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This dissertation was made in the University Hospital Centre in Nantes, France, (CHU de Nantes) in collaboration with Hematology Division of Department of Internal Medicine of School of Medicine, University of Zagreb.

Mentor 1: Prof Boris Labar
Mentor 2: Prof Mohamad Mohty

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Contents

List of abbreviations

APC	antigen presenting cells
ATG	anti–thymocyte globulin
AUC	area under curve
BAFF	B cell-activating factor
CCR	chemokine receptor
CD40l	CD40 ligand
CI	cumulative incidence
CIBMTR	international organization Center for International Bone Marrow Transplant Research
CML	chronic myeloid leukemia
CSA	cyclosporine A
CSF	colony-stimulating factor
CTL	cytotoxic lymphocytes
DC	dendritic dells
DLI	donor lymphocyte infusion
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
EBMT	European Bone Marrow Transplantation organization
EDTA	ethylenediaminetetraacetic acid
EFI	European Federation for Immunogenetics
FLT3.L	fms-like tyrosine kinaze receptor 3 ligand
GCSF	granulocyte colony-stimulating factor
GMCSF	granulocyte-macrophage colony-stimulating factor
GVH	graft-versus-host effect
GVHD	graft-versus-host disease
GVL	graft-versus-leukemia effect
GVT	graft-versus-tumor effect
HCV	hepatitis C virus
HIV	human immunodeficiency virus
HLA	human leucocyte antigen
HSV	herpes simplex virus
IFN	interferon
IL	interleukin
IL2ra	IL2 receptor alpha
IP	interferon gamma-induced protein
LIF	leukemia inhibitory factor
LPS	lipopolysacharide
MCP	macrophage chemotactic protein
MDC	macrophage-derived chemokine
mDC	myeloid dendritic cells
mHAg	minor histocompatibility antigens
MHC	major histocompatibility complex
MIF	macrophage migration inhibitory factor
MIP	macrophage inflammatory protein
MMF	mycophenolate mophetil
PAMP	pathogen-associated molecular patterns
PBMC	peripheral blood mononuclear cells
PCR	polymerase chain reaction

pDC	plasmacytoid dendritic cells
PDGFBB	platelet derived growth facor-type BB
PMA	phorbol myristate acetate
RANTES	regulated on activation normal T cell expressed and secreted
RIC	reduced intensity conditioning
ROC	receiver operating characteristic
RSV	respiratory syncytial virus
SLE	systemic lupus erythematosus
TARC	thymus and activation regulated chemokine
TBI	total body irradiation
TCR	T cell receptor
TGF	transforming growth factor
Th	T-helper lymphocytes
TLR	Toll-like receptors
TNF	tumor necrosis factor
TRAIL	tumor-necrosis-factor related apoptosis inducing ligand
Treg	T-regulatory lymphocyte
VEGF	vascular endothelial growth factor
VZV	varicella zoster virus

1. Introduction

1.1. Allogeneic transplantation of hematopoietic stem cells

The era of hematopoietic stem cell transplantation began in the wake of the first atomic bomb explosions, with landmark observations that mice could be protected from lethal effects of ionizing radiation on bone marrow by shielding their spleens with lead.[1] A short time later, scientists showed that protection against radiation could also be conferred by intravenous infusions of bone marrow of healthy animals[2,3]. Later studies in the mid 1950s confirmed that radiation protection of bone marrow was due to transplanted stem cells.[4,5] However, it was not until late 1960s that allogeneic hematopoietic stem cell transplantation was successfully used in human patients with immunodeficiencies[6,7] and aplastic anemia.[8] For patients with hematological malignancies, the initial underlying therapeutic principle was to treat them with escalating therapeutic radiation doses to levels that kill malignant cells but also cause irreversible bone-marrow toxicity, and then to rescue the patients' bone marrow with infusions of haematopoietic stem cells from healthy donors. Analyses of preclinical data, however, revealed a second powerful therapeutic effect of this approach - the antileukemic potential of transplanted hematopoietic cells. Barnes and colleagues were the first to report the *graft-versus-tumor* (GVT) effect.[9,10] This finding launched bone marrow transplantation in human leukemia patients and first results on a larger number of patients were reported in 1977.[11]

Since then, allogeneic transplantation of hematopoietic stem cells has evolved into an effective therapy for a variety of hematological and non-hematological malignancies. The treatment goal in non-hematological malignancies as in immunodeficiencies or bone marrow aplasia is to exchange inadequate hematopoesis with one derived from a healthy donor. In the treatment of hematological malignancies, high intensity myeloablative therapy (high-dose cytotoxic chemotherapy and irradiation) eradicates malignant cells and the infusion of donor stem cells enables reconstitution of the hematopoietic system in the recipient, but also shows an anti-leukemic effect (GVL; *graft-versus-leukemia effect*), mediated by donor T-lymphocytes present in the graft. The number of allogeneic transplantations continues to increase with more than 25 000 performed annualy. However, the main limitation of treating broader spectrum of diseases and patients with allogeneic stem cell transplantation is graft-versus-host disease (GVHD), a reaction closely associated to GVL effect.

1.1.1. Histocompatibility

Both GVHD and GVL occur when donor T lymphocytes respond to genetically defined proteins on host cells. The most important proteins are Human Leukocyte Antigens (HLA), which are highly polymorphic and are encoded by the major histocompatibility complex (MHC) genes, located on short arm of chromosome 6. Class I HLA (A, B, and C) proteins are expressed on almost all nucleated cells of the body and present antigenes to CD8+ lymphocytes.[12] Class II proteins (DR, DQ, and DP) are primarily expressed on hematopoietic cells (B cells, dendritic cells, monocytes), but their expression can be induced on many other cell types following inflammation or injury and they present antigenes to CD4+ lymphocytes.[13]

Beside major histocompatibility antigens, there are also "minor" histocompatibility antigens (mHA), which lie outside the HLA loci and represent host antigens also recognized as "foreign" by donor T-lymphocytes. These antigens are responsible for inducing GVHD in HLA-identical donor and recipient.[14] Some minor HAs are expressed on all tissues and are targets for both GVHD and GVL[15], while other minor HAs, are expressed most abundantly on hematopoietic cells (including leukemic cells) and may therefore induce a greater GVL effect with less GVHD.[15,16]

The donor and recipient can be both HLA and mHA identical only if they are identical twins in so-called syngenic transplantation. In this type of transplantation recipients do not develop GVHD. However, even from early murine studies it has been shown that leukemia-bearing recipients given syngeneic grafts had worse survivals and lower leukemic cure rates than recipients of allogeneic grafts, probably due to lack of GVL effect as well. In allogeneic transplantation the donor and recipient can be identical in major histocompatibility antigens but they are always different in minor histocompatibility antigens, especially for unrelated donors. Sibling donors may have some mHA compatibility.

High-resolution DNA typing of HLA with polymerase chain reaction (PCR)-based techniques have now largely replaced earlier methods. However, the routine method for typing mHA is still not used. When typing a sibling donor it is enough to have a 6/6 match. HLA-genes are inherited as haplotypes and by typing six loci (HLA-A, -B and DR) it is possible to evaluate if a sibling is HLA-identical. When typing an unrelated donor, it is necessary to have a 10/10 match (HLA-A, -B, -C, -DRB1, -DQB1). The incidence of GVHD is directly related to degree of mismatch, thus ideally donors and recipients are fully matched. However, with new improvements in immunosupressive treatment, a mismatched allogeneic transplantation (with

one or two mismatches) has become feasible as well as the use of an haploidentical donor (mother, father, son, daughter, brother or a sister).

1.1.2. Graft-versus-host disease (GVHD)

Even early experiments on mouse models showed that transplantation of bone marrow from different mouse strain caused „runting“ syndrome in transplanted mice.[9,17] GVHD was described soon after initial attempts of translating allogeneic transplantation from mouse models to clinic. It has been more than 15 years to first successful transplantation in human because the graft was either rejected by the recipient, and, if accepted, patients developed severe fatal GVHD. GVHD is an immune reaction which develops when immunologically competent donor T-cells recognize MHC antigens on host antigen-presenting cells and activate the immune response.

A major improvement in patient survival was made when the antimetabolite methotrexate was combined with the T-cell activation inhibitors cyclosporine or tacrolimus for GVHD prevention. [18-20] The same therapy is still widely used as most effective prophylactic treatment. However, GVHD continues to be a major source of morbidity and mortality following allogeneic stem cell transplantation.

Clinically, GVHD is separated into acute and chronic GVHD. Based on an early Seattle experience, acute GVHD was defined to occur prior to day 100, whereas chronic GVHD occurred after that time.[21-23] This definition is far from satisfactory, and a recent National Institutes of Health classification includes late-onset acute GVHD (after day 100) and an overlap syndrome with features of both acute and chronic GVHD.[24] The incidence of acute GVHD is directly related to the degree of mismatch between HLA proteins and ranges from 35-45% in recipients of full matched sibling donor graft to 60-80% in recipients of one-antigen HLA mismatched unrelated donor grafts.[25,26] The incidence of the severity of acute GVHD is determined by the extent of involvement of three principal target organs (skin, gastrointestinal tract and liver). The overall grades are classified as I (mild), II (moderate), III (severe) and IV (very severe). Severe and very severe GVHD occurs in 15% of patients and carries a poor prognosis, with 25% long term survival for grade III and 5% for grade IV.[27] Steroids, with their potent antilymphocyte and anti-inflammatory activity, are the gold standard for treatment of GVHD. Upon diagnosis of grades II to IV acute GVHD, all patients are primarily treated with methylprednisolone (2 mg/kg per day).

Chronic GVHD is the major cause of late non-relapse death following allogeneic stem cell transplantation.[28] Its presentation may be progressive (active or acute GVHD merging into chronic), quiescent (acute GVHD that resolves completely but is later followed by chronic GVHD) or it may occur *de novo*.[29] Older recipient age and history of acute GVHD are the greatest risk factors for chronic GVHD[30], and strategies to prevent acute GVHD may therefore help to prevent chronic GVHD. The manifestations of chronic GVHD are somewhat protean, and are often of an autoimmune nature. Clinical signs often first appear in the buccal mucosa and historically chronic GVHD was evaluated as limited or extensive. Recently, new consensus criteria for the diagnosis and staging of chronic GVHD have been developed.[24] The use of corticosteroids (with or without a calcineurin inhibitor) is the standard of care, and on diagnosis of extensive GVHD, all patients are primarily treated with methylprednisolone (1 mg/kg per day).

1.1.3. Pathophysiology of GVHD

The basic principles necessary for the development of GVHD were initially described by Billingham in 1966: GVHD requires that (i) the graft must contain immunocompetent cells, (ii) the host must be incapable of rejecting the graft the, and (iii) there must be incompatibilities in transplantation. [31] More recently, it has been defined that mature donor T cells are essential immunocompetent cells necessary for the induction and pathogenesis of acute GVHD, suggesting that GVHD is a T cell-mediated inflammatory disease, [32] and that T cell number in the transplant correlates with GVHD severity.[33] The second condition of host incapability to reject the graft is usually met after a patient receives myeloablative chemotherapy or anti-thymocyte globuline. Third condition is always met, except in syngeneic transplantation. As mentioned before, even though allogeneic transplantation is done between HLA identical donor and recipient, differences between minor histocompatibility system (mHA) induce GVHD. These differences are bigger if a donor is unrelated, which explains higher incidence of GVHD in patients treated with unrelated when compared to sibling allogeneic transplantation.

A modern perspective of GVHD pathophysiology can be summarized into a «three-step model»[34]: In step I, the conditioning regimen (high dose irradiation, chemotherapy, or both) leads to the damage of host tissues, especially the intestinal mucosa. This allows the translocation of lipopolysacharide (LPS) from the intestinal lumen to the circulation, stimulating the secretion of the inflammatory cytokines from host tissues (such as TNF alpha,

IL1, IL6). These cytokines will increase the expression of MHC antigens on host tissues, enhancing the recognition of these antigens and minor histocompatibility antigens (mHAs) by mature donor T cells. In step II, antigen-presenting cells (APC) present host antigens to donor T-lymphocytes and activate the immune response. This is characterized by the predominance of T-helper 1 (Th1) cells and the secretion of IFN gamma, as well as other cytokines. In step III, effector functions of immune cells are triggered by the secondary signal provided by LPS and other stimulatory molecules that leak through the intestinal mucosa damaged during steps I and II. Effector Th1 lymphocytes activate macrophages, along with cytotoxic lymphocytes (CTL), which secrete inflammatory cytokines that cause target cell apoptosis. CD8+ CTL also lyse target cells directly. Damage to the gastro-intestinal tract in this phase, principally by inflammatory cytokines, amplifies LPS release and leads to the so-called ‘‘cytokine storm’’, characteristic of severe GVHD.

1.1.4. Novel strategies in allogeneic transplantation due to graft-versus-leukemia effect (GVL) - donor lymphocyte infusion (DLI) and non-myeloablative conditioning

The graft-versus-leukemia effect of transplanted hematopoietic cells[9,10] was first reported in early mouse studies. It was suggested that a reaction of the donor bone marrow against the host leukemia might kill cancer cells, and this was termed ‘adoptive immunotherapy’. However, the value of GVL effect was not fully recognized untill early 1980-s, when a group from Seattle published two systematic analysis of their previous transplantations and reported that recipients of hematopoietic stem-cell transplantations who developed GVHD were less likely to suffer cancer relapse.[35,36] The importance of T cells in securing long-term engraftment and launching an attack on tumor cells was confirmed in 1990-s in a retrospective analysis of data from International Bone Marrow Transplant Registry (IBMTR). In this study, patients who received T-cell-depleted grafts experienced less GVHD, but also experienced increased graft failure and profoundly higher relapse rates (***Figure 1***).[37]

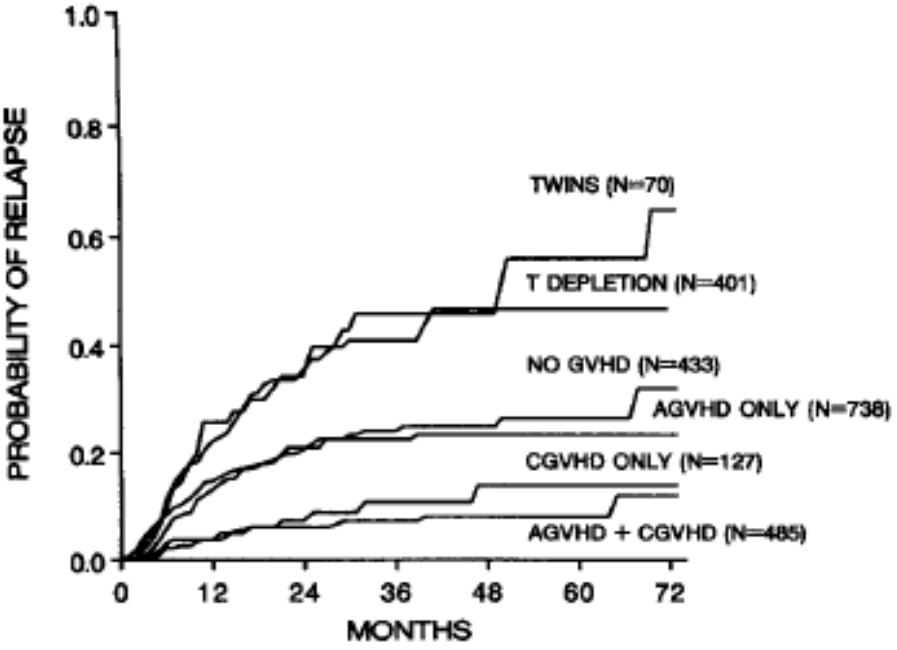

Figure 1. The probability of relapse after allogeneic transplantation in patients with leukemia in relation to type of transplant and GVHD.

Further support for this postulate came from adoptive immunotherapeutic attempts to induce GVHD and antitumor effects through the infusion of donor lymphocytes. In 1990-s, Hans-Jochem Kolb was the first to successfully treat three chronic myeloid leukemia (CML) patients with a combination of interpheron alpha and infusion of „buffy coat“ (separated leukocytes and thrombocytes) from peripheral blood of donors.[38]

Infused donor lymphocytes become sensitized to surface antigens that are expressed on the leukemic cells, either to polymorphic minor histocompatibility antigens or leukemia-associated antigens, and are transformed into cytotoxic lymphocytes that kill the leukemic cells. The most astonishing graft-versus-tumor effects after donor lymphocyte infusions (DLI) have been observed in patients with CML.[39,40] Approximately 75% of relapsed chronic leukemia patients experience remission after DLI, whereas results are less impressive in patients with acute leukemias and multiple myeloma. It is presumed that in CML part of the malignant clone are host antigen-presenting cells (dendritic cells), which activate infused T-lymphocytes.[41]

In some patients, graft-versus-tumor effects are associated with GVHD, whereas in others remissions are achieved without GVHD. The tumor responses seen in the latter patients are

probably due to expression of the polymorphic minor histocompatibility antigens only on the tumor cell surface and not on all hematopoietic cells, which serve as targets for the immune attack.[14,42-44]

All this described findings have changed the old paradigms for hematopoietic stem cell transplantation. Because of its risks, the conventional myeloablative stem cell transplantation was restricted to relatively young patients who are in good medical condition. As a result, very few patients older than 50 years and virtually none older than 60 years have been treated by conventional allogeneic transplantation. The age restriction is unfortunate, because the median ages at diagnosis of patients with most candidate diseases for allogeneic transplantation range from 65 to 70 years. However, investigators have recently developed less intensive hematopoietic stem cell transplantation programmes, also known as non-myeloablative or mini-transplant approaches. These approaches require lower levels of chemotherapy and irradiation and rely on the graft-versus-tumor effects of the bone marrow transplantation, rather than the myeloablative effects of irradiation and chemotherapy to kill cancer cells.[45-51] These regimens have been, in part, directly translated from preclinical models.[52,53] In this type of transplantation, patients receive non-myeloablative irradiation or chemotherapy, immunosuppressive drugs and hematopoietic stem cell transplantation. After receiving the transplant, the recipient typically experiences mixed chimerism, in which the blood system is made up of a combination of host and donor cells (determined by microsatellite analysis). If the patient does not experience GVHD, s/he might be infused with more donor lymphocytes, leading to a conversion of completely donor-derived hematopoietic cell populations. This leads to graft-versus-tumor effects and cure of the malignancy.

1.2. Antigen-presenting cells (APC)

APC are highly specialized cells which can process antigens and present their peptide fragments on the cell surface together with the co-stimulatory molecules necessary for T-cell activation.[13] Dendritic cells (DC) are key antigen-presenting cells that originate from bone marrow precursors and play a strong and central role in innate and adaptive immunity to infections and antigens in vivo.[54,55] DC circulate in the blood before migrating to peripheral lymphoid tissues or inflammation sites. In response to microbial stimuli or inflammatory cytokines DC go through differentiation and maturation by decreasing ability of uptaking new antigens, increasing the expression of MHC antigens and co-stimulatory molecules as well as adhesion molecules and chemokine receptors which helps them migrate to lymphoid tissues.

In lymph nodes DC take up and process antigens to prime T cell responses.[56-62] Besides T cells, cytokine-activated or mature DC activate other innate immune cells such as natural killer cells, natural killer T cells and B cells[63,64] and polarize the immune response to either Th1 or Th2 types. [65] In addition, DC can induce antigen-specific unresponsiveness or even tolerance. In the absence of an activating signal, immature DC process antigens and present them to T-lymphocytes without co-stimulatory molecules which induces anergy and deletion of T-cells. There is increasing evidence that in some clinical situations including autoimmunity and certain infectious diseases DC induce immune tolerance either by deleting self-reactive T cells or by regulating or suppressing other immune T cells with T-regulatory lymphocytes (Tregs).[66]

DC mature in response to two different signals- by recognizing the pathogen directly through pattern-recognition receptors or indirectly by recognizing the signs of infections as inflammatory cytokines. The most important surface receptors which can induce the maturation of DC are Toll-like receptors (TLR) following exposure to pathogen-associated molecular patterns (PAMPs). DC mature in response to different PAMPs, recognized by different TLR. For example, TLR4 recognizes LPS, TLR2 recognizes bacterial peptidoglicozides as part of bacterial wall in gram positive bacteria, TLR5 recognizes bacterial flageline, TLR9 recognizes CpG and TLR7 recognizes imidazoquinolones.[67]

Human blood precursor DC numbers are low, namely in the order of 0.1 – 0.7% of mononuclear cells[68-71] and peripheral blood contains at least 2 major DC subsets with distinct phenotypes and distinct allostimulatory capacity.

Myeloid DC (mDC) originate from bone marrow precursors, differentiate along the monocyte/macrophage lineage and require the presence of granulocyte-macrophage colony-stimulating factor (GMCSF) for survival. Mature mDC produce high levels of interleukin12 and other Th1-polarising signals.[65]

Lymphoid or plasmacytoid DC (or pDC) depend on interleukin 3 for their survival and after activation secrete type I IFN, activate mDC, but can also polarize T-cell differentiation into Th2 cells.[65]

1.2.1. Plasmacytoid dendritic cells

Plasmacytoid dendritic cells also known as the type I IFN-producing cells, are a major member of the innate immunity effectors in both humans and mice. pDC display plasma cell morphology, selectively express TLR7 and TLR9, and are specialized in rapidly secreting

massive amounts of type I IFN following viral stimulation. Activated pDC can promote the function of NK cells, B cells, T cells, and mDC through type 1 IFN during an antiviral immune response but inactivated pDC can also induce antigen- specific anergy.[72]

pDC accumulation in lymphoid tissues, mucosa and organs occurs during many viral infections (HSV[73], VZV[74], HCV[75,] influenza[76-78], RSV[79,80]) and several human pathologies, particularly in lymph nodes of patients affected by sarcoidosis, Mycobacterium tuberculosis infection[81] , Kikuchi's disease[82] and in the skin of patients affected by psoriasis[83,84], systemic lupus erythematosus (SLE)[85], and lichen planus[86,87]. pDC accumulation has also been observed in brain lesions of patients with multiple sclerosis[88], in the salivary glands of patients with Sjogren's syndrome[89] and the synovia or inflamed muscle tissue/skin of people afflicted with rheumatoid arthritis[90,91]or dermatomyositis[92,93], respectively.

The accumulation of pDC in several viral models and disease settings has been well defined, but the role of pDC in the pathogenesis and progression of diseases stays controversial, as their both negative and positive effects have been demonstrated.

According to several studies, pDC are considered to be the culprits in promoting SLE and psoriasis,[94-99] where they produce IFN-I in response to self-DNA.[100] Blocking IFN-I strongly inhibits the T-cell-dependent progression of psoriasis, thus implicating pDC as critical mediators of disease.[84]

On the other side, although activated pDC appear to behave as immunogenic cells, unstimulated or alternatively stimulated pDC can alleviate protective immunogenic responses through the induction of T-regulatory lymphocytes (Tregs). pDC induce Tregs via several mechanisms, and this has been particularly studied in breast cancer[101] and melanoma[101,102], as well as in HIV infection.[103,104]

1.2.2. pDC in GVHD

After allogeneic stem cell transplantation, host DC are usually rapidly replaced by donor DC precursors except for Langerhans cells of the skin, which have been shown to be of host origin for at least 18 months.[105] In murine allogeneic bone marrow transplantation as well as in humans it was shown that, despite the presence of numerous donor DC, only host-derived DC initiate acute GVHD, whereas development of chronic GVHD seems to require both donor and host DC, depending on the target organ involved.[106-109] Donor DC can intensify GVHD probably by cross-priming alloreactive cytotoxic T-lymphocytes.[106,109-111]

In mice exposed to total body irradiation (TBI) host pDC can prime donor T-cells and cause GVHD, but this process does not require TLR signaling. It is assumed that an inflammatory environment created by host irradiation has the decisive role in maturing pDC for T-cell priming.[112] In a similar model, infusion of immature pDC can effectively prevent GVHD by inducing Tregs.[113]

In human studies, delayed DC recovery and lower DC numbers, especially of pDC, are found in patients with acute and chronic GVHD and/or steroid treatment[114-116], and a low pDC count in the peripheral blood on day +28 after transplantation was shown to be predictive for both acute and chronic GVHD.[117] However, in other studies, high numbers of mDC and pDC have been observed during the early phase of acute GVHD, as well as chronic GVHD.[118-122]

These studies show that the impact of DC accumulation on immune responses is still controversial and is probably dependent on their activation state, distribution and migration patterns. More studies are needed to elucidate this problem. Another important challenge in the field is to target pDC for therapeutic purposes. BDCA2 is a molecule expressed exclusively by human pDC, which provides an attractive target for the development of human pDC depleting antibodies.[123,124] On the other hand, infusion of tolerogenic or activated pDC may be useful therapies for transplantation and cancer, respectively.

1.3. CD4+ T helper (Th) lymphocytes

CD4+ T helper (Th) lymphocytes are essential mediators of immune responses and inflammatory diseases. After being activated by professional APC, Th cells differentiate into effector cells specialized in cytokine secretion and function. In the past, effector Th cells have been classified as type 1 (Th1) and type 2 (Th2) based on their cytokine expression profiles and immune regulatory function.[125] Th1 cells produce IFN gamma and mediate cellular immunity, whereas Th2 cells produce interleukin 4 (IL4), IL5 and IL13 and mediate humoral immunity and allergic responses. Th cell differentiation is regulated by the interaction of naive CD4+ T-cells with APC that express specific peptide MHC class II complexes, costimulatory molecules and inflammatory cytokines. Naive T-cell activation normally requires two signals: T cell receptor (TCR) signals, and costimulation through several accessory molecules. The main costimulatory molecule on Th cells is CD28[126] which interacts with CD80 and CD86 expressed on mature DC. In addition to TCR and costimulatory molecules, IL12 produced by activated mDC is critical in Th1 differentiation.[127] Additional cytokines in the IL12 family, IL23 and IL27, are also important for Th cell differentiation and

function.[128,129] Other cytokines also influence the development of effector functions of Th cells; for example, IL4 produced by activated T cells (and perhaps by other innate cells as well) is crucial in driving Th2 differentiation.[130]

Recently, a third subset of CD4+ effector cells has been identified; termed Th17, because the signature cytokine produced by this subset is IL17.[131-133]

Of note, T-regulatory lymphocytes (Tregs) are another subset of the immunosuppressive CD4+ T-cell lineage responsible for inducing immune tolerance.[134,135] Thus, today we know that naive CD4 T cells can give rise to at least four distinct helper subsets of effector T cells at periphery (Th1, Th2, Th17 and Treg) depending on the presence of cytokines upon mitogenic stimulation. We now face a completely different and complex scenario, involving the new possible roles of Th17 cells and Tregs in immune responses and pathogenesis of diseases.

1.3.1.Th17 subpopulation of lymphocytes

Th17 cells are found in the systemic circulation and secondary lymphoid organs and tissues, particularly in the intestinal mucosa where they protect the host from microorganisms that invade through the epithelium.[136] Th17 cells are characterized by their expression of proinflammatory cytokines IL17, IL21, and IL22, cytokines involved in neutrophilia, production of antimicrobial peptides and tissue repair.[137-140]

IL17 is also produced by innate immune cells in an inflammatory milieu, and may have a central role in the initiation of IL17-dependent immune responses even before Th17 development.[141] Th17 cells differentiate from naive CD4+ T cells by stimulation with antigens in the presence of TGF beta and IL6 or IL21 both in mice and humans. Recent studies have shown that pDC can drive the differentiation of IL17 and IL22 producing T cells and that IL6 is implicated in this process.[142-144]

IL17 belongs to a family of six members and among them, IL17A and IL17F are by far the best characterized.[145] Many *in vitro* studies have indicated a proinflammatory function for IL17A which has been associated with many inflammatory diseases such as rheumatoid arthritis, asthma, SLE and psoriasis, respectively.[146-149] CD161+Th17 population has been demonstrated to have a proinflammatory role in Crohn's disease.[150]

1.3.2. Th subsets in GVHD

Until recently, CD4+Th1 cells were held responsible for the development of acute GVHD and CD4+Th2 for the development of chronic GVHD.[151] The role of Th17 responses in GVHD has not yet been established and research on Th17 in GVHD is rapidly developing.

Controversy exists in both mice and humans regarding the role of Th17 cells in the pathogenesis of GVHD. In murine model, some studies showed a protective role of IL17. In these studies infusion of IL17-deficient donor T cells induced more severe GVHD than that of wild-type T cells, and this was in association with enhanced Th1 differentiation of donor T cells by increased production of IL12 from DC in the absence of IL17.[152] By contrast, other studies indicated a pathogenic role of IL17 in GVHD.[153,154] Of note, infusion of Th17 cells caused lethal GVHD hallmarked by extensive tissue damage in irradiated mice.[155] Further studies in mice showed that liver and gut GVHD is largely Th1 dependent, whereas skin GVHD is more Th17 dependent and lung GVHD is Th2 dependent.[156]

Clinical studies assessing the correlation between Th17 cells with GVHD have also shown conflicting results. Th17 cells are increased in peripheral blood of patients with acute GVHD in one study[157], but not another study.[158] Ratio of Th17 to Treg was found to be increased in the biopsied samples of GVHD tissues in one study[157], whereas it was decreased in another study.[159] It was also shown that Th17 cells were not increased in the skin in contrast to a significant increase in Th1 IFN gamma producing cells at the onset of acute GVHD.[158]

Schematically, it is likely that all four populations of T-helper cells: Th1, Th2, Th17, and Tregs pathways cross-regulate in the immune system. In allogeneic transplantation depending on the initiating signal delivered to DC during or after the conditioning regimen, the IL12/IFN gamma, IL23/IL17 or Tregs axes may become a more or less prominent pathway, and the dominance of one axis to another may lead or not to breakdown of tissue-specific immune tolerance. A key question is why the regulatory mechanisms fail to shut down the stimulatory pathways in GVHD.

In order to modulate the balance of Th subsets *in vivo*, the use of biological products such as cytokine-neutralizing monoclonal antibodies has increasingly been used clinically in treating patients with cancer and autoimmune diseases, and it represents a realistic and attractive strategy because cytokine environment is critically important for T helper and Treg differentiation. To establish highly effective Treg therapy or cytokine modulation, consideration of timing of administration is particularly important in the setting of highly inflammatory allogeneic stem cell transplantation. However, as reduced intensity conditioning

(RIC) has been developed to minimize toxicity of conditioning regimen, advances in transplant medicine will synergistically facilitate development of such novel strategies. Conversely, results from animal studies suggest that a combined blockade of Th1 and Th17 differentiation pathways of donor T cells may represent a promising strategy for the prevention or treatment of GVHD, while inhibition of either pathway alone seems to be insufficient to prevent GVHD.[152,160,161]

1.4. Pro-inflammatory cytokines and chemokines

Cytokines are a group of small peptides or glycoproteins produced by a wide variety of immune and inflammatory cells with molecular weights between 8 and 30 kDa. They had been shown to play an essential role in cell comunication and modulation of the immune response.[162] The „cytokine storm“ has already been defined in the pathophisiology of potentially fatal immune reactions. This reaction consists of a positive feed-back loop between cytokines and immune cells and can also lead to severe GVHD. Chemokines are a large family of very small 8 to 12 kDa cytokines that have been discovered more recently and which primarily function as leukocyte chemoattractants.[163,164] It has been demonstrated that members of the chemokine superfamily are involved in immunological and autoimmunological diseases.[165-167]

These mediators have been classified according to several classifications. The classical classification separates cytokines into five groups; interleukins mostly produced by T-lymphocytes (IL1 to IL35), interferons (IFN types I-III), cytotoxins produced by macrophages and cytotoxic lymphocytes which cause target cell death (TNF alpha and TNF beta), colony-stimulating factors (CSF) and finally other cytokines which can not be otherwise classified (as TGF beta, leukemia inhibitory factor (LIF) or macrophage migration inhibitory factor (MIF). The second classification also separates cytokines into five groups according to the structure and mechanism of action of their receptors; type I cytokine receptor family (hematopoietin receptors), type II cytokine receptor family (interferon receptor family), TNF-receptor family, immunoglobulin receptor family and chemokine receptor family. Finally, the third classification divides the cytokines into four groups according to their function- innate immunity (pro-inflammatory) cytokines, specific (adoptive) immunity cytokines, immunosupressive (immunoregulatory) cytokines and colony stimulating factors. These cytokines and their cross reactive functions are presented in ***Figure 2.***

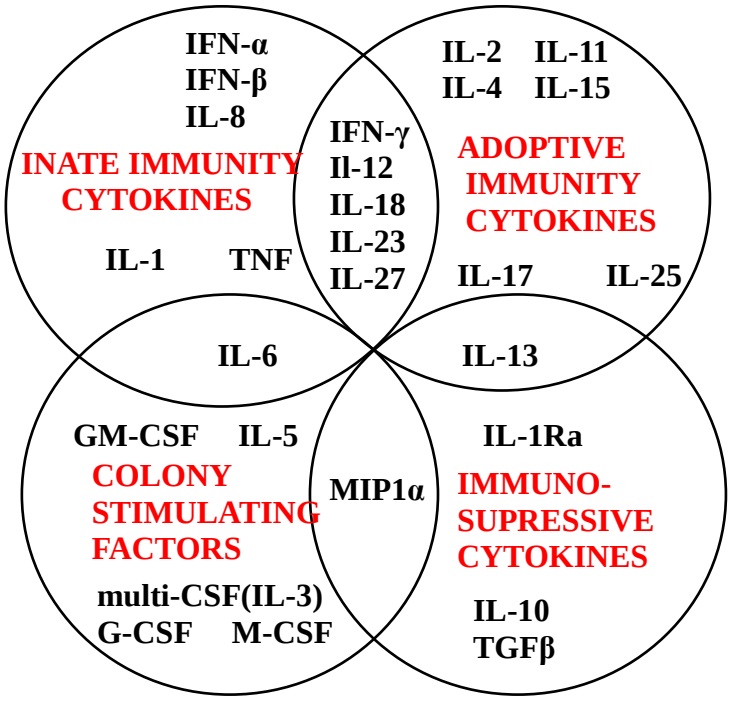

Figure 2. The functional classification of cytokines

CSF indicates colony stimulating factor (G, granulocyte; M, macrophage; GM, granulocyte-macrophage.), IL interleukin; IFN, interferon; MIP, macrophage inflammatory protein; TGF, transforming growth factor; TNF, tumor necrosis factor;

The family of chemokines is sub-divided according to the number and position of NH2-terminal cysteine (C) residues. The majority of chemokines fall into the CC (CCL1-28) and CXC (CXCL1-16) subfamilies, while the C family contains only 2 members (XCL1 and XCL2) and CX3C only 1 member (CX3CL1). There is significant redundancy in the chemokine system as shown in ***Figure 3.*** by the binding of multiple chemokines to a particular receptor and multiple receptors interacting with a particular chemokine. There are currently 10 identified CC chemokine receptors (CCR1-10), 6 CXC receptors (CXCR1-6), 1 C receptor (XCR1), and 1 CX3C receptor (CX3CR1).[168,169]

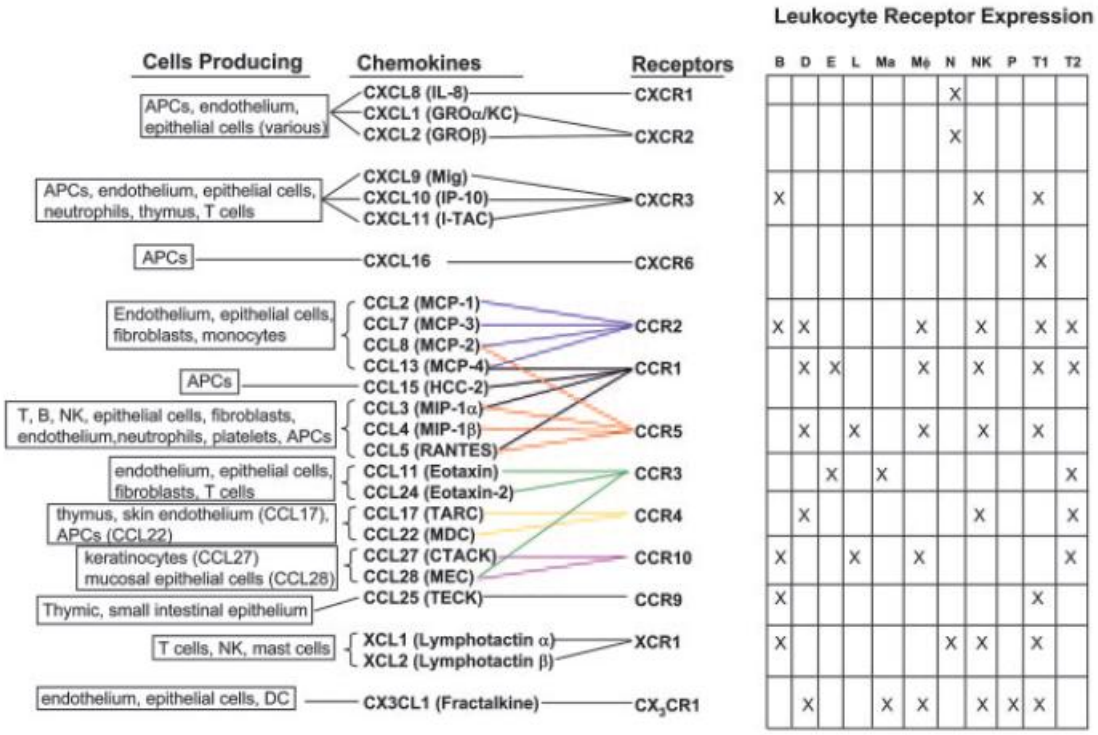

Figure 3. Inflammatory chemokines and receptors
APC indicates antigen-presenting cell; B, B cell; D, dendritic cell; E, eosinophil; L, Langerhans cell; Ma, mast cell; M_,macrophage; N, neutrophil; NK, natural killer cell; P, platelet; T1, TH1/TC1 cell; and T2, TH2/TC2 cell. DC indicates dendritic cell; GRO, growth-related oncogene; I-TAC, inducible T cell alpha chemoattractant; MCP, macrophage chemotactic protein; HCC, hemofiltrate CC chemokine; MIP, macrophage inflammatory protein; RANTES, regulated on activation normal T cell expressed and secreted; TARC, thymus and activation regulated chemokine; MDC, macrophage-derived chemokine; CTACK, cutaneous T-cell–attracting chemokine; MEC, mucosae-associated epithelial chemokine; and TECK, thymus-expressed chemokine.

1.4.1. Cytokines and chemokines in GVHD

The role of proinflammatory cytokines such as TNF alpha and IL1, has been extensively studied in murine and human studies in the context of acute GVHD.[170] The role for TNF alpha in clinical acute GVHD has been suggested by studies demonstrating elevated levels of TNF alpha in the serum of patients with acute GVHD as well as the studies in which target organ damage could be inhibited by infusion of anti-TNF alpha monoclonal antibodies.[171,172]

The other well-studied proinflammatory cytokine that plays a role in acute GVHD is IL1. Secretion of IL1 appears to occur predominantly during the effector phase of GVHD of the spleen and skin and mice receiving IL1 after allogeneic stem cell transplantation display an

accelerated form of GVHD.[173,174] Although administration of an IL1 receptor antagonist to recipients reduces GVHD mortality in animal models[175], human studies failed to demonstrate any significant benefit against acute GVHD.[176]

Among all other cytokines that have been extensively studied in GVHD, several cytokines emerged as significantly correlated with development and severity of GVHD. In one study, IL12p70, a cytokine mainly produced by mDC, was significantly associated with clinically severe GVHD development, probably through activation of Th1 subpopulation of T-lymphocytes.[177] In another study, a higher production of another cytokine, IL10 (produced by DC and Th2 cells) was associated with reduced incidence and severity of acute GVHD.[178,179] Paradoxically, high-serum IL10 levels in patients after allogeneic transplantation were associated with a fatal outcome[180], whereas administration of low doses of IL10 was found to be protective in murine acute GVHD.[181]

Recently, the role of chemokines and integrins in the migration of donor T cells to tissue sites of GVHD has been examined in mice following allogeneic stem cell transplantation.[182] Gene expression profiling of GVHD target organs has identified elevated expression of the proinflammatory chemokines CCL2/MCP1, CCL3/MIP1a, CCL4/MIP1b, CCL5/RANTES, CXCL9/MIG, and CXCL10/IP10.[183-185]

All these findings show that dose and timing of cytokine production are critical factors in the induction and development of GVHD. More studies have to be done in order to investigate other potentially important cytokines and their roles in the „cytokine storm“ dogma as they represent attractive diagnostic tool and an attractive therapeutic targets as well.

2. Hypotheses

1. The number and function of circulating pDC and Th17 cells is correlated with development and severity of GVHD.
2. The number and function of circulating pDC and Th17 cells is correlated with the outcome of patients.
3. A valuable predictive and prognostic score for the development of GVHD can be made by the use of significant serum cytokines.

3. Aim and purpose of the research

The main aim of this research was to quantify plasmacytoid dendritic cells and Th subsets (Th1 and Th17 subpopulations) in peripheral blood of patients at day 100 after allogeneic stem cell transplantation.

Specific aims of this research were:

1. To quantify specific intracellular cytokines in plasmacytoid dendritic cells and Th subsets at day 100 after allogeneic stem cell transplantation.
2. To quantify specific serum cytokines at day 100 after allogeneic stem cell transplantation.
3. To evaluate correlation of plasmacytoid dendritic cells and T helper subsets with the development of GVHD and its severity.
4. To evaluate the specific correlation of plasmacytoid dendritic cells and T helper subsets with development of relapse and outcome of patients.
5. To make a prognostic score for the development of GVHD based on the significant cytokines.

The purpose of this research was to allow better understanding of the pathophysiology of GVHD and GVL. In addition to its cognitive value, this might also identify specific molecules important for development of GVHD and GVL. These molecules could then not only be used as diagnostic markers, possibly enabling earlier treatment of GVHD, but would also offer the attractive possibility of their specific targeting *in vivo*. This could maybe pave the way for novel, more specific and less toxic therapeutic interventions in this field.

4. Material, subjects, methodology and research plan

The research was done in the University Hospital Centre in Nantes, France, (CHU de Nantes) under the mentorship of professor Mohamad Mohty and as a part of his project «Facteurs prédictifs de la reaction du greffon contre l'hôte après alogreffe de cellules souches hématopoietiques» («Predictive factors for graft versus host reaction after allogeneic stem cell transplantation»). The research was done in collaboration with Hematology Division of Department of Internal Medicine of School of Medicine, University of Zagreb, within a project „Leukemias and hematopoietic stem cell transplantation" and under the mentorship of professor Boris Labar.

4.1. Subjects

This research included 79 patients treated with allogeneic stem cell transplantation at University Hospital Centre in Nantes. Serum cytokines were additionaly evaluated in 73 more patients treated with allogeneic stem cell transplantation at the same institution (152 patients in total).

4.2. Material

Biological material (blood samples) was obtained after informed consent of the patients, and as part of the material usually collected for routine care of the patients after allogeneic stem cell transplantation in CHU de Nantes. For the purpose of this project, such "biocollection" has been declared and approved by the French Ministry of Health and regulatory authorities (Reunion de concertation pluridisciplinaire in CHU de Nantes), which is a local authority for ethical aspects of this kind of research.

4.2.1. Blood samples

All blood samples were collected in ethylenediaminetetraacetic acid (EDTA) tubes and peripheral blood mononuclear cells (PBMC) were isolated by density gradient centrifugation. All the cells, as well as serums, were then cryopreserved in 0.5-mL aliquots at -80°C.

4.3. Methodology and research plan

4.3.1. Treatment with reduced-intensity conditioning allogeneic stem cell transplantation

The majority of the patients were treated with reduced-intensity conditioning (RIC) allogeneic stem cell transplantation. The RIC regimen was mainly based on fludarabine in a dose 30 mg/m^2 daily over 4-6 days, IV busulfan 3.2 mg/kg daily over 2-3 days, and anti–thymocyte globulin (ATG) (Thymoglobuline; Genzyme, Lyon, France) for a total dose of 5 mg/kg infused over 2 days.[186]

All donor/recipient pairs were typed at the allelic level, according to the recommendations of the European Federation for Immunogenetics (EFI) Histocompatibility Laboratory standards. A single HLA mismatch of 10 (at HLA-C) was allowed at the allele level. Grafts were obtained from HLA-identical sibling donors or from HLA-matched or mis-matched unrelated

donors. Patients usually received peripheral blood stem cell allografts, mobilized from donors with granulocyte-colony-stimulating factor (GCSF) (10 µg/kg per day).

4.3.2. Prophylaxis, diagnostics and treatment of GVHD

GVHD prophylaxis was performed with cyclosporin A (CSA) alone after related allogeneic stem cell transplantation or with CSA and mycophenolate mofetil (MMF) after unrelated allogeneic stem cell transplantation. In the absence of GVHD, MMF and CSA were tapered over 4 weeks starting from day 60 and day 90, respectively.[187] Acute GVHD was evaluated according to standard Seattle criteria.[188] On diagnosis of grades II to IV acute GVHD, all patients were primarily treated with CSA and methylprednisolone in a dose of 2 mg/kg per day. Acute GVHD and chronic GVHD were arbitrarily separated by day 100 after allogeneic stem cell transplantation. Chronic GVHD was clinically evaluated as limited or extensive. On diagnosis of extensive GVHD, all patients were primarily treated with methylprednisolone (1 mg/kg per day).[189]

4.3.3. Collection of blood samples

All blood samples were collected and then cryopreserved at day 100 after allogeneic stem cell transplantation.

4.3.4. Flow cytometry analysis and intracellular cytokine staining

4.3.4.1. Plasmacytoid dendritic cells

Cryopreserved cells were thawed and stimulated for 6 hours with TLR7 ligands (R848, in a concentration 5 µg/ml) and TLR9 (CpGA, in a concentration 10 µg/ml), in the presence of 10 ng/ml IL3. Two hours after the beginning of stimulation, 10 µg/mL Brefeldin A was added to stop the extracellular secretion of intracellular cytokines.

For surface markers, cells were then stained according to manufacturer's protocol with conjugated antibodies Pacific Blue HLA-DR (from Biolegend), Pe-Cy7 CD123 (from Biolegend) and APC BDCA2 (from Miltenyi). Cells were then fixed and permeabilized using Cytofix/Cytoperm reagents (BD Biosciences) and then incubated with PE IFN alpha (from Miltenyi), FITC IL6 (from Biolegend) and PerCp-Cy5.5 TNF alpha (from Biolegend). Finally, cells were analyzed on a FACSCanto II using DIVA software (BD Biosciences).

4.3.4.2. Subpopulations of T-helper cells

The same PBMC were stained for cell-surface markers according to standard protocols with conjugated antibodies V500 CD3, APC CD8 (both from BD Biosciences), Pe-Cy7 CD4, PE CCR10, PerCp-Cy5.5 CD161 Pacific Blue CXCR3 (all from Biolegend) and FITC CCR6 (from R&D Systems).

For analysis of intracellular cytokine production, PBMC were stimulated with phorbol myristate acetate (PMA) (25ng/ml) and Ca2+ ionophore (1µg/ml) for 4.5 hours, with Brefeldin A (10µg/ml) added for the final 3.5 hours. For surface markers, cells were stained with conjugated antibodies V500 CD3, FITC CD8 (both from BD Biosciences) and Pe-Cy7 CD 4 (from Biolegend). Cells were then fixed and permeabilized by using Cytofix/Cytoperm reagents (BD Biosciences) and then stained for intracellular cytokines with conjugated antibodies PE IFNgamma (from Diaclone), PerCp-Cy5.5 TNFalpha (from Biolegend), V450 IL17A and AF647 IL21 (both from BD Biosciences) according to the manufacturer's protocols. Finally, cells were analyzed on a FACSCanto II using DIVA software (BD Biosciences).

4.3.5. Cytokine analysis in peripheral blood

Forty one different cytokines, chemokines, colony stimulating factors, cytotoxins and interferons (IL1a, IL1b, IL1ra, IL2, IL2ra, IL4, IL5, IL6, IL7, IL9, IL10, IL12p40, IL12p70, IL13, IL15, IL17, IL21, IL23, IL8 (CXCL8), IP10 (CXCL10), MCP1 (CCL2), MIP1a (CCL3), MIP1b (CCL4), RANTES (CCL5), MCP3 (CCL7), TARC (CCL17), MDC (CCL22), FRACTALKINE (CX3CR1), IL3 (multi-CSF), VEGF, PDGFABBB, TNF alpha, TNF beta, TRAIL, CD40L, BAFF, IFN a2, IFN gamma, ELAFIN, TGF alpha, FLT.3L) were quantified from the collected serums using a commercially available Luminex-xMAP system. Luminex is a system of an immune test with xMAP microspheres which are then analyzed in the Luminex analyzer. Antibodies to cytokines are coated to microspheres which are internally dyed with different intensities of two fluorophores allowing detection of a large number of cytokines from the same sample at the same time. As the microsphere passes through the cytometer the red laser excites the fluorophores while the green laser excites the reporter molecule also coated to a microsphere and makes it possible to quantify the reaction. Finally the results were analyzed with xPONENT software.

4.3.6. Statistics

The data were computed using the R package (R Development Core Team, 2006. R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. ISBN 3-900051-07-0, URL http://www.R-project.org.)

The Mann–Whitney test was used for comparison of continuous variables. Categorical variables were compared using the chi square or Fisher's exact test. The probability of developing acute and chronic GVHD was depicted by calculating the cumulative incidence treating death as a competitive risk.[190] Cumulative incidence estimates were also used to measure the probability of relapse. Probabilities of overall survival (OS) were estimated from the time of transplantation using the Kaplan–Meier product-limit estimates.[191] Differences between groups were tested using the logrank test. A multiple logistic regression with backward stepwise model selection was used to depict predictive factors for cell recovery at 100 days after allogeneic stem cell transplantation. The association of time to death with the cell counts and other relevant variables was evaluated in a multivariate analysis with the use of Cox's proportional-hazard regression model.

The association of time to GVHD with cell counts and cytokine levels was evaluated first in the univariate analysis (with CI method). The association of development of chronic GVHD with cell counts and other relevant variables was evaluated in a multivariate regression analysis, using the semiparametric proportional hazards model of Fine and Gray.[192]

The threshold value of the each serum cytokine value was evaluated with the Hothorn and Zeileis recursive partitioning method, which is available as ctree function in the R package.[193]

The association of time to GVHD with cytokine levels independent of the identified risk factors (factors with p value <0.20 in the univariate analysis) was evaluated in a multivariate analysis with the use of Grambsch and Therneau residual functional test for the Cox proportional hazards model.[194]

For making the prognostic score for development of GVHD at two years after allogeneic stem cell transplantation, we included both clinical variables and levels of cytokines which had a p-value < 0.20 in the univariate analysis. The score was made as a sum of covariables multiplied with logarithms of the relative risks obtained in the Cox multivariate analysis. The sensitivity and specificity of the prognostic score was evaluated with the Heagerty method, the Kaplan-Meier based time-dependent receiver operating characteristic (ROC)-curves.[195]

In order to avoid the "overfitting" of our prognostic score (related to the great number of the variables that will be included), and to get a reliable estimate, we used additional techniques, as 0.632 bootstrap resampling method for repeated cross-validation.[196]

5. Results

5.1. Study population characteristics

Patients' characteristics are summarized in ***Table 1.*** Briefly, the first part of the study included 42 male patients (53%) and 37 female patients (47%) that received allogeneic stem cell transplantation between years 2009 and 2011. The median age of recipients was 54 (range 25-71) years. In all, 37 patients were treated with allogeneic stem cell transplantation for acute leukemias (45%), 14 patients (18%) were treated for myelodysplastic and myeloprolipherative syndromes, 14 patients (18%) for lymphomas, 5 patients (7%) were treated for chronic leukemia, 7 patients (9%) for multiple myeloma and finally, 2 patients (3%) for aplastic anemia. Patients with acute leukemia in first complete remission, patients with chronic myeloid leukemia in chronic phase and patients with untreated disease were considered as standard risk patients while all other patients were considered as high risk patients. In total, there were 70 high risk patients (89%) and 9 standard risk patients (11%). Allogeneic stem cell transplantation was done after a myeloablative conditioning in 9 patients (11%) and after a reduced-intensity conditioning in 70 patients (89%). GVHD prophylaxis was done with cyclosporine A alone in 30 cases (38%), with a combination of cyclosporine A and mycophenolate mophetil in 40 cases (51%) and with cyclosporine A and methotrexate in 9 cases (11%). Donors were male in 49 patients (62%) and female in 30 patients (38%). In 36 patients (45%) donors were identical siblings, in 29 patients (37%) matched unrelated and in 14 patients (18%) mismatched unrelated. Twenty-three recipient-donor pairs were seronegative for CMV (29%). Stem cell source was peripheral blood in 55 patients (70%), bone marrow in 10 patients (12%) and cord blood in 14 patients (18%). Median count of stem cells infused at day 0 was 5.25×10^6/kg body weight of recipient.

5.2. Transplant-related events and outcome after day 100 after allogeneic stem cell transplantation

Transplant related events and outcomes are summarized in ***Table 2.*** Overall survival in our patients was 74% at two years, with a median follow up of 592 days for living patients. ***(Figure 4.)***

53 patients (67%) had no or had grade I acute GVHD and clinically significant grade II to IV acute GVHD (moderate to severe GVHD) occurred in 26 of cases (33%) at a median of 30 days after allogeneic stem cell transplantation (with a range 8-91 days). The cumulative incidence of acute GVHD was 50% (95%CI, 38-61) at 20 months with cumulative incidence of significant acute GVHD (grades II-IV) of 36% (95%CI, 25-47) at 20 months. ***(Figures 5. and 6.)*** Two patients died from severe (grade IV) form of acute GVHD before day 100. Chronic GVHD was diagnosed in 49 of cases (62%), with limited form of chronic GVHD diagnosed in 25 of these patients (32%) extensive chronic GVHD occurring in 24 patients (30%). The cumulative incidence of chronic GVHD was of 66% (95%CI, 53-76) at 20 months with cumulative incidence of extensive chronic GVHD of 37 % (95%CI, 25-49) at 20 months. ***(Figures 7. and 8.)*** Chronic GVHD was diagnosed at a median of 134 days after allogeneic stem cell transplantation (with a range of 90-570 days).

Median blood cell counts together with the immune status (CD4+ and CD8+ lymphocytes) and CMV antigenemia at day 100 after allogeneic stem cell transplantation are depicted in ***Table 1***.

Table 1. Baseline demographic characteristics of study population

Characteristic	n (%)
Patient age, median (range)	54 (25-71)
Patient gender	
Male	42 (53)
Female	37 (47)
Diagnosis[a]	
Myeloid malignancy	44 (56)
Lymphoid malignancy	33 (42)
Aplastic anaemia	2 (2)
Disease risk[b]	
Standard risk	9 (11)
High risk	70 (89)
Conditioning regimen	
Myeloablative	9 (11)
Reduced-intensity conditioning	70 (89)
GVHD prophylaxis	
CsA alone	30 (38)
CsA and MMF	40 (51)
CsA and MTX	9 (11)
Donor gender	
Male	49 (62)
Female	30 (38)
CMV serologic status, seronegative pair	
Yes	23 (29)
No	56 (71)
Donor type	
Matched related donor	36 (45)
Matched unrelated donor	29 (37)
Mismatched unrelated donor	14 (18)
Stem cell source	
Bone marrow	10 (12)
Peripheral blood	55 (70)
Cord blood	14 (18)
Cells infused ($x10^6$/kg), median (range)	5.25(0.06-10.1)

[a]Myeloid malignancies included 28 acute myeloid leukemias, 10 myelodysplastic syndromes, 4 myeloprolipherative syndromes and 2 chronic myeloid leukemias. Lymphoid malignancies included 12 non-Hodgkin's lymphomas, 9 acute lymphoblastic leukemias, 7 multiple myelomas, 3 chronic lymphocytic leukemias and 2 Hodgkin's lymphomas.
[b]Standard risk disease – acute leukemia in first complete remission, chronic myeloid leukemia in chronic phase and untreated disease, all others-high risk.
CMV= cytomegalovirus, CsA=cyclosporine A, MMF=mycophenolate mofetil, MTX=methotrexate

Table 2. Transplant-related events and outcome

Characteristic	n (%)
Neutrophil recovery ANC > 0,5 x 10^9 /L median (range)	16 (8-43)
Acute GVHD grade 0-I grade II -IV	53 (67) 26 (33)
Acute GVHD onset (days) after transplantation median (range)	30 (8-91)
CMV antigenemia at 100 days after allo-SCT yes no	 36 (45) 43 (55)
Blood cell counts at d+100 Leukocytes Granulocytes Lymphocytes Monocytes CD4+ CD8+	 4.65 (1.0-16.4) 2.57 (0.06-14.6) 0.94 (0.1-5.2) 0.5 (0.02-1.6) 0.114 (0.001-0.98) 0.266 (0.02-2.7)
Chronic GVHD no or limited extensive	 55 (70) 24 (30)
Chronic GVHD onset (days) after transplantation median (range)	134 (90-570)
Follow up days for surviving patients median (range)	592(225-873)

ANC=absolute neutrophil count, CMV=cytomegalovirus, GVHD=graft-versus-host disease

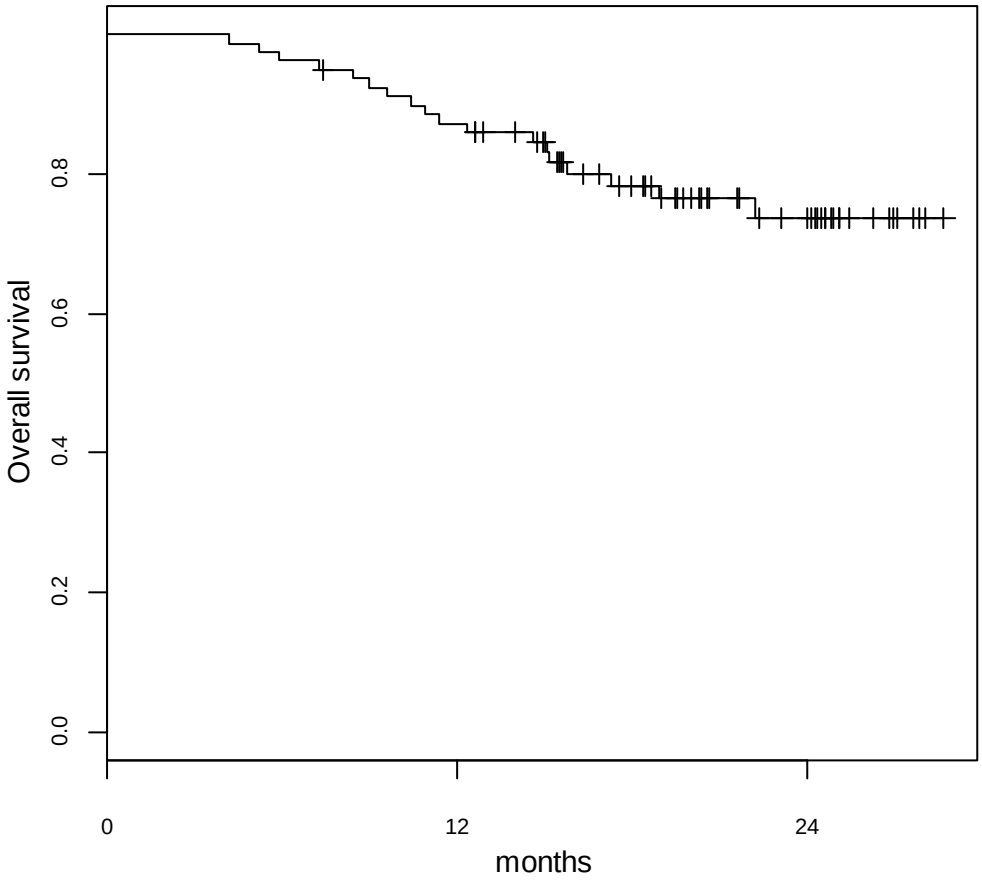

Figure 4. Overall survival in study population of 74% (95%CI 64-86%) at 22 months

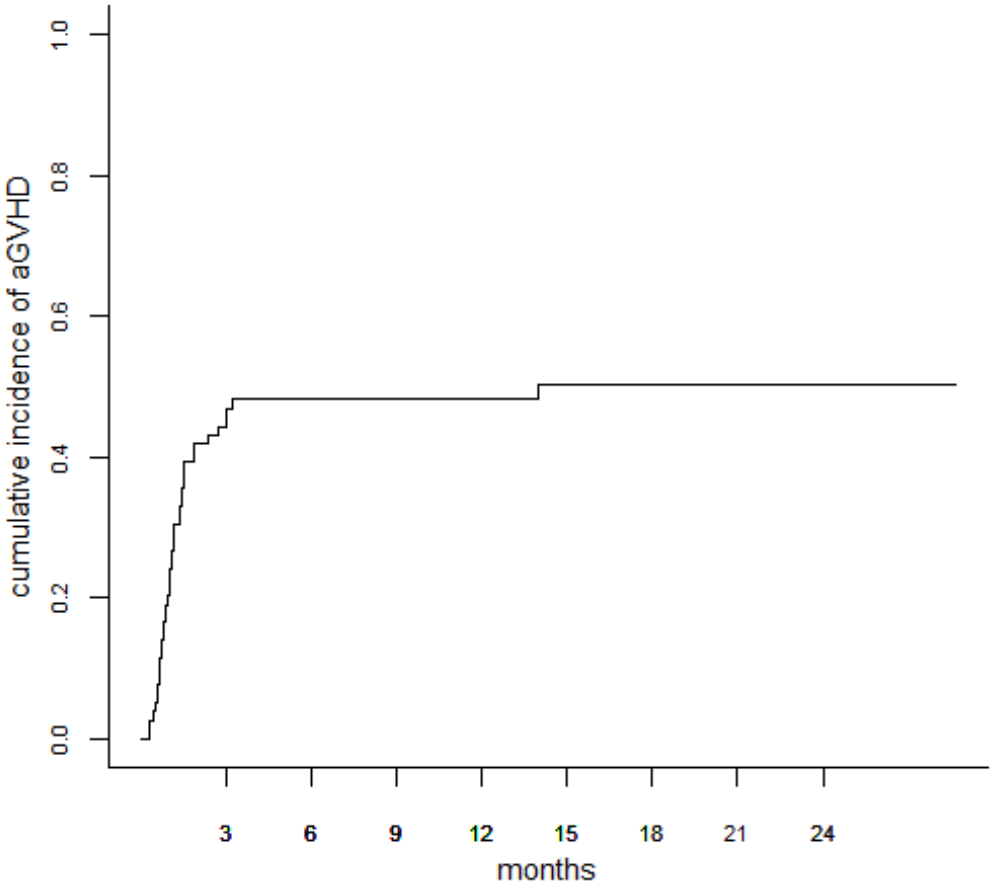

Figure 5. Cumulative incidence of acute GVHD in study population of 50% (95%CI, 38-61) at 20 months

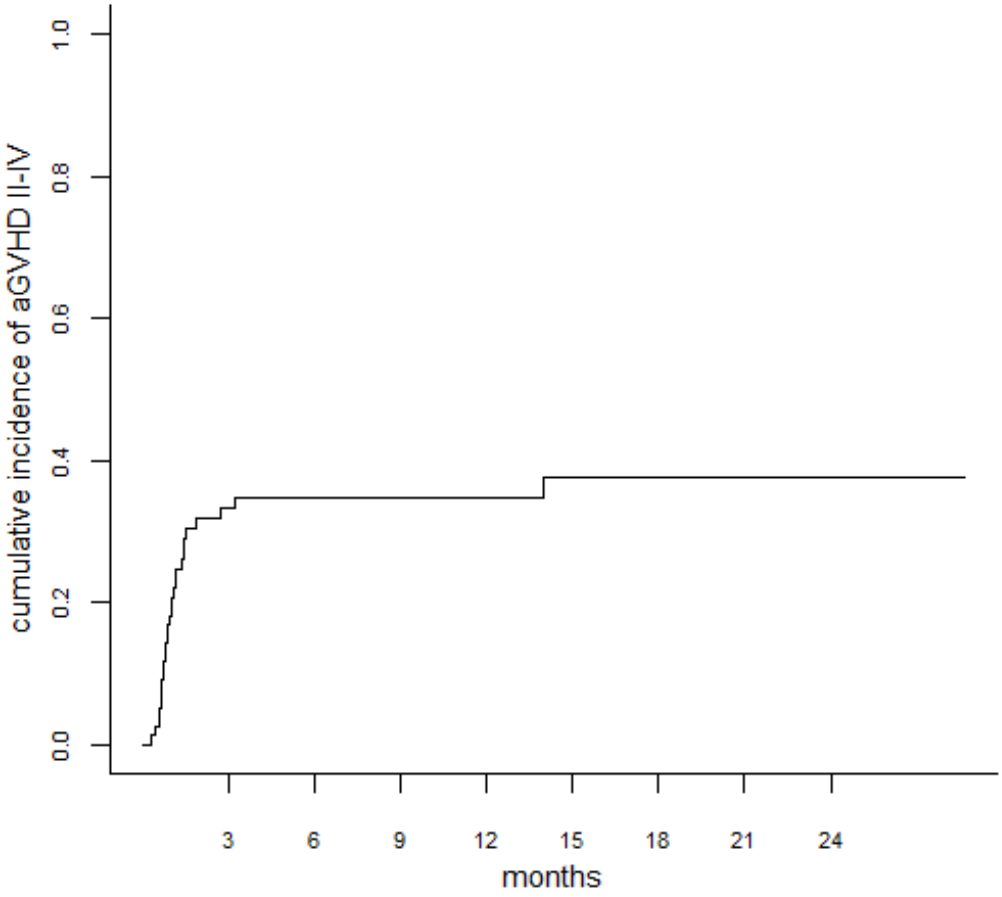

Figure 6. Cumulative incidence of II-IV acute GVHD in study population of 36% (95%CI, 25-47) at 20 months

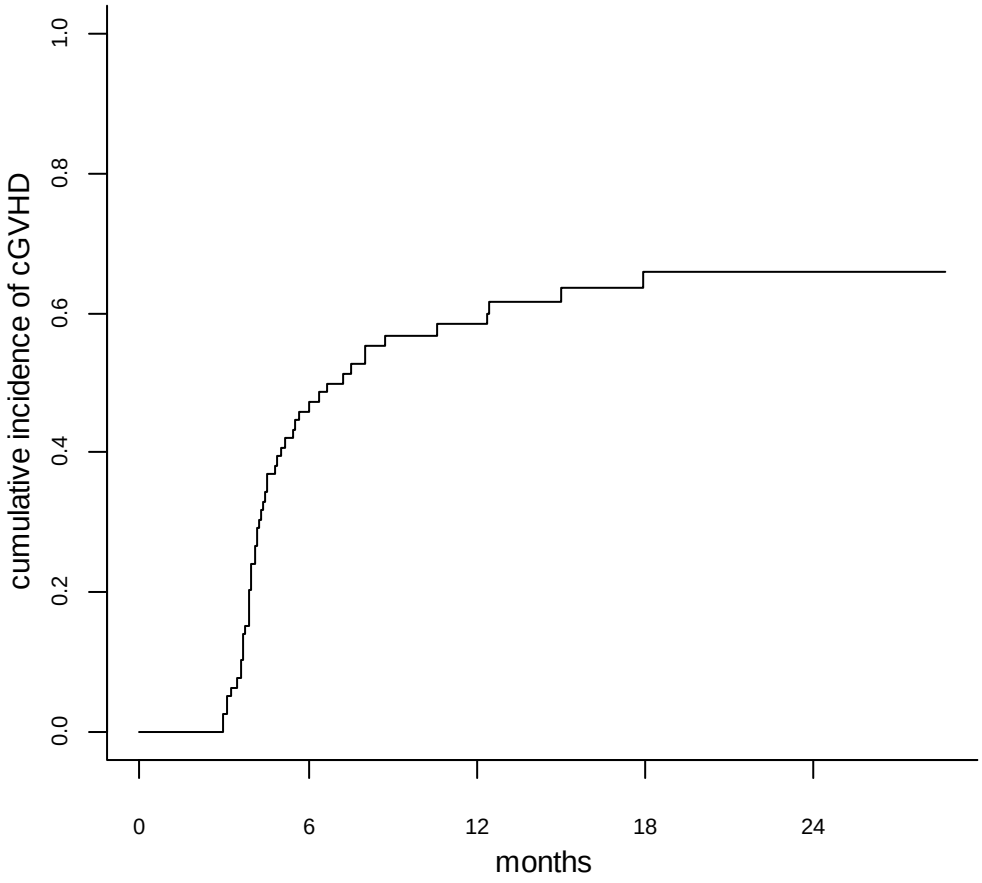

Figure 7. Cumulative incidence of chronic GVHD in study population of 66% (95%CI, 53-76) at 20 months

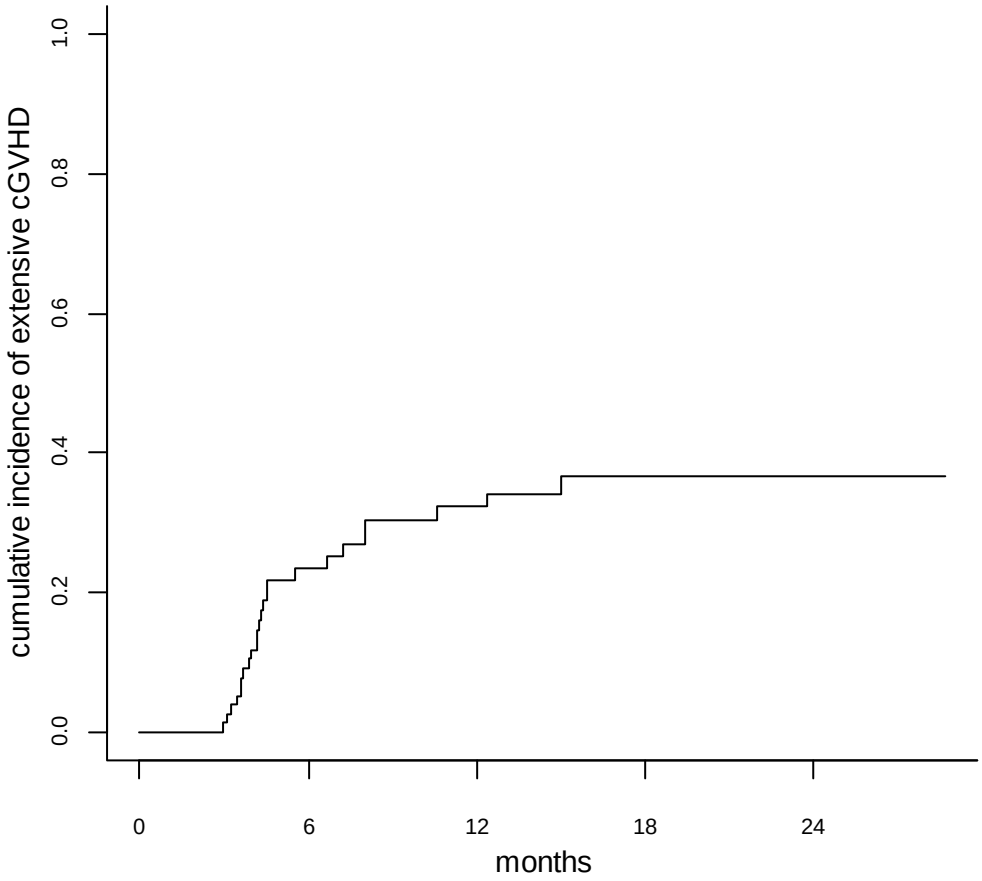

Figure 8. Cumulative incidence of extensive chronic GVHD in study population of 37 % (95%CI, 25-49) at 20 months

5.3. pDC in peripheral blood of patients at day 100 after allogeneic stem cell transplantation

5.3.1. Stimulation and analysis of pDC

For functional characterization, thawed PBMC were stimulated with three different conditions:

1. with IL3
2. with IL3 + CpGA
3. with IL3 + R848

All three conditions included IL3, as this cytokine was shown to be necessary for the survival of pDC.[67] The first condition included just IL3 and served as a negative controle. The other two conditions included ligands for specific TLR receptors present on pDC; CpGA, a ligand for TLR9 receptor and R848, a ligand for TLR7 receptor.[65] Upon stimulation of TLR9, pDC secrete large amounts of cytokines- IFN alpha and TNF alpha, and upon stimulation of TLR7, pDC secrete large amounts of both IFN alpha and TNF alpha, together with IL6.

As pDC are a rare population of cells and represent only of 0.1 – 0.7% of PBMC in the blood, a rather large number of cells needs to be stimulated to have some visible resultats on the flow cytometry at the end of the experiment. This is the reason why we used at least 10^6 of thawed PBMC cells for each condition. For already known reasons, our population of patients is a population under immunosupressive treatment and a population with sometimes lower numbers of PBMC that could be cryopreserved. Actually, a median number of cryopreserved cells at day 100 for all our patients was 3.2×10^6. It is known that, after thawing, a large number of cells is destroyed with DMSO and this is the reason why the number of cells at the beginning of stimulation was sometimes modest. It was planned to do all our experiments, both stimulation of pDC and lymphocytes, with the same PBMC from one sample per patient.

All this led us to a conclusion that all conditions could not be done in all patients. Finally, the stimulation with CpGA (TLR9 ligand) was done in all 79 patients and the stimulation with R848 (TLR7 ligand) was done in 46 patients. (58% of cases).

As described before, after 6 hours of stimulation, cell were stained for surface markers; HLA-DR (a marker specific for dendritic cells), CD123 and BDCA2 (pDC express CD123high and BDCA2, which is expressed exclusively on human pDC).

After staining for cell surface markers, cells were fixed and permeabilized and finally stained for intracellular cytokines. As mentioned before, pDC were evaluated for production of following cytokines: IFN alpha (a signature cytokine for pDC), TNF alpha and IL6.

Finally, pDC were evaluated by multicolor flow cytometry. The isotype controls in this experiment were not used because the condition with IL3 served as negative controle. We also used two healthy donors as positive controls. (an example of flow cytometry analysis of one of the patients with positive and negative controls is shown in **Figures 9. -12.**)

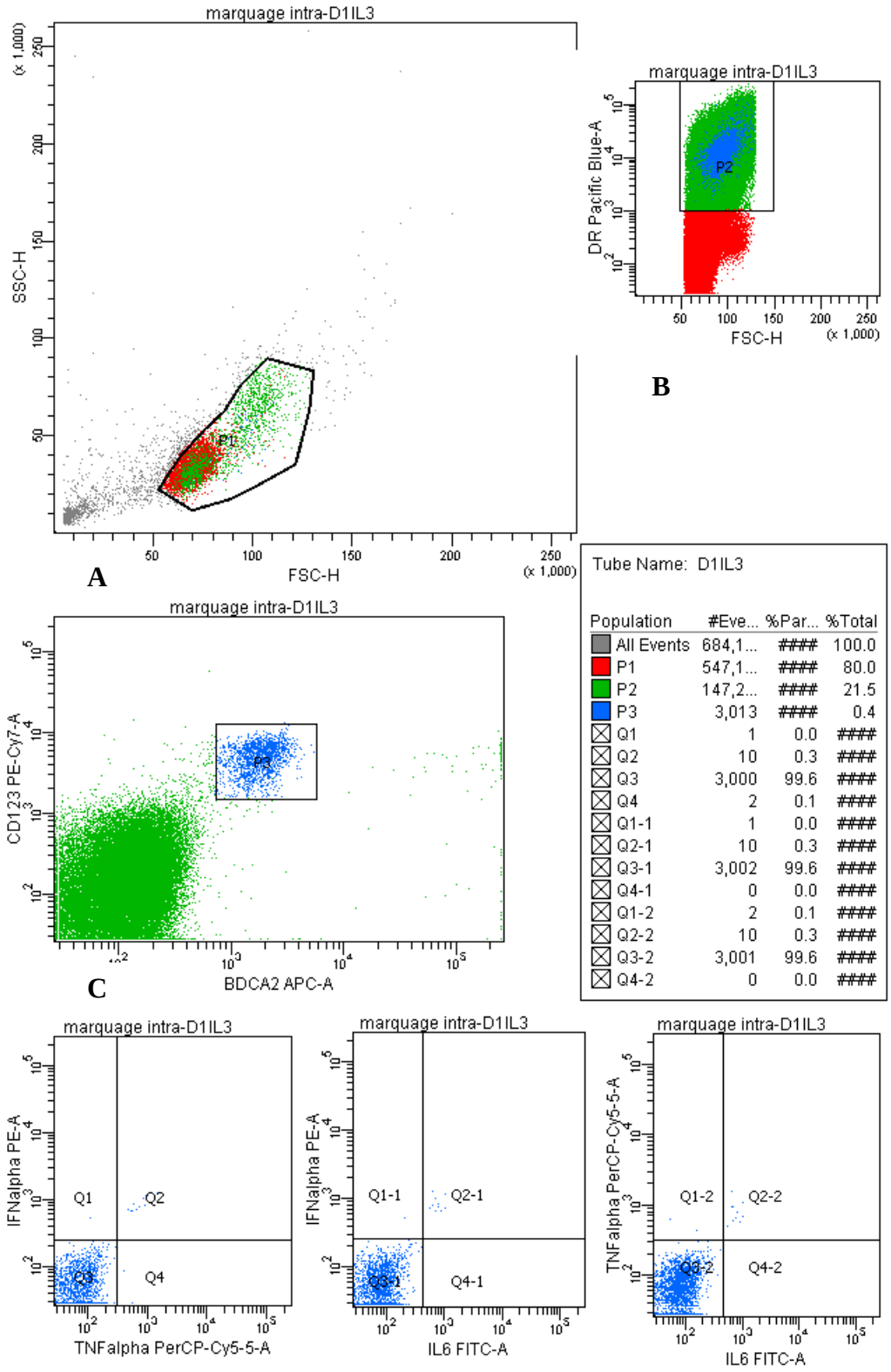

Figure 9. Stimulation with IL-3 in a healthy donor (negative control); pDC were detected among mononuclear cells (A) as HLADR+ (B), CD123+ and BDCA2 (C)

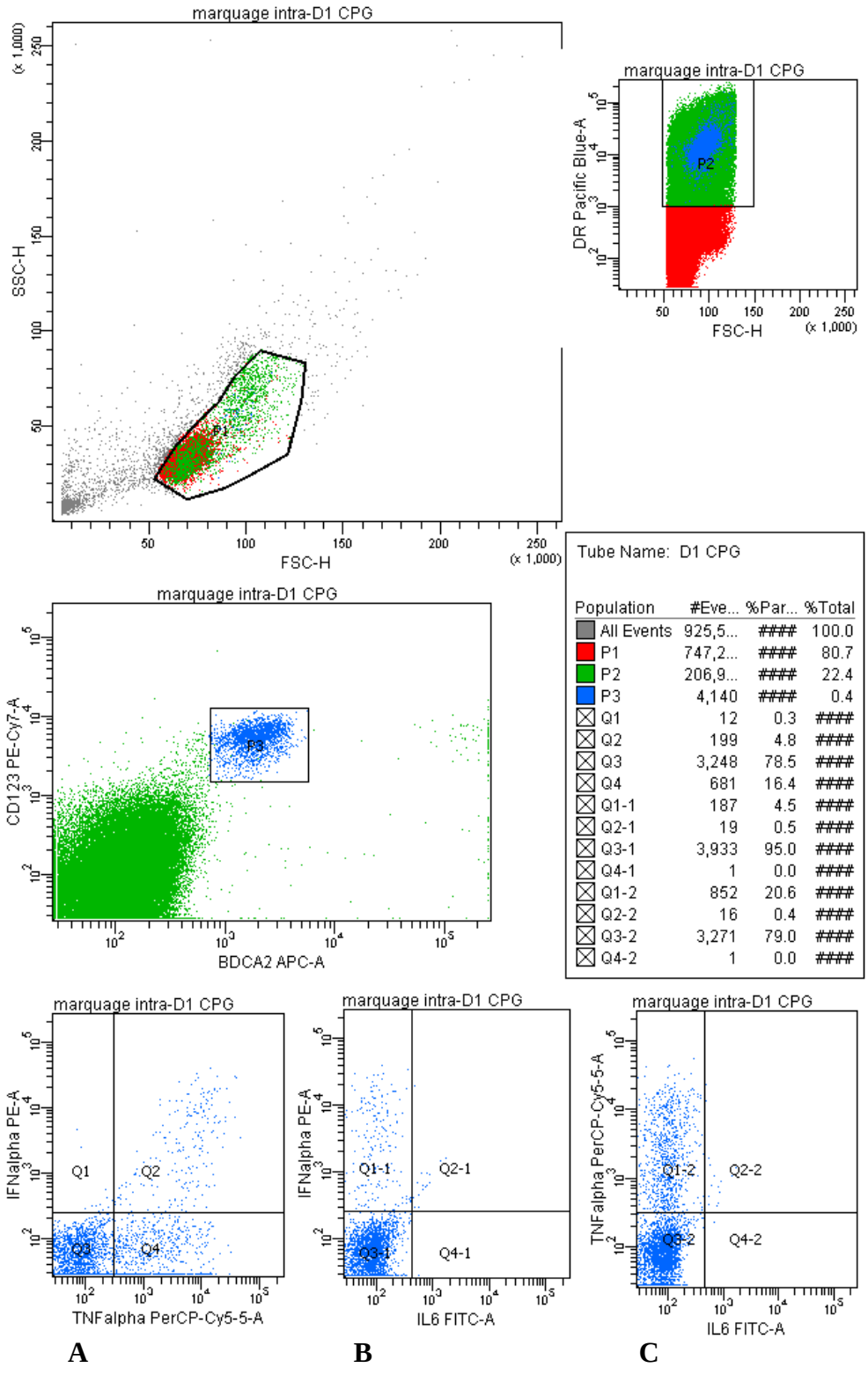

Figure 10. Stimulation with IL3 + CPG in a healthy donor (positive control); pDC were evaluated for production of IFN alpha (A, B), TNF alpha (A, C) and IL6 (B, C)

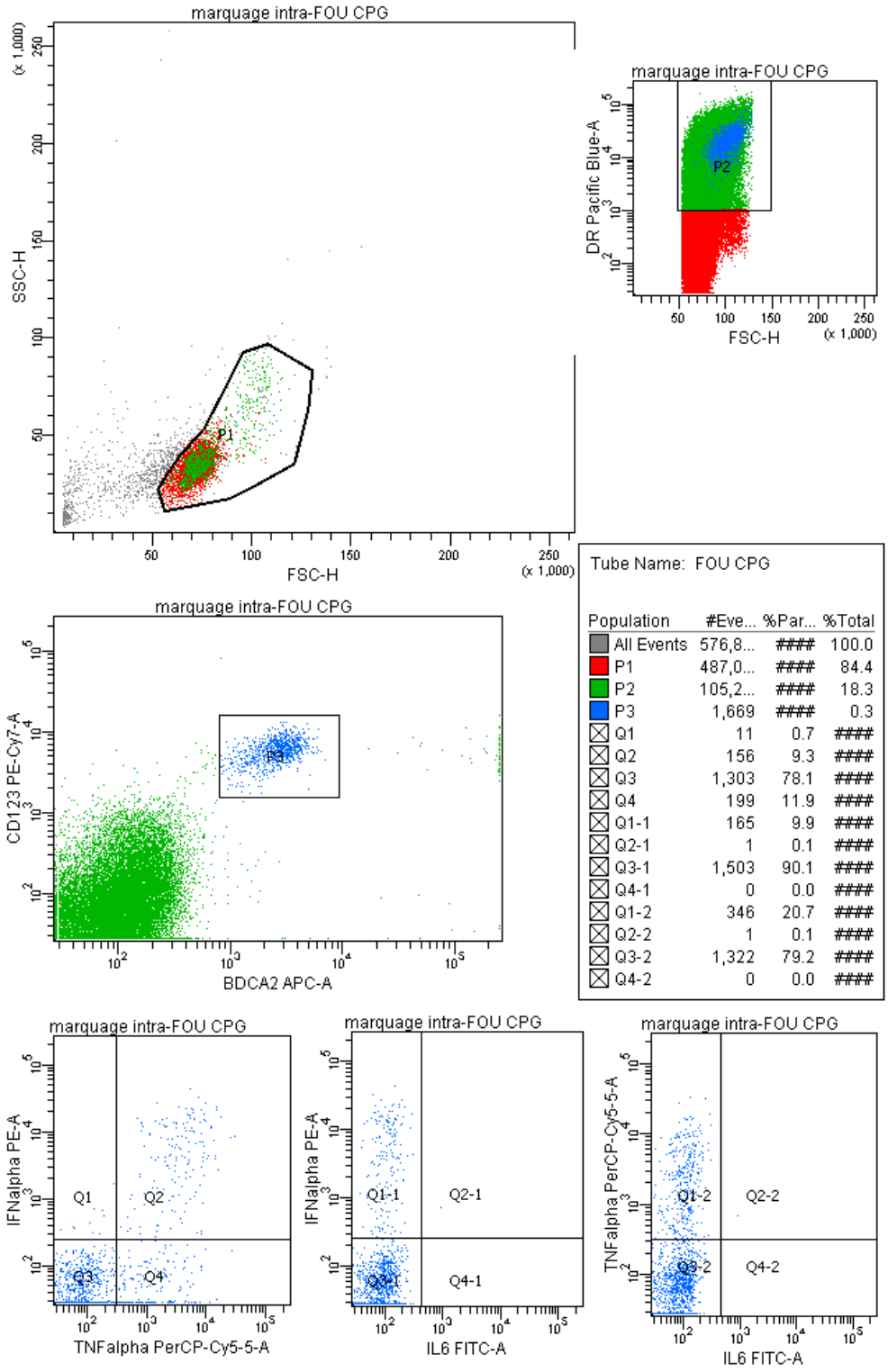

Figure 11. Stimulation with IL3 + CPG in a patient from PBMC taken at day 100 after allogeneic stem cell transplantation

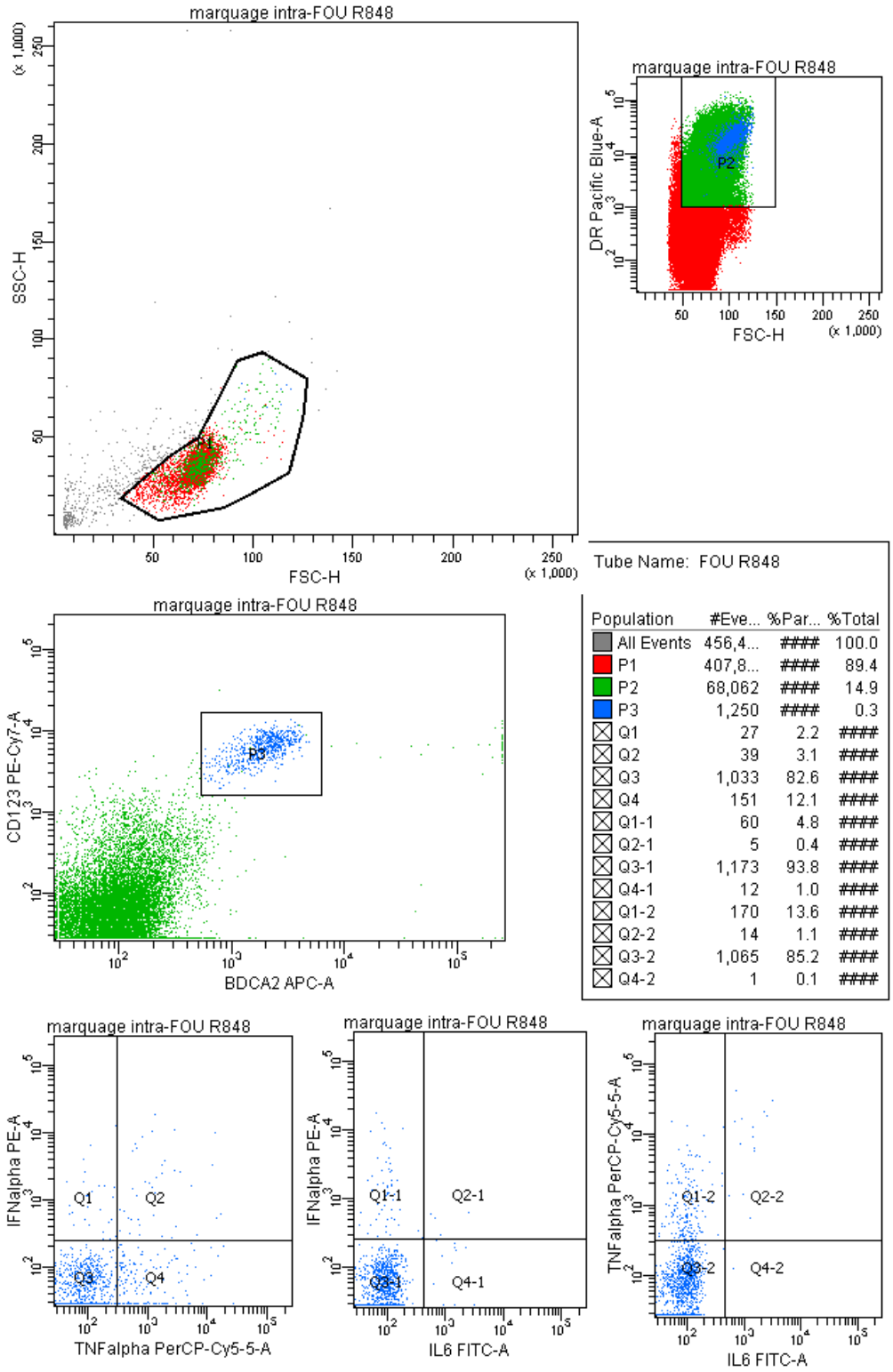

Figure 12. Stimulation with IL3 + R848 in a patient from PBMC taken at day 100 after allogeneic stem cell transplantation

5.3.2. pDC count and predictive factors for pDC recovery

The median pDC count at day 100 after allogeneic stem cell transplantation in our group of patients was 0.2% of PBMC. We used this value to allocate the patients into a „low pDC“ recovery group (patients with pDC count <0.2% of PBMC) or „high pDC“ recovery group (patients with pDC count >=0.2% PBMC). Baseline demographic and transplant characteristic of these two groups were comparable and are shown in ***Table 3.*** Transplant-related events and outcome of the patients of the „low pDC“ and „high pDC“ recovery groups were also similar as it is shown in ***Table 4.***, except of the incidence of acute GVHD. Grade II-IV acute GVHD was observed in 19 patients (59%) in the „low pDC“ recovery group while grade II-IV acute GVHD occured only in 8 patients (17%) in the „high pDC“ recovery group.

Therefore, we built a multivariate logistic regression model to explain pDC recovery in the blood of patients at day 100 after allogeneic stem cell transplantation. All variables with a p-value < 0.20 in the univariate analysis were included in the model (diagnosis, gender of the donor, type of the donor, CMV antigenemia and acute GVHD). A backward stepwise selection was performed and we obtained a model with two variables- matched unrelated donor (OR = 0.84, 95% CI 0.68-1.03 p=0.09) and occurence of grade II-IV acute GVHD (OR= 0.67, 95% CI 0.54-0.83, p = 0.0004). Finally, only the absence of clinically significant grade II-IV acute GVHD was significantly associated with an impaired pDC recovery at day 100 after allogeneic stem cell transplantation.

Table 3. Baseline demographic characteristics of "low"and "high" pDC group

Characteristi	"low pDC" n (%) n=32	"high pDC" n (%) n=47	p-value
Patient age, median (range)	54 (26-71)	54 (27-69)	0.65
Patient gender			
Male	18 (55)	24 (51)	0.65
Female	14 (45)	23 (49)	
Diagnosis[a]			
Myeloid malignancy	18 (56)	17 (36)	
Lymphoid malignancy	13 (41)	29 (62)	
Aplastic anaemia	1 (3)	1 (2)	**0.10**
Disease risk[b]			
Standard risk	13 (41)	19 (40)	
High risk	19 (59)	28 (60)	0.98
Conditioning regimen			
Myeloablative	3 (9)	6 (13)	
Reduced-intensity conditioning	29 (91)	41 (87)	0.64
GVHD prophylaxis			
CsA alone	8 (25)	18 (38)	0.22
CsA and MMF /MTX	24 (75)	29 (62)	
Donor gender			
Male	17 (53)	32 (68)	**0.18**
Female	15 (47)	15 (32)	
CMV serologic status, seronegative pair			
Yes	9 (28)	14 (30)	
No	23 (72)	33 (70)	0.87
Donor type			
Matched related donor	10 (31)	27 (57)	
Matched unrelated donor	12 (38)	13 (28)	
Mismatched unrelated donor	10 (31)	7 (15)	**0.06**
Stem cell source			
Bone marrow	5 (16)	5 (11)	
Peripheral blood	20(63)	35 (74)	
Cord blood	7 (21)	7 (15)	0.52
Cells infused ($x10^6$/kg), median (range)	6 (0.06-10.1)	4.98 (0.07-10)	0.17

CMV= cytomegalovirus, CsA=cyclosporine A, MMF=mycophenolate mofetil, MTX=methotrexate

Table 4. Transplant related events and outcome of "low"and "high" pDC group

Characteristic	**" low pDC" n (%) n=32**	**"high pDC" n (%) n=47**	**p-value**
Neutrophil recovery ANC > 0,5 x 10^9 /L median (range)	17 (11-32)	16 (8-43)	0.28
Acute GVHD			
grade 0-I	13 (41)	39 (83)	
grade II -IV	19 (59)	8 (17)	< **0.0001**
Acute GVHD onset (days) after transplantation median (range)	29 (13-97)	32 (8-91)	0.56
CMV antigenemia at 100 days after allo-SCT			
yes	24 (75)	42 (89)	**0.09**
no	8 (25)	5 (11)	
Blood cell counts at d+100			
Leukocytes	4.9 (1.7-16.4)	4.3 (1.0-13.1)	0.18
Granulocytes	3.2 (0.8-14.6)	2.4 (0.06-8.3)	0.11
Lymphocytes	0.8 (0.1-5.2)	1.0 (0.12-2.9)	0.18
Monocytes	0.4 (0.02-1.6)	0.5 (0.02-1.5)	0.64
CD4+	0.09 (0.001-0.65)	0.13(0.02-0.98)	0.13
CD8+	0.14 (0.02-2.7)	0.31 (0.02-2.4)	0.24
Chronic GVHD			
no or limited	20 (63)	35 (74)	
extensive	12 (37)	12 (26)	0.26
Chronic GVHD onset (days) after transplantation median (range)	127 (90-546)	137 (91-379)	0.49
Follow up days for surviving patients median (range)	638 (225-825)	626 (384-873)	0.72

ANC=absolute neutrophil count, CMV=cytomegalovirus, GVHD=graft-versus-host disease

5.3.3. Acute GVHD and pDC

Twenty-six (33%) of our patients developed grade II-IV acute GVHD at a median of 30 days and grade II-IV acute GVHD was found to be an independent predictor of pDC recovery.
At day 100, we observed a significant decrease of total pDC in patients with grade II-IV acute GVHD when compared to patients without clinically significant acute GVHD (grades 0-I). (p<0.0001***, Figure 13***.)

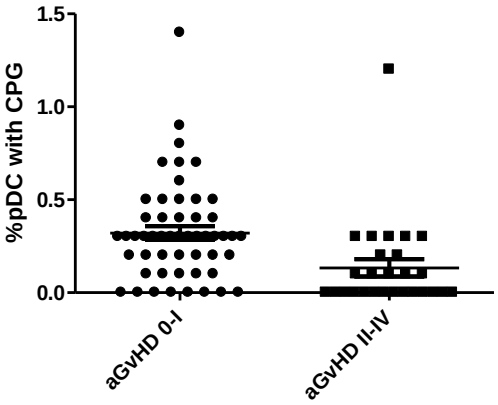

Figure 13. pDC stimulated with CpGA in patients with and without acute GVHD, p<0.0001

Moreover, we observed that pDC stimulated with CpGA in patients with grade 0-I acute GVHD secreted significantly more IFN alpha and TNF alpha than pDC in patients with grade II-IV acute GVHD. (p=0.002 and p=0.0005, respectively, in ***Figure 14***.)

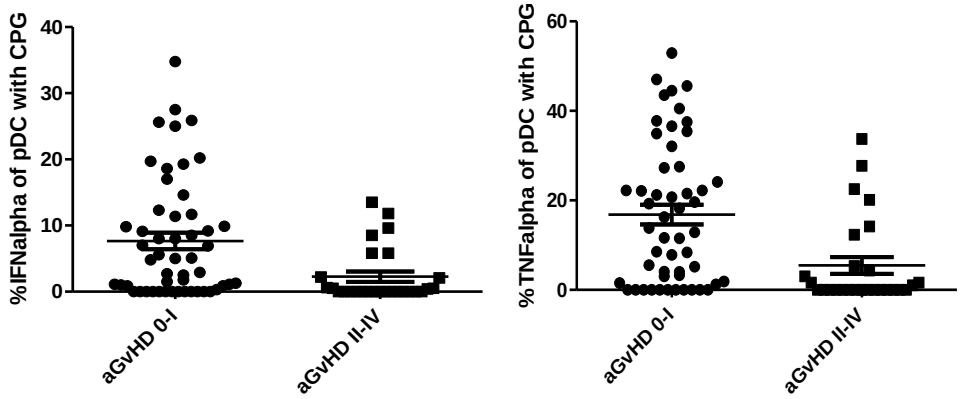

Figure 14. IFN alpha and TNF alpha-secreting pDC stimulated with CpGA in patients with and without acute GVHD, p=0.002 and p=0.0005, respectively

Similarly, in 46 patients the stimulation of PBMC was done with a TLR7 ligand R848, and here we also observed a significant decrease of total pDC in patients with grade II-IV acute

GVHD when compared to patients without clinically significant acute GVHD (grades 0-I). (p=0.001**, *Figure 15*.**)

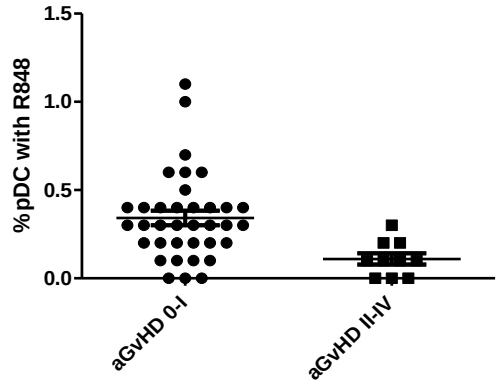

Figure 15. pDC stimulated with R848 in patients with and without acute GVHD, p=0.001

Finally, we also observed a decrease of IFN alpha-secreting, TNFalpha and IL6 secreting pDC in patients with grade II-IV acute GVHD when compared to patients without clinically significant acute GVHD (grades 0-I), even though it did not reach statistical significance. (p=0.22, p=0.08 and p=0.23, respectively, in ***Figure 16***.)

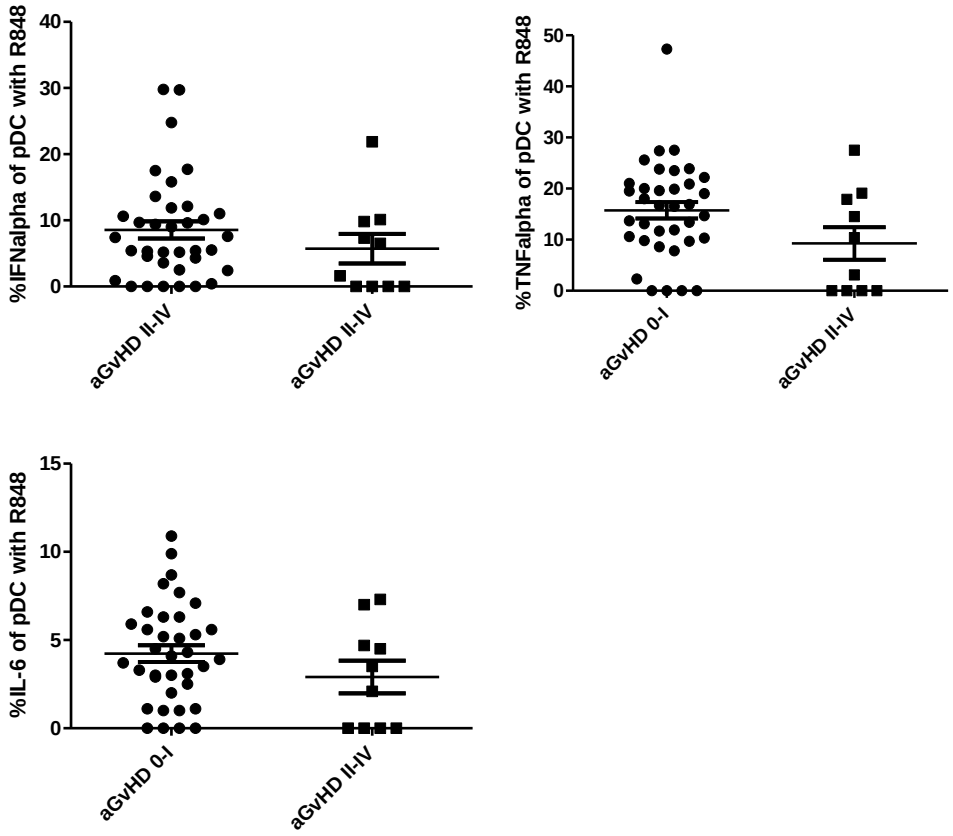

Figure 16. IFN alpha, TNF alpha and IL6 -secreting pDC stimulated with R848 in patients with and without acute GVHD, p=0.22, p=0.08 and p=0.23, respectively

Corticosteroids, either dexamethasone, prednisolone or methylprednisolone, do not affect DC viability but down-regulate the expression of costimulatory molecules on immature DCs, prevent DC maturation and impair their immunostimulatory activities.[197-202]

Therefore we excluded the patients who received high doses of corticosteroid treatment (at least 1 mg/kg) at day 100 after allogeneic stem cell transplantation and reanalysed the pDC count and function in 58 patients. In concordance with previous studies, in our study the viability of pDC in patients was similar in both patients with and without corticosteroid therapy. We still saw a significant decrease of total pDC in patients with grade II-IV acute GVHD when compared to patients without clinically significant acute GVHD (grades 0-I). ($p = 0.05$ when stimulated with pDC and $p = 0.01$ when stimulated with R848, ***Figure 17*.)**

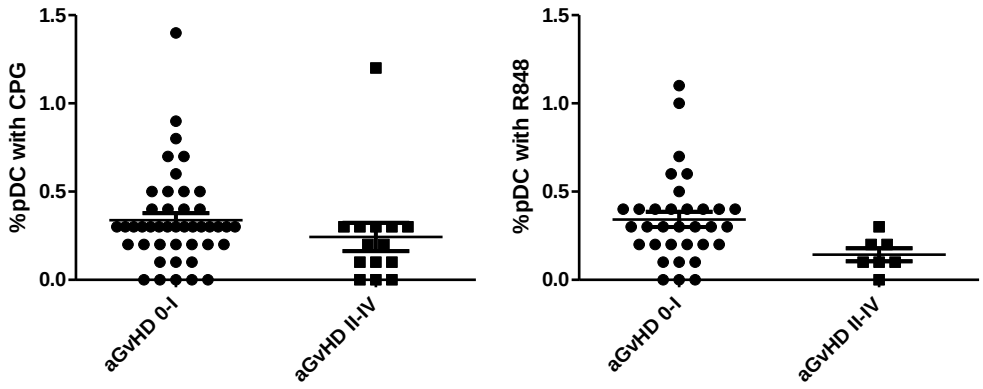

Figure 17. Total pDC count without patients who received high doses of corticosteroids (p=0.05 and p=0.01, respectively)

On the contrary, in the analysis without the patients treated with high doses of corticosteroids at day 100, we did not observe any significant difference in the function of pDC between the groups of patients with and without clinically severe GVHD. Patients without acute GVHD or grade I acute GVHD before day 100 secreted similar amounts of IFN alpha, TNF alpha and IL6 as patients with grade II-IV acute GVHD. (in both conditions- stimulation with CpGA and R848, data not shown).

5.3.4. pDC and chronic GVHD

The cumulative incidence of extensive chronic GVHD in our study group was 37% (95%CI 25-49%) at 20 months, with this incidence being higher in the "low pDC" recovery group (44%, 95% CI 24-63%) than in the "high pDC" recovery group (30%, 95% CI 17-46%) all though this did not reach statistical significance (p=0.25, ***Figure 18.)***

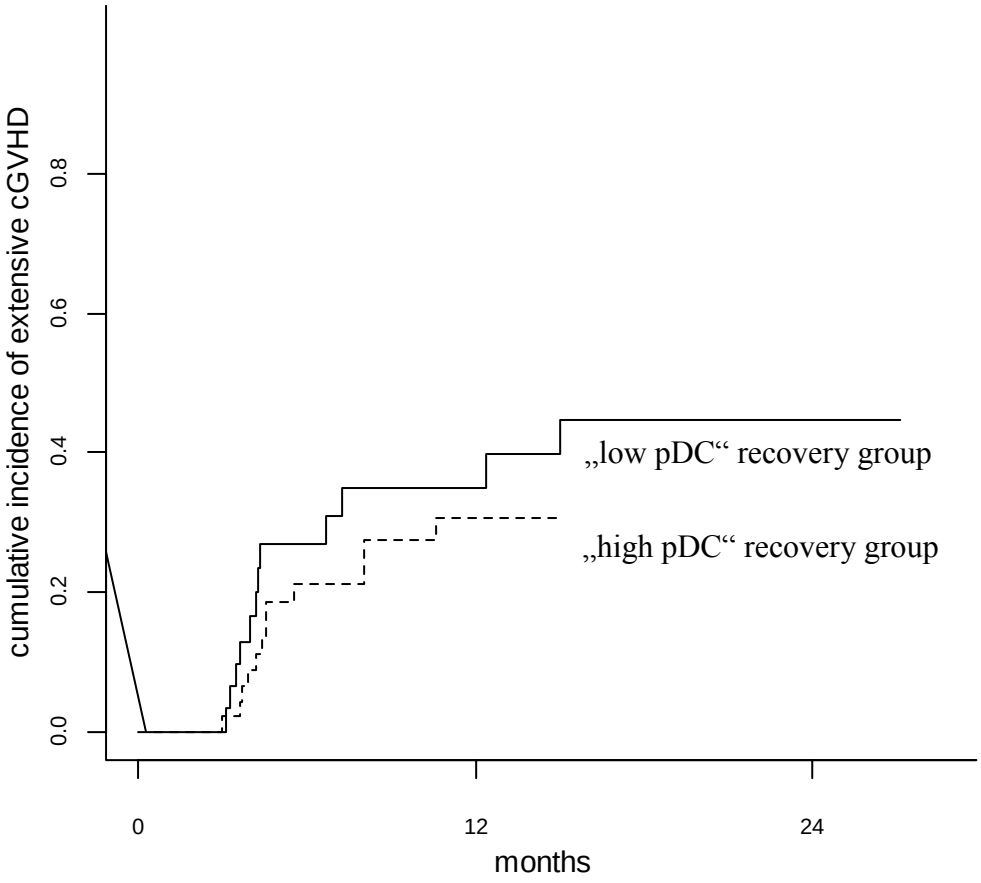

Figure 18. Cumulative incidence of extensive chronic GVHD in the "low pDC" group of 44% (95% CI 24-63%) at 20 months and in the "high pDC" group of 30% (95% CI 17-46%), p=0.25

5.3.5. pDC and relapse

Relapse related mortality in our patients was 35 (95% CI) at 20 months with this being significantly higher (p=0.018) in the "low pDC" recovery group patients (35%, 95% CI 16-54%) than in the "high pDC" recovery group patients (9.5%, 95% CI 3-21%) and this is shown in ***Figure 19.***

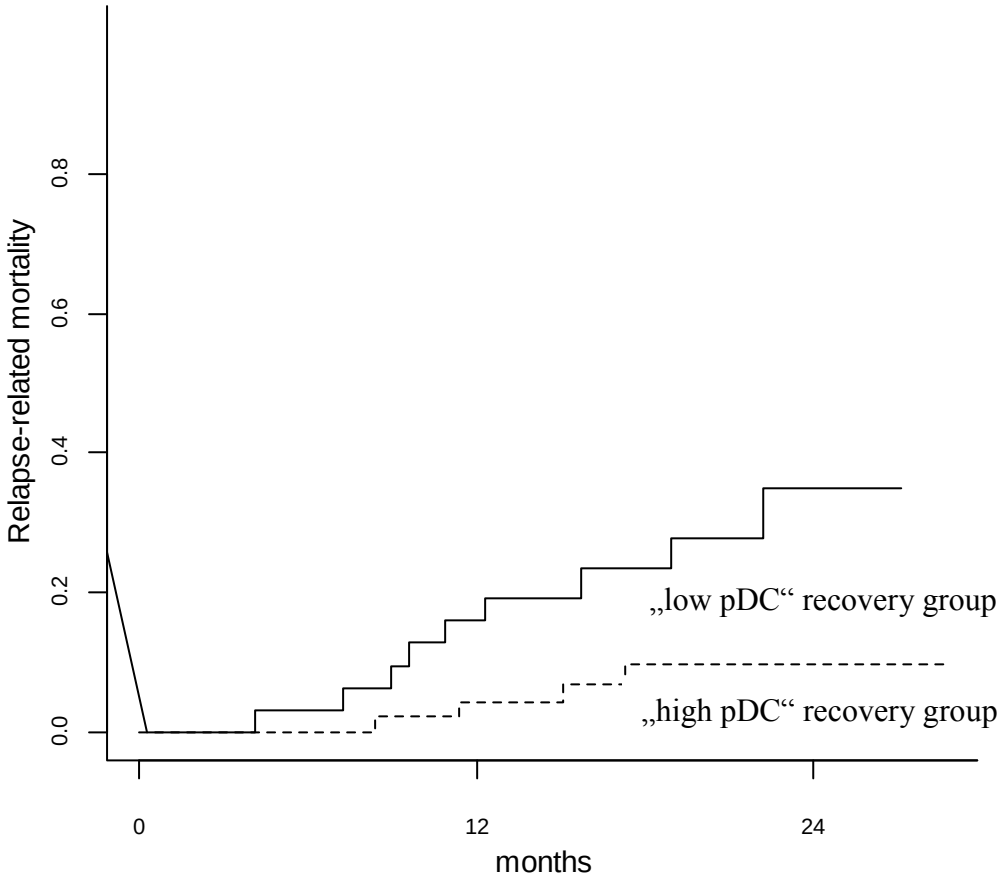

Figure 19. Cumulative incidence of relapse in the "low pDC" group of 35% (95% CI 16-54%) at 20 months and in the "high pDC" group of 9.5% (95% CI 3-21%), p=0.018

5.3.6. pDC and overall survival

Finally, "high pDC" recovery group of patients showed significantly better (p=0.007) overall survival (86%, 95% CI 76-97%) when compared with the overall survival of the patients in the "low pDC" recovery group (55%, 95% CI 38-80%). Overall survival of both group of patients is shown in ***Figure 20***.

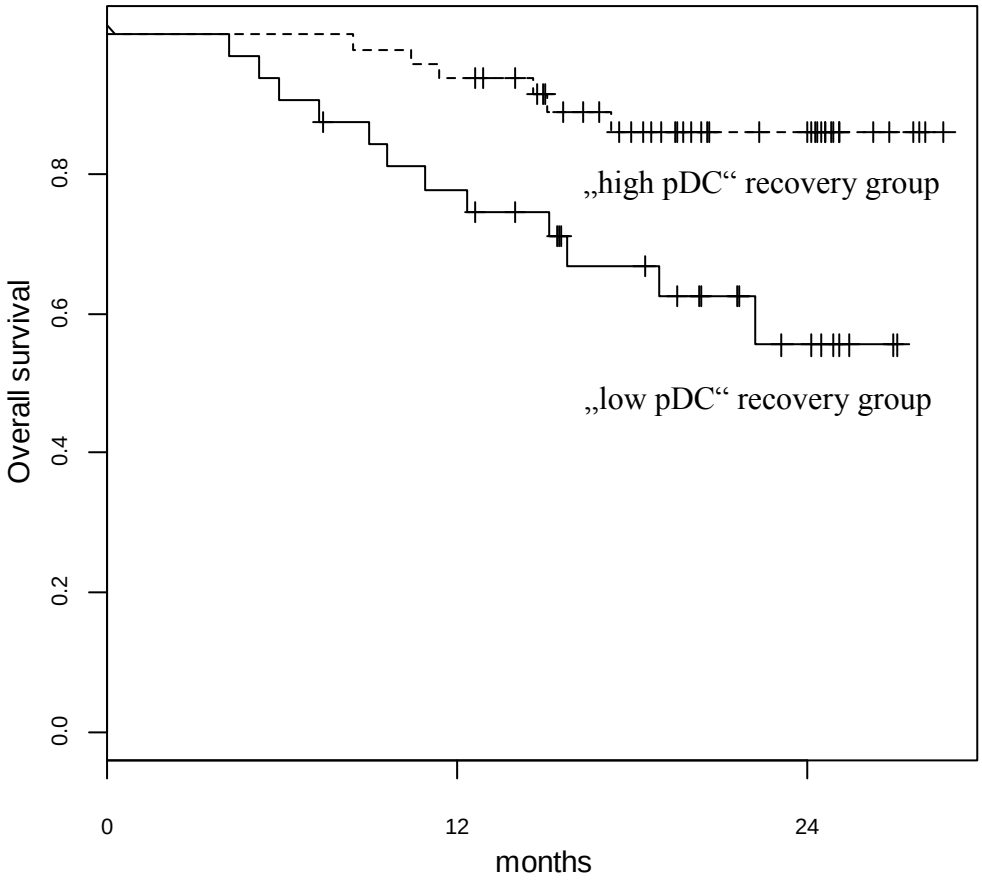

Figure 20. Overall survival in "low pDC" group (55%, 95% CI 38-80%) and "high" pDC group (86%, 95% CI 76-97%); p=0.007

5.3.7. pDC count is an independent predictor of overall survival

Finally, a multivariate analysis was done and included all relevant variables from the univariate analysis with a $p<0.20$ (donor, age of the recipient, pDC count) or previously known risk factors (risk of the disease). In this analysis, both older age of recipient and "low pDC" count stayed independent predictive factors of worse overall survival ($p=0.02$ and $p=0.03$, respectively). (***Table 5.***)

Table 5. Multivariate analysis of predictors of overall survival

Risk factor	Relative risk	Confidence interval	p
"low pDC" count	3.41	1.19-9.79	**0.02**
older age of recipient	5.16	1.15-23.14	**0.03**
donor type- MUD	2.11	0.63-7.06	0.23
high risk disease	1.14	0.43-2.98	0.79

MUD-matched unrelated donor

5.4. Th subpopulations of lymphocytes in the blood of patients at day 100 after allogeneic stem cell transplantation

5.4.1. Stimulation and analysis of Th1 and Th17

The functional analysis of Th subpopulations was done from the same PBMC samples already used for functional analysis of pDC. Blood CD4+T cells producing IL17A, IL21, TNFalpha and IFNgamma have been evaluated after 4.5 hours of stimulation of PBMC with PMA and ionomycine. The cells were first stained with the superficial markers CD3, CD4 and CD8, along with the FVD, a marker of viability. Unfortunately, we soon noticed that after stimulation only half of thawed cells stayed viable. This finding prevented us from performing an adequate flow cytometry because of the initial number of thawed cells available for this experiment. More precisely, as we already used about a million of PBMC for functional analysis of pDC for every condition and as we had 3 million of thawed cells per whole sample on average, we came to the conclusion that the available quantity of cells is not sufficient for the functional analysis of both lymphocytes and pDC at the same time. Therefore we decided to perform the functional analysis of only pDC from our samples, as it is a rare, controversial population of cells, especially in the GVHD setting. We analysed pDC together with the secretion of intracellular cytokines in three conditions of stimulation through TLR receptors. At the same time we evaluated only the number of Th1 and Th17 cells by staining the PBMC for eight specific cell surface markers; CD3, CD4, CD8, CCR6, CCR10, CD161 and CXCR3.

Th17 cells have been found to express the mucosal chemokine receptor CCR6 but not CCR10.[203,204] However, we had to exclude CCR10 antibody conjugated with PE because of

the problems with the compensation of fluorophores with the overlapping emission spectra (PE conjugated to CCR10 and FITC conjugated to CCR6). For this reason here we could not count the Th17 subpopulation by counting CCR6+CCR10-CD4+ T cells.

However, as CD161 is the hallmark of Th17 cells as well as CXCR3 of IFN gamma secreting cells[205], we were finally able to evaluate the absolute numbers of these two subpopulations of lymphocytes in the peripheral blood of our patients at day 100 after transplantation. (example or flow cytometry analysis in ***Figure 21.***) The isotype controls served as negative control and we used 3 healthy donors as positive controls.

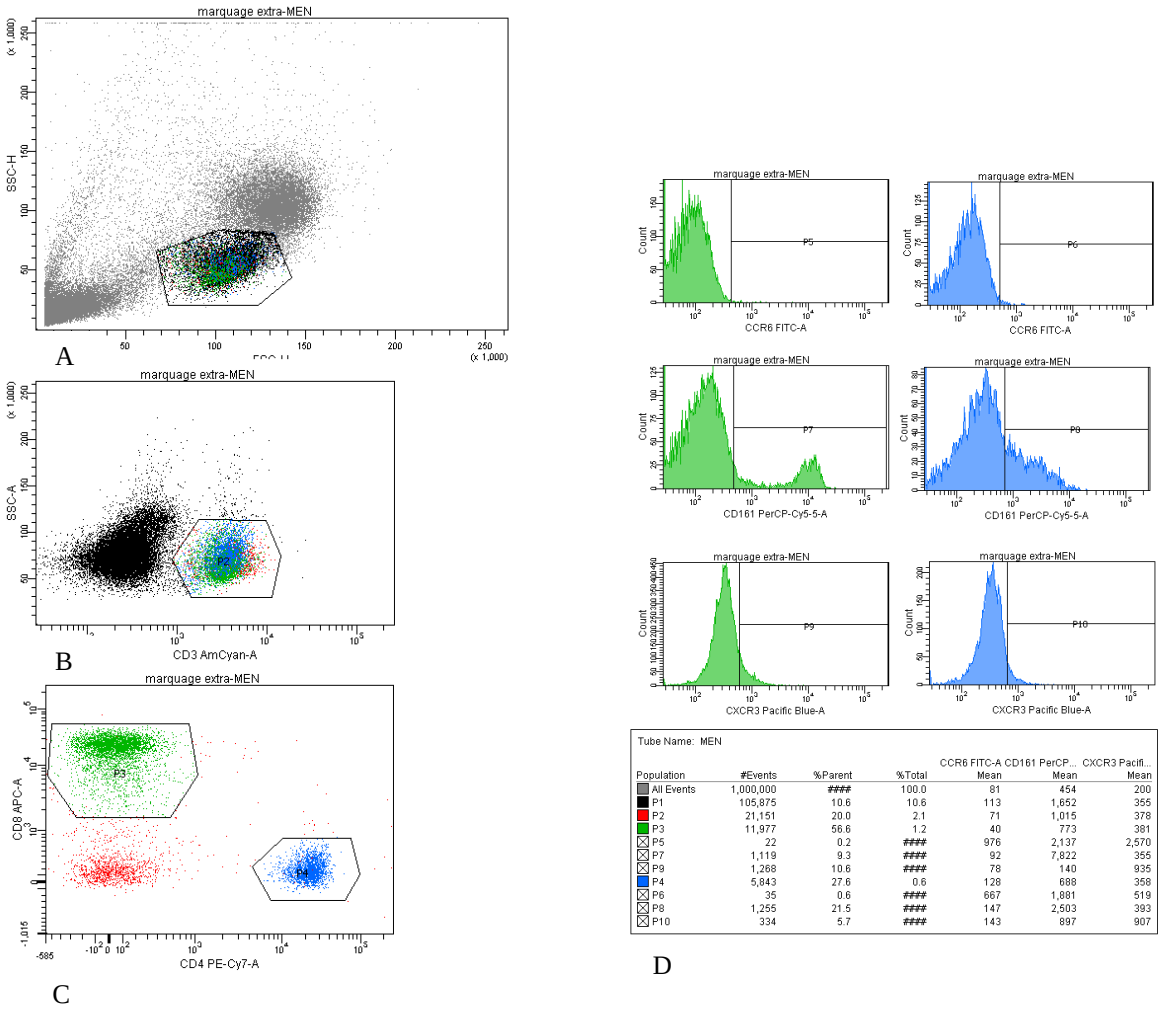

Figure 21. Flow cytometry analysis of Th17 and Th1 cells; Th cells were detected among mononuclear cells (A) as CD3+ (B) CD4+ (C) cells; CD3+CD4+cells and CD3+CD8+ cells were further evaluated for specific surface markers CCR6, CD161 and CXCR3 (D)

5.4.2 Th17 and acute GVHD

Similarly as in pDC, we observed a decrease of the percentages of both CD161+CD4+ T cells and CXCR3+CD4+ T cells at day 100 in patients who had already developed acute GVHD. (***Figure 22.***) This decrease became significant when the absolute number of these cells was correlated with acute GVHD severity. Patients who had developed grade II-IV acute GVHD

had significantly lower percentages of both CD161+ Th cells as well as CXCR3+ (IFN gamma producing) Th cells when compared to patients with no acute GVHD or with grade I acute GVHD. (p = 0.009 and p = 0.029, respectively in ***Figure 23.***)

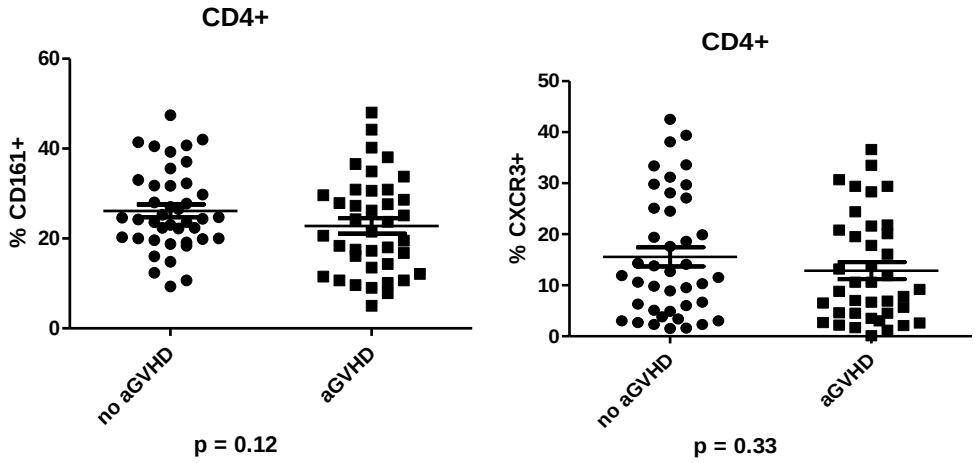

Figure 22. CD161+ Th cells and CXCR3+ Th cells in patients with and without acute GVHD, p = 0.12 and p = 0.33, respectively

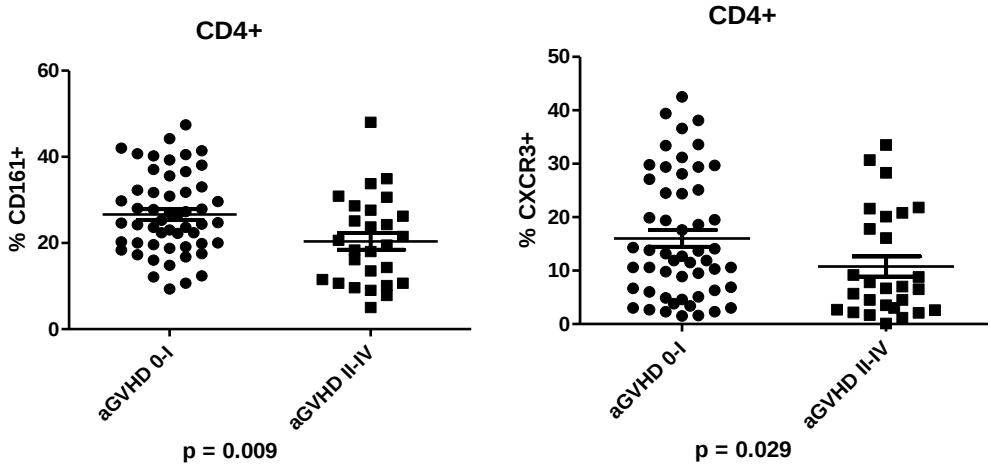

Figure 23. CD161+ Th cells and CXCR3+ Th cells in patients with grade 0-I acute GVHD compared to patients with grade II-IV acute GVHD, p = 0.009 and p = 0.029, respectively

5.4.3. Th17 and chronic GVHD

Median value of the Th17 cells at day 100 after allogeneic stem cell transplantation was 24% of PBMC. We used this value to allocate the patients into a low or a high Th 17 group in order to define a predictive value for the chronic GVHD development. Baseline demographics of these two groups were comparable and are shown in ***Table 6.***

Table 6. Baseline demographic characteristics of "low"and "high" Th group

Characteristic	**"low Th17" n (%) n=40**	**"high Th17" n (%) n=39**	**p-value**
Patient age, median (range)	54 (27-71)	54 (25-69)	0.94
Patient gender			
Male	19 (48)	21 (54)	0.50
Female	21 (53)	18 (46)	
Diagnosis			
Myeloid malignancy	20 (50)	22 (56)	
Lymphoid malignancy	18 (45)	17 (44)	
Aplastic anemia	2 (5)		0.66
Disease risk			
Standard risk	24 (60)	24 (62)	
High risk	16 (40)	15 (48)	0.98
Conditioning regimen			
Myeloablative	6 (15)	3 (8)	
Reduced-intensity conditioning	34 (85)	36 (92)	0.28
GVHD prophylaxis			
CsA alone	12 (30)	14 (36)	
CsA and MMF /MTX	28(70)	25 (64)	0.47
Donor gender			
Male	25 (63)	26 (67)	
Female	15 (37)	13 (33)	0.82
CMV serologic status, seronegative pair			
Yes	12 (30)	9 (23)	
No	28 (70)	30 (77)	0.45
Donor type			
Matched related donor	18 (45)	19 (49)	
Matched unrelated donor	12(30)	14 (36)	
Mismatched unrelated donor	10 (25)	6 (15)	0.51
Stem cell source			
Bone marrow	7 (18)	3 (8)	
Peripheral blood	25(63)	31 (79)	
Cord blood	8 (19)	5(13)	0.21

CMV= cytomegalovirus, CsA=cyclosporine A, MMF=mycophenolate mofetil, MTX=methotrexate

As shown before, the cumulative incidence of chronic extensive GVHD was 37% (95%CI 25-49%) at 20 months in the whole study group. When we separated our patients into a "low Th17" and "high Th17" group we found significantly higher incidence of chronic extensive GVHD in the "low Th17" group (53%, 95%CI 33-70%) than in the "high Th17" group (18%, 95% CI 7-34%) with a p-value 0.01 (***Figure 24***.)

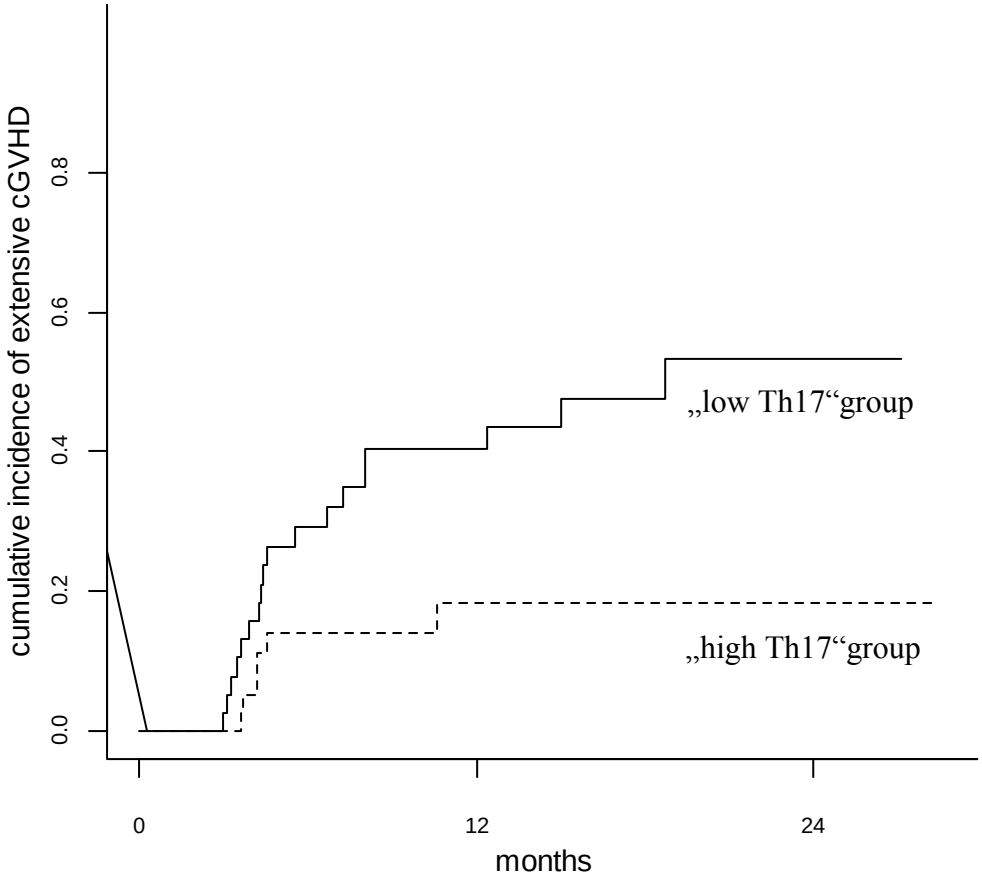

Figure 24. Cumulative incidence of extensive chronic GVHD in the "low Th17" group of (53%, 95%CI 33-70%) at 20 months and in the "high Th17" group of (18%, 95% CI 7-34%), p=0.01

5.4.4. Univariate analysis of predictive factors for extensive chronic GVHD

A cumulative incidence method was used in the univariate analysis of risk factors for the development of extensive chronic GVHD. Low count of Th17 in blood of patients at day 100 after allogeneic stem cell transplantation and development of clinically severe grade II-IV acute GVHD before day 100 (***Figure 24. and Figure 25.***) were associated with a significantly higher cumulative incidence of extensive chronic GVHD (p=0.01 and p=0.02, respectively). Female donor was also associated with a higher cumulative incidence of extensive chronic GVHD (p=0.13, ***Figure 26.***)

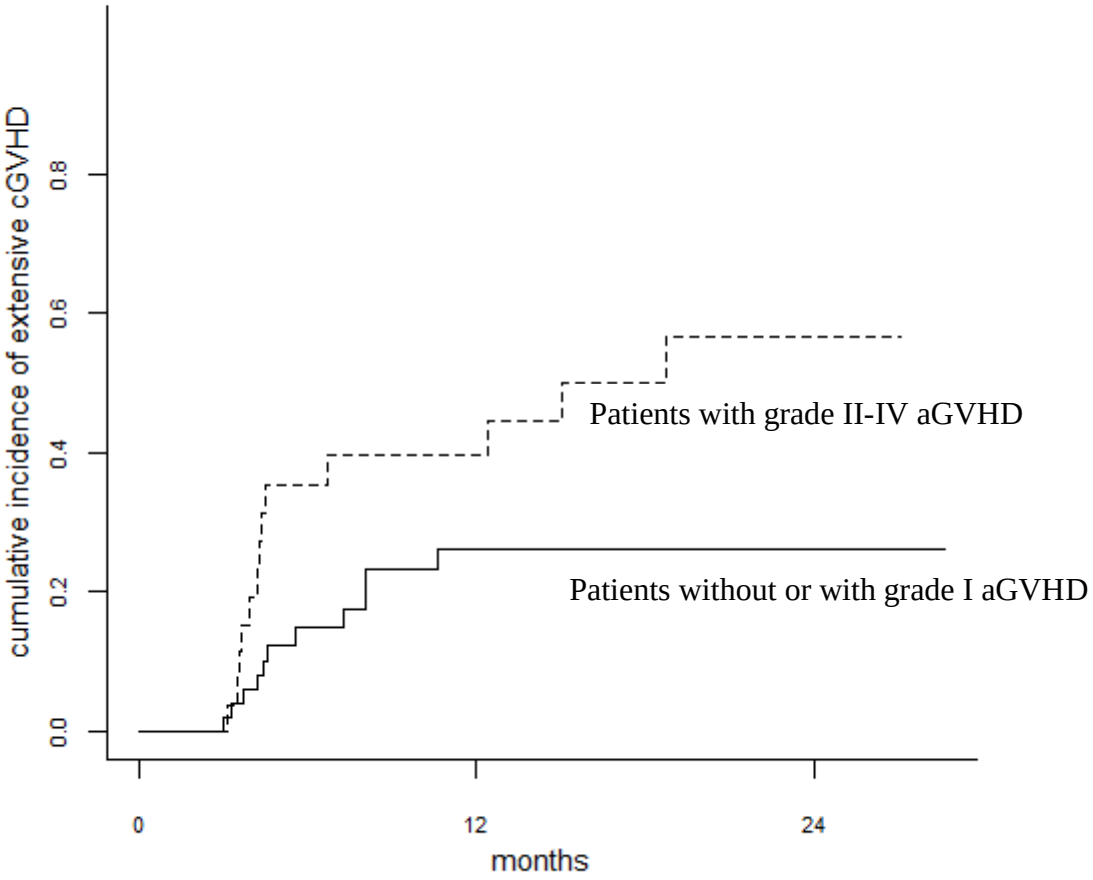

Figure 25. Cumulative incidence of extensive chronic GVHD in the group of patients with clinically severe aGVHD of 57% (95%CI 32-76%) at 20 months and in the group of patients without aGVHD of 26% (95%CI 14-40%), p=0.02

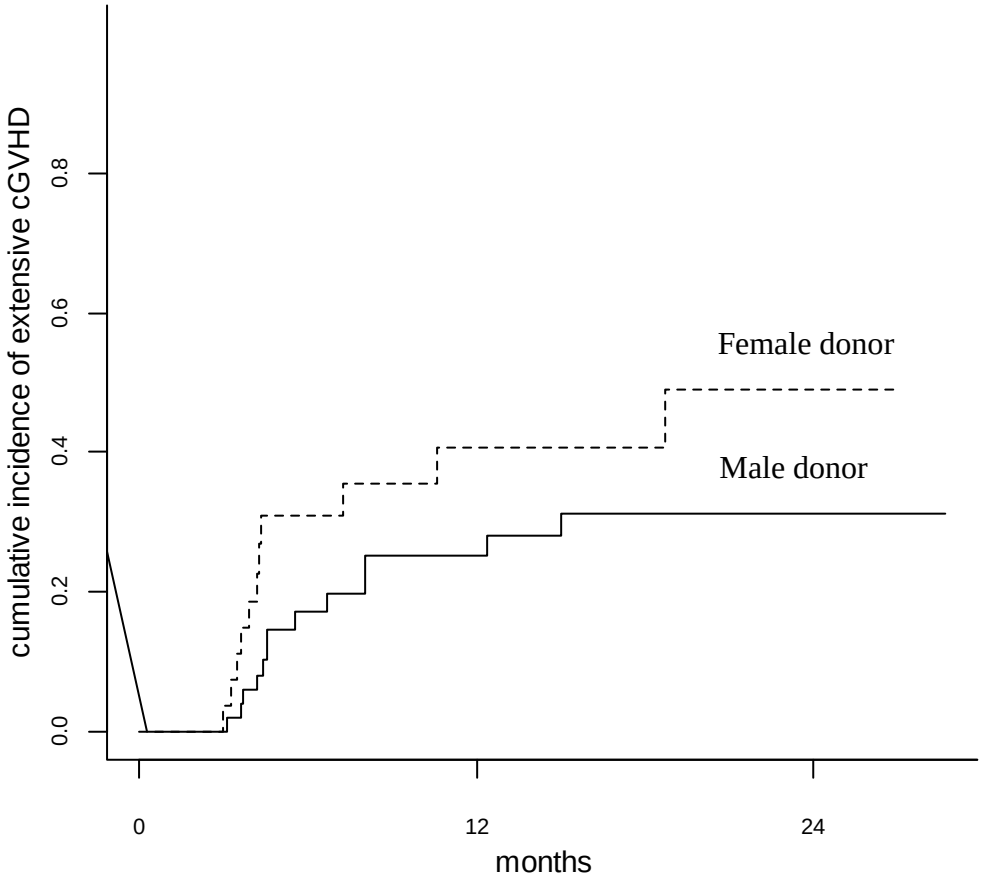

Figure 26. Cumulative incidence of extensive chronic GVHD in the group of patients with female donor of 49% (95%CI 24-70%) at 20 months and in the group of patients with male donor of 31% (95%CI 17-46%), p=0.13

5.4.5. Multivariate analysis of predictive factors for extensive chronic GVHD

All variables with a p-value < 0.20 in the univariate analysis (the presence of grade II-IV acute GVHD, „low“ Th17 count and female sex of the donor) together with previously known predictive factors of chronic GVHD (unrelated donor, older age of recipient, peripheral blood as stem cell source and active disease) were included in the multivariate analysis in order to define predictive factors for the development of extensive chronic GVHD.

In this analysis ***(Table 7.)*** only the low Th17 count stayed an independent predictor of development of extensive chronic GVHD. (RR 3.318, 95% CI 1.234-8.92, p = 0.017)

Table 7. Multivariate analysis of predictors of risk factors of extensive chronic GVHD

Risk factor	Relative risk	Confidence interval	p
"low Th17" count	3.318	1.234-8.92	**0.017**
female donor	2.180	0.917-5.19	0.078
grade II-IV acute GVHD	1.992	0.863-4.60	0.110
peripheral blood stem cells	1.689	0.650-4.39	0.280
active disease	0.773	0.304-1.97	0.590
unrelated donor	1.159	0.511-2.63	0.720
"older" age of recipient	1.070	0.396-2.89	0.890

5.5. Inflammatory cytokines in the serum of patients at day 100 after allogeneic stem cell transplantation

Using a Luminex Xmap system from one serum of each patient taken at day 100 after allogeneic stem cell transplantation we evaluated the concentrations of 41 cytokine: IL1a, IL1b, IL1ra, IL2, IL2ra, IL4, IL5, IL6, IL7, IL9, IL10, IL12p40, IL12p70, IL13, IL15, IL17, IL21, IL23, IL8 (CXCL8), IP10 (CXCL10), MCP1 (CCL2), MIP1a (CCL3), MIP1b (CCL4), RANTES (CCL5), MCP3 (CCL7), TARC (CCL17), MDC (CCL22), FRACTALKINE (CX3CR1), IL3 (multi-CSF), VEGF, PDGFABBB, TNFalpha, TNFbeta, TRAIL, CD40L, BAFF, IFNa2, IFN gamma, ELAFIN, TGF alpha, FLT.3L. In this analysis we included serums from 152 patients taken at day 100 after allogeneic stem cell transplantation and evaluated the relation of concentrations of cytokines to the development and severity of chronic GVHD.

5.5.1. Patient characteristics

This time study population included 79 patients already characterized plus additional 73 patients who received allogeneic stem transplantation between years 2005 and 2008. Finally, the study population was similar, with 83 male patients (55 %) and 69 female patients (45%), and the median age of 49 years (range, 17-71 years). Donors were matched related in 70 cases (46%) and unrelated in 82 patients (54%). Source of stem cells was peripheral blood in 108 cases (72%). Conditioning regimen was myeloablative in 48 transplantations (32%) and reduced-intensity in 104 (68%) transplantations. 77 patients (51%) had previously developed acute GVHD.

With the median follow up of 16 months, 70 patients (46%) developed chronic GVHD after a median of 6 months. This led to a cumulative incidence of chronic GVHD of 52% at 2 years and cumulative incidence of extensive chronic GVHD of 38% at 2 years. 46 patients (30%) developed limited chronic GVHD and 24 patients (16%) developed extensive chronic GVHD.

By making histogrammes of cytokine levels at day 100 after allogeneic stem cell transplantation in our patients, we saw a degenerated distribution of eight cytokines (IL1b, IL1ra, IL5, IL12p70, IL13, IL21, IL23 and TNF beta) that made us exclude these cytokines from further analysis. The caracteristics of the resting cytokines are presented in the ***Table 8.***

5.5.2. Significant cytokines at day 100 for the development of chronic GVHD

In univariate analysis (using a cumulative incidence method) we analized 33 cytokines and found 22 cytokines to be significative with a $p < 0.20$. (***Table 8****.)*

Univariate analysis for risk factors associated with chronic GVHD is shown in ***Table 9***. Eight factors were evidently different between subgroups with and without chronic GVHD ($p < 0.20$), but four risk factors emerged as clinically significant- sex and age of the recipient, number of CD34+ cells infused and previous acute GVHD. Male patients had 1.5 times higher chance of chronic GVHD ($p = 0.083$), patients older than 35 years had 2.2 times higher chance of chronic GVHD than younger patients ($p = 0.065$), patients who received more than 8.8×10^6 CD34+ cells had 1.9 times higher risk of chronic GVHD ($p = 0.019$), and the patients who have had acute GVHD previously, had a 1.9 times higher chance of chronic GVHD. ($p = 0.012$). A multivariate analysis was done next to define the cytokines related to the development of chronic GVHD independently of risk factors for chronic GVHD. (***Table 10.***) Finally, we defined 8 cytokines related to the development of chronic GVHD; IL10,

IL15, IP10, TNF alpha and FLT.3L seemed to increase the chance of chronic GVHD, while TARC, MDC and FRACTALKINE seemed to decrease the chance of chronic GVHD.

Table 8. Univariate analysis of 33 cytokines related to chronic GVHD

Cytokines	N	N_0	N_1	average.+	e.t.+	min+	max+	threshold	HR	p-value
IP10	152	93	59	2499,48	4101,53	2,92	24244,34	1448,42	3,09	<0,0001
FLT.3L	152	84	68	94,78	115,23	4,02	903,54	70,29	2,43	0,0002
IL15	152	109	43	3,61	7,70	1,67	90,14	2,61	2,42	0,0004
MDC	152	40	112	1431,89	1723,47	49,34	10053,35	504,97	0,41	0,0004
IL3	152	91	61	10,33	13,34	1,45	75,88	7,25	2,22	0,0007
IL10	152	48	104	14,39	38,73	2,08	433,61	2,08	2,57	0,0015
TARC	152	32	120	1236,32	1953,70	4,97	5144,85	60,56	0,49	0,0054
RANTES	151	74	77	41311,25	24703,62	1458,00	93825,00	41100,00	0,51	0,0056
IL2ra	152	51	101	506,22	938,44	7,72	8522,69	141,10	2,05	0,0089
FRACT-ALKINE	152	67	85	65,17	229,67	6,79	2400,55	12,91	0,60	0,0315
IL12p40	152	119	33	26,30	64,83	2,51	739,95	37,61	0,51	0,0456
MIP1a	152	88	64	26,54	20,35	3,18	97,53	24,68	0,61	0,0471
TNFa	152	97	55	12,01	7,65	0,76	49,25	12,70	1,60	0,0493
TRAIL	152	77	75	23,16	25,22	4,85	120,46	12,15	0,66	0,0817
PDGF-ABBB	152	25	127	8235,88	3073,73	1,05	9847,81	4122,41	0,59	0,0830
IL6	152	92	60	8,07	14,51	0,89	102,30	3,59	1,50	0,0872
IFN-gamma	152	97	55	18,80	30,68	2,12	240,54	11,26	0,64	0,0944
MCP1	152	75	77	758,42	857,74	2,27	4540,87	468,00	1,49	0,0988
ELAFIN	151	91	60	10028,51	8492,81	870,00	53650,00	8917,00	1,44	0,1274
MIP1b	152	133	19	65,95	48,73	2,21	462,45	108,29	0,50	0,1325
IL1a	152	118	34	15,39	45,29	3,76	362,06	4,00	0,65	0,1736
TGF-alpha	152	129	23	8,34	9,60	0,85	88,56	12,29	1,48	0,1960
IL17	152	90	62	5,66	15,40	0,60	120,19	0,60	0,73	0,2163
IL8	151	125	26	40,37	34,54	0,42	260,39	57,78	1,41	0,2634
VEGF	152	109	43	153,83	208,29	10,57	1996,39	165,97	1,32	0,2754
CD40L	152	16	136	8806,31	2850,88	2,95	10009,50	2417,11	1,66	0,3223
IL2	152	136	16	1,88	7,08	0,54	77,47	1,75	1,44	0,3320
BAFF	151	89	62	3566,80	2194,90	379,00	11960,00	3739,00	0,79	0,3467
IFN-alpha2	152	126	26	8,99	12,11	4,15	78,01	12,33	1,33	0,3495
MCP3	152	111	41	9,79	16,55	5,67	167,97	5,67	0,80	0,4419
IL7	152	134	18	2,59	3,59	1,34	26,54	4,42	1,26	0,5143
IL9	152	128	24	3,42	4,33	2,31	39,53	2,79	1,22	0,5514
IL4	152	136	16	13,55	31,94	5,96	250,42	15,98	0,80	0,5804

Table 9. Univariate analysis of risk factors for chronic GVHD

Risk factor	HR	p-value
Acute GVHD (Yes vs. No)	1,9	0,012
Number of CD34 (More than 8,8 vs.Less)	1,9	0,019
Age of recipient (More than 35 years vs.Younger)	2,2	0,065
Sex of the recipient	1,5	0,083
CMV serology of recipient (Positives vs. Negatives)	1,5	0,109
Type of the donor (Siblings vs. Others)	0,7	0,148
CMV serology of donor (Positives vs. Negatives)	0,7	0,161
Source of the transplant ("PB infused" vs. Other)	1,4	0,195
Type of disease (Myeloid vs.Other)	0,8	0,391
GVHD prophylaxis (Dual vs.Other)	1,1	0,573
Sex of the donor (Males vs. Females)	1,1	0,641
Disease status (CR vs. Other)	0,9	0,659
Type of conditioning (Myeloablatives vs. RIC)	0,9	0,7

Table 10. Multivariate analysis of cytokines to the development of chronic GVHD, indepedent of risk factors

Cytokines	Threshold	N	HRa_	Pvalue (HRa)	p-value (pH+)
IP10	1448,42	151	3,13	<0,0001	0,8987
FLT.3L	70,29	151	2,11	0,0032	0,9391
FRACTALKINE	12,91	151	0,54	0,0125	0,7806
IL15	2,61	151	2,00	0,0144	0,7319
MDC	504,97	151	0,54	0,0192	0,8081
TNFalpha	12,70	151	1,76	0,0200	0,9422
IL10	2,08	151	2,03	0,0329	0,3948
TARC	60,56	151	0,58	0,0438	0,2459
IL3	7,25	151	1,63	0,0607	0,6348
PDGFABBB	4122,41	151	0,56	0,0707	0,1800
RANTES	41100,00	150	0,64	0,0926	0,8223
IL12p40	37,61	151	0,57	0,1141	0,9133
IL1a	4,00	151	0,60	0,1141	0,8867
IL2ra	141,10	151	1,59	0,1142	0,3548
MCP1	468,00	151	1,44	0,1352	0,2166
TGFa	12,29	151	1,58	0,1480	0,0116
MIP1a	24,68	151	0,70	0,1585	0,8417
IFNgamma	11,26	151	0,69	0,1691	0,9626
TRAIL	12,15	151	0,72	0,1741	0,3059
IL6	3,59	151	1,39	0,1799	0,4129
MIP1b	108,29	151	0,59	0,2630	0,7228
ELAFIN	8917,00	150	1,18	0,5343	0,6741

5.5.3. Significant cytokines at day 100 for the development of extensive chronic GVHD

In this analysis we included only the patients who developed extensive chronic GVHD, more precisely- the patients who developed limited chronic GVHD were included in the no GVHD group. In the univariate analysis (using a cumulative incidence method) we found 25 cytokines to be significative with a $p < 0.20$. (***Table 11.***) Univariate analysis for risk factors associated with extensive chronic GVHD defined six significant risk factors with a $p < 0.20$, and previous acute GVHD, source of the transplant and number of CD34+ infused cells emerged as clinically significant. (***Table 12.***) Finally, a multivariate analysis was done to define the cytokines related to the development of extensive chronic GVHD but indepedently of the three clinical risk factors defined for the extensive chronic GVHD. In this analysis 10 cytokines emerged as significant; IL2, IL2ra, IL10, IP10, MIP1b seemed to increase the chance of extensive chronic GVHD, while IL12p40, RANTES, TARC, MDC, FRACTALKINE seemed to decrease the chance of extensive chronic GVHD. (***Table 13****.)*

Table 11. Univariate analysis of 33 cytokines related to extensive chronic GVHD

Cytokines	N	N_0	N_1	average.+	e.t.+	min+	max+	threshold	HR	p-value
MDC	152	40	112	1431,89	1723,47	49,34	10053,35	504,97	0,29	<0,0001
IL10	152	101	51	14,39	38,73	2,08	433,61	8,97	2,94	0,0001
IP10	152	84	68	2499,48	4101,53	2,92	24244,34	1166,34	2,97	0,0002
IL15	152	131	21	3,61	7,70	1,67	90,14	5,38	2,98	0,0010
TARC	152	81	71	1236,32	1953,70	4,97	5144,85	244,40	0,39	0,0025
FLT.3L	152	84	68	94,78	115,23	4,02	903,54	70,29	2,30	0,0038
IL3	152	92	60	10,33	13,34	1,45	75,88	7,64	2,28	0,0040
RANTES	151	74	77	41311,25	24703,62	1458,00	93825,00	41100,00	0,42	0,0045
IL12p40	152	85	67	26,30	64,83	2,51	739,95	13,32	0,41	0,0080
FRACTA-LKINE	152	63	89	65,17	229,67	6,79	2400,55	11,25	0,50	0,0151
IFNgamma	152	46	106	18,80	30,68	2,12	240,54	3,43	0,51	0,0207
IL2ra	152	71	81	506,22	938,44	7,72	8522,69	231,04	1,99	0,0217
TRAIL	152	130	22	23,16	25,22	4,85	120,46	50,09	1,93	0,0512
MIP1b	152	77	75	65,95	48,73	2,21	462,45	57,06	1,72	0,0652
BAFF	151	91	60	3566,80	2194,90	379,00	11960,00	3804,00	0,55	0,0663
TGFa	152	22	130	8,34	9,60	0,85	88,56	1,69	0,53	0,0725
MCP1	152	18	134	758,42	857,74	2,27	4540,87	253,63	0,52	0,0887
IL8	151	82	69	40,37	34,54	0,42	260,39	34,61	1,62	0,1008
IL2	152	136	16	1,88	7,08	0,54	77,47	1,75	1,92	0,1073
PDGFABBB	152	25	127	8235,88	3073,73	1,05	9847,81	4122,41	0,57	0,1259
MIP1a	152	88	64	26,54	20,35	3,18	97,53	24,68	0,64	0,1485
ELAFIN	151	109	42	10028,51	8492,81	870,00	53650,00	10667,00	1,53	0,1589
IL6	152	93	59	8,07	14,51	0,89	102,3	3,68	1,48	0,1823
IL9	152	136	16	3,42	4,33	2,31	39,53	4,13	0,40	0,1929
TNFalpha	152	136	16	12,01	7,65	0,76	49,25	19,97	0,40	0,1948
VEGF	152	109	43	153,83	208,29	10,57	1996,39	165,97	1,44	0,2242
MCP3	152	133	19	9,79	16,55	5,67	167,97	10,87	1,53	0,2957
IL17	152	90	62	5,66	15,40	0,60	120,19	0,60	0,75	0,3435
IL7	152	117	35	2,59	3,59	1,34	26,54	1,34	0,70	0,3519
IFNa2	152	108	44	8,99	12,11	4,15	78,01	4,34	1,29	0,4111
CD40L	152	24	128	8806,31	2850,88	2,95	10009,50	7043,93	0,77	0,5075
IL1a	152	135	17	15,39	45,29	3,76	362,06	18,71	0,72	0,5348
IL4	152	130	22	13,55	31,94	5,96	250,42	8,67	1,18	0,6748

Table 12. Univariate analysis of risk factors for extensive chronic GVHD

Risk factor	HR	p-value
Acute GVHD (Yes vs No)	2,3	0,009
Source of the transplant ("PB infused" vs. Other)	2,6	0,019
Number of CD34 (More than 0,4 vs. Less)	7	0,054
Age of recipient (More than 35 years vs.Younger)	2,2	0,127
Sex of the recipient	1,5	0,15
Type of the donor (Siblings vs. Others)	1,1	0,171
CMV serology of recipient (Positives vs. Negatives)	1,4	0,218
Type of disease (Myeloid vs.Other)	0,8	0,437
Disease status (CR vs Other)	0,8	0,48
Type of conditioning (Myeloablatives vs. RIC)	1,1	0,769
CMV serology of donor (Positives vs.Negatives)	1	0,87
GVHD prophylaxis (Dual vs.Other)	1	0,921
Sex of the donor (Males vs. Females)	1	0,942

Table 13. Multivariate analysis of cytokines to the development of extensive chronic GVHD, indepedent of risk factors

Cytokines	Threeshold	N	HRa_	p-value (HRa)	p-value (pH+)
IP10	1166,34	151	3,00	0,0003	0,5906
MDC	504,97	151	0,43	0,0103	0,7040
FRACTALKINE	11,25	151	0,47	0,0110	0,9492
IL10	8,97	151	2,09	0,0193	0,4118
IL2ra	231,04	151	2,04	0,0212	0,1836
RANTES	41100,00	150	0,47	0,0214	0,5811
MIP1b	57,06	151	1,90	0,0315	0,5459
TARC	244,40	151	0,51	0,0389	0,8166
IL12p40	13,32	151	0,48	0,0417	0,1160
IL2	1,75	151	2,32	0,0478	0,7293
IFNgamma	3,43	151	0,57	0,0597	0,0389
FLT.3L	70,29	151	1,76	0,0701	0,6670
TGFa	1,69	151	0,53	0,0821	0,1964
IL15	5,38	151	1,87	0,0923	0,3436
BAFF	3804,00	150	0,61	0,131	0,5073
IL3	7,64	151	1,60	0,1311	0,6893
PDGFABBB	4122,41	151	0,57	0,1327	0,0643
MCP1	253,63	151	0,57	0,1467	0,9683
IL6	3,68	151	1,41	0,2499	0,9248
ELAFIN	10667,00	150	1,40	0,2801	0,8500
TRAIL	50,09	151	1,44	0,3020	0,6001
IL9	4,13	151	0,49	0,3233	0,5347
IL8	34,61	150	1,34	0,3374	0,3649
TNFalpha	19,97	151	0,57	0,4625	0,4620
MIP1a	24,68	151	0,82	0,5266	0,3550

5.5.4. A prognostic score for the development of chronic GVHD

Using the multivariate Cox model combined with the statistical approach called "time-dependent ROC curves" we tried to establish a practical prognostic score for the development of chronic GVHD. All the parameters that were found to be significant in the univariate analysis ($p<0.20$) were included in the calculation of the score and the score was finally composed of 30 parameters – eight clinical factors and 22 cytokines. (***Table 14.***) For every patient the score was calculated as a sum of coefficients (logarithms of relative risks) for every risk factor multiplied with 1 if the parameter was equal or higher than the threshold or with 0 if not. Accordingly, when the coefficient was positive, the score and the risk of chronic GVHD augmented when the factor was 1. In the contrary, if the coefficient was negative, the score and the risk of chronic GVHD decreased when the factor was 1. Further on, we evaluated this scoring system and its predictive value by using the ROC curves, shown in the ***Figure 27***.

The first histogram shows the problem of overfitting and overestimation of the predictive power if there are no corrections (AUC=0.96, 95%CI, 0.93-0.99). On the contrary, cross-validation by bootstrap underestimates the prognostic power (AUC=0.77, 95% CI, 0.63-0.89). The last graph is based on 0,632 bootstrap resampling method for repeated cross-validation, and is the most interesting with the AUC 0.80 (95%CI, 0.70-0.89).

Table 14. Prognostic score for the development of chronic GVHD

Parametres	Coef.	CI95%inf.	CI95%sup.	Value
IP10	2,23916112	0,79759320	4,23724068	1 if IP10>1448,42
FLT.3L	0,40374927	-1,53750513	2,05893911	1 if FLT.3L>70,29
IL15	1,18686042	-0,74788538	3,57690213	1 if IL15>2,61
MDC	-1,87466497	-4,15841103	0,02710002	1 if MDC>504,97
IL3	0,83257559	-0,77387295	2,62119197	1 if IL3>7,25
IL10	-0,61229720	-2,66460604	1,09402341	1 if IL10>2,08
TARC	1,16168225	-0,60674783	3,42480191	1 if TARC>60,56
RANTES	-0,45332960	-2,06756804	0,97900221	1 if RANTES>41100,00
IL2ra	-0,37738579	-2,12602193	1,39214229	1 if IL2ra>141,10
FRACTALKINE	-0,70487796	-2,26464985	0,70393524	1 if FRACTALKINE>12,91
IL12p40	-2,21646362	-4,37689538	-0,64984374	1 if IL12p40>37,61
MIP1a	-0,81412769	-2,54714373	0,58623723	1 if MIP1a>24,68
TNFalpha	1,29784277	-0,21616966	3,61206427	1 if TNFa>12,70
TRAIL	-0,86428069	-2,19104771	0,40640364	1 if TRAIL>12,15
IL6	0,86064206	-0,70139761	2,48980162	1 if PDGFABBB>4122,41
IFNgamma	-0,72052015	-2,43529138	0,81057035	1 if IL6>3,59
MCP1	1,18352587	-0,02360060	2,62437113	1 if IFNgamma>11,26
ELAFIN	-0,42643423	-2,24982683	1,15489178	1 if MCP1>468,00
MIP1b	-1,51953280	-3,59925344	0,30048601	1 if MIP1b>108,29
IL1a	-1,41822684	-3,15136537	0,12025628	1 if IL1a>4,00
TGFa	0,96051419	-0,94231936	2,70213910	1 if TGFa>12,29
Sex of Recipient	1,09819942	-0,31457606	2,80232758	1 if recipient is male
Type of Donor	0,07893726	-1,11936261	1,42853186	1 if donor is identical sibling
CMV Donor	-0,78967092	-2,26220464	0,48160984	1 if recipient is CMV positive
CMV Recipient	0,78343149	-0,60365179	2,27149181	1 if donor is CMV positive
Source of Cells	0,27156417	-1,72166762	2,09964677	1 if PB infused
Acute GVHD	-0,16325185	-1,83509242	1,33543387	1 if acute GVHD before
Age of Recipient	0,37478191	-1,70664429	2,79312051	1 if age of recipient>35,00
Number of CD34	0,39777467	-1,14524435	2,06809033	1 if CD34>8,80

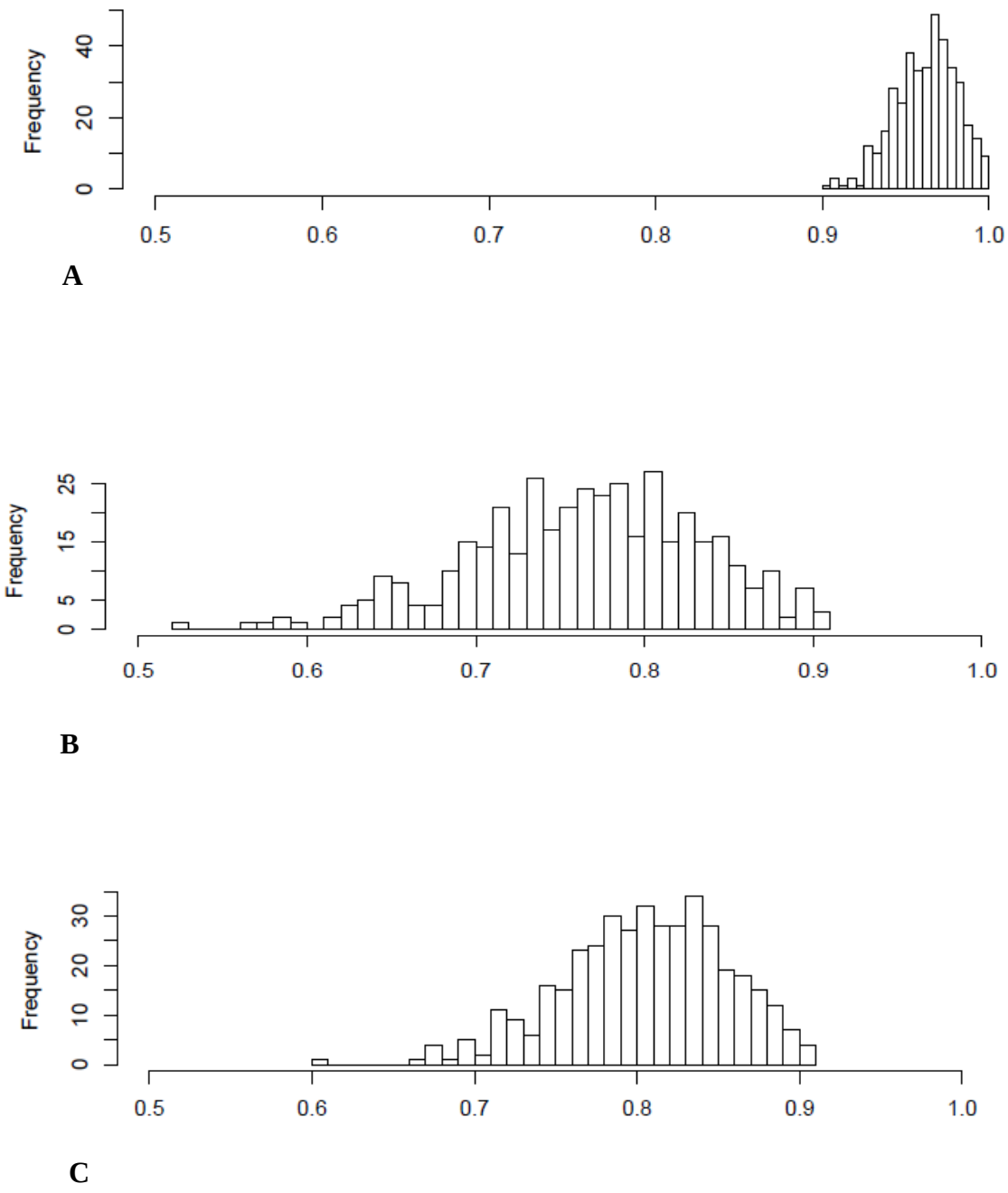

Figure 27. Prognostic score for the development of chronic GVHD at 2 years after allogeneic stem cell transplantation; predictive power of 96% (95%CI 93-99%) if there are no corrections (A); predictive power of 77% (95% CI, 63-89%) estimated with cross-validation by bootstrap (B); predictive power of 80% (95%CI, 70-89%) with the use of 0,632 bootstrap resampling method for repeated cross-validation (C)

5.5.5. A prognostic score for the development of extensive chronic GVHD

Using the same tests we tried to establish a similar prognostic score for the development of extensive chronic GVHD. All the parameters significant in the univariate analysis ($p<0.20$) (***Table 15.***) were included in the calculation of the score (31 parameter – six clinical variables and twenty five cytokines) and the ROC curves are shown in the ***Figure 28***. The first histogram shows the problem of overfitting which is even more evident and overestimates the predictive power to 98% (AUC=0.98, 95%CI, 0.96-1.0). However, using the 0,632 bootstrap resampling method for repeated cross-validation, the AUC is 0.78 (95%CI, 0.66-0.88) and shows the power of the test in reality among the comparable but independent individuals.

Table 15. The prognostic score for extensive chronic GVHD development

Parametres	Coef.	CI95%inf.	CI95%sup.	Value
MDC	0,26367659	-4,8337435	5,5303094	1 if MDC>504,97
IL10	-0,19731159	-4,8977038	3,8524482	1 if IL10>8,97
IP10	2,43238655	-1,3497585	7,9599239	1 if IP10>1166,34
IL15	2,43678023	-2,0233360	7,9312229	1 if IL15>5,38
TARC	-0,52852715	-4,7447792	3,7705134	1 if TARC>244,40
FLT.3L	0,34026638	-3,8500076	5,8886953	1 if FLT.3L>70,29
IL3	0,84907886	-2,5964658	4,6315737	1 if IL3>7,64
RANTES	-1,15303387	-6,7333993	1,7969284	1 if RANTES>41100,00
IL12p40	-2,33306453	-9,0162553	1,0591098	1 if IL12p40>13,32
FRACTALKINE	-2,14486388	-6,8269798	0,5762474	1 if FRACTALKINE>11,25
IFNgamma	-1,23429345	-6,8607339	1,9398523	1 if IFNgamma>3,43
IL2ra	0,80002705	-4,2925700	5,7382398	1 if IL2ra>231,04
TRAIL	0,82921537	-3,7890205	6,4874130	1 if TRAIL>50,09
MIP1b	2,97522835	-0,3396939	12,0788208	1 if MIP1b>57,06
BAFF	-2,48149497	-9,5570088	1,1197671	1 if BAFF>3804,00
TGFalpha	-0,15994565	-5,2852718	5,4871899	1 if TGFalpha>1,69
MCP1	-1,22616057	-7,6681998	5,2281949	1 if MCP1>253,63
IL8	-1,41458658	-7,9819421	2,3840474	1 if IL8>34,61
IL2	0,53327738	-4,0063708	5,2598009	1 if IL2>1,75
PDGFABBB	0,17681959	-6,8965569	6,8507727	1 if PDGFABBB>4122,41
MIP1a	0,04088765	-3,6220374	3,7556950	1 if MIP1a>24,68
ELAFIN	1,94733210	-1,1841098	6,5251781	1 if ELAFIN>10667,00
IL6	1,49156156	-2,3507186	6,8333935	1 if IL6>3,68
IL9	-4,07205383	-22,0987445	5,1544495	1 if IL9>4,13
TNFalpha	-1,13177124	-21,1773142	7,9925181	1 if TNFalpha>19,97
Sex of Recipient	0,21588724	-3,5736196	3,7790536	1 if recipient male
Type of Donor	1,23695302	-1,8167660	4,9934955	1 if Identical sibling
Source of cells	3,32039370	-1,2149629	11,8415079	1 if PB infused
aGVHD	0,95830143	-3,4919826	4,9309722	1 if acute GVHD before
Age of Recipient	0,72704734	-4,9769957	9,3507852	1 if Age recipient>35,00
Number of CD34	20,83204323	13,5454600	33,7111661	1 if CD34>0,40

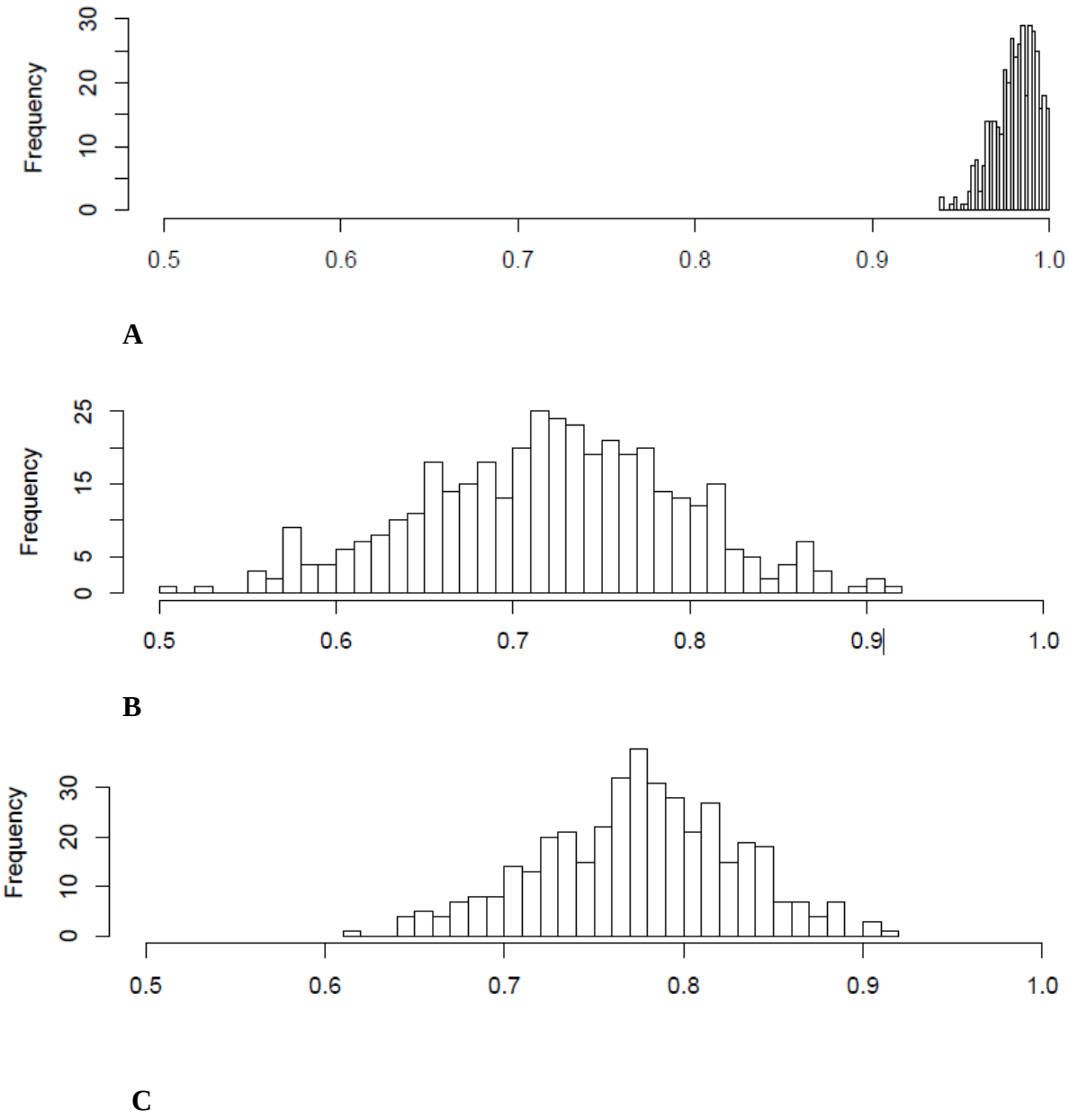

Figure 28. Prognostic score for the development of extensive chronic GVHD at 2 years after allogeneic stem cell transplantation; predictive power of 98% (95%CI 96-100%) if there are no corrections (A); predictive power of 72% (95% CI, 55-88%) estimated with cross-validation by bootstrap (B); predictive power of 78% (95%CI, 66-88%) with the use of 0,632 bootstrap resampling method for repeated cross-validation (C)

6. Discussion

6.1. The pDC recovery at day 100 in peripheral blood of transplanted patients is impaired by the development of clinically significant acute GVHD

It has been previously shown that both mDC and pDC recover quickly in the peripheral blood of patients after allogeneic stem cell transplantation and represent bone marrow recent emigrants since they reach their highest proportions among white blood cells in the earliest phase of immune reconstitution.[114] However, patients recover to the pretransplant mDC and pDC levels within day +60 after allogeneic stem cell transplantation but do not reach a normal absolute number in the blood until one year after allogeneic stem cell transplantation.[120] The release kinetics of DC is found to be more rapid in patients treated with G-CSF, but their count decreases gradually after G-CSF withdrawal and reaches the values similar to patients untreated with G-CSF. Furthermore, the source of stem cells does not seem to influence mDC recovery either but the level of pDC is significantly decreased in the blood of patients who receive bone marrow instead of peripheral blood stem cells. Moreover, DC recovery may be faster after RIC as compared to myeloablative preparatory regimens.[206] Finally, patients with acute GVHD present with a less rapid DC recovery on day +30 and on day +100 than patients without acute GVHD.

Our study evaluated pDC proportions in the peripheral blood of patients at day +100 after allogeneic stem cell transplantation and analyzed all relevant clinical parameters related to this counts. The recovery of pDC in our study was not influenced by the patients' or graft characteristics, conditioning regimen or infections, as it has been shown before. On the contrary, in our group of patients, only the occurence of grade II-IV acute GVHD in the multivariate analysis was found to be significantly associated with an impaired pDC recovery. However, treatment with corticosteroids has been shown to efficiently deplete pDC from the blood of transplant recipients[207] as well as healthy donors.[197] Since the glucocorticoids represent the standard treatment of grade II-IV acute GVHD in our institution, it is possible that the drop in the pDC level at day +100 in our group of patients was at least in part due to the corticosteroids and not only to acute GVHD itself. It has been shown before[114] that number of pDC even increases in the blood during the early phase of acute GVHD and consistently and significantly decreases soon after the beginning of therapy with steroids.

Our data would be in concordance with the finding that the reconstitution of blood pDC levels in patients after allogeneic stem cell transplantation can be delayed because of clinically significant acute GVHD and its treatment with corticoids.[198]

6.2. Corticosteroids have a deleterious impact on the function of pDC, but do not affect their viability

Together with the finding of a significant decrease of pDC count in patients with grade II-IV acute GVHD in comparison to patients without clinically significant acute GVHD, we also observed a significant decrease of functional pDC (IFN alpha and TNF alpha-secreting pDC) in patients with clinically significant grade II-IV acute GVHD when compared to patients with grade 0-I acute GVHD.

As mentioned before, all our patients with grade II-IV acute GVHD were treated with high doses of corticosteroids (2 mg/kg) at the time of GVHD ocurrence and, if an adequate response was observed, the dose was rapidly tappered down. Consequently, the lower pDC proportion in grade II-IV acute GVHD could partially be related to the imunosupressive treatment.

Therefore we reanalyzed our results by excluding the patients who were still receiving high doses of corticosteroids at day +100 after allogeneic stem cell trasplantation. This left out 21 patients from our study group for further analysis. In the new study group, we still saw a significant decrease of total pDC in patients with grade II-IV acute GVHD when compared to patients without clinically significant acute GVHD (grades 0-I). Therefore we presumed that the lower pDC proportions in the peripheral blood of our patients at day +100 were directly associated to the development of the acute GVHD, and not to the corticosteroid treatment itself.

However, in the new functional analysis of activated pDC, without the patients under the treatment of corticosteroids, we did not see a significant decrease of IFN alpha, TNF alpha and IL6-secreting pDC. This finding suggested that the resting pDC in the blood of patients were functionally less activated because of the corticosteroid treatment. This observation is in concordance with previous studies where corticosteroids seemed to prevent DC maturation and impair their immunostimulatory activities even though they did not affect DC viability.[199-202]

As it is known that residual host DC after conditioning regimen[107-109] are crucial in initiating acute GVHD, we could here hypothesize that administering corticosteroids to patients shortly

before the beginning of the conditioning regimen might selectively inactivate recipients' DC and thus reduce the risk of acute GVHD.

6.3. The reduced pDC number in peripheral blood of transplanted patients correlates with clinically significant acute GVHD

A possible mechanism underlying the decrease in the number of pDC in the peripheral blood at day +100 in patients with clinically significant acute GVHD might be the recruitment of pDC from the peripheral blood to the affected tissuses during the occurrence of acute GVHD. In accord with this assumption, in a previous study from our study group[208] we determined proportions of pDC in intestinal biopsies in patients with and without acute GVHD. This study showed a significant increase of CD123+ pDC in the intestinal mucosa of patients with acute GVHD compared with mucosa of patients without acute GVHD. Furthermore, the number of CD123+cells paralleled the histological grade of acute GVHD (the higher the grade, the higher was the number of pDC). The recruitment of pDC to inflammatory lesions of target tissues is not exclusively related to GVHD, but it has also been shown in several autoimmune diseases, as systemic lupus erythematosus.[85]

On the other hand, a tolerogenic potential of human pDC has already been suggested, and it has been proposed that pDC might be responsible for the induction and maintenance of self-tolerance. It has been shown that antigen-specific CD4 T-cell lines in humans become anergic when exposed to immature pDC previously pulsed with specific antigen.[209] CCR9+pDC were identified as tolerogenic pDC capable of inhibiting acute GVHD by inducing Tregs.[113] In this context, a reduced pDC number in the blood of our patients with clinically significant acute GVHD provides evidence for the concept that resting pDC in the blood are tolerogenic and have the potential to prevent GVHD. This is in concordance with the finding that our patients with severe acute GVHD had also lower proportions of IFN alpha and TNF alpha-secreting pDC than the patients without clinically significant acute GVHD.

In summary, we have demonstrated that pDC decrease in the peripheral blood of patients with severe acute GVHD. However, since we did not analyze the proportions of pDC in the GVHD target tissues, we cannot conclude whether activated pDC migrated to the acute GVHD organs and have a role in maintenance of acute GVHD or if reduced numbers of tolerogenic pDC in blood may correlate with enhanced donor T-cell responses thus promoting GVHD, as it has been proposed before.[210] Consequently, we conclude that the role of pDC in promoting or supressing GVHD should also be evaluated in the context of their activation status.

6.4. The reduced pDC number in peripheral blood of transplanted patients is an independent predictor of worse long-term outcome of patients

Given the central role of DC in the immune system, several previous studies have hypothesized that the recovery of DC after allogeneic stem cell transplantation could be related to long-term outcome of patients' relapse and survival. Reddy et al.[115] measured circulating DC in the peripheral blood at engraftment with a presumption that this represents the earliest period of donor immune reconstitution and found that low mDC count was independently associated with death, time to relapse and acute GVHD, while low pDC number was associated with acute GVHD only.

Mohty et al.[116] evaluated pDC count in the peripheral blood of 54 patients at the third month after reduced-intensity conditioning HLA-identical sibling allogeneic stem cell transplantation and separated the patients according to the pDC recovery profile into a „high“ and „low“ pDC group. In this study, „high“ pDC group had an improved overall survival in comparison to the „low“ pDC group due to the higher non-relapse mortality, notably from GVHD and late infections. In contrast, patients from the „low“ and „high“ pDC group had comparable rates of relapse and this was explained by a high predominance of high-risk patients with a high-tumor burden in the study group. In a multivariate analysis, a „high“ PDC count stayed an indepedent factor predictive of a decreased risk of death and this result suggested that quantification of pDC at 3 months after transplantation could be a simple but useful tool for predicting patients' outcome.

In our study we wanted to further evaluate the pDC count at day +100 after allogeneic stem cell transplantation as an indicator of long term outcome.

By allocating the patients according to the median value of pDC at day +100 we obtained two comparable groups according to demographic and transplant characteristics, as well as transplant related events, except of the acute GVHD. As already mentioned, the „low“ pDC group had significantly more grade II-IV acute GVHD than the „high“ pDC group.

The Kaplan-Meier estimate of overall survival was 86% in the „high“ pDC group compared to 55% in the low „pDC“ group. This difference stayed significant in the multivariate analysis, together with a known factor of worse survival - older age of the recipient.

Better survival of patients in the „high“ pDC group was mostly due to significantly lower cumulative incidence of relapse-related mortality in this group of patients (9% vs 35%).

Our results indicate that higher number of pDC reconstituted after transplantation may reduce relapse and, to our knowledge, we were the first to potentially show the clinical antitumor

activity of pDC. However, there have been conflicting resultes published before, as in an earlier study by Waller et al.[211] who associated higher number of pDC with higher rate of relapse in transplanted patients. However, there are key differences between this study and our observations. The number of pDC evaluated in this study was the number in the graft and our results concern the immune reconstitution of pDC in the peripheral blood after transplantation. More importantly, most of our patients (70%) received peripheral blood instead of bone marrow as stem cell source, and G-CSF administration used to mobilize peripheral blood grafts may have changed cytokine release and DC activation status.[211]

Rajasekar et al.[117] hypothesized that graft and early postengraftment DC counts would have an important impact on development of acute and chronic GVHD as well. However, they did not find a correlation between cell counts in the graft and GVHD, but they found the peripheral blood low pDC count on day +28 after allogeneic stem cell transplantation to be an independent predictor for both acute and chronic GVHD. Lau et al.[212] found severity of GVHD to be associated with the low mDC and pDC numbers and their activation status.

As with other analyses, there have been conflicting results as one of the studies[122] associated high pDC number with chronic GVHD, although this was at a median of 14.5 months after transplantation.

In our study, „low“ pDC count was associated with a higher cumulative incidence of extensive chronic GVHD (44% vs 30% in the „high“ pDC group) at a median of 20 months after transplantation, even though this difference did not reach statistical significance. We speculate that the difference was not reached because of the small number of patients with extensive chronic GVHD in our study population (12 patients per each pDC group).

In summary, our findings are in concordance with previous studies and support the role of enumerating pDC count at day 100 after allogeneic stem cell transplantation as a simple, fast and reproducible indicator of adverse clinical outcome of patients, as relapse, death and GVHD. Monitoring pDC count could allow for early classification of patients according to the risk for adverse events. This finding would also allow for potential early therapeutical interventions or even influencing the pDC number in the blood of patients to improve their outcomes.

6.5. pDC may have a role in supressing GVHD while promoting GVL effect

In summary, the results from our study indicate that low pDC count at day +100 after allogeneic stem cell transplantation predicts both relapse and higher incidence of GVHD in stem cell recipients.

This finding made us hypothesize about the potential pathophysiological role of pDC in supressing GVHD while promoting GVL affect, contrary to the observations with T cells, the depletion of which decreases GVHD but increases relapse.

Two major questions emerge from this hypothesis – pDC chimerism and pDC activation status of pDC at day +100 in our patients after allogeneic stem cell transplantation.

As it has been established before[106], DC populations in the recipient after transplantation represent a combination of residual host DC which have survived the conditioning regimen and donor DC which have been infused as part of the graft or have diferentiated from donor hematopoietic cell. In a murine model, the presence of host DC is a prerequisite for the development of CD8+ T cell-mediated acute GVHD, while donor DC amplify the process. In contrast, donor DC seem to be operative in a CD4+ T cell-mediated chronic GVHD, but skin chronic GVHD can be induced by host DC as well.[109,213]

Rapid establishment of full donor chimerism has been observed by day 14 after both myeloablative and nonmyeloablative allogeneic stem cell transplantation[214], but mixed DC chimerism and persistence of host DC was detected as well at day +100 after allogeneic stem cell transplantation and was correlated with the development of severe acute and chronic GVHD.[107]

pDC chimerism was not analysed in our study, due to the rarity of pDC in the peripheral blood (<1% of PBMC). However, according to the previous studies, we presume that most of our patients had a full donor DC chimerism at day +100 after transplantation. If we assume that pDC have migrated to the GVHD target organs, our finding is intriguing because it suggests the role of donor pDC in both maintenance of acute GVHD and predicting chronic GVHD.

However, the role of DC in the immune response depends of their activation status, and TLR-activated pDC secrete high levels of IFN alpha and stimulate CD4+ and CD8+ T cells, while in the steady state, pDC have intrinsic tolerogenic properties, induce Tregs and have been implicated in the regulation of disease in the experimental models of autoimmunity.[215]

The finding that BDCA2, a pDC specific marker is downregulated upon pDC activation and is preserved in our pDC from transplanted patients, might suggest that circulating pDC in our

study were in resting state.[124] This assumption does not contradict the hypothesis that activated pDC may have migrated to the activated GVHD tissues.

Additional mechanisms for a protective impact of circulating pDC against relapse and GVHD can be extrapolated from the study of Sato et al.[216] which suggest the role of immune regulatory cells. More precisely, administration of regulatory DC after allogeneic bone marrow transplantation protected mice from relapse and acute GVHD, respectively. Mechanisms in this model include activating Tregs through DC and supressing the effector functions of CD4+ and CD8+ cells. This study also showed that regulatory DC induce a more potent tolerance in CD4+ T cells than in CD8+ T cells. CD8+ T cells were previously reported to participate in the GVL effect, so they hypothesized that DC-regulated cytotoxicity which failed to cause acute GVHD may be sufficient to cause GVL effect.

In seems possible that activated pDC could have migrated to the acute GVHD target tissues but circulating donor pDC in the blood of patients could be in the resting state and could have a role in preventing chronic GVHD while supporting GVL effect. However, more functional studies are needed to decipher the exact role of pDC in antitumor immunity and GVHD. This could especially be done in the RIC setting, where the role and the impact of these rare population of immune cells logically tends to be more evident.

6.6. pDC represent important targets for future therapies

Current therapeutical approaches in GVHD are targeted against T cells, but given the importance of DC in the pathogenesis of GVHD, mDC and pDC represent important targets for future therapies.[217] However, existing therapies already affect DC function. More precisely, calcineurin inhibitors (as cyclosporine) supress antigen presentation, corticosteroids inhibit DC maturation and activation, while ATG induces complement-mediated lysis of DC and decreases their capacity to stimulate allogeneic T cells.[218]

Innovative treatment approaches target DC *in vivo* and *in vitro*, latter by producing negative DC vaccines or tolerogenic DC in culture conditions by pharmacological modification or cell sorting. *In vivo* DC manipulation comprises of pharmacological interventions which block DC maturation, reduce expression of co-stimulatory molecules and cytokine release and decrease T-cell allostimulatory capacity. In an experimental setting, anticancer drugs as HDAC inhibitors[219], proteasome inhibitors[220] and NF-κB[221] inhibitors were shown to attenuate GVHD while preserving GVL effect. A biologic intervention that has been shown to act in the

same way in an experimental model is targeting activated DC by a monoclonal antibody against CD83, a cell surface molecule usually upregulated upon DC maturation.[222]

Furthermore, DC can be targeted *in vivo* by cell therapies which have been shown to have immunosupressive properties as well, as mesenchimal stem cells[223], myeloid-derived supressor cells[224] and T-regs[225], respectively.

Importantly, cell therapies have begun in humans and recently a DC based vaccine has been approved in treatment of prostate cancer by FDA.[226] Moreover, a phase I safety study of tolerogenic DC in autoimmune diabetes [227] is being conducted, as well as many clinical trials for the prevention and treatment of GVHD which target DC.[218]

Therefore, our and similar studies are important in further understanding of the precise immunoregulatory properties of pDC and pave the way for the new targeted therapies in GVHD and hematological malignancies, given the importance of the GVL effect.

6.7. The reduced number of Th17 in peripheral blood of transplanted patients correlates with clinically significant acute GVHD.

Previous studies established an important role of effector Th1 cells in acute GVHD pathophysiology. The identification of proinflammatory Th17 cells which contribute to autoimmune diseases, especially inflammatory bowel disease[228] raised the question of the role of Th17 cells in human acute GVHD. However, the contribution of Th17 cells in acute GVHD has been explored in humans with conflicting results. Of note, in peripheral blood of patients with acute GVHD, Th17 subpopulation of cells was found to be both increased[157] and decreased.[158] In acute skin GVHD, Th1 and not Th17 cells were found to be significantly increased.[158] In one study Th17 were not found to be significant in the acute intestinal GVHD[159], while in the recent study of our study group[208] we showed a significant increase of Th17 population in the intestinal mucosa of acute GVHD patients.

In this study we investigated the role of Th17 cells in the peripheral blood of patients at day 100 after allogeneic stem cell transplantation. Here we observed that patients with grade II-IV acute GVHD at day 100 had significantly lower percentages of CD161+ Th cells than patients without clinically significant acute GVHD (grades 0-I).

Our results are in concordance with the recent study of van der Waart et al[229], who also evaluated the count of CD161+CD4+ T-cells as well as CD161+CD8+ T-cells and observed a significant decreased frequency in peripheral blood samples 3 months after transplantation in patients with acute GVHD. We speculate that the decrease in circulating CD161+ Th cells is

due to their specific recruitment into GVHD affected tissues. This is in concordance with the results from the previous study[208] of our study group based on the same group of patients, where Th17 cells counts increased in acute GVHD-affected intestinal mucosa. Consequently, at day 100 after transplantation these cells may have migrated from the peripheral blood to GVHD target tissues in patients with clinically significant acute GVHD.

The abbility of Th17 cells to migrate to GVHD organs has been associated with the high expression of CCR6 on their surface. CCR6 is a chemokine with only one ligand, CCL20, which is constitutively expressed in organs such as liver, colon, small intestine and skin.[230] Furthermore, damage of the epidermal barrier, as well as stimulation with IL1 alpha and IL1 beta which are part of the cytokine storm created after conditioning regimen, were shown to up-regulate CCL20.[231,232] All together, these findings support the hypothesis of the pathogenic role of CD161+ Th17 cells in the acute GVHD.

Importantly, we also observed a decrease of CXCR3+ cells at day +100 in the peripheral blood of patients with clinically significant acute GVHD, highlighting the role of these cells in the acute GVHD pathophisiology. CXCR3+ is a hallmark of IFN gamma secreting and most Th1 cells are CXCR3+. However, previous clinical studies have shown that Th17 phenotype is unstable and that Th17 can convert to Th1 cells [229] as well it has been clear both preclinically[153] and clinically [229] that, during acute GVHD, IL17A producing cells can also coproduce IFN-gamma. By following our previous findings, we can speculate that, in the context of GVHD, Th17 cells infiltrate target tissues of acute GVHD, and then either conserve their pathologic properties, either convert into a Th1 phenotype.

In a similar study[157], Th17 subpopulation increased in the peripheral blood of patients with the occurence of acute GVHD, but in patients recovering from GVHD progressively declined reaching the levels lower than in the healthy donors. The low proportion of Th17 in these patients was explained with a higher proportion of circulating Tregs in patients with inactive forms of GVHD.[233] In a similar context, in our study population, a lower count of CD161+ and CXCR3+ Th cells at day 100 after stem cell transplantation could be also in relation to an increased Treg population in the controlled GVHD setting.

6.8. The reduced number of Th17 cells in the peripheral blood of transplanted patients at day 100 is an independent predictor of extensive chronic GVHD

As mentioned before, the pathophisiology of chronic GVHD is poorly understood. In general, CD4+Th1 cells were held responsible for the development of acute GVHD and CD4+Th2 for

the development of chronic GVHD. However, chronic GVHD manifests with features characteristic of autoimmune diseases, including sclerodermatous skin disease, and Th1 cytokine IFN gamma can clearly play a causal role.[158,234] Consequently, chronic GVHD does not fit easily into either Th1 or Th2 paradigms.[29] In systemic sclerosis, a condition closely resembling scleroderma, the predominant clinical feature of chronic GVHD, fibrosis, is mediated by Th17 cells infiltrating the skin and serum IL17 levels positively correlate with disease severity.[235,236] In the setting of chronic GVHD, recent preclinical and clinical data support a role for IL17A as a central mediator of pathology, particularly within the skin.[155,157]

In our study, we hypothesized that number of Th17 cells at day 100 after allogeneic stem cell transplantation could serve as a predictor for the development of chronic GVHD. In the univariate analysis we found acute GVHD and low Th17 count to be significantly associated with the development of extensive chronic GVHD, and observed more extensive chronic GVHD in patients who received a transplant from a female donor. The female donor and acute GVHD are known risk factors for chronic GVHD, but when we performed a multivariate analysis of our data, only a low Th17 count at day +100 after allogeneic stem cell transplantation in the peripheral blood of patients retained its predictive value for extensive chronic GVHD.

Our results are in concordance with a previously mentioned study where the association between decreased CD 161 expressing cells at 3 months after transplantation and later-on chronic GVHD also remained independent from the known confounders such as age, gender combination and graft source in the multivariate analysis.

Therefore, we conclude that enumerating Th17 cells at day +100 after allogeneic stem cell transplantation may be a simple, fast, and reproducible method to predict the future development of extensive chronic GVHD. By allocating the patients into a „low“ or a „high“ Th17 group at this time point after stem cell transplantation, it would potentially be possible to stratify the patients into risk groups for developing extensive chronic GVHD. It would also become feasible to further stratify them for early therapeutic interventions to enhance or negate the impact of chronic GVHD and modify the outcome of the patients.

6.9. Th17 represent a promising target for the prevention and treatment of GVHD

Previous animal studies suggested that a combined blockade of Th1 and Th17 differentiation pathways of donor T cells may represent a promising strategy for the prevention and treatment of GVHD, while inhibition of either pathway alone seems to be

insufficient.[156,160,161] In this context in humans, novel strategies which target Th1 and Th17 promoting cytokines represent a new, attractive method for the control of GVHD.

Moreover, cytokine-neutralizing monoclonal antibodies are increasingly being investigated in the treatment of patients with cancer and autoimmune diseases.

Ustekinumab is a monoclonal antibody against p40, a common component of IL-12 and IL-23, both cytokines important for the differentiation of effector Th1 and Th17 cells. The efficacy and safety of ustekinumab has been demonstrated in Phase II studies for multiple sclerosis, sarcoidosis and Crohn's disease[237,238] and in Phase III trials for treatment of psoriasis.[239-241]

Furthermore, clinical trials using anti–IL17 monoclonal antibodies are emerging, aiming to directly block the Th17 response. Several studies are currently evaluating the IL17 blockade in the treatment of psoriasis.[242] Ixekizumab and secukinumab are human monoclonal antibodies to IL17A that were proven to be effective in the treatment of psoriasis in Phase II clinical trials[243-245], while brodalumab binds to IL17RA and is currently studied in the Phase III clinical trials so far showing promising clinical improvement in more than 75% of patients.[246-248]

Modulation of the Th17/Treg balance by cytokine blockade is another attractive strategy to prevent GVHD. Tocilizumab, monoclonal antibody against IL-6R, has been shown to be effective against several autoimmune and inflammatory diseases such as rheumatoid arthritis and Castleman's disease.[249-251] A hypothesis developed from animal studies suggests that IL-6 blockade attenuates acute GVHD by shifting Th17 responses toward Tregs. A single case report showed the effectiveness of tocilizumab in a patient with refractory gastrointestinal acute GVHD.[252] Furthermore, a recent study demonstrated tocilizumab efficacy for skin lesions in patients with systemic sclerosis, suggesting that tocilizumab could also be effective for treatment of sclerodermatous skin lesion in chronic GVHD.[253]

In conclusion, monoclonal antibodies against cytokines important in effector cell pathways represent an attractive future perspective for prevention and treatment of GVHD.

Importantly, in the setting of allogeneic stem cell transplantation, timing of administration of anti-cytokine antibodies will be particularly important for cytokine modulation. However, reduced intensity conditioning that has been developed to minimize toxicity of conditioning regimen will pave the way for the development of such novel strategies.

6.10. There is a potential pathophysiological link between decrease of the number of pDC in peripheral blood of transplanted patients and the decrease of the number of Th17

The decrease of the number of pDC in the peripheral blood of our patients at day 100 after allogeneic stem cell transplantation was paralleled by the decrease of the number of Th17 cells as well, suggesting a potential pathophysiological link between pDC and Th17 response in the context of both acute and chronic GVHD.

From the pathophysiological standpoint, our findings support a GVHD induction model that can mimic data from other autoimmune diseases.[100,254-256] It has already been shown that the induction of Th17-related cytokines is abrogated in pDC-depleted mice.[254] Moreover, type I IFN produced by pDC after stimulation with a TLR7 agonist are able to drive Th17 responses in vivo.[257,258] Therefore we can speculate that activated pDC recruit to GVHD target tissues following local damage (e.g. conditioning regimen) and drive the differentiation of Th17 cells, although the exact mechanism that links type I IFN production to pDC-mediated Th17 responses remains unclear and needs to be adressed in future.

However, data described in this paper do not contradict the established role of Th1 cells in GVHD pathophysiology, as the decrease of peripheral pDC at day +100 in our study was also paralleled by the decrease of peripheral blood CXCR3+CD4+ T cells. Similarly, it has already been shown that human Th17 cells may exhibit a close developmental relationship with Th1 cells.[177] As Th17 cells can easily convert to a Th1 phenotype, as well as they can coproduce IFN gamma, a likely scenario is a synchronized or reciprocal interplay between Th1 and Th17 as previously reported in autoimmune arthritis.[259]

It is clear that our study was done retrospectively and the patients' blood samples were frozen and then thawed at one time point. Moreover, our study group was rather heterogenous, as for diagnosis and transplant characteristics, precluding establishing the definitive role of pDC and Th17 after allogeneic stem cell transplantation. Therefore, more prospective studies with more homogenous groups of patients are needed. However, our results altogether provide further evidence for an important impact of pDC and Th17-mediated responses in human GVHD. Moreover, our data raise the prospect of future innovative approaches to optimize immunosuppression regimens for the treatment or prophylaxis of GVHD by targeting pDC and the Th17 response.

6.11. Th1 cytokines and chemokines in peripheral blood of transplanted patients at day 100 have a pathogenic role for the development of chronic GVHD while Th2 cytokines and chemokines have a protective role

It is widely accepted that Th1 subpopulation and Th1-related proinflammatory cytokines such as IFN gamma and TNF alpha are important in the initiation of the acute GVHD. As previously mentioned, chronic GVHD does not fit easily into either Th1 or Th2 paradigms and its pathophysiology stays poorly understood. It is also known that acute GVHD primarily involves the skin, liver, gastrointestinal tract and lymphoid tissues, while chronic GVHD is a systemic and multiorgan syndrome that has many features suggestive of a range of spontaneous autoimmune diseases. Previous studies with animal models of autoimmune diseases have characterized the pathogenic role of Th1 cells and the possible protective role of Th2 cells.[260,261] A recent study in humans examined the expression of cytokines, chemokines and chemokine receptors in oral lesions from chronic GVHD patients.[262] In this study, Th1 cytokines such as IL2 and IFN gamma were consistently expressed in nearly all of the oral lesions examined in chronic GVHD patients, and the degree of such cytokine expression showed no relationship to the degree of lymphocytic infiltration or to the clinical severity. In contrast, Th2 cytokines such as IL4 and IL5 were expressed in association with strong lymphocytic infiltration and severe tissue damage in the oral lesions in chronic GVHD patients. These findings suggested that Th1 cytokines might be primarily involved in the initiation and/or maintenance of chronic GVHD, while Th2 cytokines are involved in the progression of the disease process.

In our study, we extensively studied 41 cytokine and chemokine at day 100 after transplantation and their relation to the development of chronic GVHD after day 100. In the multivariate analysis we defined 8 cytokines and chemokines related to the development of chronic and 10 cytokines and chemokines related to the development of chronic extensive GVHD. IP10, FLT.3L, IL15, IL10 and TNF alpha seemed to increase the chance of chronic GVHD, while FRACTALKINE, MDC and TARC seemed to decrease the chance of chronic GVHD. Furthermore, IP10, IL10, IL2ra, MIP1b and IL2 seemed to increase the chance of extensive chronic GVHD, while FRACTALKINE, MDC, RANTES, IL12p40 and TARC seemed to decrease the chance of extensive chronic GVHD.

In our study Th1 cytokine IL2 and IL2 receptor alpha (IL2ra) were consistently elevated in the serum of patients who developed extensive chronic GVHD, confirming the role of the Th1 subpopulation in chronic GVHD ($p = 0.047$ and $p = 0.0212$, respectively).

IL2ra is one of previously validated biomarkers with diagnostic and prognostic value for acute GVHD[263], which was also prospectively evaluated and proven to be a good predictor of clinical outcomes as death and nonresponsiviness to therapy. [264] Moreover, IL2 production by donor T cells remains the main target of many current clinical therapeutic and prophylactic approaches for acute GVHD, such as cyclosporine A, tacrolimus and monoclonal antibodies.[265,266] Daclizumab is a humanized monoclonal antibody, which inhibits competitively the IL2ra, and has been found to be effective in the second-line treatment of refractory acute and chronic GVHD.[267,268]

As already mentioned, the role for TNF alpha in clinical acute GVHD has been suggested by studies demonstrating elevated levels of TNF alpha in the serum of patients with acute GVHD.[171] Target organ damage could be inhibited by infusion of anti TNF alpha monoclonal antibodies.[172] In our study, we confirmed TNF alpha as a valuable indepedent predictor of chronic GVHD development ($p = 0.02$).

Dose and timing of cytokine production are critical factors with regard to their role in the induction of GVHD. This is illustrated by the case of IL10 produced by DC and Th2 cells which is critical for the induction of Tregs. Higher production of IL10 or the presence in recipients of a polymorphism linked with increased IL 10 production is associated with reduced incidence and severity of acute GVHD.[178,179] Paradoxically, high-serum IL10 levels in patients after allogeneic stem cell transplantation are associated with a fatal outcome[180], whereas administration of low doses of IL10 is protective in murine acute GVHD[181], highlighting the pleiotropic, but also opposing, nature of cytokines during the different phases of GVHD pathogenesis. In our study IL10 was shown to increase the chance of chronic GVHD ($p = 0{,}03299$) .

Both GVHD and GVL effect depend on T-cell reconstitution after allogeneic stem cell transplantation. T-cell reconstitution initially depends on homeostatic peripheral expansion of donor T cells, and IL7 and IL15 are known to be key homeostatic cytokines in this process. Accordingly, studies which analysed blood levels of these cytokines showed that high levels of these cytokines on day 14 after allogeneic stem cell transplantation could positively predict the development of acute GVHD, while a reduced level of IL15 was associated with a greater risk of malignant relapse.[269] In our study, the high concentration of IL15 on day 100 after allogeneic stem cell tranplantation was also strongly associated with the development of chronic GVHD ($p = 0{,}0144$) and could therefore be used as a marker of both acute and chronic GVHD development.

Flt3L is a nonredundant cytokine required for DC homeostasis in lymphoid tissues. In a recent study pretreatment with lymphocyte only transplantation and continued Flt3L treatment with sublethal irradiation followed by combined bone marrow and lymphocyte transplantation led to full and partial protection from GVHD, respectively. The latter was correlated with higher relative host DC, and host and donor Treg numbers after transplantation.[270]

However, this cytokine in our study increased the chance of chronic GVHD development. (p = 0.0032).

IL12 is a heterodimeric cytokine that is composed of two subunits (p35 and p40) and acts to promote both NK cell and CTL activity. In addition, IL12 induces undifferentiated Th0 cells to commit to the Th1 phenotype and reduces Th2 activity.[271,272] For this reason, IL12 has been tested as an immunotherapeutic agent for the treatment of a variety of Th2-mediated diseases.[273-275] Both anti-IL12 Ab and the homodimeric p40 subunit of IL12 act as IL12 antagonists and have been used to prevent Th1-mediated diseases.[276,277] One study showed that the use of IL12-encoding plasmid improved Th1/Th2 balance and prevented the development of murine chronic renal GVHD, without causing acute GVHD.[278] Importantly, our study also showed that a higher level of this cytokine reduces the incidence of development of extensive chronic GVHD (p = 0.0417).

In identifying the significant chemokines at day 100 after allogeneic stem cell transplantation, our study confirmed most evaluated Th1 chemokines as pathogenic and Th2 chemokines as protective for chronic GVHD.

IP10 is one of chemokines toward CXCR3, has a chemotaxis for monocytes and Th1 cells, and is produced by fibroblasts and vascular endothelial cells[279-281] while CXCR3 and CCR5 are specific chemokine receptors for Th1 cells.[282,283] Serum CXCL10 (IP10) was proposed to be an accurate marker for the development of acute GVHD.[284] In our study, we also found CXCL10 (IP 10) to be the strongest indepedent marker for the development of chronic GVHD as well (p<0,0001). One of the chemokines overexpressed in GVHD target organs[285] and another CCR5 ligand, MIP 1b, was also elevated in the blood of our patients and positively predicted the development of extensive chronic GVHD (p = 0.0315) .

TARC and MDC are natural ligands for CCR4 and serve as chemokines which attract Th2 cells.[286,287] TARC is produced by vascular endothelial cells and DC, while MDC is produced by APC.[288,289] In our study, these chemokines had a protective role for the development of both chronic and extensive chronic GVHD (p = 0.0192 and p = 0103 for MDC and p = 0.0438 and p = 0.0389 for TARC, respectively) and this finding would be consistent with the

hypothesis that Th2 subpopulation of lymphocytes has a protective role in the pathogenesis of chronic GVHD.

In murine models, the systemic release of inflammatory cytokines such as TNF alpha and IFN gamma by T cells activated within lymphoid tissue leads to the release of the chemokines CXCL9-11 at tissue sites.[182] These recruit CXCR3+ T cells, which are believed to mediate the tissue damage characteristic of GVHD. A second wave of T cells that produce CCR5 ligands, such as CCL5 (RANTES), is then induced and may play a role in limiting tissue damage.[290]

In our study, higher levels of blood RANTES also seemed to decrease the chance of extensive chronic GVHD ($p = 0.0214$).

Finally, Fractalkine is the unique ligand for the chemokine receptor CX3CR1, which is expressed on monocytes, natural killer cells, T cells, and smooth muscle cells, where it mediates functions including migration, adhesion, and proliferation.[291-293] A pro-inflammatory role of this cytokine has been shown in autoimmune diseases, as rheumatiod arthritis[294] and inflammatory bowel disease.[295] However, in our study higher blood levels of this chemokine decreased the chance of extensive chronic GVHD development ($p = 0.0214$).

In general, our results suggested that an elevated concentration of Th1 cytokines and chemokines at day 100 in transplanted patients could be predictive of chronic GVHD development while an elevated serum concentration of Th2 cytokines and chemokines could negatively predict the chronic GVHD development.

6.12. A prognostic score made of the most signficant serum cytokines and chemokines at day 100 in transplanted patients can accurately predict the development of chronic GVHD and chronic extensive GVHD at 2 years after allogeneic stem cell transplantation

We next sought to establish a practical prognostic score capable of predicting chronic and chronic extensive GVHD. This score was calculated using the traditional multivariate Cox model combined with the statistical approach called "time-dependent receiver-operator characteristic (ROC) curves. Here, we have focused on the significant clinical variables and the most significant cytokines found in the multivariate analysis in order to obtain a useful tool in the daily medical practice. Based on 0,632 bootstrap resampling method for repeated cross-validation, the area under the time-dependent ROC curve was 0.80 (95%CI, 0.72-0.87) for chronic GVHD and 0.78 (95%CI, 0.66-0.88) for chronic extensive GVHD indicating that such composite score is a powerful predictor of the risk of GVHD at 2 years. These results do not seem exceptional when known that there are studies with ROC curves with AUC between

0.9 and 1.0 which give exceptional predictive markers. However, our score is prognostic, while the most of the previous scores are diagnostic. The prognostic scores are time dependent, which has to be taken into consider when the statistics is done. Also, unlike previous studies, our results are more realistic because we kept the „overfitting“ in mind and used a bootstrap 0.632 correction. Of course, this score has to be also validated externally. Results from this study allowed to build a new noninvasive score to accurately predict the risk of chronic and chronic extensive GVHD occurrence after allogeneic stem cell transplantation. Such score could be used as a decision tool in the clinical management after allogeneic stem cell transplantation.

7. Conclusions

7.1. Acute GVHD is the only independent predictor of impaired blood pDC recovery at day 100 after transplantation

The recovery of pDC in the peripheral blood at day 100 after allogeneic stem cell transplantation in our patients was not influenced by the patients' or graft characteristics, conditioning regimen or infections, as it has been shown in previous studies. On the contrary, in our group of patients, only the occurence of grade II-IV acute GVHD in the multivariate analysis was found to be significantly associated with an impaired pDC recovery in the peripheral blood at day 100 after allogeneic stem cell transplantation.

7.2. Corticosteroid therapy downregulates function of activated pDC

When we excluded patients who at day 100 still received high doses of corticosteroids, blood pDC count stayed significantly higher in the acute GVHD group of patients, while activated pDC secreted similar amounts of IFN alpha and TNF alpha in both patients with and without acute GVHD. This finding suggested that the resting pDC in the blood of patients were functionally less activated due to the corticosteroid treatment. This observation is in concordance with previous studies where corticosteroids seemed to prevent DC maturation and impair their immunostimulatory activities, without affecting their viability.

7.3. Activated pDC and effector Th17 cells have a pathogenic role in acute GVHD

Patients with grade II-IV acute GVHD had significantly lower percentages of peripheral blood pDC, IFN alpha-secreting pDC and Th17 than patients with grade 0-I acute GVHD. This is in concordance with our previous study where we found both of these populations increased in the intestinal biopsies of patients with acute GVHD. Our hypothesis is that activated pDC and Th17 migrated from peripheral blood to target GVHD tissues. Moreover, a decrease of pDC paralleled with the decrease of Th17 could suggest a potential pathophysiological link between these two populations in acute GVHD. Our findings support earlier studies where pDC seemed to have a role in triggering Th17-related cytokines.

7.4. Low blood pDC count at day 100 after transplantation is an independent predictor of relapse and overall survival

By allocating the patients according to the pDC recovery profile into a „high“ and „low“ pDC group, we found significantly better overall survival in the „high“ pDC group of patients. Consequently, we presumed a potential „protective“ role of blood resting pDC and at the same time we showed significantly higher relapse-related mortality in the „low“ pDC group. Therefore, our results also highlighted the clinical antitumor activity of the blood resting pDC. In a multivariate analysis, a „high“ pDC count and younger age of patients stayed indepedent favorable prognostic factors for longer survival. This is in concordance with previous studies and suggests that quantification of pDC at 3 months after transplantation could be a simple and useful tool for predicting patients' outcome.

7.5. Low blood Th 17 count at day 100 after transplantation is an independent predictor of chronic extensive GVHD

By allocating the patients according to the Th17 count into a „high” and „low“ Th17 group we found significantly higher cumulative incidence of extensive chronic GVHD in the „low“ Th17 group. Moreover, “low” Th17 count stayed the only independent predictor of extensive chronic GVHD in the multivariate analysis. This is in concordance with a recent study which also showed that decreased CD 161+ cells at 3 months after transplantation can independently predict later-on chronic GVHD. Enumerating Th17 cells at day +100 after allogeneic stem cell transplantation could therefore be a simple and reproducible method to predict the future development of chronic GVHD.

7.6. Ten serum cytokines at day 100 after transplantation predict the development of chronic extensive GVHD

In accordance with previous studies, our results suggested that elevated concentrations of Th1 cytokines at day 100 could be predictive of chronic GVHD development while elevated serum concentrations of Th2 cytokines could negatively predict chronic GVHD development. More precisely, we found 10 cytokines significantly correlated with the incidence of extensive chronic GVHD. High levels of IP10 (CXCL10), IL15, IL10, IL2RA and MIP-1beta (CCL4) were associated with higher incidence of extensive chronic GVHD, while high levels of Fractalkine (CX3CL1), MDC (CCL22), RANTES (CCL5), TARC (CCL17), IL12p40 were

associated with a lower risk of developing extensive chronic GVHD. These cytokines could serve as potential biomarkers of chronic GVHD development.

7.7. A noninvasive score derived from serum cytokines levels at day 100 after transplantation accurately predicts the development of chronic extensive GVHD

By focusing on the significant clinical variables and the most significant cytokines at day 100 after stem cell transplantation, we obtained a useful tool for the daily medical practice. We succeeded in making a prognostic blood test for extensive chronic GVHD development, while most previous studies evaluated diagnostic tests. AUC of 80% makes our prognostic score a powerful predictor of the risk of extensive chronic GVHD at 2 years. An additional validation of this score should be done on another independent cohort.

8. Summary

GVHD continues to be a major source of morbidity and mortality following allogeneic stem cell transplantation (allo-SCT). Previous studies established the role of Th17 subpopulation of lymphocytes and the plasmacytoid dendritic cells (pDC) in the pathophysiology of several autoimmune diseases. The aim of this research was to evaluate the role of pDC and Th17 in the peripheral blood of patients at day 100 after allo-SCT in acute and chronic GVHD and GVL effect. The other aim of the study was to expand the search for chronic GVHD biomarkers by validating cytokines in the blood of patients at day 100 after allo-SCT and to determine a composite prognostic score for prediction of chronic GVHD.

This study included peripheral blood mononuclear cells (PBMC) of 79 patients taken at day 100 after allo-SCT and 152 serums from patients who underwent allo-SCT between 2005 and 2011 in Centre Hospitalier Universitaire de Nantes, France. For functional analysis of pDC, PBMC from 79 blood samples were stimulated with TLR7 and TLR9 ligands in the presence of IL-3 over 6 hours, and then stained for surface markers and intracellular cytokines (IFN alpha, TNF alpha and IL6). Th17 were evaluated quantitatively on the same PBMC by staining for specific surface markers. Forty-one serum cytokine and chemokine was studied in all 152 serums using Luminex xMAP technology.

We observed a significant decrease of total and activated pDC, as well as Th17 and IFN gamma producing cells in the blood of patients with grade 2-4 acute GVHD as compared to patients with grade 0-1 acute GVHD. After dividing patients into 2 distinct groups, using the median value of pDC and Th17, we observed that a low pDC count predicts relapse and worse overall survival and low Th17 predicts more extensive chronic GVHD. In the multivariate analysis, low pDC count retained it's predictive value for worse overall survival together with the older age of the patients while low Th17 count stayed the only independent predictor of extensive chronic GVHD.

Independently from all relevant clinical factors, 10 cytokines were found to be significantly correlated with the development of extensive chronic GVHD. Based on the significant cytokines and clinical factors, we established a practical prognostic score using the traditional multivariate Cox model combined with"time-dependent ROC curves". The area under the time-dependent ROC curve of 0.80 indicated that such composite score is a powerful predictor of the risk of extensive chronic GVHD.

In concordance with previous GVHD studies, the significant decrease of both pDC and Th17 cells in peripheral blood on day 100 after allo-SCT in patients with clinically severe acute GVHD could be the result of the migration of these cells to target tissues of GVHD. Therefore, this study provides evidence for a potential new pathophysiological link between pDC and Th17 in human acute GVHD, and identifies these cells as potential new targets for prophylaxis and treatment of GVHD. Moreover, we established pDC and Th17 counts in the peripheral blood of patients at day 100 after allo-SCT as valuable predictors of overall survival, relapse and chronic GVHD and have proposed that these cells, have an important role in both GVHD and GVL effect. Monitoring pDC and Th17 count could allow for early classification of patients according to the risk for adverse events and allow for potential early therapeutical interventions to improve their clinical outcomes.

Furthermore, new noninvasive score developed in this study to accurately predict the risk of extensive chronic GVHD after allo-SCT could be used as a decision tool in the clinical management of allo-SCT.

9. Sažetak

GVHD je vrlo česta komplikacija transplantacije alogenične koštane srži (alo-TKS), i dalje povezana uz visoku smrtnost. Prijašnje studije su utvrdile ulogu Th17 populacije i plazmacitoidnih dendritičkih stanica (pDC) u mnogim autoimunim bolestima. Cilj ovog istraživanja je bio procijeniti ulogu pDC i Th17 u perifernoj krvi bolesnika stoti dan nakon alo-TKS, u akutnom i kroničnom GVHD-u te GVL učinku. Također, cilj ove studije je bio procijeniti ulogu citokina kao potencijalnih biomarkera za kronični GVHD u krvi bolesnika stoti dan nakon alo-TKS kao i načiniti praktičan prognostički skor za pojavu kroničnog GVHD-a.

U ovo je istraživanje uključeno 79 bolesnika kojima su periferne mononuklearne matične stanice (PMMC) uzete stoti dan nakon alo-TKS i 152 seruma uzetih stoti dan nakon alo-TKS od bolesnika koji su alotransplantirani između 2005. i 2011. u Centre Hospitalier Universitaire de Nantes, u Francuskoj. Za analizu funkcije pDC, PMMC izolirane iz 79 uzoraka krvi, stimulirane su ligandima za TLR7 i TLR9 receptore u prisutnosti IL3 kroz 6 sati, a potom bojane za površinske markere i unutarstanične citokine (IFN alfa, TNF alfa i IL6). Th17 su kvantitativno određene na istim uzorcima bojanjem na specifične površinke markere. Četrdeset i jedan citokin i kemokin je određivan u serumu 152 bolesnika korištenjem Luminex xMAP tehnologije.

Primijetili smo značajno smanjenje aktiviranih pDC, Th17 te stanica koje proizvode IFN gama u bolesnika koji su stoti dan nakon alo-TKS imali razvijen akutni GVHD stadija II-IV u odnosu na bolesnike bez ili sa stadijem I akutnog GVHD-a. Kada smo bolesnike podijelili u dvije skupine, uzimajući kao razdjelnicu medijan vrijednosti postotka pDC i Th17, primijetili smo da niže vrijednosti pDC u krvi predviđaju relaps i lošije ukupno preživljenje bolesnika, a niže vrijednosti Th17 predviđaju razvoj ekstenzivnog kroničnog GVHD-a. U multivarijatnoj analizi, niže vrijednosti pDC su zadržale prediktivnu vrijednost za lošije preživljenje bolesnika, kao i starija dob bolesnika, dok su niže vrijednosti Th17 bile jedini neovisni čimbenik za predviđanje ekstenzivnog kroničnog GVHD-a. Deset se citokina, neovisnih o važnim kliničkim faktorima, pokazalo značajnima za razvoj ekstenzivnog kroničnog GVHD-a. Pomoću značajnih citokina i kliničkih čimbenika, stvorili smo praktičan prognostički skor na temelju Coxove multivarijatne analize i vremenski ovisnih ROC krivulja. Prema AUC vrijednosti od 0.8, radi se o relativno moćnom predskazatelju rizika ekstenzivnog kroničnog GVHD-a.

U skladu s našom prijašnjom studijom uloge pDC i Th17 u GVHD-u, značajno sniženje ovih populacija stanica stoti dan u perifernoj krvi bolesnika s teškim akutnim GVHD-om bi moglo označiti migraciju aktiviranih stanica u ciljna tkiva GVHD-a. U svakom slučaju, istovremeno značajno sniženje broja obiju populacija u krvi bolesnika može ukazivati na njihovu povezanost u patofiziologiji GVHD-a, te ih identificirati kao potencijalne nove ciljne molekule u profilaksi i liječenju GVHD-a. Također, utvrdili smo da su brojevi ovih stanica u perifernoj krvi stoti dan nakon alo-TKS vrijedni prediktori za ukupno preživljenje, relaps i kronični GVHD, te tako pretpostavili da ove stanice imaju značajnu ulogu i u GVHD-u i GVL-u. Monitoriranje broja ovih stanica bi moglo omogućiti klasifikaciju bolesnika prema riziku za razvoj neželjenih događaja te potencijalnu ranu terapijsku intervenciju za poboljšanje njihovog kliničkog ishoda. Novi prediktivni skor za razvoj kroničnog ekstenzivnog GVHD-a mogao bi postati vrijedan alat u kliničkom odlučivanju nakon alo-TKS.

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11. Curriculum vitae

I was born in 1983. in Zagreb, where I graduated in natural sciences and mathemathics. In 2001. I obtained a medical degree from School of Medicine, University of Zagreb. I work as a postdoctoral research fellow from 2008., currently on the project of Croatian Ministry of Science „Acute leukemias and bone marrow transplantation“. I enrolled in PhD programme of Biomedicine and Health Sciences at School of Medicine, University of Zagreb in 2009. In the same year I spent three months in the University Hospital Centre in Nantes, France, doing a clinical research on a project “Features of EBV reactivation after a reduced intensity conditioning after allogeneic stem cell transplantation” under the mentorship of professor Mohamad Mohty. In 2011. I spent six months in the same institution doing a basic research on a project which resulted in this PhD thesis. For these efforts I received a reward from School of Medicine, University of Zagreb for notable science productivity. In 2012. I started residency in hematology at University Hospital Centre Zagreb. My main scientific interests are bone marrow transplantation immunology and acute leukemia treatment.