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Graft-versus-host disease: from experiments to clinical insight

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Abstract

The pathophysiology, prevention, and treatment of acute graft-versus-host disease (GVHD) occurring, mainly, after allogeneic hematopoietic stem cell transplantation (allo-HSCT), should be understood, in order to exploit its potential benefits while avoiding certain clinical risks. Many studies have shown haematopoietic cells to be primary targets, as well as skin, gut, and liver containing macrophage-derived cells. The latters produce pro-inflammatory cytokines that stimulate donor T cells and induce HLA class II antigens in host tissue. Dendritic cells (DCs) boost CD 8 cells to react against HLA class I peptides. Hence, GVH reactions of the graft are directed against histocompatibility antigens of the recipient that are foreign to the donor. Polymorphic non-HLA proteins may also cause severe GVH reactions. The reactions against minor histocompatibility antigens require a longer phase of activation than reactions against MHC antigens.

The preconditions of acute GVHD (aGVHD) are given before transplantation (the s.c. "cytokine storm" liberated by intensive conditioning treatment and probable infections). However, in human patients, donor lymphocyte transfusion may produce GVHD without conditioning treatment. In general, the host's immune system is continuously suppressed by the graft and; the graft becomes tolerant towards the host. The mechanism of tolerance has been related to the occurrence of non-specific and specific suppressor cells followed by clonal deletion, being also mediated by mesenchymal stromal cells, NK-T cells, and regulatory T cells. Selecting an HLA-identical sibling as donor was the major step towards successful HSCT (generally, definition of 10 HLA-loci is required to prevent severe GVHD). Several TNF-a and TNF-a receptor alleles are associated with an increased risk of GVHD. The well-known clinical features of aGVHD are also described, including skin, liver, and gut lesions. The issues of chronic GVHD are also described. Its clinical and pathological signs resemble autoimmune diseases in many aspects.

GVHD prophylaxis is well established, and should be used in any clinical setting. Special attention is given to T cell depletion and modern immunosuppressive therapies post-transplant. Current schedules of GVHD treatment are described including calcineurin inhibitors, and some novel suppressive drugs. The role of various treatment regimens is considered in view of regulatory T cell (Treg), mesenchymal stem cells and UV-A irradiation as possible means of GVHD management.

Special attention is drawn to induction of a graft-versus-host tolerance in clinical HSCT. In the majority of patients, the peripheral (thymus-independent) form of tolerance prevails. Specific selective effects of Rapamycin upon T cells are discussed.

Keywords: graft-versus-host disease, prophylaxis, treatment, conditioning therapy, dendritic cells, T-lymphocytes, immune suppression, immune therapy

Introduction

Allogeneic hematopoietic stem cell transplantation has become one of the most frequent forms of transplantation, with currently more than 6000 transplants being performed annually. Its use is still increasing in the treatment of hematological and other malignancies. In addition there are a large number of patients with debilitating and life threatening hematological dis-eases, thalassemia, sickle cell anemia, and other non-malignant diseases that may benefit from transplantation. However, the major obstacle to the wider use of transplantation is graft-versus-host disease (GVHD); still a serious threat to these patients. However, at the same time graft-versus-host reactions directed at leukemia, lymphoma, myeloma, and other tumors of the host may be beneficial. Therefore it is necessary to understand GVHD in order to ex-ploit the potential advantages without incurring the risks. Allogeneic stem cell transplantation conveys tolerance toward organs of the donor. As a rule, immunosuppressive therapy can be discontinued after several months without the risk of rejection and GVHD. This tolerance with chimerism allows the transplantation of cells and organs of the same donor without life-long immune suppression. The success of immunotherapy with donor cells and of transplantation of solid organs from the stem cell donor depends on whether or not GVHD can be controlled.

Early observations

Mice protected from hematopoietic failure following total body irradiation by bone marrow transplantation succumbed to a "secondary disease" if the bone marrow was taken from a different strain [1]. This disease was related to an immune reaction of donor cells against the host rather than a delayed radiation syndrome: cells of diseased mice induced hepato-splenomegaly when transferred to non-irradiated newborn mice [2]. Further proof was the oc-currence of this secondary disease in F1-hybrid mice transplanted with parental marrow, but not in parental mice transplanted with F1-hybrid marrow [3]. Finally, organs containing more immunologically competent cells such as those from the spleen produced more secondary disease than bone marrow [4]. Eventually, the principle requirements for GVHD were defined by Billingham [5]: 1. the graft must contain immune reactive cells, 2. the recipient must be im-munogenetically different, and 3. the recipient cannot reject the graft. The first patients with acute GVHD were described by Mathé and colleagues [6]. A major step towards successful transplantation was the selection of marrow donors within the family according to major his-tocompatibility antigens (HLA) [7]. HLA had been previously detected in humans with pre-formed antibodies [8,9]. Most preconditions for allogeneic transplantation in humans have been elaborated in animal experiments, particularly in dogs [10].

Therefore the principles for prevention of GVHD are 1. selection of a histocompatible donor, 2. adequate immune suppression for the patient before and after transplantation, and 3. ma-nipulation of the graft. In more recent years much has been learned about the regulation of the T cell response and mechanisms of tolerance, which may guide the way for immune suppression [11].

Animal models

The manifestation of GVHD in every species investigated so far involves skin, gut, and liver; primarily however hematopoietic tissue (Fig.1). Acute GVHD is a syndrome with similar fea-tures in mice, rats, monkeys, and humans; without prevention or treatment it can be rapidly fatal. Therefore pathophysiology, prevention, and treatment of acute GVHD can be studied in animal models. Chronic GVHD cannot be readily studied in animal models; it is not known why certain organs are involved and others are spared. Obviously hematopoietic cells are the primary targets, and the skin, gut, and liver may contain cells of hematopoietic origin such as dendritic cells and macrophages. These cells produce pro-inflammatory cytokines including interferon-gamma (IFN-g), tumor necrosis factor-alpha (TNF-a), interleukin 6 (IL-6), and others that stimulate donor T cells and induce expression of HLA class II antigens in host tissue (Fig.2). Dendritic cells activated by CD4 cells may stimulate CD8 cells to react against HLA class I presented peptides (Fig.3). Recent studies, however, showed that deficient production of IFN-g can increase GVHD in the skin, and failure of IFN-g induction of B7-H1 enhanced TH2 cells can produce idiopathic pneumonia [12]. TH2 cells and TH17 cells were guided to lungs and skin by the expression of chemokine receptors.

Figure 1. *Host target tissues affected in the course of graft-versus-host disease*

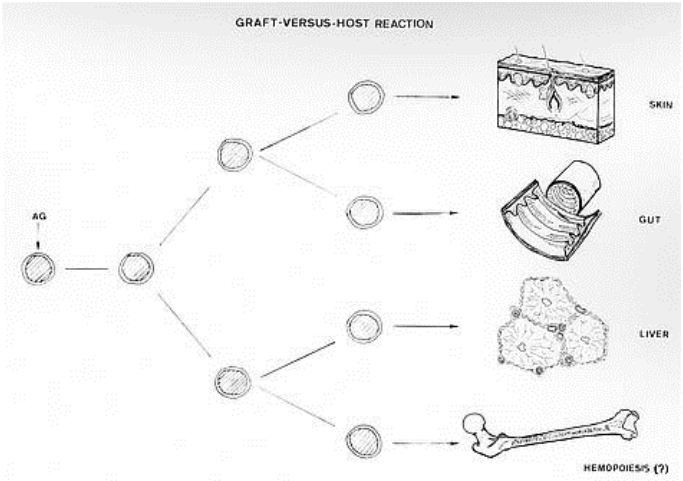
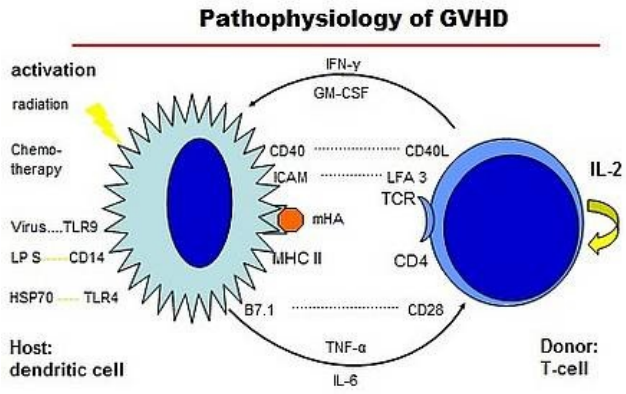


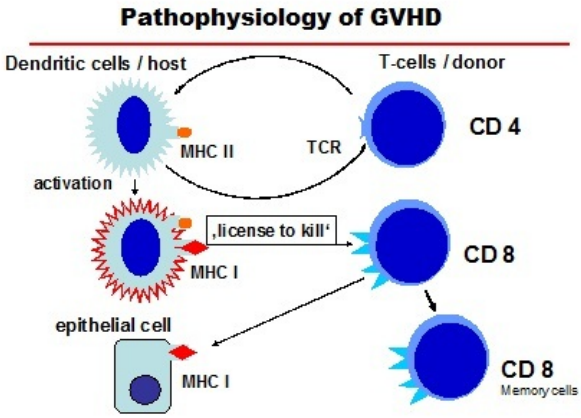
figure1

Figure 2. *A proposed role of cytokine network and specific receptors of immune cells at initiation of GvHD (for details see text)*



Cell Ther Transplant. 2012;2:e.000089.01. doi:10.3205/ctt-2012-en-000089.01-figure2

Figure 3. *Dendritic cells boost CD8+ cells to react against host target tissues*



Cell Ther Transplant. 2012;2:e.000089.01. doi:10.3205/ctt-2012-en-000089.01-figure3

GVH reactions of the graft are directed against histocompatibility antigens of the recipient that are foreign to the donor. These antigens can be defined by the major histocompatibility complex, a highly polymorphic genetic region determining class I and class II antigens. Class I antigens are present in all cells of the organism, and class II normally only in hematopoietic cells. They may be expressed in other cells if these are affected by inflammation or injury. CD4-positive T cells exert GVH reactions against cells expressing class II antigens, and CD8-positive T cells act against class I antigens [13]. Differences in both antigen classes can induce severe and rapidly fatal GVHD. Polymorphic proteins not encoded by the major histo-compatibility complex may also cause severe GVH reactions. Peptides of these proteins can be presented by MHC class I and class II antigens. In general, MHC class I presents peptides of endogenous proteins of the cell, whereas class II antigens present peptides of exogenously acquired proteins [14,15]. Here, minor histocompatibility (mHA) directed CD8 T cells require help from CD4 T cells for expansion and generation of memory T cells [16]. Therefore, reactions against mHA require a longer phase of immune recognition and activation than reactions against MHC antigens. Class II antigens are mainly expressed in hematopoietic progenitor cells, and in the case of injury and inflammation they may be expressed in non-hematopoietic cells as well. Reactions directed against class II antigens may induce severe marrow aplasia [17].

The mechanism of initiation of acute GVHD is not entirely clear; the preconditions are given before transplantation [18]. Much has been explained and published on cytokines and the cyto-kine storm liberated by intensive conditioning treatment, including high dose radiation and chemotherapy [19]. The role of cytokine release is confirmed by the suppression of acute GVHD using TNF-a antibodies [20]. There is some evidence that the systemic release of IFN-g leads to the secretion of chemokines in organs affected by GVHD and attracts activated T cells. In transgenic mice carrying the T cell receptor for ovalbumin the distribution of T cells was dependent on whether the antigen was given alone or together with lipopolysaccharide (LPS). Intravenous injection of antigens alone homes the T cells to secondary lymphoid tissue where they produce IL2, whereas injection of a combination of antigens and LPS homes the T cells to the lung, liver, gut, and skin where they produce IFN-g [21]. Systematically activated T cells produce interferons and induce chemokines in GVHD target organs [22]. However, the "danger signal" brought about by LPS may not be necessary, since in human patients donor lymphocyte transfusion may produce GVHD without conditioning treatment and infection [23].

The host's antigen presenting cells survive the conditioning treatment for various periods of time, with the

most efficient cells being dendritic cells, but B cells, macrophages and other cells present antigens as well. Whereas dendritic cells in the blood of the host are rapidly re-placed by those of the donor, data on chimerism of dendritic cells in tissues are controversial [24]. Cytokine release by the host's activated dendritic cells and the graft's T cells is part of the initiation of GVH reactions (Fig. 2), and may be powerful enough to induce fatal GVHD even in the absence of histoincompatibility [25]. In general however, histocompatibility differences are necessary to induce and maintain GVH reactions. These histocompatibility differences may be of the major histocompatibility complex (MHC) class I or class II involving CD4- or CD8-positive T cells of the graft, and minor histocompatibility differences requiring profes-sional antigen presentation by dendritic cells of the host. GVHD occurring in the skin, liver, and gut requires dendritic cells expressing class I [26]. There is a possibility of cross presenta-tion of host antigens by donor dendritic cells, but their effects are inferior to direct presentation [27].

In contrast to cases involving the transplantation of solid organs, immunosuppressive therapy can be discontinued 3–6 months after transplantation in most patients receiving hematopoietic stem cell transplants, although patients who develop chronic GVHD may require therapy for several years. The host's immune system is continuously suppressed by the graft, and the graft becomes tolerant towards the host. The mechanism of tolerance has been related to the occurrence of non-specific and specific suppressor cells followed by clonal deletion [28-30]. In DLA-identical canine chimeras tolerance could not be abrogated by the transfusion of donor lymphocytes unless the donors were immunized against the recipient [31]. Refractoriness to donor lymphocytes inducing GVHD develops at about two months after T cell depleted transplantation [32]. It may occur earlier in dogs transplanted with marrow depleted of T cells by CD6-antibody sparing NK cells [33]. NK cells can inactivate host dendritic cells and thereby prevent GVHD in mice [34]. Besides depletion of T cells and dendritic cells in the graft and the host, responder cells to antigen stimulation may respectively be eliminated by subsequent chemotherapy with methotrexate or cyclophosphamide. Cyclophosphamide can be given in rather high doses after transplantation without jeopardizing engraftment [35]. Modulation and suppression of GVH reactions has been shown for fractions of marrow cells such as mesenchymal stromal cells [36], NK-T cells (NKT1.1) [37], and regulatory T cells [11].

The results of animal models are highly informative with respect to the principles and me-chanisms of GVHD, but they also have their limitations. Apart from species-specific regulato-ry mechanisms of hematopoiesis and the immune system, animals are mostly young, have grown up in a protected environment, and are free of disease for which clinical transplantation is undertaken. In contrast, human patients are commonly older, have a history of infections and most likely a number of latent viral infections, and are possibly allo-immunized by previous transfusions and pregnancies, as are their donors. Moreover the primary disease and its treatment have a major impact on the transplant course.

The role of the immune repertoire of donor and host is still poorly defined. Female donors produce more GVHD and GVL in male recipients; most likely due to immunization during pregnancies by antigens derived from the fetuses' father [38]. Conversely, central memory T cells produce less GVHD than naïve T cells, indicating that the GVH reaction in most cases is a primary reaction [39]. Presumably central memory T cells cannot be involved in new primary reactions; there is also a risk that central memory T cells may produce vigorous GVHD when they recognize the antigen against which they developed. Alternatively they could be regulated by regulatory T cells.

Genetics

Selecting an HLA-identical sibling as donor was the major step towards successful stem cell transplantation. Selecting the donor within a family by typing for HLA-A, -B and DR-antigens is sufficient for successful transplantation, since antigen typing defines the haplotypes inhe-rited from the parents. Unlike identity by inheritance, selection of an unrelated donor relies on the most accurate typing of as many loci as possible. In general genetic definition of alleles of 10 HLA-loci is required to select a matched donor [40]. Severe GVHD can occur with any form of mismatch, but graft failure is less serious with mismatches for HLA-alleles than for the broader HLA-antigens [41]. In multiple mismatches the impact of various HLA-loci (A, B, C, DR) was similar, with the possible exception of HLA-DQ, which was less important. Notewor-thy is a possible racial difference in the role of HLA-C; in Japanese populations HLA-C has a lesser effect on GVHD than other HLA-loci [42]. In Caucasian populations HLA-C is as impor-tant for GVHD as other HLA-antigens [43]. The linkage disequilibrium, i.e. the occurrence of two antigens together, is more frequent than expected by the antigen frequency, is high for HLA-B and -C as well as for HLA-DRB1 and DQB1; therefore isolated mismatches are infre-quent. The linkage disequilibrium of HLA-DP with HLA-DRB1 is rather low, and differences of HLA-DP do not carry an additional risk for GVHD. They may, however, have an effect on the graft-versus-leukemia activity [44].

Presently little is known about permissible HLA-mismatches that allow for the development of tolerance. There may be racial differences as shown for HLA-C in Japanese as compared to Caucasian populations. In general HLA-mismatches are more permissible in patients with advanced disease than in patients with early disease. An allele mismatch may produce se-vere GVHD in a patient in chronic phase CML, but it may not have an effect in a patient with relapse of leukemia [43]. Cytokine levels and cytokine receptors are coded for by genes of the major histocompatibility complex. Sequence polymorphisms of genes for tumor necrosis fac-tor alpha (TNF-a), IL-6 and interferon-gamma (IFN-g) are different in persons with different racial backgrounds, i.e. Caucasians, Africans, and Cubans [45]. There have been several al-leles defined for both the TNF-a locus and the TNF-a receptor II locus that are associated with an increased risk of GVHD. Contrary to the pro-inflammatory cytokine TNF-a, IL-10 has anti-inflammatory effects. Polymorphisms of the promoter of IL-10 had an impact on GVHD. High levels of IL-10 correlated with a lower risk of GVHD.

Genetic factors outside of the HLA-complex may also be involved in the pathogenesis of GVHD. In the analysis of the gene expression profiles of donor cells, a particular role of transforming growth factor beta for chronic GVHD has been found [45]. In patients transplanted for chronic myelogenous leukemia [46] polymorphic alleles

GVHD has been found [18]. In patients transplanted for chronic myelogenous leukemia [18] polymorphic alleles of TNF-receptor in the patient and certain alleles in IL10 and IL1 receptor in donor lymphocytes were associated with an in-creased risk of GVHD and decreased survival. A genetic factor associated with inflammatory bowel disease had an impact on GVHD (NOD/Card1) [47]. However, the effect could be dimi-nished if the gut was microbiologically well decontaminated. Antimicrobial prophylaxis de-creases the risk of GVHD without the GVL effect deteriorating.

There is good evidence that minor histocompatibility antigens play a role in GVHD and GVL reactivity [48,49]. However, a recent analysis of the role of minor antigens in HLA-matched unrelated transplants by the NMDP did not find an impact of minor HA differences on the out-come of allogeneic stem cell transplantation [50].

Clinical features

Acute GVHD

GVHD was described and classified in the '70s [51,52], when most patients were conditioned with total body irradiation. Skin is the organ most frequently affected; a maculopapular rash is common. This rash starts frequently in the upper thorax, arms, and face, but it can occur elsewhere and spread over the whole body. Features range from a maculopapular rash to general dermatitis with blisters and epidermal necrolysis. Histological findings are degenera-tion and apoptosis of the basal cells, dyskeratosis and lymphocytic infiltration. Involvement of the gastrointestinal tract is clinically characterized by diarrhea, malaise and vomitus; diarrhea may be severe with several liters of liquid and bloody stools. Histological findings are flatten-ing of the mucosa with debris in crypts (crypt abscesses); the most frequently affected part is the ileum. GVHD of the liver is characterized by jaundice and increases of liver enzymes. Histologically the Glisson triads are infiltrated, and the bile ducts are destroyed by infiltrating lymphocytes. Unfortunately none of the histological signs are diagnostic — viral infections and drug reactions may present similar features. Nevertheless biopsies may be indicated in order to exclude other diagnoses with characteristic signs and to obtain material for microbio-logical studies.

Despite prophylactic treatment with immunosuppressive drugs the prevalence of acute GVHD of all grades of severity is high, with a rate of 40–60% in patients with an HLA-identical sibling donor and 60–90% with a matched unrelated donor. Only at a severity of grade 2 and higher is additional immunosuppressive treatment required: this equates to 40–70% of patients. Another grading system was designed by the International Bone Marrow Transplant Registry IBMTR and validated in two studies [53,54]. This grading system does not take into account the clinical performance as does the system of H. Glucksberg [51]. No advantage of one system over the other has been shown [54]. In both grading systems microangiopathy has not been scored as a form of acute GVHD; microangiopathy is characterized by red cell fragmentation, high levels of serum lactate dehydrogenase and thrombocytopenia. It is more frequent in patients treated with calcineurin inhibitors [18] or sirolimus, and resembles thrombotic thrombocytopenic purpura, but polymers of von Willebrand factor have not been found [55].

Table I. *Acute GVHD. Diagnostic criteria according to H. Glucksberg*

Stage	Skin maculopapular rash	Liver bilirubin	Gut diarrhea
+	< 25% body surface area	2 - 3 mg/dl	> 500 ml
++	25 - 505 BSA	3,1 - 6 mg/dl	> 1000 ml
+++	Generalized erythroderma	6,1 - 15 mg/dl	> 1500 ml
++++	General erythroderma with bulla formation and desquamation	> 15 mg/dl	Severe abdominal pain w/wo ileus

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Table II. *Acute GVHD. Diagnostic criteria according to H. Glucksberg*

Cell Ther Transplant. 2012;2:e.000089.01. doi:10.3205/ctt-2012-en-000089.01-table2

Grade of aGVHD	Skin	Liver:	Gut:	Clinical performance
I	+ - ++	bilirubin < 2,0 mg/dl	No diarrhea	Ok
II	+ - +++	3,1 - 6 mg/dl	Diarrhea > 500 ml	Mild decrease
III	++ - +++	6,1 - 15 mg/dl	> 1000 ml	Marked decrease
IV	++ - ++++	> 6,1 mg/dl	> 1000 ml	Severe decrease

Cell Ther Transplant. 2012;2:e.000089.01. doi:10.3205/ctt-2012-en-000089.01-table2

Chronic GVHD

Acute GVHD may resolve completely with immunosuppressive treatment or it may lead to chronic GVHD. Chronic GVHD may also develop de novo without prior acute GVHD within a year from transplantation. Chronic GVHD involves most frequently the skin with lichenoid and sclerotic changes, the nails with dystrophy, the eyes with keratoconjunctivitis, the mouth with dryness and paradontosis, the vagina with dryness and sclerosis, liver and lungs. The clinical features of chronic GVHD resemble autoimmune diseases like lupus erythematodes, Sjögren syndrome, and biliary cirrhosis in many aspects. Characteristically there is hypogammaglo-bulinemia with loss of IgA, and lymphopenia, but there may also be hypergammaglobulinemia and eosinophilia. Thrombocytopenia is a sign of poor prognosis; another factor of poor prognosis is involvement of the lungs

Thrombocytopenia is a sign of poor prognosis; another factor of poor prognosis is involvement of the lungs, which may be in the form of late interstitial pneumonitis and fibrosis or obliterating bronchiolitis. As a rule lung involvement is progressive and carries the risk of severe infections. The skeletal system may be involved in form of fasciitis, muscle dystrophy, tendinitis, and contractures. Transplant vasculopathy is a problem of solid organ transplants: in stem cell transplanted patients vasculitic changes in the CNS have been observed and vascular events can be seen in young patients [56] without other risk factors.

Overlapping GVHD

Besides the clinical features, acute and chronic GVHD have been defined by the time of oc-currence: acute GVHD in the first weeks and months, and chronic GVHD after day 100. This definition has been challenged by the introduction of cyclosporine A for immune suppression and conditioning with reduced intensity. Following discontinuation of cyclosporine A, a flare of acute GVHD may occur, and following reduced intensity conditioning, acute GVHD may occur late. Similarly, late onset of acute GVHD has been observed after prophylactic treat-ment with TNF-antibody during conditioning [20]. Obviously the activation of T cells is delayed by reduced intensity conditioning and prophylactic treatment, with TNF-antibodies leading to late acute GVHD.

Prophylaxis of GVHD

Some form of prophylaxis of GVHD is absolutely necessary even in HLA-identical sibling transplants, as hyperacute GVHD was seen in every patient with engraftment [57]. T cells are responsible for GVHD and depletion of T cells from the transplant was very successful in an-imal models [58,59]. In the clinical setting GVHD could be prevented or suppressed [60,61] effec-tively. Antithymocyte globulin (ATG) has a broad specificity, recognizing not only T cells, but other mononuclear cells as well. The monoclonal antibody alemtuzumab recognizes CD52, an antigen that is present in many leukocytes including lymphocytes, monocytes, and den-dritic cells; alemtuzumab has broad activities despite its specificity. In humans [62] as in dogs [63] the number of clonable T cells should be below 10^5/ kg body weight for effective prevention of GVHD. So far more selective depletion of T cells has not improved the overall results of transplantation [64], and depletion of CD8 has been insufficient in preventing GVHD [65]. CD6 has the advantage of sparing most of the NK cells in the transplant [64]. In dogs CD6-depleted marrow suppresses alloresponses [66] and recipients of CD6-depleted marrow tolerate donor lymphocyte transfusions earlier than recipients of marrow treated with absorbed ATG [33].

However, the advantage of ex vivo T cell depletion was offset by a high rate of graft rejection, relapse, infections, and EBV-associated post transplant lymphoproliferative disease (PTLD) [67,68]. Treatment of the patient prior to transplantation with ATG prevents rejection; T cell anti-bodies persist in the patient for 4–5 weeks and deplete T cells of the graft in vivo. A rando-mized study comparing standard post-grafting immune suppressive treatment with and with-out ATG prior to transplantation showed lower rates of acute and chronic GVHD in the group treated with ATG [69]. A beneficial effect of ATG in the conditioning treatment for chronic GVHD has also been observed in Italian studies [70] and in retrospective analyses of non-randomized studies (own unpublished observations).

Alemtuzumab also persists in the patient for a prolonged period of time, and reconstitution of T cells is delayed for 6–9 months [71]. Severity of GVHD is low in patients treated with alemtu-zumab, but graft failures have been observed [72]. There is also an increased risk of viral infec-tions, particularly cytomegalovirus, and insufficient response of the malignant disease. These deficiencies can be compensated at least partially by the transfusion of donor lymphocytes [73].

In the last decade G-CSF mobilized peripheral blood stem cells (PBSC) have replaced mar-row in most instances. PBSC contain enormous amounts of T cells and depletion of T cells has been largely unsuccessful. Surprisingly, transplantation of PBSC is not associated with an increased risk of acute GVHD, but is instead associated with a more rapid engraftment and an increased risk of chronic GVHD [74]. PBSC may be preferable for patients with advanced disease and elderly patients. Conversely, T cell depletion and marrow transplantation may be the preferred treatment for patients with early disease, non-malignant disease, and patients who are younger.

Other approaches to prevent GVHD use specific conditioning regimens [37] or specific cells to induce transplantation tolerance. Low dose total lymphoid irradiation in combination with ATG may spare natural killer T cells in the marrow and regulatory T cells suppressing GVHD, but allow graft-versus-leukemia/lymphoma effects. The addition of regulatory T cells to the graft has suppressed GVHD without inhibiting GVL effects in mice [75] and recently in humans (Martelli F, Plenary session ASH 2009). Another immunosuppressive cell product are me-senchymal stromal cells, which have been successful in the treatment of severe GVHD [76]. Co-transplantation of mesenchymal stromal cells prevented rejection in HLA-haploidentical transplants [77] and GVHD was less severe, but the difference did not reach significance be-cause of low numbers. We have used CD6-depleted PBSC transfused 6 days after trans-plantation of unmodified marrow from HLA-haploidentical donors with a low rate of acute GVHD [78].

Post-graft immunosuppressive treatment with either methotrexate or cyclophosphamide has been used since the early days of stem cell transplantation. Both agents preferably kill proli-ferating cells and should be started early after grafting. These drugs suppress donor cells proliferating in response to host antigens as well as residual host cells responding to the graft. They sustain engraftment and suppress GVHD at the same time. They induce transplantation tolerance by killing the responsive cells, and therefore patients with incomplete responses usually take a disastrous course. A recent application of this principle is the use of large doses of cyclophosphamide 3 and 4 days after HLA-haploidentical transplantation [35,79].

The introduction of the calcineurin inhibitors cyclosporine A and tacrolimus has also changed the outlook for these patients. Both drugs inhibit the activation and proliferation of T cells by inhibiting dephosphorylation and translocation of the nuclear factor of activated T cells (NFAT). The continuous inhibition is effective in suppressing GVHD and rejection, but the effect is not necessarily maintained after discontinuation of treatment; calcineurin inhibitors are less potent in the induction of transplantation tolerance [80]. Treatment should be started prior to transplantation in order to avoid antigen recognition and T cell activation. Tacrolimus is a somewhat stronger immunosuppressive than cyclosporine A and possibly less neurotoxic. However, in controlled studies comparing tacrolimus and cyclosporine A less severe GVHD was not associated with improved survival [81].

The combination of cyclosporine A and methotrexate is better than either drug alone [82]. It has become the gold standard of GVHD prophylaxis. In recent years mycophenolate mofetil (MMF) has been introduced to replace methotrexate [83]. MMF inhibits the purine synthesis and the de novo pathway of guanosine nucleotide synthesis; it kills not only proliferating T cells, but also T cells in the interphase. MMF produces less mucositis and less marrow toxicity than methotrexate. However the best regimen and timing (2–3 times per day) remains unknown.

Sirolimus binds to the tacrolimus binding protein FKBP12 and forms a complex with mTOR (target of rapamycin) that inhibits several signal transduction pathways including PTEN, PI3kinase and AKT as well as the JANUS kinase pathway. Thereby it produces several ef-fects including immunosuppression of T cells, anti-angiogenesis and inhibition of tumor growth [84]. Its immunosuppressive activity is presumably linked to the suppression of the second signal of T cell activation. This way T cell apoptosis and specific peripheral non-responsiveness may be induced [85]. Th1 cells and their cytokines are more affected by siroli-mus than Th2 cells and regulatory T cells [86,87]. The sirolimus/mTOR complex inhibits the ac-tivation signals of CD28 and CD40L stimulation and thereby the second signal essential for T cell activation [88], a situation that may lead to transplantation tolerance. The combination of sirolimus and tacrolimus is synergistic and has shown little toxicity [89], but veno-occlusive dis-ease of the liver and thrombotic microangiopathy have been observed [90]. The combination of sirolimus and MMF was promising in a smaller group of patients, where VOD and TMA were not observed [91].

The goal of preventing GVHD is the induction of tolerance in both directions, the host-versus-graft and graft-versus-host direction. Contrary to transplantation of solid organs, stem cell transplantation induces self-sustained tolerance without life-long immunosuppressive therapy. As a rule, a period of 4–6 months of immunosuppressive therapy is sufficient for tolerance to become established. In clinical terms tolerance is evident by persistent chimerism without GVHD and without severe infections more than 30 days after discontinuation of immunosuppression.

Treatment

Glucocorticoids

Despite prophylactic treatment with immunosuppressive agents, acute GVHD requiring addi-tional treatment occurs in 40–80% of patients within 3–4 weeks of transplantation [92]. Corti-costeroid therapy is the standard of treatment for acute GVHD, but the regimen and the do-sage is still under discussion. Originally, treatment with large doses was favored [93], but there are no controlled studies to support this treatment. Similarly, in organ transplantation, rejection crises are treated with bolus methylprednisolone without prospective randomized trials supporting this. Despite this general use there are only a few studies on the schedule and the dosage rates. A randomized Italian trial comparing 2mg/kg per day with 10mg/kg per day showed no advantage for the higher dose [94], however 50% of patients were switched to a high dose because of insufficient response. Recently, a retrospective study from Seattle indi-cated that even lower doses of corticosteroids (1mg/kg instead of the standard 2 mg/kg) can be given without disadvantage [95]. However the patients of the low dose group had more fa-vorable risk factors and less severe GVHD; in addition oral non-absorbable corticosteroids were given more frequently.

The mechanisms of the actions of glucocorticoids are still not fully understood, lymphopenia is mainly due to sequestration of lymphocytes, and less to lympholysis. However, glucocorti-coids exhibit strong anti-inflammatory effects in several ways including genomic and non-genomic pathways [96]. Glucocorticoids are bound to a receptor from which heat shock protein 70 is released. The glucocorticoid complex activates anti-inflammatory proteins directly and their production genomically. Inhibition of nuclear factor kB is highly sensitive to glucocortico-ids preventing the production of inflammatory proteins. Sensitivity to the treatment with glu-cocorticoids may be determined by the relative levels of glucocorticoid receptor α and ß. This may explain interpatient variation of sensitivity [97]; memory T cells [98] as well as mature den-dritic cells are less sensitive to glucocorticoids. In macrophages low doses of glucocorticoids stimulate the production of proinflammatory cytokines, whereas high doses suppress it [99]. High dose glucocorticoid therapy given for few days has shown little immune suppression in vivo [100].

Commonly treatment is started in patients with clinical grade II–IV severity of GVHD. About 40–50% of patients respond with resolution or improvement of clinical symptoms [92]. The re-mainder are classified as "steroid-refractory". The time until refractoriness to glucocorticoids is stated may vary from 5 to 14 days [101]. Many centers increase the dose of steroids in re-fractory patients prior to the addition of other agents. We prefer to start with rather high doses of glucocorticoids (1–2mg/kg every 8 hours) and score the response after three days of treatment for refractoriness. This way we initiate secondary treatment early in refractory pa-tients. The decision to start the treatment is made by two physicians. In the case of a pro-gressive and characteristic skin rash the diagnosis is not difficult, but in cases of isolated ga-strointestinal GVHD with diarrhea and nausea or

rash the diagnosis is not difficult, but in cases of isolated ga-strointestinal GVHD with diarrhea and nausea or isolated hepatic GVHD the diagnosis may be more difficult. Persistent toxicity of the conditioning treatment, veno-occlusive disease of the liver, drug-induced changes and viral infections are considered as differential diagnosis. In our centre skin biopsies are regularly performed, biopsies of gut and liver are only made in patients that do not respond to the treatment. This way we obtain not only histological con-firmation of the clinical suspicion, but also information about viral infection. Concomitant vi-rostatic treatment is given to patients with biopsies positive for viral infection as well as those that are seropositive for cytomegalovirus. Another option is the use of high doses of iv immu-noglobulins that may inhibit the deleterious effects of FAS by their blockade of FAS-L [102]. Al-though their immune modulating effects are far from understood [103], 20–30% of patients with skin GVHD do respond to the treatment with iv immunoglobulins. In any case early treatment is important as delay of the start of treatment until the results of laboratory investigations are available may jeopardize the response to glucocorticoids.

The effect of systemic glucocorticoids on gastrointestinal GVHD can be improved by local treatment with beclomethasone [104] and budesonide [105].

Antibodies

In many instances the first choice in patients with steroid refractory GVHD has been immu-nosuppressive antibodies. Antithymocyte globulin (ATG) has been used in several uncon-trolled studies with some success [106], but in controlled studies a beneficial effect could not be demonstrated [107]. Similarly, OKT3 is a monoclonal antibody against CD3 on T cells: it dep-letes T cells and stimulates proliferation by its mitogenic activity. Even though many patients have responded to the treatment with OKT3 with complete remission of GVHD, better surviv-al could not be demonstrated in controlled clinical trials [108]. Alemtuzumab has been used mainly for prophylaxis of acute GVHD by treating the patient in vivo or the graft prior to transplantation ex vivo: recently beneficial outcomes of treatment of established GVHD have been reported in two uncontrolled studies [109,110]. Viral infections may complicate treatment with alemtuzumab; therefore regular control and pre-emptive treatment is necessary. ATG and OKT3 both stimulate proliferation of lymphocytes that are not killed by cytolysis; there-fore the combination of antibody treatment with chemotherapy (methotrexate, Cyclophos-phamide, mycophenolate mofetil, etc.) may be beneficial. A humanized CD3-antibody (visili-zumab) produced good first results [111] which unfortunately were not confirmed in multicenter trials [112]. In those patients the reactivation of EBV and the incidence of post transplant lym-phoproliferative disease (PTLD) increased.

Encouraging results were also reported with ABXCBL, an antibody against CD147 that is ex-pressed in activated T cells [113]. However in a comparative study ABXCBL was not better than ATG, where survival was even inferior [114].

Antibodies against tumor necrosis factor α (TNF-a) and soluble receptors of TNF-a (etaner-cept) have been studied in the prophylaxis of GVHD [20] and the treatment of steroid refractory GVHD [115]. There has been a high rate of complete response to infliximab even in gastrointes-tinal GVHD, but this is complicated by an increased risk of fungal infections [116,117]. Contrary to infliximab etanercept neutralizes soluble TNF-a without affecting TNF-a in phagocytic cells. Etanercept is associated with a lower risk of fungal infections. The combination of etanercept with an anti-IL2-receptor antibody showed high response rates to acute GVHD, but the long-term survival was rather poor [118]. In comparison, a pilot trial of etanercept in combination with tacrolimus and steroids gave a 75% complete response and a 50% survival rate [119]. When comparing etanercept combined with steroids to steroids alone a significantly better response to the combination was observed [120]. The combination of etanercept with ATG and tacrolimus was compared to ATG and tacrolimus alone [121]; considering the limited number of patients the response and the survival of patients given etanercept was better. Neutralization of TNF-a released by the ATG treatment by etanercept may have been contributing to the better outcome.

Antibodies against IL-2 receptor have been studied early [122] with some transient success. The importance of an early treatment start was stressed. Several studies with humanized anti-IL2-receptor antibodies were encouraging [123,124], but a randomized study was stopped prematurely because of inferior survival of the antibody (daclizumab) group [125]. There is little doubt that the IL2- receptor antibody is effective in suppressing GVHD of the skin and the gut when started early, but it may have an adverse effect on the generation of regulatory T cells expressing high levels of the IL-2 receptor.

Alefacept is a fusion protein of the CD2-binding domain of LFA-3 and the Fc portion of IgG with specific activities against memory T cells [126]. Promising results in steroid refractory acute GVHD and in chronic GVHD have been reported, but there may be an increased risk of viral and fungal infections [127].

Recently, the role of B cells has been discussed more frequently, although the role of T cells in GVHD is not disputed. However, cytotoxic antibodies may be produced in HLA-mismatched chimeras, and depletion of B cells may prevent EBV-induced B cell lymphoma. Single patients have been reported to show a response to steroid refractory GVHD to the treatment with rituximab [128].

Drugs

As a rule the treatment given for prophylaxis is continued during the treatment of established GVHD, and includes glucocorticoids at a low level. Depending on the prophylactic regimen, cyclosporine A may be substituted by tacrolimus and new drugs may be added. In most Eu-ropean centers a calcineurin inhibitor is combined with methotrexate or mycophenolate mofe-til. In patients not treated prophylactically a trial with mycophenolate mofetil may be justified; a response rate of 47–48% has been reported in steroid refractory GVHD, but the survival at 6 and 12 months was not improved [129]. Methotrexate as well as basiliximab

GVHD, but the survival at 6 and 12 months was not improved [129]. Methotrexate on a weekly basis in low doses has been helpful in single cases. Mucositis and myelosuppression are limiting factors.

Similarly, sirolimus can be used for patients not treated prophylactically, as response rates of 77% overall and 44–72% complete response have been reported [130,131]. Again microangiopa-thy has been a problem, but could be controlled by discontinuation of the calcineurin inhibitor (CNH) or both sirolimus and CNH. A small study suggests a good response of acute GVHD to sirolimus without prior treatment with glucocorticoids [132]. Due to its anti-tumor activity siro-limus is preferred to calcineurin inhibitors and glucocorticoids by many investigators [133], par-ticularly in patients with lymphoma [134].

Pentostatin is an inhibitor of the salvage pathway of thymidine kinase that is specific for T cells. Phase I studies have shown efficacy in the treatment of steroid-refractory GVHD [135]. A retrospective analysis has shown activity comparable to other immunosuppressive regimens [136]. However, pentostatin has shown activity in the treatment of chronic GVHD [137,138]. Pentostatin may have better effects in patients with chronic GVHD.

Thalidomide [139,140] and more recently lenalidomide [141] have been studied in the treatment of GVHD. The initially positive results of treatment with thalidomide in chronic GVHD [139] were not confirmed in a randomized study [140]. The treatment of recurrent myeloma with lenalido-mide suggested an immune modulatory effect of lenalidomide in producing regulatory T cells [141].

Bortezomib has been tested in mice [142] and patients with HLA-mismatched unrelated donors [143]. The immunomodulatory effect has been related to the suppression of monocyte-derived dendritic cells and modified antigen presentation and release of TNF-a from CD4-positive T cells [142]. It has shown promising activity in the prophylaxis of GVHD [143].

After the description of activating antibodies against the receptor of platelet derived growth factor (PDGF) [144] in patients with systemic sclerosis similar antibodies were found in patients with sclerodermatous chronic GVHD [145] and several groups have treated sclerodermatous chronic GVHD [146,147], as well as obliterating bronchiolitis with imatinib [148,145]. In one study more than 70% of patients with sclerotic chronic GVHD responded with partial and complete remissions [147].

Cells

Many treatment regimens of GVHD favor the development of regulatory T cells characterized by the expression of CD 4 and CD25 in high density [149]. The suppressive activity is limited to cells of the CD4/CD25 immune phenotype that are positive for FoxP3 mRNA. Typically regulatory T cells should be negative for the IL7 receptor (CD127). Immunomagnetically selected regulatory T cells have been tested in vitro for immunosuppressive effects [149,150], and preliminary applications for the treatment of refractory GVHD have been promising (M. Edinger, pers. comm.). The first results of preventive application have been reported (Di Ianni et al. ASH 2009); 17 of 20 evaluable patients did not produce GVHD after HLA-haploidentical stem cell transplantation despite admixture of a limited amount of conventional T cells to the CD34-selected graft.

More information is available on the treatment of refractory GVHD with mesenchymal stromal cells [76]. The results were confirmed in a multicenter study of the EBMT involving [151] 55 patients with steroid-refractory GVHD. Twenty-seven patients received one dose, 22 two doses and 6 three doses and more from HLA-mismatched or HLA-matched donors for treatment; 30 patients had a complete response, and an improvement was seen in 9 patients. Responders had a better chance of survival than non-responders. Mesenchymal stem cells have multiple properties including differentiation into bone, cartilage, tendon and muscle cells, repair of damaged tissue and modulation of immune responses [36].

UV light

Ultraviolet light has immunosuppressive properties [152]. UV-A in combination with 8-methoxypsoralen (PUVA) has been used to treat chronic GVHD [153]. UVA may be applied to the skin in combination with oral psoralen or with a bath in psoralen containing water. PUVA treatment was studied in 103 patients with steroid-resistant acute GVHD [154] with good res-ponses in GVHD of the skin and sparing of glucocorticoid doses. The treatment was well to-lerated, but it may induce a flare before lichenoid skin changes respond to the treatment. In chronic GVHD 31 of 40 patients had an improvement following PUVA treatment, but partial and complete responses were limited to the skin [155]. Best responses were seen in the liche-noid phases of chronic GVHD, and less in the sclerodermatous phases. However, the com-bination of PUVA bath with oral isotretinoin has been effective in a small study of scleroder-matous chronic GVHD: 11 of 14 patients responded, four of these with complete remission [156].

Alternatively PUVA may be applied directly to the blood resp. leukocytes separated by a dis-continuous blood cell separator (extracorporeal photopheresis, ECP). Responses to ECP have been reported for steroid-refractory, acute GVHD [157-159] and chronic GVHD [160]. Complete resolution of acute GVHD of the skin in 82%, liver in 61% and gut in 61% of pa-tients has been reported [158]. Response was associated with better survival. In our own study of 30 patients with acute GVHD, 20 patients responded with CR and PR defined as steroid discontinuation and reduction to 10 mg or less per day respectively (unpublished). Eleven of 20 responders survived as compared to only one non-responder. Steroid treatment was a major risk factor in the treatment of acute GVHD of pediatric patients [161]. In a single centre study on steroid refractory chronic GVHD 22% of patients could discontinue steroid therapy after one year, with response to ECP and absence of thrombocytopenia being the favorable factors for survival [160]. A randomized prospective multicentre study [162] comparing standard treatment with standard treatment plus ECP showed improvement of the skin score and sig-nificant steroid sparing. ECP is a good treatment option in patients with steroid-refractory acute and

chronic GVHD with little side effects. The mechanism of the immunosuppression by ECP is not completely understood as only 5–10% of all T cells may be reached by extra-corporeal irradiation. However, a shift of dendritic cells from activating DC1 to down-regulating DC2 and from Th1 to Th2 has been described in the course of ECP [163]. Ex vivo a decrease of T cells producing pro-inflammatory cytokines was described [164]. In a murine model ECP-treated T cells induced regulatory T cells in recipients with established GVHD [165]. An increase in the proportion of regulatory T cells was observed in patients that responded to ECP [166]. Therefore ECP may be one method to induce GVH-tolerance without too many side effects.

Induction of graft-versus-host tolerance

Unlike transplantation of solid organs, transplantation of hematopoietic stem cells induces transplantation tolerance, enabling immunosuppressive therapy to be discontinued. In the form of central tolerance, lymphoid progenitor cells derived from transplanted stem cells tra-vel to the thymus where T cells tolerant to the host's tissue are produced [167-169]. However, the thymus shows progressive involution in adulthood; central tolerance may be the major form of tolerance in children and young adults. The majority of patients subjected to stem cell transplantation are older, and the thymus has shrunk to a small remnant. Therefore in the majority of our patients a peripheral form of tolerance prevails, but function of the thymus can be recovered even in elderly individuals [170]. Several studies have been performed to speed up recovery of the thymus, mostly without convincing success [171], but new agents may give better results [172,173,169]. However, GVHD may affect the thymus [174] and thereby may inhibit the induction of central tolerance in both young and adult patients. Peripheral tolerance is a first step and may be replaced by central tolerance with time. The mechanisms of tolerance may be similar, clonal deletion, clonal anergy, and suppression.

Clonal deletion is a mechanism of self tolerance occurring in the thymus [175]; in the case of allogeneic stem cell transplantation T cells of donor origin derived from lymphoid progenitors may be eliminated by the same mechanism and primed towards host MHC antigens in se-miallogeneic hosts [176]. Deletion in the periphery may be accomplished by the treatment with antimetabolite drugs such as methotrexate, or cycle active drugs like cyclophosphamide; both of which have been shown to induce tolerance in stem cell transplanted patients [177,178]. The principle of selective depletion of responsive lymphocytes has been applied more recently in HLA-haploidentical transplantation [179]. Unlike these cytotoxic agents calcineurin inhibitors do not kill the responsive cells, but inhibit cytokine production and thereby the progress of the immune response. However they may not favor the induction of tolerance; flares of GVHD have been observed after discontinuation of cyclosporin A, and late rejection of marrow grafts have been reported in single patients with aplastic anemia. Activation induced cell death (AICD) is a natural decrease of the clone size by IFN-g secretion of mature Th1 T cells and death of immature T cells, which may be achieved by the external pathway.

Clonal anergy may be the result of competitive inhibition by anergic T cells or active sup-pression by a variety of suppressor cells. Formerly, CD8-positive T cells were considered "cytotoxic/suppressor" cells, but the evidence for specific suppression was weak. Instead, several mechanisms of suppression have been described including "veto" cells suppressing the immune reaction against themselves [180]. The veto mechanism, described as the effector cells inhibiting or killing themselves has been primarily ascribed to CD8-positive T cells, but later also to other cells including stem cells. CD8-positive suppressor cells may not only func-tion as veto cells, but they may also suppress third party reactions by the secretion of FAS [181]. Other cells with suppressor function are myeloid derived suppressor cells [182], NKT cells in the marrow [183], NK cells [184], dendritic cells type 2 [185] and mesenchymal stromal cells [76]; all of them suppress activated T cells more or less specifically. Some of these have already shown clinical effectiveness [183,76], others are still in a developmental state. In recent years the detection of FoxP3 (forkhead transcription factors) showed suppressive function of CD4, CD25 positive T cells and even CD8 T cells [186]. Naïve CD4, CD25-positive regulatory T cells are able to down regulate allogeneic immune responses without inhibiting graft-versus-leukemia responses [75]. These may be naïve and non-specifically down regulating dendritic cells or adaptive and directed against specific antigen. Recently the Perugia group has reported the use of naïve regulatory T cells suppressing GVHD in patients given HLA-haploidentical transplants including small amounts of conventional T cells (ASH 2009, New Orleans).

Rapamycin exerts differential effects on T cells, inhibiting CD8 positive cells more than CD4 positive cells [86]; CD4 T cells spared by Rapamycin may become regulatory T cells without compromising GVL reactions [133]. Long-term observations of patients treated with Rapamycin and tacrolimus are encouraging with regard to control of acute GVHD and GVL [89]. Chronic GVHD still remains a problem despite tolerogenic effects of Rapamycin. Recently, the Milan group [187] (EBMT 2010) has reported generation of regulatory T cells in patients with HLA-haploidentical transplants. After conditioning with treosulfan, fludarabine, ATG and rituximab, and GVH prophylaxis with Rapamycin and mycophenolate mofetil, immune reconstitution was better than after transplantation of CD34-selected transplants, and regulatory T cells were detected early after transplantation.

Tolerogenic effects have also been described for the treatment with extracorporeal photo-pheresis (ECP) [188]. In acute GVHD ECP was applied with good results [158]. In most patients the effects of ECP are not immediate, but occur after some weeks. ECP has also beneficial effects against chronic GVHD [162] and may be preferable to other treatments for GVHD.

The main goal of prophylactic and therapeutic treatment of GVHD should be the induction of transplantation tolerance. Therefore treatment protocols interfering with tolerance should be avoided in protracted periods in favor of regimens allowing the development of tolerance. Glucocorticoids and calcineurin inhibitors are effective in controlling the acute disease, but they do not support the development of tolerance. Similarly, IL2-R antibodies may be effec-tive in the acute control of GVHD, but may not support the development of tolerance. Toler-ance may be achieved by depletion of mature T cells from the graft, killing of antigen respon-sive T cells with cell cycle active chemotherapy as Cyclophosphamide, methotrexate, or my-cophenolate mofetil, activating CTLA4 receptors by CTLA4-Ig or using drugs like Rapamycin that block the co-stimulatory pathway or ECP producing apoptotic cells that induce tolerance.

Future prospects

The time point to initiate treatment of acute and chronic GVHD is of paramount importance. Therefore, early diagnosis tests, before clinical diagnosis is possible, may improve the out-come significantly. Several proteins have been found in the urine of patients that developed GVHD [189]; a prospective study will help to demonstrate the value of early treatment. Similarly, elafin has been identified as a prognostic marker in the plasma of patients developing skin GVHD [190]. Early diagnosis will allow early treatment and thereby avoid the development of memory T cells or T stem cells with memory that are extremely difficult to suppress.

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