Overview of T-cell depletion in haploidentical stem cell transplantation

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Introduction

Graft-versus-host disease (GvHD) is a common complication of allogeneic stem cell transplantation in which functional immune cells in the transplanted graft recognise the recipient as "foreign" and mount an immunological attack. Clinically, GvHD is divided into acute and chronic forms. The acute form of the disease is normally observed within the first 100 days after transplantation[1]. The chronic form of GvHD normally occurs after 100 days[2]. However, this arbitrary distinction based on the time of onset fails to reflect the different pathophysiological mechanisms and clinical manifestations of acute and chronic GvHD. Acute GvHD can occur after day 100 in patients who received a non-myeloablative conditioning regimen or donor lymphocyte infusions. In addition, GvHD with typical clinical features of chronic GvHD can develop well before day 100 and concurrent with acute GvHD[3]. The National Institutes of Health consensus development project has, therefore, defined new criteria for the diagnosis, staging, and response assessment of chronic GvHD. The current consensus recommends that acute and chronic GvHD should be distinguished by clinical manifestations and not by time after transplantation. The consensus conference recognises two main categories of GvHD, each with two subcategories. The broad category of acute GvHD includes classic acute GvHD (maculopapular erythematous rash, gastrointestinal symptoms, or cholestatic hepatitis), occurring within 100 days after hematopoietic stem cell transplantation or donor leucocyte infusion. The broad category of acute GvHD also includes persistent, recurrent or late-onset acute GvHD, occurring more than 100 days after transplantation or donor leucocyte infusion; for brevity, this subcategory is henceforth designated as "late acute" GvHD. The presence of GvHD without diagnostic or distinctive chronic GvHD manifestations defines the broad category of acute GvHD. The broad category of chronic GvHD includes classic chronic GvHD, presenting with manifestations that can be ascribed only to chronic GvHD. The broad category of chronic GvHD also includes an overlap syndrome, which has diagnostic or distinctive chronic GvHD manifestations together with features typical of acute GvHD[4].

Donor T-cells play a fundamental role in the immunological attack on host tissues in both acute and chronic GvHD. While the cytokine production pattern of acute GvHD is mostly TH1 type, TH2 cytokines predominate in chronic GvHD[5].

In particular, acute GvHD is mediated by donor lymphocytes infused into the recipient, in whom they encounter tissues profoundly damaged tissues by the effects of the underlying disease, prior infections, and the transplant conditioning regimen. The allogeneic donor cells encounter a foreign environment that has been altered to promote the activation and proliferation of inflammatory cells. Thus, acute GvHD reflects an exaggerated response of the normal inflammatory mechanisms that involve donor T-cells and multiple innate and adaptive cells and mediators. Three sequential phases can be conceptualised to illustrate the complex cellular interactions and inflammatory cascades that ultimately evolve into acute GvHD: (i) activation of antigen-presenting cells; (ii) donor T-cell activation, proliferation, differentiation and migration; and (iii) target tissue destruction[6]. Understanding of the pathophysiology of chronic GvHD is not so advanced as that of acute GvHD. Alloreactive T-cells have been implicated in the pathogenesis; however, the precise roles of specific T-cell subsets, autoantigens, alloantigens, and B-cells, and interactions of chemokines and cytokines have not been fully elucidated. The clinical manifestations of chronic GvHD are often

similar to an autoimmune process, suggesting similar pathophysiology[7,8].

Patients with GvHD can manifest sclerodermatous skin changes, keratoconjunctivitis, sicca syndrome, lichenoid oral mucosal lesions, oesophageal and vaginal strictures, liver disease and respiratory failure[9-11].

Removal of T-cells from the donor graft (T-cell depletion) offers the possibility of preventing GvHD and, thereby, reducing transplant-related morbidity and mortality. The probability of acute GvHD ≥grade 2 after HLA-identical stem cell transplantation varied from 25-60% for patients transplanted with unmanipulated grafts and from 0-35% after T-cell-depleted stem cell transplants. Approximately 30-50% of patients develop chronic GvHD after an HLA-identical sibling stem cell transplant. The incidence of chronic GvHD may be even higher after allogeneic transplantation using unmanipulated peripheral blood stem cells because of the higher number of T-cells in these grafts. Extensive chronic GvHD requires prolonged immunosuppressive treatment and is associated with a mortality of more than 50%: most of the deaths are secondary to infections resulting from severe immune dysfunction[12-14].

T-cell depletion reduces the risk of GvHD in patients with either HLA-matched or partially matched donors and also allows the transplantation of haploidentical stem cells without increased incidence of GvHD[15]. Haploidentical stem cell transplantation is a valid approach for patients at high risk of disease progression without HLA-matched donors. In a haploidentical setting, most donors will share only one HLA haplotype with the patients. These haploidentical donors are readily available within a few days, are highly motivated to donate large numbers of stem cells (parental donors) and, with respect to further adoptive cellular therapy, are available during the post-transplant course[16]. A number of attempts to use haploidentical T-cell-replete unmanipulated bone marrow and myeloablative conditioning were made in the past. It should be highlighted that these attempts were associated with early severe and often fatal side-effects, including multi-organ failure and pulmonary oedema, a clinical picture resembling hyperacute GvHD[17]. While initial attempts at haploidentical stem cell transplantation used bone marrow as the source of stem cells, the possibility of mobilising and collecting peripheral stem cells and the development of graft-engineering methods for peripheral blood stem cells has allowed the design of strategies to overcome some of the obstacles of haploidentical transplantation, such as engraftment failure and the high incidence of GvHD[18].

A major advantage for patients transplanted with T-cell-depleted grafts is a better quality of life because of the less morbidity caused by acute and chronic GvHD. The lower probability of transplant-related morbidity and mortality offers a larger number of patients the possibility of becoming eligible for various forms of additional immunotherapy such as donor leucocyte infusions with disease-specific cytotoxic T-lymphocytes, activated donor natural killer cells and dendritic cell vaccination strategies. An increase of the graft-versus leukemia effect without introducing significant GvHD may result in lower relapse rates and increased probabilities of leukemia-free and overall survival[19-21].

A trip into the past among the techniques of T-cell depletion

Lymphocytes can be depleted from the stem cell grafts by various selection techniques. These techniques can be divided into physical, immunological, and combined physical/immunological separation methods.

Physical separation techniques

The first example of a physical separation technique is the differential agglutination with lectins followed by rosetting with sheep red blood cells described by Reinser *et al*. They used this procedure for the pre-transplantation purification of parental haploidentical bone marrow in three different cases. This procedure involves four main steps. The first is selective removal of red blood cells by gravity sedimentation in hetastarch, yielding a leucocyte-rich fraction which contains 68-76% of the original bone marrow nucleated cells. The second is agglutination of bone marrow cells with lectin soybean agglutinin (SBA) and differential sedimentation of agglutinated cells (SBA+). This step removes 70-90% of the bone marrow nucleated cells, including B-cells, monocytes, and helper T-cells, as well as most of the polymorphonuclear leucocytes.

The red blood cells remaining after the hetastarch separation are also agglutinated by SBA and removed from the remaining cell fraction (SBA-). The third step is removal of E-rosette-forming T-cells from the SBA- fraction by centrifugation over Ficoll-hypaque. In this step residual bands and polymorphonuclear leucocytes are also removed, together with the rosetted T-cells, during centrifugation. The final step is removal of the trace of residual T-cells (less than 0-5%) in the SBA-E- fraction by separation of cells forming E-rosettes with neuraminidase-treated red blood cells[22].

The second example of a physical separation technique is counterflow centrifugal elutriation. Wagner *et al*. described the use of this technique for the removal of donor T-lymphocytes before allogeneic bone marrow transplantation in 38 patients. In particular, the donors' bone marrow was processed by the Apheresis Unit at the Johns Hopkins Oncology Center in order to prepare buffy coat. Subsequently, the bone marrow buffy coat cells were filtered through an 80 μm blood filter (Abbott Laboratories, North Chicago, IL, USA) and loaded into a Beckman JE-IOX elutriation rotor and chamber (Beckman Instruments) at a total flow rate of 70 mL/min, rotor speed of 2,040 rpm ($1.26x10^3$ g) and temperature of 20 °C. An important note of the authors is that the elutriation medium was tested for sterility and absence of endotoxin and medium flow was monitored continuously. After the cells had been loaded into the chamber, the medium flow rate was increased to 110 mL/min with the rotor speed held constant. The small-sized cells were eluted to exhaustion. Subsequently, the flow rate was raised to 140 mL/min when intermediate-sized cells were collected. Cells remaining in the chamber were collected by continuing medium flow after stopping the rotor (designated the rotor-off [R/O] fraction). Calcium chloride (USP 10%, 0.8 mL/L) was added to all collection bags except the R/O fraction. The highly lymphocyte-depleted R/O fraction (mean volume, 367 mL; range, 299 to 425 mL) was immediately issued to the Bone Marrow Transplantation Unit and was passed through an 80 μm blood filter during infusion for each patient[23,24]. In another work, de Witte *et al.* described the combined use of density flotation centrifugation followed by counterflow elutriation for the removal of donor T-lymphocytes before histocompatible sibling bone marrow transplantation in 22 patients. In this study, the authors obtained a mean depletion of 98% for the lymphocytes[25].

The third example of a physical separation technique is fractionation on density gradients. Löwenberg *et al*. described the use of this technique, based on discontinuous albumin gradient fractionation, in a study in which nine patients were enrolled. The average T-lymphocyte content obtained was $50x10^5$/kg body weight[26].

Immunological techniques

Generally, immunological techniques involve the use of monoclonal antibodies, antibodies in conjunction with homologous, heterologous or rabbit complement factors, directed against T-cells. Monoclonal antibodies against human T-lymphocytes have the potential to play a major role in the prophylaxis of GvHD. In this context, Filipovich *et al*. used OKT3, an IgG_{2A} complement-binding mouse monoclonal antibody which recognises immunocompetent T-cells. In their study, the authors indicated that the administration of bone marrow pre-treated with a purified mouse monoclonal antibody was a relatively safe procedure which did not delay engraftment, but the incidence of acute GvHD was 50%[27].

In another study published in 1985 by Martin *et al*., a mixture of eight murine anti-T-cell monoclonal antibodies was used for the T-cell depletion. It is important to highlight that these antibodies bound non-competitively, with one exception, and thus this mixture was selected in order to provide optimal antibody binding to all T-lymphocytes[28].

A very clever strategy of T-cell depletion was adopted by Soiffer *et al*. In their study, they used a single antibody, anti-T12 (CD6), to remove T-cells from the donor's bone marrow. This antibody is specific for mature T-cells and does not react with closely related cells such as natural killer cells. As the authors report, complement-mediated lysis with anti-T12 can remove approximately 1.5 to 2.0 logs of T-cells from donor bone marrow. The aim of Soiffer's study was not to produce exhaustive depletion of all T-cells, but rather to deplete only mature T-cells selectively without affecting other bone marrow elements that may play important roles in engraftment and in the prevention of relapse. The data from this study indicated that this technique is

an effective strategy for the prevention of both acute and chronic GvHD. Moreover, the incidence of graft rejection in this population of patients was low, which suggests that a more selective depletion of T-cells can avoid graft failure after allogeneic bone marrow transplantation. This study was conducted on 112 consecutive adult patients with HLA identical, mixed lymphocyte culture non-reactive, sibling donors who underwent bone marrow transplantation[29].

In 1984, Waldmann *et al*. described the use of a unique monoclonal anti-human lymphocyte antibody CAMPATH-1 for removal of all immunocompetent lymphocytes, including T-cells. In this study the authors found that mature T-lymphocytes can be effectively removed from the bone marrow before transplantation using CAMPATH-1 with autologous human serum as a source of complement. Moreover, the risks of infection and technical mishaps are minimised in graft manipulation with CAMPATH-1, because this technique involves few steps[30].

Other antibody-based methods that do not depend on complement include immunotoxins, e.g. anti-CD5-ricin and immunomagnetic beads.

A pioneering study in the use of immunological separation techniques was conducted by Filipovich *et al*. They illustrated the use of three anti-T-cell monoclonal antibodies. The three antibodies were: (i) TA-1, which recognises a p-90/175 dimeric glycoprotein on the majority of peripheral T-cells, monocytes, and some myelomonocytic bone-marrow precursors, (ii) T101, which recognises a p65 antigen on most peripheral T-cells, and (iii) UCHT-l, which recognises a p19 structure on immunocompetent late thymic and post-thymic T-cells (also recognised by OKT3). In addition, preclinical studies indicated that the mixture of these three immunotoxins was more efficient in depleting proliferative T-cell responses than equivalent amounts of any individual immunotoxin alone[31,32].

As far as concerns immunomagnetic beads, a milestone study was conducted by Geisler *et al*. In this study, bone marrow mononuclear cells were incubated with F101.01, a monoclonal antibody that recognises an epitope of the T-cell receptor-CD3 complex, and subsequently with immunomagnetic beads. Interestingly, flow cytometric analysis demonstrated that mature T-cells were efficiently depleted and that natural killer cells and pre-thymic T-cells were preserved in the bone marrow mononuclear cells[33].

Combined immunological/physical methods

From 1990 onwards, combined immunological/physical methods using CD34+, CD3+ and CD19+ monoclonal antibodies loaded with iron particles have been developed and can be used for positive stem cell selection or negative T- and B-cell depletion using magnetic columns[34,35].

Perotti *et al*. described the results of immunoselection performed using the Isolex 300i device (Baxter; software version 2.5) for haploidentical transplantation from nine donors. In all these procedures for haploidentical transplantation, combined CD34+/- cell selection was performed by using a cocktail of murine monoclonal anti-human CD34-CD4-CD8-CD19/20 (Nexell Therapeutics Inc. Irvine, CA, USA) antibodies. In addition, regarding the ability of the device to reduce lymphocyte contamination, the median log of T and B depletion was 3.87 (range: 3.5-4.3) and 2.9 (range: 2.5-3.5), respectively[36,37].

The leading company in immunomagnetic selection is currently Miltenyi Biotec. In positive selection techniques, cells of interest are magnetically labelled with MACS® MicroBeads (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany). MACS MicroBeads are superparamagnetic particles with a diameter of approximately 50 nm and are composed of a biodegradable matrix. Cells are separated in a MACS Column (Miltenyi Biotec) placed in a MACS Separator (Miltenyi Biotec). The flow-through fraction can be collected as a negative fraction depleted of the labelled cells. Finally, the column is removed from the separator and the retained cells are eluted as the enriched, positively selected fraction. In contrast, in the negative selection techniques, non-target cells are magnetically labelled with a biotinylated antibody cocktail and Anti-Biotin MicroBeads (Miltenyi Biotec). In this case, undesidered cells are retained in the MACS® Column placed in the MACS® Separator. Finally, the target cells pass through the column and are collected as the enriched, unlabelled fraction, depleted of non-target cells. These methods offer several advantages over the immunological and physical methods. In particular, they are easy to perform in closed systems under good laboratory practice conditions. Selected cell populations are very pure with excellent yields of the target cells. Furthermore, these methods are less laborious than

physical separation techniques such as counterflow centrifugation. A fixed number of T-cells can be used, including the opportunity to manipulate the number of T-lymphocytes in the graft. Finally, effective B-cell depletion can be accomplished preventing the risk of development of Epstein-Barr virus (EBV)-lymphoproliferative diseases. Worldwide, the majority of transplant centres that perform T-cell depletion have gradually introduced these new combined physical/immunological positive and negative selection techniques into clinical practice.

As mentioned previously, passive T- and B-cell depletion using enriched CD34+ stem cells may reduce the incidence of GvHD and EBV-induced lymphoproliferative disease in allogeneic transplantation settings. This has been demonstrated in various studies[38,39].

In contrast to strategies using enriched stem cells, the CliniMACS® Miltenyi system enables the operator to perform clinical-scale magnetic enrichment of target cells, but also depletion of unwanted cells, together with the CliniMACS® CD3/CD19 Combination to simultaneously deplete CD3+ T-cells and CD19+ B-cells from the graft. This method preserves the stem cells, natural killer cells, myeloid precursors, monocytes and other progenitor cells, which might have engraftment-facilitating effects[40].

Grafts depleted of CD3+ and CD19+ cells have been used in allogeneic transplants, particularly haploidentical stem cell transplants for paediatric patients with haematological malignancies or solid tumours[41-43]. Sodani *et al*. described a study in which eight patients with thalassaemia major received T-cell-depleted peripheral blood progenitors cells (CD34+ immunoselection) and CD3+ and CD19+ depleted bone marrow stem cells. The median infused cell doses per kilogram of recipient body weight were $15.2x10^6$ (range: $8.2\text{-}26x10^6$) for CD34+ cells; $1.8x10^5$ for CD3+ T-cells and $0.27x10^6$ CD19+ cells[44]. In a haploidentical transplant setting, a non-extensive B-cell depletion, as emerged from the data presented, could lead to the onset of post-transplant EBV- related lymphoproliferative disorders[15]. Counterintuitive to the literature data, in the work by Sodani *et al*. no association was found between the number of CD19+ cells infused and occurrence of EBV reactivation[44]. At any rate, with efficient CD19+ depletion the onset of GvHD could also be prevented. In fact, recent evidence supports a role of B-cells in the development of GvHD. Speculation on the role of B-cells in the pathogenesis of GvHD includes the direct cellular cytoxicity of alloantibodies and the ability of B-cells to function as antigen presenting cells and facilitate presentation and processing of antigens to effector T-cells[45]. The mechanisms by which B-cells contribute to acute and chronic GvHD currently are still incompletely understood[3].

Finally, Sodani *et al*. found that stromal interleukin-7 production was decreased in transplant recipients, suggesting an important role for bone marrow accessory cells in immunohaematological reconstitution after transplantation. The authors hypothesised that the recovery of the T-cell compartment resulted from deregulated production of new T-cells from haematopoietic stem cells under the influence of the stromal microenvironment. The results of their work suggest that it may be possible to boost engraftment and immune recovery via the administration of specific cytokines (e.g., interleukin-2+interleukin-7) and/or mesenchymal stem cells[44].

In adults, the combination of a reduced intensity regimen with a T-cell-depleted and B-cell-depleted graft results in fast engraftment, also without the need of megadoses of stem cells. Likewise, in the adult transplant setting a trend to faster T-cell recovery and immune reconstitution has been described[46-49] (Table I).

Conclusions and future prospects

It has been clearly demonstrated that the T-cells in a graft are directly correlated to the risk of developing a life-threatening GvHD in allotransplanted patients[50]. Furthermore, depletion of donor alloreactive T-cells is particularly remarkable when an HLA-mismatched or haploidentical transplant is performed[51,52]. Many approaches have been explored in the attempt to improve recovery and purity of the progenitor cells to be infused. Different techniques have been employed for this purpose, including physical, immunological and combined immunological/physical methods with different results in terms of purity, recovery, and time consumption[53].

Focusing on haploidentical transplantation, we should highlight that in the early 1980s all attempts to carry out transplants from donors incompatible

Table I - T-cell depletion techniques: analysis of the literature data.

	Physical separation techniques		Immunological techniques		Combined immunological/physical methods	
Graft Manipulation Method	Density flotation centrifugation followed by counterflow elutriation (CD3+ depletion)	Discontinuous albumin gradient fractionation (CD3+ depletion)	T depletion with monoclonal antibody OKT3 (CD3+ depletion)	In vitro T depletion with a mixture of eight murine monoclonal antibodies and rabbit serum complement	ICS performed with Isolex 300i (Baxter) (CD34+ positive selection)	CD34+ ICS and CD3+/ CD19+ depletion with CliniMACS (Miltenyi Biotec)
Number of donors	22	9	10	20	9 HLA haploidentical donors and 20 patients (39 consecutive ICS) ***	8
Cell source	Bone marrow	Bone marrow	Bone marrow	Bone marrow	Peripheral blood	Bone marrow and peripheral blood
Stem cell purity (%) (mean)	n.r. **	n.r. **	n.r. **	n.r. **	95,3 (93 - 99)	n.r. **
Stem cell recovery (%) (mean)	82,3±35,2 (CFU-GM)	43±15 (CFU-GM)	n.r. **	63 (16-130) (CFU-GM)	55,1 (41,8 – 68,2) (CD34+ cells)	n.r. **
T-cell depletion	98% of mature T-lymphocytes have been removed	1 (log depletion)	n.r. **	2,2 to 3,2 (log depletion)	3,87 (3,5 – 4,3) (log depletion)	n.r. **
B-cell depletion	n.r. **	n.r. **	n.r. **	n.r. **	2,9 (2,5 – 3,5) (log depletion)	n.r. **
CD3+ total or / Kg of recipient*	0,64 ($x10^6$/Kg)	616 ($x10^4$/Kg)	0,57±2 ($x10^8$/Kg)	n.r. **	n.r. **	1,8 ($x10^5$/Kg)
CD19+ total or / Kg of recipient*	n.r. **	n.r. **	n.r. **	n.r. **	n.r. **	0,27 ($x10^6$/Kg)
Reference	de Witte T. *et al*. (1986)[25]	Löwenberg B. *et al*. (1986)[26]	Filipovich AH. *et al*. (1982)[27]	Martin PJ *et al.* (1985)[28]	Perotti C. *et al*. (2004)[36]	Sodani P. *et al*. (2010)[44]

Legend: *: in the graft after manipulation (mean); **: not reported; ***: in this case the data reported refer to both haploidentical haematopoietic stem cell transplants and autologous transplants; ICS: immunomagnetic cell selection.

for three HLA loci gave negative results because of the high incidence of severe GvHD in patients who received transplants that not were manipulated[54,55]. Conversely, graft rejection was observed especially in patients undergoing massive T-cell-depleted transplantation[56,57].

An interesting strategy to overcome the rejection of T-cell-depleted grafts is to increase the stem cell dose. Following the pioneering experiments with the "megadose" method in mice, clinical trials were started in 1993 after the harvest of megadoses of stem cells had been made possible by the availability of haematopoietic growth factors. A graft containing a megadose of CD34+ cells was achieved by combining a bone marrow graft with granulocyte colony-stimulating factor-mobilised peripheral blood progenitor cells and T-cell depletion of the graft by soybean agglutination and E-rosetting[58].

More recently, Aversa *et al*. adopted other T-cell depletion methods such as the positive selection of CD34+ cells from peripheral blood by magnetic beads using the CliniMACS system (Miltenyi)[59]. With regards to the *ex vivo* positive selection of CD34+ stem cells, which results in excellent T-cell depletion and is currently a widely used method of T-cell depletion, CD34+ positively selected stem cells can be associated with delayed immune reconstitution and, therefore, with an increased risk of viral and fungal infections[60]. Moreover, this positive selection may be associated with an increased risk of relapse of the underlying malignancy because of an impaired anti-malignancy effect exerted by a T-cell-depleted graft[61]. Furthermore, it should be highlighted that positive selection of CD34+ cells is also associated with the removal of other non-stem cells that might have beneficial effects after transplantation into patients, such as donor-derived, natural killer cells[62].

To overcome this problem, Chaleff *et al*. recently described a pioneering large-scale clinical method using the CliniMACS® TCR α/β System (Miltenyi Biotec) for the depletion of α/β T-lymphocytes from peripheral blood stem cells while retaining all other cells, which could be used in a clinical setting for haploidentical transplantation. The CliniMACS® TCR α/β System uses murine monoclonal antibodies specific for the T-cell recptor α/β antigen conjugated to biotin in combination with the CliniMACS® Anti-Biotin reagent. Interestingly, as already mentioned, the T-cell receptor α/β cell depletion with immunomagnetic negative selection retains other potential beneficial effector cells in the graft, such as γ/δ T-cells, natural killer cells and stem cells. These "facilitating" cells might promote engraftment, exert graft-versus-leukemia effects and reduce the risk of infections[62]. This new method of immunoselection is one of the most ambitious challenges in the field of stem cell transplantation.

Keywords: immunomagnetic selection, haploidentical transplantation, graft-versus-host disease, cellular processing.

The Author declares no conflicts of interest.

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