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Determination of Th1/Th2/Th17 Cytokines in Patients Undergoing Allogeneic Hematopoietic Stem Cell Transplantation

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1. Introduction

Allogeneic hematopoietic cell transplantation emerges as a therapy option to treat the sequels of exposure to radiation, a great concern at the beginning of the atomic age and cold war (Welniak et al., 2007). Hematopoietic cell transplantation emerged as a rescue strategy since there were already antecedents, like the study of Lorenz et al. in 1952 (as cited by Welniak et al., 2007), who showed that infusion of the bone marrow after lethal irradiation healed radiation disease in mice. This lay the foundations for the current consideration of allogeneic hematopoietic cell transplantation as the first-line therapy for many life-threatening oncological and hematological diseases. Today, it is primarily used to treat patients with hereditary anemias or immunological deficiencies through replacement of the hematopoietic system with cells from a healthy individual. It also allows cancer patients to be treated with myeloablative radiation and/or chemotherapy (known as myeloablative conditioning) in an attempt to eliminate tumoral cells, and although this strategy brings loss of bone marrow function, the latter can be recovered with infusion of normal hematopoietic cells (Jenq & Van den Brink, 2010).

2. Hematopoietic stem cell transplantation

Hematopoietic stem cell transplantation is today a simple procedure involving infusion of these cells intravenously. Once in the bloodstream, stem cells are able to migrate to the bone marrow in order to thus restore hematopoiesis during the first two weeks following transplantation (Léger & Nevill, 2004).

Stem cells giving rise to hematopoietic cells are known as hematopoietic stem cells due to their capacity for self-renewal, division and differentiation into a variety of specialized hematopoietic cells. This is the principle underlying hematopoietic stem cell transplantation (Hows, 2005).

One of the major difficulties in using hematopoietic stem cells has been their identification, since they are morphologically very similar to lymphocytes. This problem was solved with the use of biomarkers such as CD34, a transmembrane glycoprotein expressed by hematopoietic stem cells which is currently the main biomarker used to identify these cells.
Hematopoietic cell transplantations are classified as isotransplantation, allotransplantation or autotransplantation. These are not the only types of transplantation, but they are the most commonly used in medical practice. It calls allotransplantation when donor and recipient are the same species but are not identical twins. The advantages of this type of transplantation compared to the allogeneic type are that the cells infused are normal cells and therefore the incidence of relapse is lower, as well as the fact that the graft cell is infused with immunocompetent cells that are able to induce a graft-versus-leukemia effect. The major concern with this type of transplantation is development of graft-versus-host disease (GVHD) or infections caused by opportunistic microorganisms, since patients are treated with immunosuppressant drugs (Léger & Neville, 2004; Vela-Ojeda et al., 2005).

2.1 Hematopoietic stem cell mobilization by G-CSF

At present peripheral blood hematopoietic stem cells are preferably used since grafting (particularly of blood platelets) is faster. This procedure is not excessively invasive – as is bone marrow procurement – and better results are obtained when mobilized peripheral blood is used as the hematopoietic stem cells source (Jaime et al., 2004). Normally, peripheral blood contains only a small amount of hematopoietic stem cells (<0.1% of nucleated cells). Different methods are therefore used to induce their egress from the bone marrow into the bloodstream in order to be able to collect them by apheresis for subsequent infusion in the patient. Hematopoietic stem cells mobilization was an innovative development in the 1990s, in particular after it was seen that the number of stem cells obtained from mobilized peripheral blood contained 1-log more lymphocytes than the number obtained from bone marrow (Champlin, 2000). The established, widely-used method of mobilization involves the use of G-CSF, which induces mobilization by initiating a stress process through neutrophil and osteoclast activation. This results in dissociation of the cell membrane unions between stem cells and the stroma cells as well as stem cell proliferation and activation and/or adhesion molecule degradation. Hematopoietic stem cells mobilization is also seen when chemotherapy is exclusively used (Devetten & Armitage, 2007).
The mechanism through which G-CSF mobilizes $CD34^+$ hematopoietic stem cells from the bone marrow into the peripheral blood involves a series of steps. First of all, there is increased hematopoietic stem cell proliferation followed by exit of these cells from the bone marrow. Increased proliferation has been shown to occur with cytokines such as GM-CSF that temporarily increase the cell adhesion of $CD34^+$ hematopoietic stem cells to the bone marrow stroma, a process that in turn increases cell proliferation. The mobilization of hematopoietic stem cells from the bone marrow to peripheral blood comprises several mechanisms. One hypothesis is modification of the cellular interactions occurring between hematopoietic stem cells and the bone marrow stroma. Analyses of peripheral blood mononuclear cells mobilized by G-CSF reveal a decrease in the expression of VLA-4 (*Very late antigen 4* [CD49d/CD29]) integrin which normally binds firmly to its ligand VCAM-1 (*Vascular cell adhesion molecule-1*) as well as to an extracellular-matrix fibronectine fragment. Other molecules in which a marked decrease occurs are LFA-1 (*Leucocyte functional antigen 1*

[CD11aCD18]) and c-kit. These molecules are expressed in most hematopoietic stem cells and are involved in binding of these cells to bone marrow stroma, a process that is expressed by the ligands of cells of the latter VCAM-1, ICAM-1 (*Intercellular adhesion molecule-1*) and ICAM-2 for LFA-1 and c-kitL. G-CSF can also initiate mobilization through neutrophils, by secretion of gelatinase B, breaking extracellular matrix molecules and

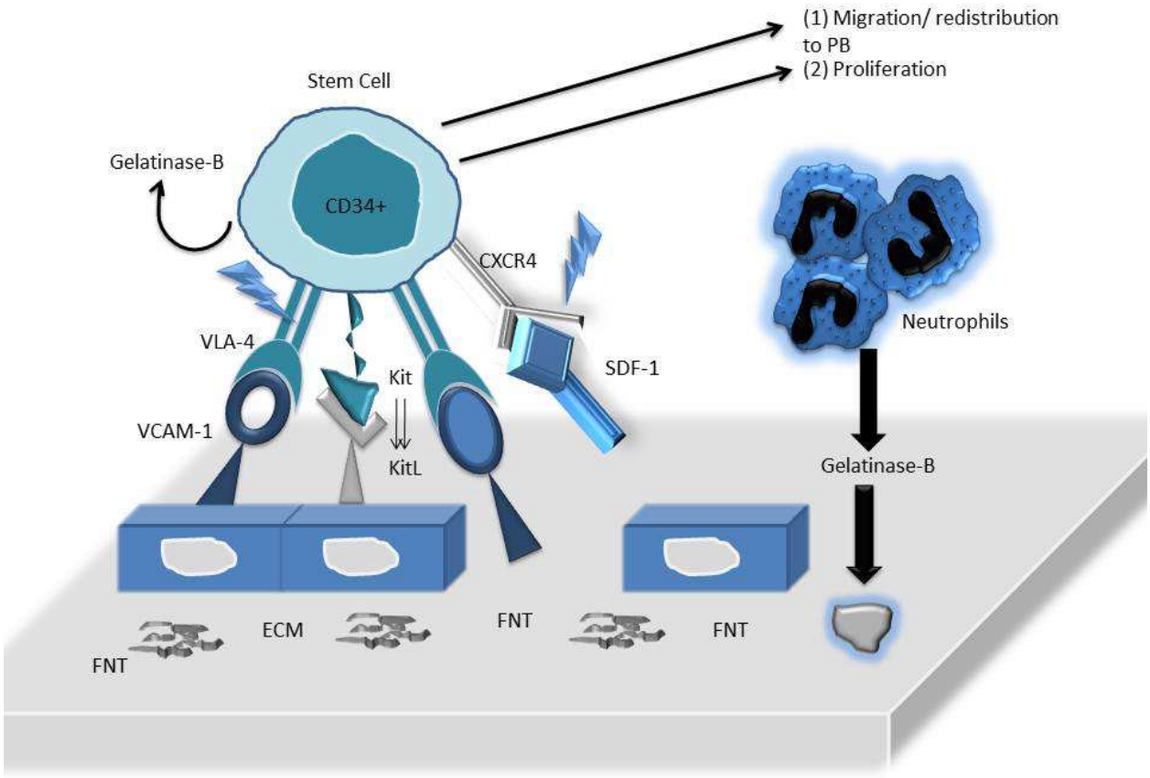

Fig. 1. Mechanism of hematopoietic stem cell mobilization from bone marrow to peripheral blood by stimulation with G-CSF. This process involves several steps, including modification of interactions occurring between stem cells and the stroma. Affected interactions include decreased expression of VLA-4 integrin, which normally binds to its ligand VCAM-1 and to FNT. Another molecule in which expression is decreased is c-kit; this process reduces the bond between this molecule and its ligand kit-L-. Decreased expression of these molecules reduces the bond between stem cells and the stromal cells. Another means by which G-CSF promotes stem cell mobilization is through neutrophils, by releasing gelatinase B, breaking extracellular matrix molecules and weakening adhesive interactions between stem cells and stroma cells. Yet another mechanism is stem cell secretion of gelatinase B, promoting faster migration of these cells to peripheral blood. A further mechanism involves indirect interaction of G-CSF with ligands, receptors/factors and stimulation of stem cell proliferation. PB: peripheral blood. G-CSF: granulocytic-colony stimulating factor. VCAM-1: vascular cell adhesion molecule-1, VLA-4: very late antigen 4 [CD49d/CD29] FNT: fibronectina (Modified from Gyger et al., 2000)

weakening adhesive interactions between stem cells and stroma cells. Stem cells have also been shown to secrete gelatinase B, a mechanism which may improve migration of these cells to peripheral blood (Figure 1). Finally, experimental evidence indicates that G-CSF may interact indirectly with a stem cell ligand to stimulate proliferation of these cells (Cashen et al., 2007; Cottler-Fox et al., 2003; Gyger et al., 2000).
In recent years, it has been demonstrated that G-CSF is also able to break one of the most important interactions between stem cells and stromal cells, which is formed by CXCL12 (formerly called SDF-1) and CXCR4 (Cashen et al., 2007).

2.1.1 Which stem cell source is best: bone marrow or mobilized peripheral blood?

This is one of the most commonly asked questions, since using mobilized peripheral blood rather than bone marrow in allogeneic transplantation is increasingly frequent. A retrospective study by Champlin et al. in 2000 examined the evolution of 288 allogeneic transplants in which mobilized peripheral blood was used and 536 in which bone marrow was used. They were followed-up during one year. The study found that in patients who had received mobilized peripheral blood, neutrophil engraftment was faster (14 days vs. 19) as was also platelet engraftment (19 days vs. 25). No significant differences were observed in development of acute GVHD. Chronic GVHD development was significantly higher in patients receiving mobilized blood, with a mean of 65% vs. 53%. The incidence of relapse did not differ significantly. Treatment-related mortality and leukemia-free survival were higher with mobilized blood transplants and hospitalization time was shorter. Additional studies similar to this one examining the benefits and drawbacks of allogeneic hematopoietic cell transplantation set the basis for mobilized peripheral blood being currently the most commonly used source in hematopoietic stem cell transplantation.

2.2 Points to consider before hematopoietic stem cell transplantation

When considering transplantation in patients in remission, two important aspects should be taken into account: whether medical evidence indicates that hematopoietic stem cells transplantation is more likely to heal the disease than other therapy forms and whether a suitable donor is available as a stem cell source. Although these are the major aspects to consider they are not the only ones. Other factors requiring consideration include biological characteristics at diagnosis, the specific disease that is being treated, presence of comorbidities that may complicate transplantation, and patient age (Deeg, 2010; Léger & Nevill, 2004).

2.3 Immunological typing of human leukocyte antigens

One reason for the progress that has taken place in hematopoietic cell transplantation is human leukocyte antigen (HLA) immunotyping. This is one of the major points to consider in allogeneic hematopoietic cell transplantation since, as already stated, it is important to have a suitable donor.
HLA proteins were first identified in 1950 by Jean Dausset upon observing that in many individuals, particularly those who previously received multiple blood transfusions or who were multiparous women, blood serum contained antibodies that reacted against a new kind of glycoprotein present on the outer surface of leukocytes of other members of the population; these glycoproteins were named human leukocyte antigens (HLAs). The latter

behave as immunogenic markers making a person's cells distinct and are the major barrier to histocompatibility; they are therefore also called major histocompatibility complex (MHC) molecules. The importance of HLA molecules is not limited to the histocompatibility barrier, they are also essential in T-cell activation since HLA-molecules bind peptides to be presented to T cells. HLA class I molecules present peptides primarily to CD8+ T cells while CD4+ T cells recognize mainly peptides presented by class II molecules (Appelbaum, 2001; Bleakley & Riddell, 2004).

MHC genes are encoded on the short arm of chromosome 6 at locus p21. There are three different groups of genes called HLA-A, HLA-B and HLA-C, which individually code for the α chain of MHC class I. Similarly, there are three loci for the genes of class II MHC molecules known as HLA-DP, HLA-DQ and HLA-DR. Each of these includes genes coding for the α polypeptide chain and at least one β polypeptide chain. A person normally inherits two copies of the locus of each gene, one from each parent. Statistical data provided by the European Bioinformatics Institute (EBI) and the International Immunogenetics Organization Database (IMGT) suggest that the number of HLA class I and class II alleles discovered is on the increase. These data indicate that in human population there are 4,946 different class I alleles and 1,457 class II alleles, of which 1,601 are known alleles for HLA-A, 2,125 for HLA-B, 1,102 for HLA-C and 1,027 for HLA-DRβ of which 928 are HLA-DRβ1 alleles (these are the ones most commonly used to determine histocompatibility due to their high polymorphism) (Table 1).

Numbers of HLA Alleles	
HLA Class I Alleles	4,946
HLA Class II Alleles	1,457
HLA Alleles	6,403

HLA Class I

Gene	**A**	**B**	**C**	**E**	**F**	**G**
Alleles	1,601	2,125	1,102	10	22	47
Proteins	1,176	1,641	808	3	4	15

HLA Class II

Gene	**DRA**	**DRB**	**DQA1**	**DQB1**	**DPA1**	**DPB1**	**DMA**	**DMB**	**DOA**	**DOB**
Alleles	7	1,027	44	153	32	149	7	13	12	13
Proteins	2	774	27	106	16	129	4	7	3	5

HLA Class II- DRB Alleles

Gene	**DRB1**	**DRB2**	**DRB3**	**DRB4**	**DRB5**	**DRB6**	**DRB7**	**DRB8**	**DRB9**
Alleles	928	1	57	15	19	3	2	1	1
Proteins	704	0	46	8	16	0	0	0	0

(Modified from http://www.ebi.ac.uk/imgt/hla/stats.html)

Table 1. Statistical data from the European Bioinformatics Institute (EBI) and the International Immunogenetics Organization database (IMGT) showing the number of each of the HLA alleles.

Historically, HLA immunotyping was performed by serological methods but now, with the advent of polymerase chain reaction (PCR), molecular immunotyping of the donor and recipient is possible. A study by Petersdorf et al. in 2001 examined patients who had previously undergone transplantation and were compatible by serological methods. When reexamined by molecular immunotyping, about 30%of these individuals were found to be incompatible in one or more alleles. These differences were correlated with increased GVHD and poor survival, indicating that a compatible donor and reliable HLA immunotyping are extremely important. The number of class I and class II HLA antigens is relatively large and therefore the probability of HLA matching between the recipient and an unrelated donor is extremely small.

2.4 Graft-versus-host disease and Th1/Th2/Th17 cytokines

Graft-versus-host disease (GVHD) may develop after hematopoietic stem cell transplantation. It is a reaction of immune cells from the donor against tissues of the host. Damage induced on epithelial cells of the host by activated T cells occurs after an inflammatory cascade that is unleashed by the conditioning regimen. Approximately 35-50%of allogenic hematopoietic cell transplantation recipients develop GVHD. The risk of developing the disease depends on several factors, primarily the stem cell source and donor cytokines, patient age, existing conditions and GVHD prophylaxis. GVHD involves mainly the skin, liver and gastrointestinal tract. Despite GVHD-related morbility and mortality, its development is often desirable since it has been found to be associated with a lower recurrence of malignant disease, in other words, it is important for establishment of the graft-versus-tumor effect (Ferrara & Levine, 2008; Léger & Nevill, 2004; Saliba et al., 2007; Weisdorf 2007).

The physiopathology of acute GVHD described by Ferrara & Levine (2008) is a three-stage phenomenon. The initial stage involves damage to tissues of the host due to inflammation derived from chemo- and/or radiotherapy during the recipient conditioning. In the second stage, antigen-presenting cells (APC) of both donor and recipient as well as inflammatory cytokines unleash the activation of donor-derived T cells, with expansion and differentiation of the latter into effector cells. Antigens (Ag) of the minor histocompatibility complex have a central role in this activation. The pathway of T-cell activation results in activation of genes coding for cytokines such as IL-2 and interferon gamma (IFNγ). Cells that produce these cytokines are considered to be Th1 profile, as opposed to cells producing predominantly IL-4, IL-5, IL-10 and IL-13 which are considered to be Th2 phenotype and are assumed to be the ones that modulate GVHD. During the third stage, also known as the effector stage, donor-derived activated T cells mediate cytotoxicity against target cells of the recipient through FasL-Fas, perforin and granzyme B interactions as well as additional production of tumor necrosis factor α (TNFα). This cytokine is produced by monocytes and macrophages, and secondarily by T lymphocytes and natural killer (NK) cells (Figure 2). (Ferrara & Levine, 2008; Jacobsohn & Vogelsang, 2007; Socie & Blazar 2009).

TNFα is firmly involved in GVHD physiopathology at several steps of the process including induction of apoptosis in target tissues through the TNFα receptor. It also induces the activation of macrophages, neutrophils, eosinophils, and B and T cells; stimulates production of inflammatory cytokines such as IL-1, IL-6, IL-12 and TNFα itself; increases the expression of HLA molecules; and promotes lysis by T lymphocytes. High levels of TNFα are associated with a higher incidence of GVHD in bone marrow transplant recipients. This

allogeneic dysregulation, in addition to dysregulation of cytokines, leads to the acute tissue damage produced by GVHD.

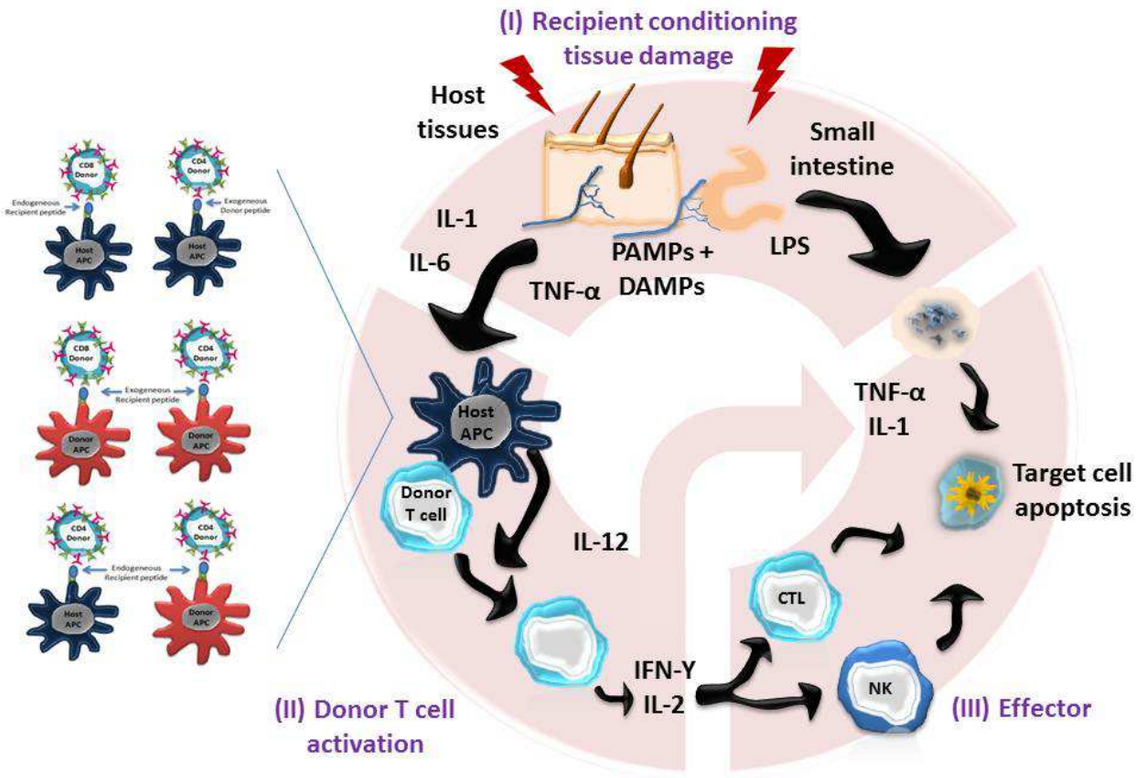

Fig. 2. Physiopathology of graft-versus-host disease. (I) Damage to host tissues by inflammation derived from the chemotherapy and/or radiotherapy conditioning regimen. (II) Antigen-presenting cells (APC) of both donor and recipient as well as inflammatory cytokines unleash the activation of donor-derived T cells, with expansion and differentiation of the latter into effector cells. The pathway of T-cell activation results in activation of the genes coding for cytokines such as IL-2 and IFNγ. (III) Effector stage: donor-derived activated T cells mediate cytotoxicity against target cells of the recipient through FasL-Fas, perforin and granzyme B interactions as well as additional production of tumor necrosis factor α (TNFα) (Modified from Ferrara & Levine, 2008).

In our laboratory, we have studied patients who underwent allogenic hematopoietic cell transplantation and developed GVHD, finding a correlation between these patients and an increased of CD14+ TNFα+ cells. Up to 32% of CD14+ cells secreting TNFα were found in a patient with stage II GVHD, increasing to 47% when the patient progressed to stage III, while in patients who did not develop GVHD this behavior was not observed. In patients with GVHD development, TNFα may promote increased expression of adhesion molecules such as VCAM-1, ICAM-1 and E-selectin in endothelium, therefore also promoting diapedesis of leukocytes in affected areas and degranulation of these cells with subsequent damage to tissues in which they are infiltrated (Aggarwal et al., 2000). On the other hand,

activation of endothelium, release of nitric oxide, vasodilation and increased vascular permeability, and blood platelet activation are also promoted and, most importantly, increased expression of MHC-I and MHC-II molecules as well as co-stimulatory molecules such as CD40, CD80 and CD86 in dendritic cells, thus also activating T lymphocytes and giving rise to more effective antigen presentation, and along with this, more effective allorecognition (Steinman et al., 1998).

In addition to this, TNFα has a major role in the promotion of apoptosis (De Freitas et al., 2004) and due to the previously mentioned properties, TNFα overexpression contributes to GVHD emergence and severity (Figure 3). Recent studies reveal an increase not only in TNFα levels but also in TNF receptors (TNFR1 and TNFR2), and the latter are more stable and easier to quantify (Choi et al., 2009; Kitko et al., 2008).

The role of other cytokines such as those of the Th1 profile is worth noting. Diverse research groups initially correlated this profile with GVHD emergence, since large quantities of cytokines such as IFNγ, IL-2 and IL-12 were found in patients with GVHD development and this is correlated with GVHD severity (Das et al., 2001; Ju et al., 2005). However, these cytokines have a controversial role as they are necessary for development of both GVHD and the graft-versus-leukemia effect. We have found that mononuclear cells from patients with GVHD development in co-culture secrete large amounts of IFNγ and IL-2, but we have also observed that the capacity of these cells to secrete these cytokines is correlated with graft success unaccompanied by relapse or development of infections by opportunistic microorganisms, which tells us these cytokines have a dual role. Regarding the significance of the Th2 cytokine profile in GVHD control, some study teams point to the overexpression of IL-4, IL-5 and IL-10 as a positive prognostic factor (Das et al., 2001; Ju et al., 2005). In our laboratory, however, a correlation has been observed only between the overexpression of IL-10 by mononuclear cells of patients and control of GVHD.

There are new lymphocyte subsets to which great significance has been ascribed in inflammatory processes as well as in many pathologies previously thought to be associated with the Th1 profile. The subpopulation of Th17 cells discovered in 2005 is now known to have a controversial role as they are implicated in rejection of solid organ grafts (Kappel et al., 2008; Carlson 2009; Coghill et al., 2010).

In murine models, differences in GVHD development have been found between mice that were transferred CD4+ IL-17$^{-/-}$ T cells and mice that were transferred normal CD4+ cells. In the former, GVHD development took longer. However, no significant differences were noted between these groups in relation to mortality due to GVHD or in graft-versus-tumor activity. Another major finding was the fact that mice that were transferred CD4+ IL17$^{-/-}$ cells had fewer Th1 cells during early stages of GVHD. Also, a reduction occurred in the number of IFNγ-secreting macrophages and granulocytes as well as a decrease in the amount of pro-inflammatory cytokines. IL-17 is therefore believed to be essential for GVHD development and graft-versus-leukemia activity as it promotes pro-inflammatory cytokine production – all this in murine models (Kappel et al., 2008). These data and others showing the importance of the Th17 profile in inflammatory processes made several researchers think that this profile might be involved in GVHD development and severity in humans (Coghill, 2011). Our study team recently found that the Th17 profile is not relevant for GVHD development. We conducted a pilot study on the importance of this profile in six patients who underwent allogenic hematopoietic cell transplantation, following them for six months. This group was divided into patients with GVHD development and patients without GVHD. Peripheral blood and mononuclear cell cultures were analyzed at 30, 60, 100 and 180

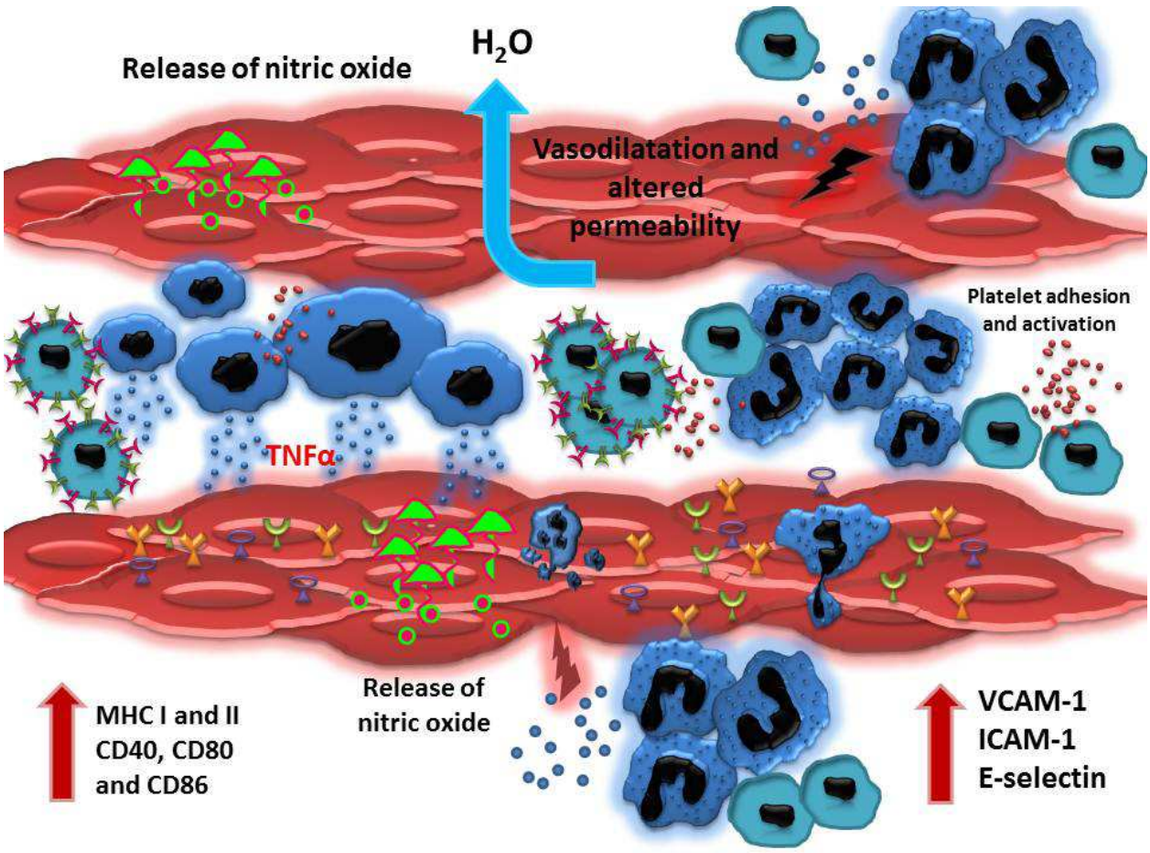

Fig. 3. The role of TNFα in GVHD emergence and exacerbation.

days after transplantation. Overproduction of Th17 profile was not observed neither in patients with GVHD nor in individuals without GVHD, as opposed to its production in healthy volunteers and in a patient who received a syngeneic transplant. Few months after concluding our study, Broady et al. (2010) published a similar study on patients who underwent allogenic hematopoietic cell transplantation, in which they evaluated Th1 and Th17 cells in tissue and peripheral blood in a cohort of 34 patients, of which 20 developed acute GVHD and 14 did not develop GVHD. The authors did not find an increase in the number of Th17 cells in patients with acute cutaneous GVHD as compared to healthy donors, but they did detect increased production of IFNγ-secreting cells (Broady, 2010).

GVHD diagnosis is suspected when the recipient develops all or some of the signs and symptoms that are characteristic of this disease such as dermatitis (rash), epidermal blistering, stomach cramps, abdominal pain with or without diarrhea which may be accompanied by passage of blood, nausea, persistent vomiting, and hepatitis (elevated bilirubins and/or liver enzymes). Typically, these signs and symptoms occur 100 days after allogenic hematopoietic cell transplantation but may also appear later. Because many of them are not specific of this complication, diagnosis must be supported with suitable biopsies, particularly if the symptoms are atypical or involve only the liver or gastrointestinal tract, since histological confirmation is extremely useful. A further reason

for biopsy-taking is to help differentiate GVHD from other diseases with similar symptoms, such as viral infections or reactions to pharmaceutical agents (Ferrara & Levine 2008; Jacobsohn & Vogelsang, 2007).

In GVHD, mature T cells of the donor that accompany the graft attack host tissues, particularly the skin, liver and gastrointestinal tract. This explains the signs and symptoms that characterize this disease. To prevent GVHD development, all patients receive some type of prophylaxis with immunosuppressors and in some cases T cell depletion , being one of the most commonly used methods although its effectiveness has not been fully proven since T-cell elimination contributes to absence of GVHD development but patients die from relapse because lack of GVT effect. Another prophylactic method involves pharmacological treatment with agents that affect T-cell function. These types of prophylaxis elicit adverse effects since mature T cells of the donor have a major role in mediating the reconstitution of the adaptive immune system, particularly in adults with low thymus function (Jacobsohn & Vogelsang, 2007).

GVHD is classified according to the number and extent of the organs involved (Table 2). In the current classification system, established in 1994, GVHD is divided into four groups (I – IV). Skin damage is evaluated by the percentage of the body surface area involved, liver damage by elevated bilirubins, and gastrointestinal tract damage by the amount of diarrhea (Jacobsohn & Vogelsang, 2007; Vela-Ojeda et al., 2008).

Stage	Skin	Liver (bilirubin)	Gut (stool output/day)
0	No GVHD rash	< 2 mg/dl	< 500 ml/day or persistent nausea
1	Maculopapular rash < 25% BSA	2-3 mg/dl	500-999 ml/day
2	Maculopapular rash < 25 - 50% BSA	3.1-6 mg/dl	1000-1500 ml/day
3	Maculopapular rash > 50% BSA	6.1-15 mg/dl	Adult: >1500 ml/day
4	Generalized erythroderma plus bullous formation	>15 mg/dl	Severe abdominal pain with or without ileus
Grade			
I	Stage 1-2	None	None
II	Stage 3 or	Stage 1 or	Stage 1
III	-	Stage 2-3 or	Stage 2-4
IV	Stage 4 or	Stage 4	-

(Modified from Jacobsohn & Volgelsang 2007).

Table 2. Classification of acute graft-versus-host disease.

Chronic GVHD (cGVHD) is one of the most common and significant problems affecting recipients of allogenic hematopoietic cell transplantation at long term. It appears approximately 100 days after allogenic hematopoietic cell transplantation, which is the critical time at which close to 50% of patients develop some degree of cGVHD. Increased use of hematopoietic stem cells obtained from peripheral blood rather than bone marrow,

increased age of recipients, and the use of busulfan in the conditioning regimen have led to a higher incidence of cGVHD. Other clinical risk factors for cGVHD development include previous acute graft-versus-host disease (aGVHD) and a second transplant. cGVHD most commonly affects the skin, liver, eyes and mouth, although other sites may also be affected. Death from severe cGVHD is generally a result of infectious complications. The standard treatment for severe cGVHD is a combination of cyclosporine and prednisone. An alternating daily regimen of these two agents prolongs survival and reduces drug-related adverse effects. Topical therapy in affected areas is recommended for patients with grade 1-2 cutaneous disease. Survival at 10 years in patients who develop light aGVHD is approximately 80%, but it is drastically reduced in patients with severe cGVHD, in whom this rate is reported to be 5% (Horwitz & Sullivan 2006; Lee 2005; Vela-Ojeda et al., 2008).

Alloreactivity is the basis for cGVHD pathogenesis. However, the exact phenotype and the origin of alloreactive cells remain somewhat ambiguous. Donor-derived alloreactive T-cells transplanted with hematopoietic stem cells play a key role in acute and chronic GVHD. Current animal models of cGVHD implicate Th2 cells as the first cell type to induce damage. However, in humans with cGVHD, Th1/Th2-polarized $CD4^+$ cells have alloreactive properties. The formation of antibodies has been observed in experimental models and clinical studies of cGVHD. This suggests that B cells are implicated in the physiopathology of cGVHD as shown by antibody production in allogenic hematopoietic cell transplantation patients with donor of different sex, since antibodies against minor histocompatibility antigens are encoded in the Y chromosome. The presence of anti-nuclear, anti-double strand DNA and anti-smooth muscle antibodies in a frequency range of 11-62% has also been detected in patients with cGVHD as well as the presence of anti-cytoskeletal and anti-nucleolar antibodies. However, despite these findings, the role of antibodies in cGVHD remains unclear (Horwitz & Sullivan 2006; Lee 2005; Vela-Ojeda et al., 2008).

cGVHD can be classified according to type of clinical manifestations or extent of the disease. Most patients with cGVHD have previously had aGVHD. cGVHD may also be observed after achieving control of aGVHD. Similarly, patients may develop cGVHD without a previous history of aGVHD (*de novo* cGVHD). Classification according to the type and extent of clinical manifestations is shown in Table 3.

The International Center for Research on Bone Marrow and Blood Cell Transplantation estimates that 50,000 – 60,000 hematopoietic cell transplantations are performed each year throughout the world. Bone marrow is the main source of grafts for transplantation in children, although peripheral and umbilical cord blood are being increasingly used. Between 2004 and 2008 peripheral blood accounted for 27% and umbilical cord blood for 32% of transplants in patients fewer than 20 years in age. In patients over 20 years the most common source for allogenic hematopoietic cell transplantation is peripheral blood. Currently, very few adults receive grafts of umbilical cord blood but its use, although infrequent, increased 2-4% between 2004 and 2008. Mobilized peripheral blood is the main source for autologous transplantation, representing 91% of autotransplantations in children and 98% in adults. In recent years the number of hematopoietic cell transplantations, both allogeneic and autologous, in patients over 50 years old has increased. Approximately 40% of allogeneic transplants are unrelated donor transplantations. There has been a change lately: before 2002 the most commonly used source of hematopoietic stem cells was bone marrow but its use has declined since 2003 and peripheral and umbilical cord blood have been increasingly used.

Classification of GVHD	
Limited chronic GVHD	
Either or both:	
1	Localized skin involvement
2	Hepatic dysfunction due to chronic GVHD
Extensive chronic GVHD	
Either:	
1	Generalized skin involvement, or
2	Localized skin involvement and/or hepatic dysfunction due to chronic GVHD
Plus:	
3a	Liver histology showing chronic aggressive hepatitis, bridging necrosis, or cirrhosis, or
b	Involvement of eye (Schirmer test with <5-mm wetting), or
c	Involvement of minor salivary glands or oral mucosa demonstrated on labial biopsy, or
d	Involvement of any other target organ

Table 3. Classification of chronic graft-versus-host disease (Horwitz & Sullivan, 2006)

The most common cause of death after allogenic hematopoietic cell transplantation is relapse, although in unrelated donor transplants it is followed by GVHD and in HLA-matched sibling transplants by death from infections. Another major cause of death is interstitial pneumonitis (Pasquini & Wang, 2007).

2.5 Graft-versus-tumor effect

Existence of the graft-versus-tumor effect was first suggested in 1956 by Barnes et al., upon noting eradication of leukemia in irradiated mice receiving allogeneic bone marrow transplants, but not in those that after irradiation received syngeneic bone marrow transplants. The first evidence of this also occurring in humans came from studies reporting that the incidence of relapse was markedly lower in patients who developed GVHD than in those who did not (as cited in Appelbaum, 2001), and that just as in murine models, human allogenic hematopoietic cell transplantation recipients were at lower risk of relapse than recipients of syngeneic stem cell transplantations or allogenic hematopoietic cell transplantations in which T cells are previously depleted. Male patients receiving allogenic hematopoietic cell transplantation grafts from female donors are also seen to be a special group in that donor-derived T cells specific for receptors of minor histocompatibility antigens may contribute to development of the graft-versus-tumor or the graft-versus-leukemia effect as well as GVHD since the graft-versus-leukemia effect is associated with presence of GVHD. The potential impact of the graft-versus-leukemia effect can be observed

when the patient is administered infusions of donor-derived lymphocytes. Graft versus tumor effect formed the basis of the so-called non-myeloablative transplants in which intensive chemotherapy regimen are substituted by the antitumoral effect of lymphocytes (Jacobsohn & Vogelsang, 2007).

Factors inducing GVHD or the graft-versus-leukemia effect are not well defined and may to some extent be considered speculative. Minor histocompatibility antigens have been suggested to play a key role since some of them have limited expression in hematopoietic cells, including leukemic cells. These antigens are perhaps targeted by the selective effect of the graft-versus-leukemia effect while minor histocompatibility antigens that are expressed in general may be targeted by GVHD, but this is still not firmly established (Jacobsohn & Vogelsang 2007; Kolb, 2008; Riddell & Appelbaum, 2007).

Various minor histocompatibility antigens are encoded in Y-chromosome genes that exhibit significant polymorphism with their homologues genes of the X chromosome. The former genes are responsible for the immune reactions occurring between men and women. Differential expression of the genes encoding for minor histocompatibility antigens in tissues has been proposed for potential use as a basis to separate the graft-versus-leukemia effect from GVHD. Cells that recognize minor histocompatibility antigens which are expressed only in hematopoietic cell receptors, including leukemic cells, may have a major role in the elimination of the latter without GVHD development. Several minor histocompatibility antigens such as HA-1, HA-2, HB-1 and BCL2A1 are expressed by hematopoietic cells and are therefore being examined as potential targets in order to promote the graft-versus-leukemia effect (Randolph et al., 2004; Kolb, 2008).

2.6 Clinical relevance of regulation and induction of tolerance after allogenic hematopoietic cell transplantation

The potential benefits of allogenic hematopoietic cell transplantation are offset by the moderate survival rate of the graft at long term. This is to a large extent due to immunosuppressant agents that unspecifically inhibit immune response in order to prevent rejection, but bring multiple adverse effects which are responsible for chronic rejection. Therefore, one of the major goals of allogenic hematopoietic cell transplantation is to achieve absence of immune response in the face of donor-derived alloantigens without requiring prolonged administration of immunosuppressants and to promote the graft-versus-leukemia effect. To this end, research has focused on the study of regulatory T cells (Treg), particularly those of the CD4+CD25highFoxP3+ phenotype (natural Treg, nTreg) as they have been shown to be able to control immune response in the face of donor-derived alloantigens and therefore possess great potential to establish in vivo tolerance to the transplant. These cells have been studied the most but are not the only ones described as having a regulatory role since regulatory functions have also been observed in other subpopulations of CD4+ cells, in T CD8+ cells, Tγδ cells, NK cells and CD3+CD4-CD8-CD16+56+ cells, also known as NKT cells. The latter have been shown to have a powerful anti-tumoral effect and are important in the maintenance of tolerance. This is why many studies are focusing on transfer of these cells. The strategy that is being sought for use in the near future is ex vivo stimulation of Treg purified with alloantigens of the donor or even FoxP3-mediated transfection of alloreactive $CD4+CD25^{-}$ cells (Bryceson & Ljunggren, 2007; Fehérvari & Sakaguchi, 2005).

Natural regulatory T cells (nTreg) express from the time of their differentiation in the thymus, $CD4^+$, $CD25^+$ and FoxP3 (*forkhead box P3 transcription factor*) expression patterns. They were identified by Sakaguchi et al. (1995) as a natural subset of $CD4^+$ T lymphocytes (approximately 5-10% of the T lymphocytes present in peripheral blood) that constitutively express the CD25 molecule and suppress the response of effector T lymphocytes ($CD4^+$ and $CD8^+$) *in vivo*. Another lymphocyte subpopulation in peripheral blood are $CD4^+CD25^-$ cells, which through the action of TGF-β and IL-2 may come to express CD25 and FoxP3, and are known as induced Treg (iTreg). Treg lymphocytes are also associated with low expression of CD127 which is positively correlated with regulatory function acquisition and negatively correlated with expression of FoxP3 (Curiel et al., 2004, Horwitz et al., 2008; Korn 2009; Liu et al., 2006; Seddiki et al., 2006).

CD4+CD25+FoxP3+ Treg cells have a key role in the maintenance of peripheral tolerance, and Treg deficiencies give rise to progressive autoimmune disorders. Similarly, improved Treg function can prevent graft rejection and suppress tumor immunity. In other words, an adequate balance between Treg and effector T cells is essential for maintenance of tolerance. In the context of allogenic hematopoietic cell transplantation, Treg have also been shown to have a major role in the establishment of tolerance between tissues of the recipient and donor-derived immunity. This was initially shown to occur in murine models where Treg depletion in the stem cell graft produced increased in GVHD and increased Treg numbers resulted in suppression of GVHD after transplantation (Lee 2005). In humans, patients with active cGVHD are reported to have a lower frequency of Treg than patients without cGVHD. These findings suggest that robust reconstitution of Treg post-allogenic hematopoietic cell transplantation is required to establish immune balance in order to be able to maintain adequate levels of peripheral tolerance. However, the mechanisms responsible for Treg reconstitution post-allogenic hematopoietic cell transplantation have not been adequately characterized and the factors contributing to inadequate recovery of Treg in patients who develop cGVHD are unknown (Matsuoka 2010).

Based on data obtained by Matsuoka et al. (2010) in patients examined during the first year after allogenic hematopoietic cell transplantation, thymus-dependent generation of Treg was considerably affected, but this subpopulation maintained high levels of cell proliferation in comparison to constitutive T cells. Such *in vivo* proliferation is apparently driven mainly by lymphopenia of CD4 cells. Among other findings, this study team has also shown that high levels of Treg proliferation were offset by higher susceptibility to apoptosis. Depletion of Treg in periphery in these patients was associated with development of extensive cGVHD.

On the other hand and in reference to lymphocyte populations that may take part in development of tolerance to alloantigens, the subpopulation of CD8+ Treg has been recently characterized. These lymphocytes, despite a long history in the field of immunology as described by Gershon & Kondo in 1970, have been difficult to characterize, and this factor combined with the discovery of CD4 Treg by Hall et al. (1990) and Sakaguchi et al. (1995) have considerably limited this area. The importance of CD8 Treg lies in the regulatory role observed in experimental autoimmune encephalitis (Lu & Cantor, 2008; Wang & Alexander 2009; Zheng et al., 2004).

The latter subpopulation has been said to be increased in peripheral blood analyses and infiltrated tissue of patients with colorectal cancer. Increased expression of TGF-β has also been found in these lymphocytes as compared to samples from healthy donors. These cells were able to suppress CD4+CD25- cell proliferation and cytokine Th1 production. The paper mentions the significance of immunological vigilance and the fact that CD8 Treg may promote tumoral growth. It also describes this cell subpopulation in patients with multiple sclerosis in whom lower numbers of these cells were correlated with relapse, a fact that may evidence their immunosuppressant role in the control of autoimmune diseases (Giovanni Frisullo, 2010; Chaput, 2009; Kiniwa, 2007).
Recently described populations of CD8+ Treg include CD8+ IL-10+ cells present in ovarian carcinoma, which are induced by plasmocytoid dendritic cells infiltrating the tumor. This differentiation towards CD8+ Treg was shown to be independent of $CD4^+$ Treg. All these antecedents combined with the generation of CD8+ Treg through continuous antigen stimulation may indicate the importance of the latter in GVHD control.

3. Conclusions

TNFα levels have a major role in development and severity of graft-versus-host disease and can be used as a negative prognostic factor.
Th1 response is essential and required in post-transplant patients to prevent relapse, graft loss and appearance of infections caused by opportunistic microorganisms.
The Th17 profile has no essential role in development or severity of GVHD and is therefore not a target for therapy in these patients.

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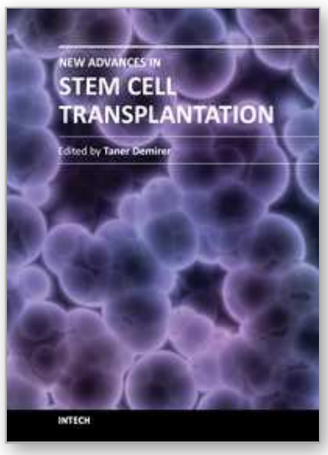

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