to recognize patterns in large data sets. The difference between the more traditional partial component analyses (PCA) is that this method allows one to determine if the data set can discriminate between different study groups by help of Y-variables.[188] For example, in study II, the method was used to see if the data set of eosinophilic surface receptors (X-variables) could discriminate transplanted patients with acute GVHD from those without GVHD; patients with acute GVHD were set as Y=1 and patients without GVHD were set as Y=2 and the median FI expression levels of different eosinophilic receptors were set as X-variables. The OPLS-DA could then find differences and similarities between these two groups.

Before the analysis by help of the SIMCA transformation tool, all X-variables were normalized by log-transformation, mean-centering and unit variance scaling. The purpose is to give every variable an equal chance of influencing the model. After calculation, a valid model might be generated. The quality of the model is defined by R2Y, a measure of the amount of variance in Y that is explained by the X-variables. The R2Y-value therefore indicates to what extent that the X-variables can or cannot explain the differences between the studied groups. Internal validation of the models has been done using cross-validation, in which one of seven samples is removed and the analysis is done without that sample. When all objects have been removed once, the cross-validation will calculate a Q2Y value. A high value indicates that the model will give the same results even if one or many samples are removed. In univariate analyses, a p-level is used as a significance level. In OPLS-DA this is not possible, but instead permutation tests can be used as validation. The purpose is to exclude that the model is "overfit". An overfit model occurs when there are few samples and many variables and the model is correct for the actual data, but will not be transferable or adequate in other populations.

3.2.3 Survival analysis and competing risks.

In study IV, we followed the EBMT statistical guidelines[189] to choose patient-related outcomes. For the survival analyses, when only two outcomes were possible, e.g. disease-free survival (DFS) and overall survival (OS), when the patient could be alive (event=0, censor) or dead (relevant event=1), Kaplan-Meier analysis was done.[190] These analyses were done in SPSS Statistics 21. A log-rank test was used to see if there were significant differences between groups. When we

analyzed acute GVHD, we used the same variant of Kaplan-Meier analysis and log-rank test, because we only had two possible outcomes: acute GVHD=1 (relevant event) and no acute GVHD (censored event= 0) . We did not have any competing events, as patients who had died during the first 100 days were excluded.

Concerning non-relapse mortality (NRM) or relapse incidence (RI), the Fine and Gray's method[191] for competing risks was used. The reason for this was that there were three possible outcomes, exemplified by relapse incidence: event= 1, censored= 0 and death from non-relapse cause= 2. This analysis was performed using the software R, (http://www.r-project.org/), the package "cmprsk" and the CumIncidence function.[192]

4 RESULTS

4.1 Eosinophils can suppress allogeneic T cell proliferation (Study I)

The first study is based on the hypothesis that the eosinophilic granulocytes from peripheral blood can be T cell immunoregulatory in patients after allogeneic HSCT. It consists of functional studies of the eosinophilic granulocytes after isolation from peripheral blood. We used an *in vitro* model of GVHD, the mixed lymphocyte reaction, and co cultured eosinophils with proliferating T cells.

We started with studies of eosinophils from healthy persons and we could see that eosinophils could suppress allogeneic T cell proliferation. This effect was only seen when eosinophils were added 1:1 to PBMC and it seemed to be specific for eosinophils, as neutrophils did not have this effect. (Figure 1A). The suppressive effect was also a feature of eosinophils from patients after HSCT (Figure 1C and D), but there was not any significant difference in suppressive capacity between eosinophils derived from the transplanted patients with GVHD compared to those without GVHD.

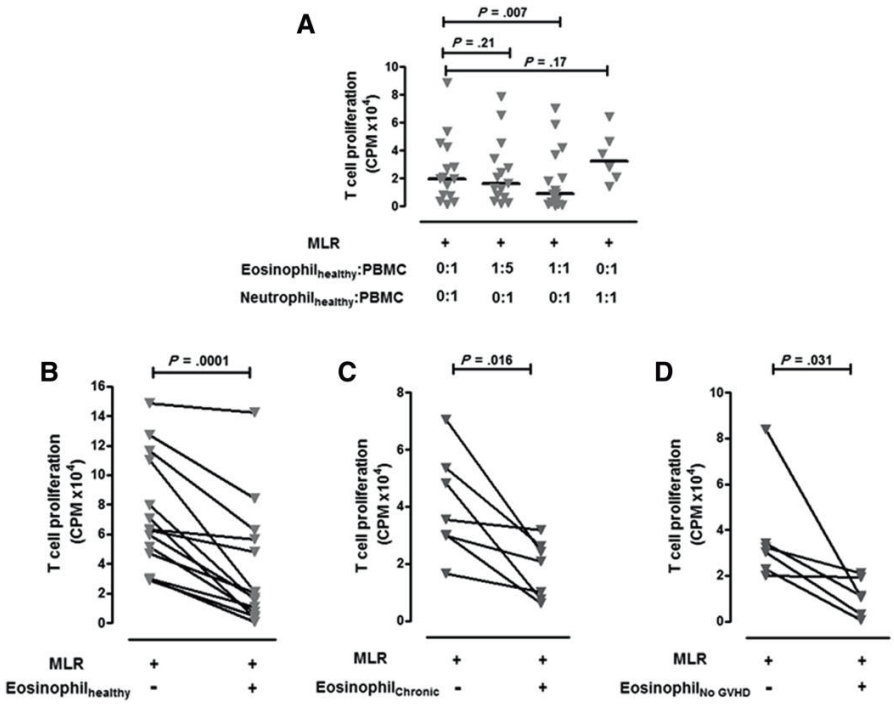

Figure 1. *T cell proliferation measured as counts per minute in MLR/eosinophil co cultures with eosinophils from healthy persons (A,B) or HSCT transplant recipients with chronic GVHD (C, n=7) or no GVHD (D, n=6). MLR/neutrophil co cultures were used as controls (A). The ratio of eosinophils (or neutrophils) to peripheral blood mononuclear cells (PBMC) in the co cultures is indicated below the x-axis (A). Reproduced with permission from Elsevier.*

We continued to see if cell-cell contact between eosinophils and T cells was compulsory for the T cell regulatory effect of eosinophils. To test this, we cultured the eosinophils in transwell inserts. These inserts had permeable pores, enabling eosinophils to reach the T cells or non-permeable inserts where eosinophils would only be able to influence the T cells through cytokines or other soluble mediators. As seen in Figure 2, the suppression only occurred in the cell culture plates with permeable inserts. Our conclusion is that cell-cell contact is compulsory.

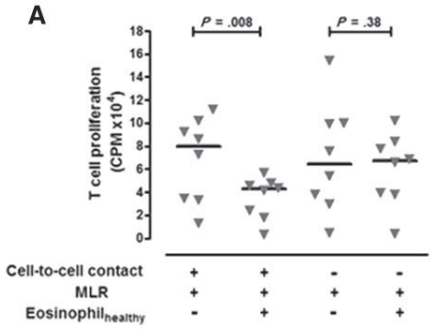

Figure 2. *Contribution of cell-cell contacts on the inhibitory effect of eosinophils on allogeneic T cell proliferation. (n= 8 peripheral blood blood eosinophil donors in each group). Each data point represents the median counts per minute value of 3-4 replicate cultures derived from one individual. Reproduced with permission from Elsevier.*

We also tried to examine by which mechanisms the eosinophil practiced their suppression. Antibodies were used to block various molecules to try to diminish the inhibitory capacity of eosinophils. We could not identify the inhibitory mechanism, but in our hands, the eosinophilic granule proteins ECP, EDN, regulatory cytokine IL-10 and its receptor CD210, did not seem to be inhibitory mediators, as addition of the corresponding to the MLR did not diminish the inhibition. We also excluded the enzyme indoleamine 2,3-dioxygenase (IDO), as we could not measure signs of IDO-activity in the supernatants from the MLR. IDO is an enzyme, which can act immunosuppressive and inhibit T cell proliferation by metabolizing the essential amino acid tryptophan into kynurenine.[193] An interesting finding was that RT-PCR analysis showed that eosinophils from allogeneic HSCT recipients expressed very elevated levels of galectin-10 mRNA, compared to healthy individuals, see Figure 3. However, the addition of antibodies to galectin-10 did not abolish or diminish the suppressive effect of eosinophils to a statistically significant degree, median inhibition before addition of antibodies to galectin-10 was 70% and after addition 47%.

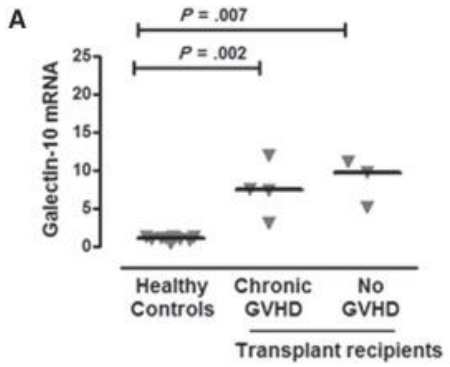

Figure 3. *Expression levels of mRNA for galectin-10 in eosinophils from peripheral blood from healthy control persons and in eosinophils from allogeneic hematopoietic stem cell transplanted patients with and without chronic GVHD. Shown are the transcript levels of galectin-10 as determined by the Pfaffl method. For all patient eosinophils, each data point represents the mean of duplicate PCR reactions. Reproduced with permission from Elsevier.*

4.2 Blood eosinophils from patients with acute and chronic GVHD have an activated phenotype (study II)

In study II, our aim was to examine the hypothesis that blood eosinophils are activated in GVHD. To do this, we analyzed blood eosinophil counts and percentage of leukocytes together with 15 surface markers on eosinophils by help of flow cytometry performed on 78 blood samples from 35 HSCT recipients. As we hoped to identify different patterns of receptors on the eosinophils, we used multivariate statistical methods to analyze the data.

First, we could see that the eosinophils in the blood of patients with chronic GVHD had a different phenotype compared to the eosinophils from patients without chronic GVHD. It may be seen in Figure 5a that the majority of chronic GVHD patients (dark square) form a cluster above the horizontal line, whereas the majority of the patients without GVHD (indicated by open triangles) cluster below the line. This segregation is based on eosinophilic expression of surface markers. A column loading plot was done, of the eosinophilic receptors, see Figure 5b. The plots show that eosinophils from patients with chronic GVHD had relatively higher levels of surface receptors. Univariate analysis showed higher levels of CD11c, CD69, CD9 and CD18.

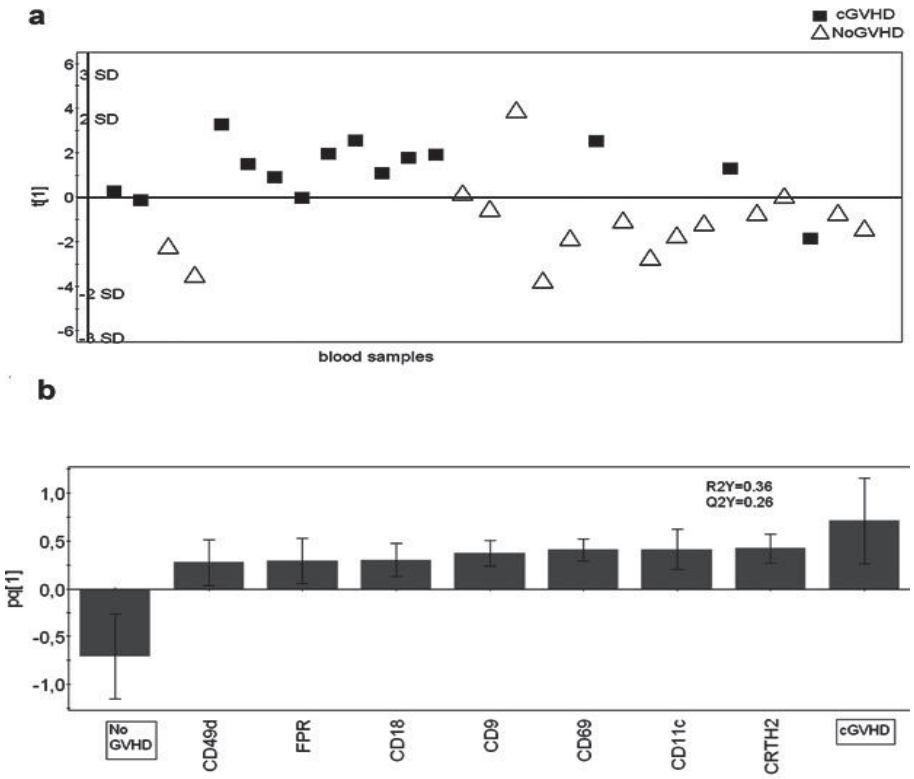

Figure 5. *The pattern of surface receptors in eosinophils from peripheral blood samples from transplanted patients with chronic GVHD (cGVHD) differs from those of transplanted patients without GVHD (NoGVHD). A) OPLS-DA score plot based on flow cytometry analysis in patients with cGVHD (n=9; sampled on 14 occasions) and transplanted patients without GVHD (n=9; sampled on 15 occasions). Only variable with VIP-values>1.0 are included in the model. The y-axis indicated the degree of separation of the study groups, and the x-axis indicates the arbitrary order in which the samples were entered into the model. B) Column loading plot of the eosinophilic variables. Only parameters with VIP-values>1.0 are included. X variables that project in the same direction as the "cGVHD" column are positively associated with cGVHD, and inversely related to the "NoGVHD" column, which projects in the opposite direction. Reproduced with permission from John Wiley and Sons.*

After the chronic GVHD versus no GVHD comparisons, we next compared acute GVHD versus no GVHD. Here we could see that eosinophil markers could help to differentiate these two patient categories, and moreover, that the percentage of blood eosinophils (eos%) and the activation markers CD69 were the most important factors for this differentiation.

Next, we examined if there was a stereotypical kind of activation of the eosinophils in GVHD or if they had different patterns of surface markers in acute and chronic

GVHD. A new OPLS-DA analysis could conclude that there were different molecular patterns in the groups of patients with acute and chronic GVHD, respectively. The markers CD18, CD11c, CRTH2 and CD9 were the molecules that differed most between the two types of GVHD, and were relatively more elevated in chronic GVHD than in acute GVHD.

Systemic corticosteroids are the cornerstone of treatment of more severe forms of acute as well of chronic GVHD. Our next aim was to examine how glucocorticoid therapy affected the activated eosinophil phenotype displayed by patients with GVHD. As controls, we also investigated transplanted patients treated with systemic steroids for other causes than GVHD. In patients with GVHD, the results were striking: after systemic introduction of steroids several molecules, notably CD11c, CRTH2, CD44, CD9, CD18 and CD49d were all significantly down-regulated.

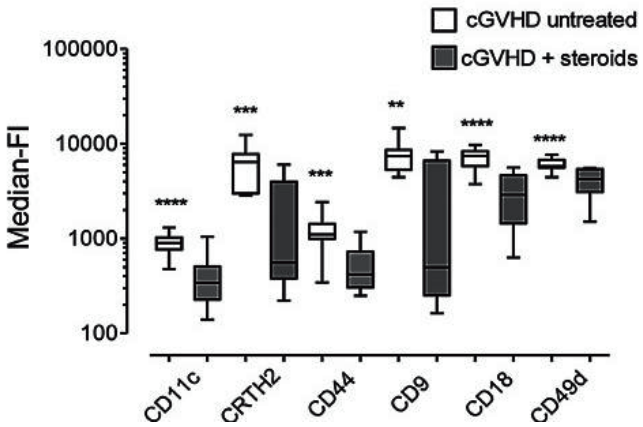

Figure 6. *Systemic therapy of corticosteroids results in a down-regulation of surface markers in patients with chronic GVHD (n=12, sampled on 17 occasions) compared to eosinophils in patients with untreated chronic GVHD (n=9; sampled on 14 occasions). Univariate Mann-Whitney statistical analyses . Data are shown as boxed with median horizontal lines and min/max whiskers. **P<0.01, ***P<0.001,**** P<0.0001. Reproduced with permission from John Wiley and Sons.*

We also compared the pattern of eosinophilic receptors from patients with chronic GVHD under glucocorticoid treatment with transplanted patients under glucocorticoid treatment, without a diagnosis of GVHD. The OPLS-DA analysis revealed that they had different phenotypes, as seen in Figure 7.

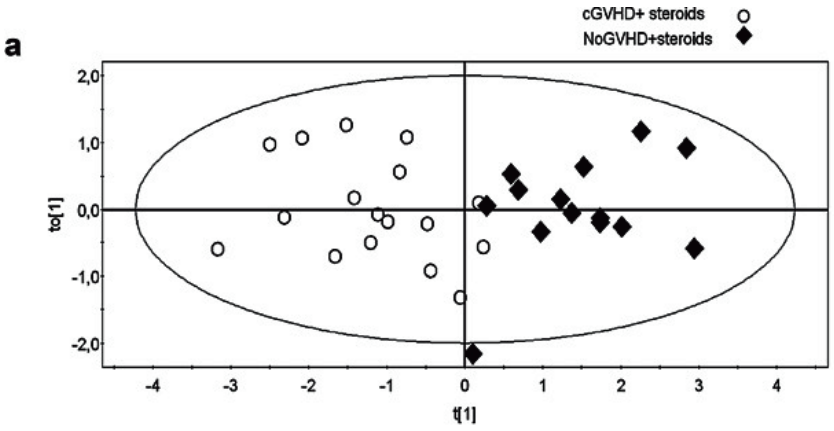

Figure 7. *Eosinophils in blood samples from patients with chronic GVHD under systemic glucocorticoid therapy (cGVHD+ steroids) have a different phenotype compared to steroid-transplanted patients without GVHD (NoGVHD+steroids). OPLS-DA score plot based on flow cytometry analysis of eosinophils in blood samples from patients with treated cGVHD (n=12; sampled on 17 occasions) and patients without GVHD under systemic glucocorticoid treatment (n=8, sampled on 14 occasions). Only variables with VIP-values >1.0 are included in the model. Reproduced with permission from John Wiley and Sons.*

4.3 A tumor diagnosis or chemotherapy are possible risk factors for acute GVHD after MVTX (Study III)

The third study is a clinical retrospective study of the incidence of acute GVHD and possible risk factors for GVHD in a cohort of intestinal and multivisceral organ transplanted patients in Scandinavia. We also wanted to study the clinical features of GVHD and outcome of treatment strategy. We chose to examine the impact of the following parameters on the risk of GVHD: graft type (intestinal or multivisceral), malignancy as a cause of transplantation, chemotherapy or radiotherapy as neoadjuvant therapy, and inclusion of the spleen in the graft combined with recipient splenectomy. All 26 patients transplanted at Sahlgrenska University Hospital from January 1 1998 until December 31 2014 were investigated.

Here we could see that 5/26 (19%) patients developed acute GVHD. No one incurred chronic GVHD. Fischer's exact test showed that a tumor diagnosis was a possible risk factor (P=0.001) as well as neoadjuvant chemotherapy/radiotherapy (P=0.004). However, graft type or a spleen-containing graft was not risk factors.

The clinical features included skin rash in four patients and hemolysis in the fifth patient (passenger lymphocyte syndrome). The diagnosis of acute skin GVHD was supported by the finding of macrochimerism (a maximum of 70% donor T cells in blood) in all four patients when they presented with the skin rash. The diagnosis of GVHD was difficult in most cases, especially as acute GVHD occurred at the same time as kidney failure, septicemia, acute rejection, pleural effusions and secondary hemophagocytosis in different patients. A complicating factor was that chimerism analysis was not always indicative, illustrated by one patient who had macrochimerism (27% donor T cell chimerism) without a diagnosis of GVHD. It was also difficult to choose the level of immunosuppression, as one patient incurred acute GVHD, acute rejection and EBV-driven PTLD at the same time. The treatment strategy also varied, such that immunosuppression was increased in 4/5 (80%) patients when GVHD was confirmed, but in one case it was decreased following recommendations by the consultant hematologist who wanted to strengthen the patient's own immune system in the fight against acute GVHD.

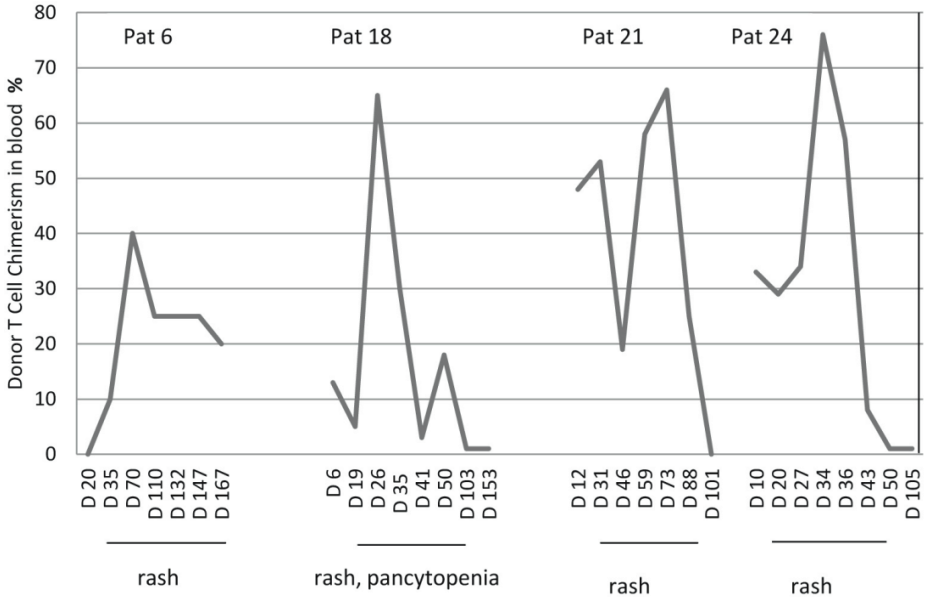

Figure 8. *Donor T cell chimerism in four patients with acute GVHD. D indicates the post-transplantation day. The level of donor T cell chimerism is expressed as a percentage of donor-derived cells of total $CD3^+$ cells in peripheral blood. The different patients (Pat) are marked by their different number. Reproduced with permission from Elsevier.*

4.4 Patients with GVHD or mixed chimerism have longer treatment time with cyclosporine A (Study IV)

In study IV, we wanted to see if chimerism status on day 100 after allogeneic HSCT can predict overall survival, disease-free survival and GVHD. We also focused on how clinicians interpreted chimerism data 100 days after transplantation, and to what extent and in what direction the laboratory data influenced the clinician's choice of immune suppression. First, we examined if overall survival and disease-free survival in patients with mixed chimerism on day 100 (≤95% donor T cells) was shorter than in patients with complete chimerism. Here we could not see any significant difference between the groups.

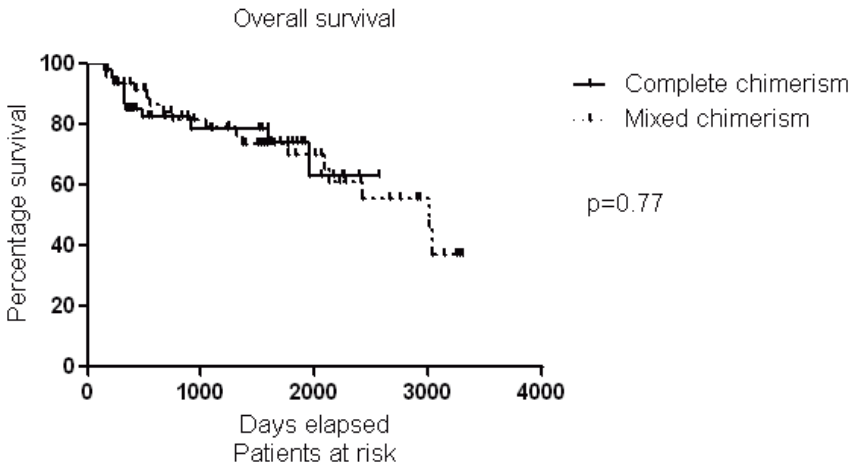

Figure 9. *Kaplan-Meier analysis of overall survival (OS) among patients (n=105) with complete (n=52) and mixed chimerism respectively (n=53) 100 days after transplantation. Log-rank test, p=0.77. The survival curve is terminated when less than five patients are at risk in each group.*

We also examined how biopsy-verified acute GVHD during the first three months was associated with chimerism status on day 100. There were more patients with mixed chimerism who incurred acute GVHD (n=15), than with complete chimerism (n=9), but the difference was not significant, p=0.22.

The next step was to see whether the clinician was guided by chimerism status at day 100 in choice of treatment, especially if they withdrew the immunosuppression or prescribed donor lymphocyte infusions (DLI:s). Here we did a Cox regression analysis and found that patients with complete chimerism had shorter duration of

cyclosporine A therapy (hazard ratio 1.86, p=0.01), see Figure 10. The patients with chronic GVHD had a longer treatment time with CsA (hazard ratio=0.43, p<0.01). Bone marrow cells as donor source, a related donor or a diagnosis of acute GVHD were not associated with a significant longer or shorter time of CsA treatment. Patients with a related donor, had significantly more chronic GVHD compared to those with an unrelated donor, p=0.02.

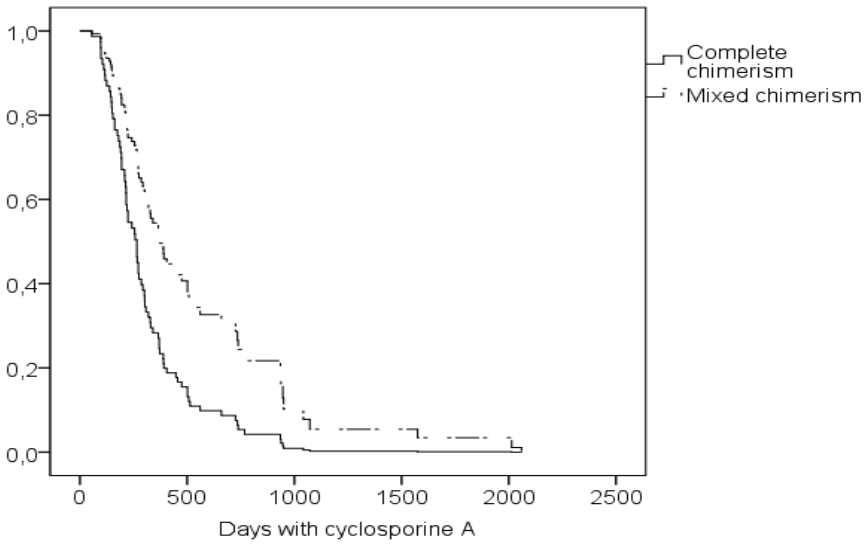

***Figure 10**. Days with cyclosporine A among patients with complete (n=52) or mixed (n=53) chimerism status at three months. Y-axis shows fraction of patients. Complete chimerism; ≥ 96% donor T cell chimerism, Mixed chimerism ≤95% donor T cell chimerism. Cox regression analysis, complete chimerism, hazard ratio 1.86, confidence interval: 1.19-2.93, p-value =0.01. The curve is terminated when less than five patients are at risk in each chimerism group.*

During the first 100 days, the immunosuppression was completely withdrawn in 5/105 (4.8%) patients because of suspected relapse. Four of these patients subsequently developed mixed chimerism and one developed complete chimerism on day 100 after transplantation. Altogether, 14/105 patients (13%) received one or several donor lymphocyte infusions (DLI:s), eight of these were due to relapse (therapeutic DLI), and six were due to mixed chimerism (preemptive DLI). Here 2/8 (25%) with relapse survived compared with 5/6 (83%) in the group with preemptive DLI , however it did not reach statistical significance p=0.10 (Fischer's exact test).

In summary, our results indicate that chimerism status on day 100 could not

predict overall survival, disease-free survival, relapse incidence, or non-relapse mortality. Patients with mixed chimerism and a diagnosis of chronic GVHD had longer time with CsA. We also found that the clinicians were influenced by chimerism results in their choice of immunosuppression such that CsA therapy was stopped in several patients with suspected relapse during the first 100 days, or preemptive DLI were given due to worrying mixed chimerism status.

5 DISCUSSION

The focus of this thesis is on GVHD after allogeneic HSCT, ITX or MVTX. We chose to study the role of the eosinophilic granulocyte, as it has been proposed to be a biomarker in chronic GVHD[37] and to have immunoregulatory properties.[100, 127]

In the first two articles, we focused on the role of the eosinophilic granulocyte in GVHD. Our first hypothesis was that the eosinophils could be immunoregulatory. Therefore, we focused on functional *in vitro* studies. In study I we saw that the eosinophils could inhibit allogeneic T cells proliferation, unlike the neutrophilic granulocytes. Our second hypothesis was that blood eosinophils would have different patterns of surface receptors in acute and chronic GVHD because the immunopathogenesis is different in these diseases, and possibly, the function of the eosinophil might differ in these two conditions. In study II, we found that patients with acute GVHD had higher levels of blood eosinophils and that there was a pattern of up-regulation of CD9, CD11c, CD18, CD69 on eosinophils from patients with chronic GVHD compared to transplanted patients without chronic GVHD. Both CD11c and CD18 belong to the leukocyte integrin subfamily, which mediates important cell-cell and cell-extracellular matrix interactions.[114] Our interpretation is that eosinophils can act immunoregulatory in GVHD (study I) and that they have different phenotype in acute and chronic GVHD(study II). Probably this up-regulation of eosinophilic receptors facilitates for the eosinophil to bind to activated endothelial receptors like ICAM-1 and VCAM-1 and then enter the tissues, where it inhibits the GVHD reaction. Supporting data behind this is that eosinophil counts in our study (paper 2) were higher in patients with acute GVHD and also other studies have shown eosinophilia to be a presenting sign before acute GVHD.[160,162] In contrast, a recent study could not find any association between hypereosinophilia and GVHD.[38]

However, the unique and interesting finding in our study was that we not only examined the eosinophilic counts, but also evaluated the eosinophilic phenotypes (study 2) and functional properties (study 1). Among the very few studies of surface markers in human blood eosinophils in transplanted patients there is one article, which indicates that CD25 was up-regulated in patients with untreated treated acute GVHD.[112] In common with us, they saw an effect of systemic steroid therapy, as CD25 was down-regulated on the surface of eosinophils after start of systemic steroid therapy.[112] The most striking result was the global down-regulation of eosinophilic surface molecules after start of systemic steroid therapy. The decrease in blood eosinophilic counts that we documented was also seen in the article by Mortensen[38] and it was expected, as it is well-known that systemic corticosteroids give rise to eosinopenia by inhibition of IL-3-, GM-CSF- and IFN-

γ-mediated eosinophil survival[48] and by induction of apoptosis of the eosinophils.[49] By help of multivariate analysis we also could see that the esoinophils from patients with and without chronic GVHD differed with regard to their patterns of surface receptors. To our knowledge this has not been examined in GVHD before, but it is in agreement with recent studies that the eosinophils have different phenotypes in patients with eosinophilic esophagitis, inflammatory bowel disease, airway allergy[115,194], systemic sclerosis[195] and other diseases with eosinophil involvement. Our interpretation is that the eosinophils can adapt to different microenvironment such as in acute and chronic GVHD, respectively, two complex conditions with partly different immunopathogenetic background. Possibly, the eosinophils have a function in GVHD. These different molecular patterns could perhaps be exploited as a diagnostic aid in GVHD. It is an attractive option to identify blood-based biomarkers for the diagnosis of GVHD. In addition, we saw that the eosinophils from transplanted patients also reacted differently to systemic glucocorticoid treatment it was only the eosinophils from the patients with GVHD that altered their phenotype, which we take to indicate that it was only the activated eosinophils in GVHD that responsed, whereas the non-activated, resting eosinophils in the circulation of patients with other causes were not influenced by the steroids. We did not study patients with glucocorticoid-resistant GVHD. However, it would be interesting in further studies to see if the eosinophils express different surface markers in patients without response to glucocorticoids. If the eosinophils have different patterns of surface receptors also in these patients, it could shed light on the mechanisms underlying glucocorticoid-resistant GVHD, which is a disease with still a high mortality and morbidity.[196]

In study 2 we examined the functional properties of the eosinophils. This is an area not much studied. Here we could see a significant inhibition of allogeneic T cell proliferation in our *in vitro*-model of GVHD. Interestingly, this effect was restricted to eosinophils and did not include the granulocyte suspension (most consisting of neutrophils). This control was done to exclude the possibility that it was merely a lack of nutrients that accounted for the reduced proliferation in eosinophil: PBMC co-cultures due to overcrowding. Nevertheless, other studies have reported the existence of particular neutrophil subsets with ability to inhibit T-cell proliferation.[197]

We attempted to identify the T cell suppressive mechanism used by eosinophils and found that cell-cell contact was required. This does not preclude the involvement of soluble suppressive molecules, as it may be necessary to reach high local concentrations for such putative molecules to be suppressive. Earlier studies from the 80:s[126], have shown that purified granule proteins ECP and EPX could inhibit lymphocyte proliferation. However, when we used whole eosinophils we could not abrogate their T cell suppressive capacity by administration of antibodies

to these granule proteins. Nor could we verify the neutralizing capacity of the selected antibodies as we were unable to reproduce the T cell suppressive capacity of purified granule proteins. As galectin-10 has been proposed to be responsible for the immunosuppressive function of Tregs[142] we examined its expression in blood eosinophils from transplanted patients by help of RT-PCR. Interestingly, we found that galectin-10 mRNA increased in eosinophils from patients after allogeneic HSCT, compared to in healthy controls. However, we could not detect any differences in galectin-10 mRNA between patients with and without chronic GVHD. Other studies have documented elevations of galectin-10 in other diseases like in eosinophilic esophagitis,[105] celiac disease[198] and in asthmatic airway inflammation.[199,200] Our result showing elevated galectin-10 mRNA levels in transplanted patients suggests that galectin-10 is required for some reason, possibly to help regulate a very dysregulated immune system as seen in the post-transplant setting. However, we could not clearly document that blockade of galectin-10 abrogated the inhibitory capacity of eosinophils.

However, additional later analyses in a thesis done by a member of our group indicated that part of the eosinophil-mediated suppression of allogeneic T cell proliferation could partly be restored (31%) by blocking galectin-10 using a larger number of study subjects and matched statistical analyses.[201] Our conclusion is that galectin-10 may mediate part of the T cell suppression exerted by human eosinophils. Interestingly the inhibiting effect of the eosinophils on allogeneic T cell proliferation was seen also in eosinophils from transplanted patients, indicating that eosinophils in transplanted patients have preserved T cell suppressive capacity, at least in vitro. However, there were no differences in suppression between patients with chronic GVHD and not. One reason could be our small sample size. Another reason could be that our *in vitro*-model was not too robust and therefore did not have enough sensibility for this kind of experiments. These experiments are also technically advanced as the eosinophils can degranulate if they experience temperature differences. Another explanation could be that the eosinophil uses several mechanisms when inhibiting T cells, including both cell-cell contact and inhibition by help of soluble factors. If that is the case, it would be in common with other immunomodulatory cell types like human mesenchymal stromal cells, which use multiple mechanisms like IDO[202] galectin-1[203] and both soluble factors and cell-cell contact.[204]

A challenge when studying allogeneic transplanted patients and especially GVHD is the large amount of possible confounding factors, which are known to influence the immune cells. The most evident are: different diseases before transplantation, different conditioning protocols and different post-transplantation complications like graft rejection, graft failure, relapse and therefore different immunosuppression. Another possible confounder when studying blood

eosinophils is that systemic glucocorticoids are known to decrease eosinophilic counts[205] due to apoptosis of eosinophils.[206, 207] As we were aware of this effect, we excluded patients with GVHD under systemic glucocorticoids in this MLR study. Therefore, we could unfortunately not include patients with severe GVHD, as they are under systemic treatment, but the advantage was that we could exclude this possible confounder. However, there is a need of studies of steroid resistant GVHD. As the blood eosinophil is rare in steroid-treated patient, we think it is important to include tissue samples and examine the role of the eosinophil as a tissue cell in future studies of GVHD and especially when studying steroid resistant GVHD.

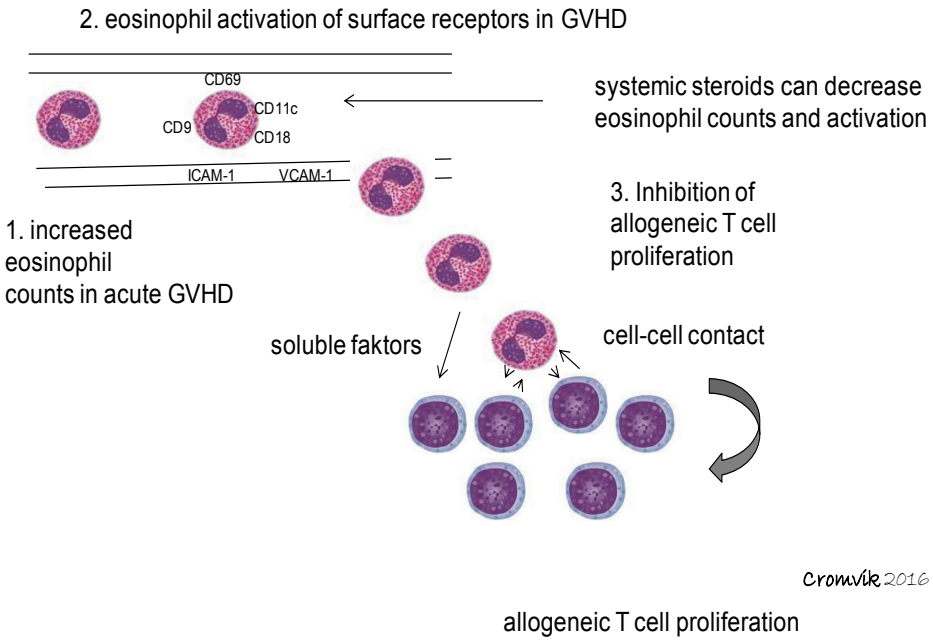

Figure 11.. *Our model of the eosinophilic granulocyte in GVHD. The first reaction is increased eosinophil counts in acute GVHD. During GVHD, the eosinophil gets an activated phenotype with up-regulation of CD69, CD11c, CD9 and CD18 in the blood vessel. By help of these activation receptors, it can bind to the receptors ICAM-1 and VCAM-1 on the activated endothelium. In the tissues, the eosinophils can inhibit allogeneic T cell proliferation in acute and chronic GVHD by help of cell-cell contact and unknown soluble factors. The cells are reproduced with permission from DocCheck pictures.*

After these functional studies, we did a clinical study of GVHD after solid organ transplantations. This is a unique cohort as Sahlgrenska Transplant Institute was the only institute among the Scandinavian countries to perform this type of

transplants during the study period. A unique feature of this study population is that many patients with malignant diseases like pancreatic tumors with unresectable liver metastases were included. The most important finding was that patients transplanted because of a malignant disease had an increased risk of acute GVHD. Neoadjuvant chemotherapy was also a risk factor. To our knowledge, this has not been examined in other studies. Our interpretation is that the chemotherapy may have damaged the intestinal mucosa before transplantation. This resembles the situation before an allogeneic HSCT, in which the conditioning therapy damages organs with rapidly proliferating cells such as the GI tract, believed to be an essential first step in the development of acute GVHD. It likely that there is an increased permeability in the transplanted intestine and viscera, making it possible for bacterial products to enter the bloodstream and induce T cell activation. As a routine at our transplant center, the intestine is also placed as a stoma in the newly transplanted individual. This procedure facilitates examinations of the intestine, but also facilitates for bacterial products or contaminants to enter the bloodstream.

It has been debated whether a spleen-containing graft and a recipient splenectomy could be a risk factor for acute GVHD in organ transplanted patients. One American studies indicates that a spleen containing graft could be a risk factor.[20] Others have not found that spleen-containing grafts are associated with an increased risk of GVHD.[208-210] The spleen is a lymphatic organ and the hypothesis is that the transferred lymphocytes will react to foreign tissue antigens in the donor (who is under immunosuppression). However, we could not find an increased incidence of GVHD among the patients with a spleen-containing graft. This may be a false negative result due to our modest number of patients.

None of our patients incurred chronic GVHD. This is in agreement with studies from other groups.[20, 211] It is unclear why chronic GVHD is less common after ITX or MVTX than after allogeneic HSCT. A likely explanation is that the solid organ transplanted patients maintain their own immune system, and are less immunologically deranged compared to the HSCT transplanted patient. Fortunately, all of our patients survived their episodes of acute GVHD. Due to the small sample size, it is difficult to make analyses of the underlying causes. However, one possible explanation could perhaps be that we used a quite generous definition of GVHD, as clinical features compatible with GVHD and a positive DNA chimerism analysis was enough to define GVHD. Therefore, it is likely that patients with a less severe acute GVHD were included, compared to if we would have included only those with a positive biopsy.

Chimerism analyses are used to facilitate the diagnosis of GVHD after solid organ transplantation. However, in our study of patients after MVTX, one patient had macrochimerism (27% donor T cells) without signs of acute GVHD. This is in

congruence with another study[75] which also reports macrochimerism without GVHD after ITX or MVTX. In addition, GVHD can also occur without detectable donor lymphocytes in blood.[212] This reflects the difficulties to diagnose GVHD after ITX or MVTX. Our interpretation is that macrochimerism has to be interpreted in combination with clinical features. At the moment, routine chimerism analyses are not done after solid organ transplantation at our transplant center. However, as also the histopathological diagnosis of skin GVHD is difficult, we think it would be valuable to do more regular chimerism analyses after ITX or MVTX, to follow trends and thereby facilitate the diagnosis and regulation of immunosuppression.

After these chimerism studies in GVHD following MVTX, we continued to focus on chimerism analyses after HSCT. In this setting, chimerism analyses are done routinely. The difference here is that it is desirable for the patient to achieve complete chimerism status with 100% donor T cells as opposed to mixed chimerism. This is a difference compared to after solid-organ transplantation when the recipient is supposed to maintain their own immune system and their recipient T cells.

In study IV we analyzed whether T-cell chimerism status on 100 days after allogeneic RICT could predict overall survival, relapse incidence, disease-free survival, non-relapse mortality and GVHD. In this retrospective study, we could not see an association between chimerism status and these patient-related outcomes. This is in contrast to some other studies, which have documented a shorter overall survival in patients with mixed chimerism on day 30 or day 100. [213, 214]. A recent study also found that mixed chimerism with recipient cells among the $CD34^+$ and $CD8^+$ cell subsets after a period of complete chimerism, predicted an increased risk of relapse in patients with acute lymphoblastic leukemia.[215] Other studies have shown increased risk of relapse after MAC conditioning.[216-218] However, there is also a study of MAC treated patients showing a connection with rejection instead of relapse.[219] Another study of RICT treated patients indicates that mixed chimerism can be associated with relapse.[220]

The reason why we could not see shorter survival in our studies of chimerism could be that all our patients were treated with RICT. It is more prevalent with mixed chimerism after RICT, than after MAC[221], and we suppose that mixed chimerism after MAC therefore is a more sensitive test for prediction of rejection and relapse. Another difference in our study was that we did not analyze cell subsets and that we used a cut-off level for mixed chimerism of >4% recipient T cells, which is higher than in some of the other studies. In a Chinese study, they used a lower cut-off level of >1% recipient T cells.[222] Concerning relapse incidence and non-relapse mortality, we only had 25 relapses among the patients, and

therefore the study probably is under-powered to answer the question about relapses. Our modest number of relapses is because patients with a verified relapse before day 100 were excluded.

In study IV, we only included one chimerism value on 100 days. It is possible that another study design with inclusion of several chimerism samples and analysis of "trends" of chimerism towards more complete or mixed chimerism, would have shown more results. This type of study design would be easier to perform in a prospective study design, as chimerism samples then could be done both on regular interval and on demand after suspected signs of GVHD. Another advantage with a prospective study design is that the diagnosis of GVHD would be more assured, when it is reported instantly. In retrospective studies, it is difficult to interpret clinical signs of GVHD via medical records several years afterwards.

An unexpected finding was that patients with mixed chimerism had longer treatment time with CsA. We had expected that many clinicians would interpret mixed chimerism as sign of threatening relapse and taper CsA more rapidly. A probable explanation can be that CsA initially was tapered faster in patients with mixed chimerism and they therefore achieved signs of chronic GVHD. Then the chronic GVHD implicated a prolonged CsA treatment, Another reason could be that several patients with mixed chimerism received donor lymphocyte infusions (DLI:s), which is a known risk factor for acute and chronic GVHD.[223,224] Alternatively, the prolonged CsA treatment induced chronic GVHD as CsA can induce a GVHD-like reaction after autologous or syngeneic HSCT.[225]. Experiments in mice have shown that CsA can induce chronic GVHD by an inhibited reconstitution of bone marrow derived Tregs.[226] The patients with grafts from related donor had significantly more chronic GVHD. One possible explanation is the use of ATG after grafts from unrelated donor at our hospital. A recent randomized multicenter study showed a significant reduction of chronic GVHD two years after transplantation with ATG.[227]

Unfortunately, we could not find any association between neither classical acute GVHD nor chronic GVHD and chimerism status. Several other studies have shown an association between complete chimerism on day 90[228] and GVHD. Sometimes mixed chimerism on day 30[229-231] is thought to "protect" against GVHD. One reason for why we could not find an association between complete chimerism and acute GVHD is that it was not always clear if the patients had contracted acute GVHD in retrospect. To be sure that we only included certain cases of acute GVHD, we only included the cases with a verified biopsy. It is therefore a possibility that we missed some cases. Concerning chronic GVHD, the statistical analysis was insufficient as we did not find the debut date of chronic GVHD. Therefore, we only could do logistic regression instead of Kaplan-Meier tests.

In conclusion, chimerism status on day 100 could not be used to predict GVHD, overall survival or relapse in our population of patients with malignant hematological diagnoses after RICT. Prospective studies of selected patients and more frequent chimerism sampling of cell subsets would probably clarify the possible utility of chimerism analyses.

6 CONCLUSION

The eosinophilic granulocytes have activated surface receptors in peripheral blood from patients with acute and chronic GVHD. Our hypothesis is that these receptors facilitate the binding of the eosinophils to the vascular endothelium and their further migration into the affected tissues. The first-line treatment of GVHD is glucocorticoids. Start of treatment with systemic glucocorticoids decreases the numbers of eosinophils in blood and down-regulate their surface receptors.

Functional studies of the eosinophils also show that it can be immunosuppressive and inhibit allogeneic T cell proliferation *in vitro.* This effect is dependent of cell-cell contact between T cells and eosinophils.

Patients with a tumor diagnosis or neoadjuvant chemotherapy or radiotherapy are at an increased risk of GVHD after a multivisceral transplantation. Among 26 patients, (5/26) 19% incurred acute GVHD and the mortality was lower than expected as all survived their episode of acute GVHD.

Chimerism status on day 100 after allogeneic HSCT cannot be used to predict overall survival, GVHD status or relapse incidence. Patients with mixed chimerism and chronic GVHD have prolonged treatment with cyclosporine A, (CsA). It is probable that the CsA initially was tapered faster in patients with mixed chimerism. Therefore, they were at higher risk of incurring chronic GVHD and signs of chronic GVHD implicated a prolonged treatment of CsA. Other explanations could be that patients with mixed chimerism more often are treated with donor lymphocyte infusions, which is a risk factor of GVHD or that the CsA in itself elevates the risk of incurring chronic GVHD.

7 FUTURE PERSPECTIVES

My ideas of futures project in the field of preventing, diagnosing and treating GVHD after allogeneic hematopoietic stem cell transplantation and multivisceral transplantation:

- Improve functional studies of the inhibiting effect of the eosinophil on T cells

Do more anti-inhibiting studies to identify one or several inhibiting mechanisms together. An evaluation of whether the inhibition effect is done by help of apoptosis or necrosis.

- Better prevention of GVHD

Identification of high-risk individuals to make a more individual treatment. Prospective large cohort studies to identify possible risk factors for GVHD.

- Improved diagnostics of GVHD

Prospective studies to follow immune status and chimerism status directly in the post-transplant period after HSCT and MVTX. For example examination of blood and tissue eosinophils' interactions with other immune cells before and after debut of GVHD, to better understand the post-transplant microenvironment.

As GVHD is a relatively new and rare disease, it is very important with international collaboration concerning diagnosis, treatment and nomenclature. Here multi-center studies will be needed to succeed with these questions.

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