T deplete or T replete: which is better?

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A B S T R A C T

Family stem cell donors who share one HLA haplotype with the recipient are referred to as haploidentical donors (HAPLO). These are increasingly used in patients who lack an HLA matched sibling (SIB) or a well matched 8/8 unrelated donor (UD). There are two ways of performing such transplants. One is with graft manipulation, involving one or another form of T-cell depletion (TCD), and the other is with unmanipulated bone marrow (BM) or peripheral blood (PB), also referred to as T-cell replete. Both methods have been improved with time. TCD transplants have recently been improved with selective removal or addition of T-cell subpopulations. Similarly, unmanipulated HAPLO grafts have been improved by effective measures of preventing acute and chronic graft-*versus*-host disease (GvHD). The outcome of HAPLO transplants has recently been compared with UD and CB grafts, suggesting that HAPLO donors are an important alternative option for patients who lack an HLA identical donor. For this reason HAPLO transplants are rapidly increasing in numbers worldwide.

Learning goals

At the conclusion of this activity, participants should have an understanding of:
- the methods and outcome of haploidentical transplants using *ex vivo* T-cell depletion;
- the methods and outcome of haploidentical transplants using unmanipulated bone marrow or peripheral blood;
- survival of patients undergoing transplants from haploidentical donors, as compared to transplants from HLA identical siblings, unrelated donors and cord blood;
- future directions in this setting.

T-cell depletion

Severe GvHD and graft rejection have been significant obstacles for the use of transplants from HLA-haploidentical family donors. These immunological reactions are mediated by alloreactive T-cell responses, which are particularly strong in the context of an HLA haplo-mismatch donor. The first successful haploidentical transplants were carried out in the early 1980s in severe combined immunodeficiency (SCID) patients, in whom host-*versus*-graft (HvG) reactivity is minimal, enabling the study of new approaches for GvH prevention.[1] Using TCD by combining soybean agglutinin (SBA) and erythrocyte (E)-rosetting with sheep red blood cells, GvHD was prevented in the absence of any additional post transplant GvHD prophylaxis.[1,2]

The megadose of progenitor cells

The same approach of TCD (E-rosetting) was not as successful in leukemia patients due to a high rate of rejection.[3] This was due to the immune competence of leukemia patients mediated by cytotoxic T-lymphocyte precursors (CTLp) surviving standard conditioning regimens. In this case, the activity of host T cells dominates over the small number of donor T cells, and leads to rejection of the graft.[3] One way of overcoming this problem is to increase the number of CD34+ cells infused, the so-called megadose of hematopoietic progenitor cells.[4,5] Indeed, *in vitro* studies had shown that CD34+ cells exhibit specific "veto" activity, quenching a mixed lymphocyte reaction (MLR) against self antigens, but not against third-party antigens.[6]

In the early 1990s, the Perugia group developed a platform for TCD haplo-mismatched transplants. This included a novel myeloablative conditioning regimen combined with a "megadose" of CD34+ cells, selected via microbeads from granulocyte-colony stimulating factor (G-CSF) mobilized peripheral blood of healthy donors.[7] The conditioning regimen had two major changes: the introduction of thiotepa (at that time not used for preparation to transplant), and the substitution of cyclophosphamide with fludarabine as an immunosuppressive agent.[8,9] Both these changes were bound to last and are currently used in many preparative regimens. The regimen consisted of single dose total body irradiation (sTBI), thiotepa, anti-thymocyte globulin (ATG), and fludarabine, followed by the infusion of 10 million or more $CD34^+$ cells/kg with a very low T-cell content ($\beta 1x10^4$ $CD3^+$ cells/kg), and no post-graft immunosuppression. Engraftment was seen in 95% of leukemia patients with no acute or chronic GvHD.[8,9] Despite the absence of GvHD in these high-risk patients, the leukemia relapse rate was 18% in acute myeloid leukemia

(AML) and 30% in acute lymphoid leukemia (ALL) patients who were transplanted in any complete remission (CR).[9] This is probably due to the strong myeloablative conditioning regimen compensating the lack of T-cell mediated graft-*versus*-leukemia (GvL) effect. In addition, an important role of natural killer (NK cells) was highlighted by the Perugia group.

Natural killer alloreactivity

Human NK-cell function is regulated by a balance between activating and inhibitory receptors.[10-12] Clonally distributed inhibitory receptors termed "killer cell immunoglobulin-like receptors" (KIRs) recognize human leukocyte antigen (HLA) class I allele groups ("KIR ligands"): HLA-C alleles with a Lys80 residue ("Group 2" alleles), HLA-C with an Asn80 residue ("Group 1" alleles) and HLA-B alleles sharing the Bw4 specificity. All KIR genes are randomly expressed and KIR distribution varies on NK cells. Only NK cells which express inhibitory KIRs for self HLA ligands become "licensed/educated".[10-12] When confronted with an allogeneic target, educated NK cells do not recognize the allogeneic HLA as self HLA: the missing inhibitory ligand mediates alloreactions ("missing self" recognition). Combined evidence from *in vitro* studies, murine models and clinical trials indicated the ability of NK cells to mediate donor-*versus*-recipient alloreactivity rested on "missing self recognition".[13,14] In haploidentical hematopoietic stem cell transplantation (HSCT), engrafted progenitor cells gave rise to an NK-cell repertoire of donor origin that included alloreactive clones that killed recipient cryopreserved leukemic cells.[14,15] Thus, these donor-derived NK cells matured in a bone marrow microenvironment where they were predominantly exposed to, and "licensed/educated" by, donor HLA. This process shaped their repertoire to be both donor-tolerant and recipient-alloreactive. They are, therefore, able to recognize and react to missing self on recipient targets.[14,15]

In the clinic, the Perugia group could show that donor-*versus*-recipient NK alloreactivity significantly prevented relapse in patients with AML.[13,14] Indeed, relapse was 7% if the grafts came from NK-alloreactive donors as compared to 36% in grafts from non-NK alloreactive donors, and this translated into improved survival (59% *vs*. 33%).[9] Thus, at least for patients with AML, donor-*versus*-recipient NK alloreactivity should be exploited when selecting the optimal donor from among the mismatched family members. Results are more controversial for patients with ALL.[9]

Post-transplant immunological reconstitution

One major problem, at least in adults, that appears to be common to all TCD transplants, is the slow recovery of the anti-microbial and anti-viral responses. Because of: a) the small number of residual T lymphocytes in the graft; and b) the ATG-linked *in vivo* T-cell depletion, post-transplant immunological reconstitution is delayed, and haplo-transplant recipients remain susceptible to life-threatening opportunistic infections for several months.[3,9] Overall non-relapse mortality was 36% for 145 patients in CR at transplant and 58% for 110 patients transplanted in relapse.[8,9] To reduce treatment-related mortality (TRM), several groups are focusing on rebuilding post-transplant immunity to improve clinical outcomes separating GvHD from favorable donor immune responses. Adoptive immunotherapies were explored with pathogen-specific T lymphocytes or broad repertoire T cells that were depleted of alloreactive T cells.[16-20] Another method for adoptive transfer of T cells with a wide T-cell receptor repertoire is the use of engineered T cells to express suicide genes (namely, the herpes simplex thymidine kinase (HSV-TK) gene) to allow lysis of engineered cell if they triggered GvHD.[21-23] Although TK-engineered T-cell engraftment appeared necessary and sufficient to promote a robust T-cell recovery, the immune reconstitution was progressively enriched by T lymphocytes negative for transgene expression and retroviral integration.[24] Needless to say these methods require a dedicated laboratory and good manufacturing product (GMP) facilities, and are thus very expensive and labor intensive.

From CD34+ selection to T-/B-cell depletion

More recently, negative selection of G-CSF mobilized peripheral blood cells has been used instead of CD34+ cell immunoselection. The difference when compared to CD34+ cell selection, lies in the fact that by removing T and B cells, the graft then contains hematopoietic stem cells together with immune components, such as NK cells, dendritic cells and monocytes, and these may be exploited to generate anti-leukemic, anti-viral or graft-facilitating effects. The removal of B cells is important to prevent the emergence of Epstein-Barr virus (EBV)-driven lymphoproliferative disorders. CD3+/CD19+ depleted haploHSCT were used in children with acute leukemia after a conditioning that included fludarabine, thiotepa, melphalan and OKT-3 or ATG.[25] Primary engraftment was achieved in 88% of patients, acute GvHD grade III-IV occurred in 7%, chronic GvHD in 21%. TRM was 20% at five years.[26] Event-free survival (EFS) at three years was 25% for the whole group and 46% for patients in remission. A concern with this approach is the higher incidence of GvHD in comparison with the CD34+ immunoselection.

TCR α/β depletion

An innovative approach was pioneered by Handgretinger's group who depleted G-CSF mobilized peripheral blood cells of TcR α/β+ cells, thus retaining large numbers of effector cells such as TcR$\gamma\delta$+ T cells and NK cells.[25-27] TcR$\gamma\delta$+ T cells combine conventional adaptive features with direct, rapid responses against sterile stresses and a variety of pathogens.[27,28] Furthermore, TcR$\gamma\delta$+ T cells appear to exert anti-leukemic activity since they directly recognized stress-induced self antigens expressed by malignant cells. They are not expected to initiate GvHD since they do not recognize specific processed peptide antigens as presented on major histocompability complex (MHC) molecules. To remove TcR$\alpha\beta$+ T lymphocytes a biotinylated anti-TcR $\alpha\beta$ antibody was employed, followed by an anti-biotin antibody conjugated to magnetic microbeads. CD19+ B lymphocytes were also immuno-depleted to prevent post-transplant EBV-associated lymphoproliferative disorders.[29] Recovery of CD34+ cells (74%) was similar to CD34+ enrichment procedures. In Tubingen, children with advanced acute leukemia were transplanted with TcR$\alpha\beta$+/CD19+ depleted grafts follow-

ing chemotherapy-based conditioning. Locatelli in Rome employed the same method for graft processing and a TBI-based conditioning.[30] In both cohorts, no post-transplant GvHD prophylaxis was given, although anti-T antibodies (OKT3 or ATG) in the conditioning exerted additional *in vivo* T-cell depletion of the inoculum. Very rapid full donor type engraftment occurred in all patients. Few had acute grade I and II GvHD, and no patient developed chronic GvHD, confirming that TcRγδ+ T cells do not cause GvHD. Immune reconstitution was rapid in these children. A longer follow up and more patients are required to assess the post-transplant relapse rate. This novel graft manipulation strategy has been also tested in 23 children with non-malignant disorders undergoing haploHSCTs.[31] With no post-transplantation pharmacological prophylaxis for GvHD, 3 patients experienced skin-only grade I/II acute GvHD. No cases of visceral acute or chronic GvHD were observed. The 2-year probability of disease-free survival is 91.1%.[31]

This approach of selective TcR α/β+ and CD19 depletion has been recently tested in 16 adults with high-risk acute leukemia (AML n=12; ALL n=4).[32] All but one patient, who required a second graft from the same donor to boost hematopoietic reconstitution, achieved a full donor sustained engraftment. Tending to confirm the working hypothesis, there was a rapid, sustained increase in peripheral blood T-cell subpopulations. Two patients had skin grade I/II acute GvHD, one grade III-IV. Cytomegalovirus (CMV) reactivation occurred in only 2 cases; both were successfully treated with ganciclovir. No patient had EBV-related post-transplant lymphoproliferative disease (PTLD) and no invasive fungal disease occurred. Relapse was the main cause of failure (6 of 7) for patients transplanted with active disease.

Regulatory T cells

In the search for an alternative strategy to improve immune recovery without the risk of GvHD, attention focused on thymic-derived CD4+CD25+ FoxP3+ regulatory T cells (Tregs) that play a physiological role in maintaining immunological self-tolerance and immune homeostasis;[33] this was carried out in the setting of the Perugia platform. Adoptive immunotherapy with a high number of broad repertoire T cells, infused together with freshly isolated CD4+CD25+ FOXP3+ Tregs, has been recently exploited in a cohort of high-risk leukemia patients.[34] Only 6 of 43 evaluable patients developed grade II-IV acute GvHD; no patient had chronic GvHD.

The infusion of T cells under the protective umbrella of Treg, and the absence of post-transplant pharmacological immunosuppression, resulted in a significant control of AML and ALL relapse. The leukemia relapse rate was 0.05 at a medium follow up of 46 months. The probability of DFS was 0.56 at a median follow up of 46 months. These results demonstrate that the immunosuppressive potential of Tregs can be used to suppress GvHD without loss of the benefits of GvL activity.

Unmanipulated bone marrow and/or peripheral blood grafts

In the 1980s, several attempts were made to overcome HLA disparity between donors and recipients in the context of unmanipulated bone marrow transplants (in those days that was the only stem cell source available); a combination of cyclosporine A and methotrexate (CyA+MTX) with or without ATG was used to prevent GvHD.[35-37] TRM was very high and the procedure remained extremely toxic. Very few centers dared to use mismatched family donors and, as a consequence, the number of family mismatched allogeneic transplants performed in Europe remained stable until around 2005.[37]

Intensive graft-versus-host disease prophylaxis

A breakthrough came in 2006, when a Chinese group compared the outcome of patients grafted from HLA identical siblings with patients grafted from haploidentical family members.[38] Overall survival and disease free survival were identical. So what was the secret? Nothing new in the conditioning regimen [a modified busulfan cyclophosphamide (BU CY) for both groups], but all patients received intensive GvHD prophylaxis with CyA, MTX and mycophenolate (MMF), and the HAPLO group received in addition ATG.[38] The stem cell source was a combination of G-CSF (G) mobilized bone marrow (G-BM) and G mobilized peripheral blood (G-PB). There was a higher rate of acute GvHD and a border-line increase of TRM in the HAPLO group, but overall survival was comparable This was the first report on a relatively large number of family mismatched grafts, with outcome comparable to sibling grafts, and this led to the development of other programs.

Basiliximab and G-CSF mobilized bone marrow

Another Chinese group developed a program incorporating unmanipulated G-CSF mobilized-BM (G-BM) and intensive GvHD prophylaxis, with basiliximab, ATG, CyA, MTX and MMF.[39] This approach has recently been reported by an Italian co-operative group.[40] Acute GvHD grade II-IV and III-IV was, respectively, 24% and 5%, which is extremely low in a setting of unmanipulated family HLA haplo-mismatched grafts. TRM was not negligible, being 30% for standard and 45% for high-risk patients.[40] Overall 3-year survival was 54% for standard and 33% for high-risk patients. There was no difference in the incidence of infections to that reported for unrelated donor grafts: 47% bacterial, 70% CMV, and 16% invasive fungal infections**.** For most patients, this group received a conditioning regimen combining thiotepa, intravenous busulfan and fludarabine (TBF), originally described by Sanz and co-workers for cord blood (CB) transplants.[41]

Rapamycin and G-CSF mobilized peripheral blood

The Milan group has reported HAPLO transplants using unmanipulated PB cells with a protocol of GvHD prophylaxis consisting of ATG and rapamycin.[42] Acute GvHD grade II-IV was reported to be 40% and GvHD III-IV 20%, with an overall TRM of 30% and actuarial 3-year survival of 45% for early and 20% for advanced leukemias.

Post-transplant cyclophosphamide and non-myeloablative conditioning

The Baltimore group has brought to the clinic a protocol of GvHD prophylaxis originally described many years ago in rats.[43] The hypothesis is that after infusion of unmanip-

ulated haplo-mismatched graft on day 0, alloreactive T cells will become activated and start proliferating in the absence of a calcineurin inhibitor, and on day +3 most of the alloreactive T cells will be in an S phase. Patients are then given a large dose of CY (50 mg/kg) on days +3 and +4, killing T cells in the S phase. This post-transplant purging would spare T cells not undergoing active proliferation, which may include antigen specific T cells, and hopefully some T cells exerting a GvL effect. This was the original idea, and the results seem to confirm the hypothesis. In 2008, Baltimore in collaboration with the Seattle group, reported a first group of patients receiving unmanipulated HAPLO marrow following a non-myeloablative regimen of low-dose CY, fludarabine and TBI 2 Gy, CY was given at the dose of 50 mg/kg on days +3 and +4 and tacrolimus MMF started on day +5.[44] Engraftrment was achieved in 87% of patients; acute GvHD grades II-IV was diagnosed in 34%, and chronic GvHD was 4%. Transplant-related mortality was less than 20%, though relapse was significant at over 60%.[44] Survival was superior for lymphoid disorders as compared to myeloid leukemias. This study proved that post-transplant CY (PT-CY) was feasible, prevented GvHD, and allowed engraftment, though relapse was high. The question remains: is the high relapse rate due to elimination of GvL with PT-CY, or is it due to the non-myeloablative regimen?

Post-transplant cyclophosphamide and bone marrow: myeloablative conditioning

It has been shown that PT-CY can effectively prevent GvHD also in the context of a myeloablative regimen, with relapse as expected with a cyclosporine A (CyA) + methotrexate (MTX) GvHD prophylaxis.[45] The overall survival of leukemia patients in remission was close to 70%. An update of those results on a larger number of patients confirms encouraging overall survival for patients in 1st complete remission (77%), for patients in 2nd remission (49%), and for patients with advanced disease (38%) (Figure 1).

Post transplant cyclophosphamide and peripheral blood

Some centers have been using unmanipulated PB instead of unmanipulated marrow with PT-CY. It may be that the outcome depends on the intensity of the conditioning regimen. With a non-myeloablative regimen, rates of GvHD and mortality have been reported to be low.[46] However, when patients have been prepared with a myeloablative regimen, acute GvHD grade III-IV has been reported to be as high as 22%.[47]

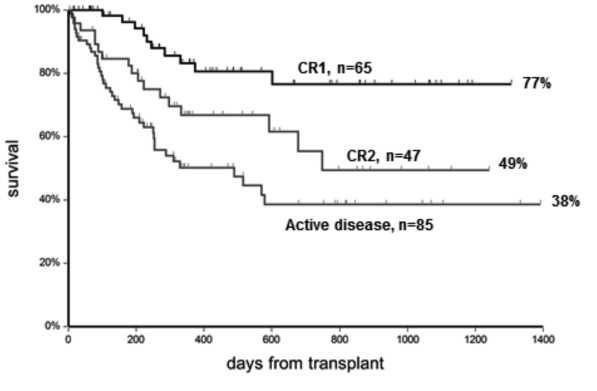

Figure 1. Actuarial survival of 197 patients receiving a MA regimen, haploidentical BM, andPT CY+CyA+MMF.

Platforms for unmanipulated haploidentical transplants

There are several platforms in place for unmanipulated HAPLO transplants: different conditioning regimens, different stem cell source, and different GvHD prophylaxis. A high rate of engraftment has been reported for all platforms but severe acute GvHD can vary from 3% to 30%, which of course may impact on quality of life. In particular, in the absence of a demonstrated GvL effect of peripheral blood cells, every measure should be taken to reduce to a minimum the immune complications, which can lead to life-long side-effects.

Dose and timing of post-transplantation cyclophosphamide

We currently do not know whether PT-CY 50 mg/kgx2 (total dose 100 mg/kg) is necessary to achieve the required protection against GvHD; there are already some ongoing trials using a lower dose. However, in the original Luznik paper, acute and chronic GvHD was significantly higher in patients receiving 50 mg/kg as compared to patients given 100 mg/kg. Also the day of administration of PT-CY can be modified. Most reports are based on the original day +3, day +4 regimen, with a calcineurin inhibitor starting on day +5.[44] However, one group has used PT-CY on days +3 and +5,[45] together with CyA and MMF starting on day 0. Patients have shown excellent engraftment and very low incidence of acute and chronic GvHD, and standard relapse rates have been observed, suggesting the platform can be modified.[45]

How do unmanipulated HAPLO grafts compare with other donor transplants?

The original LU paper[38] showed comparable outcome of unmanipulated HAPLO and sibling grafts. A report from the Atlanta group compared 53 HAPLO grafts with 117 sibling and 101 MUD donor grafts.[47] TRM was 7%, 13%, and 16%, respectively, acute GvHD grades III-IV occurred in 8%, 11%, and 11%, respectively, and extensive chronic GvHD in 38%, 54%, and 54%, respectively. There was no difference in overall survival or disease-free survival.[47] A similar report on 459 patients has recently been reported.[48] In a multivariate Cox analysis, disease phase was the strongest predictor of survival, and transplants from family haplo-mismatched donors had outcome similar to matched unrelated donors and HLA identical siblings.[48] We have up-dated that study. Figure 2 shows overall survival of patients receiving HAPLO mismatched family grafts compared with transplants from other donors, in the period 2007-2014, in the San Martino Transplant Unit, Genoa, Italy. No difference in survival was observed (Figure 2).

The Center for International Blood and Marrow Transplant Research (CIBMTR) has recently presented a comparison of 104 HAPLO mismatched grafts receiving PT-CY with 1245 matched (8/8) unrelated donor transplants, following a myeloablative regimen. The analysis

also included 88 HAPLO *versus* 737 matched unrelated donors (MUD) receiving a reduced intensity conditioning.[49] In addition, in this study, overall survival and leukemia-free survival was comparable in the 4 groups, although acute and chronic GvHD were lower in the HAPLO mismatched transplants.[49]

These initial reports suggest that HLA identical siblings, 8/8 matched unrelated individuals and family HAPLO mismatched members, are alternative options to perform a transplant when indicated. Matched unrelated donor and cord blood transplants have perhaps a somewhat inferior outcome, due to a combination of more GvHD (in the mismatched unrelated setting) and more infections (CB transplants).

Haploidentical transplants for Hodgkin disease

One of the original papers from the Baltimore group reported on patients with Hodgkin disease (HD).[50] In that study, the Authors compared the outcome of HD patients grafted from HLA identical siblings, unrelated donors and haploidentical relatives. Transplant protocols differed and GvHD prophylaxis in the haploidentical recipients was PT-CY.[50] Survival was comparable but disease-free survival was somewhat superior in recipients of haploidentical donors. A recent paper has confirmed these encouraging results in a series of 26 HD patients grafted from unmanipulated marrow and PT-CY.[51] The transplant-related mortality seems to be very low (5%), and both survival and disease-free survival are excellent. These results should be tested in a prospective study, possibly using the same transplant protocol also with different donors.

Conclusions and future perspectives

After a pioneering decade led by the Perugia group, haploidentical mismatched family transplants are rapidly increasing in numbers because of two major advances: selective α/β T and CD19 B-cell depletion on one hand and several platforms for unmanipulated stem cell grafts on the other. Results are currently similar, if not identical, to grafts from siblings and matched unrelated donors, and this has led to an increase in numbers. This is particularly true in Italy, as shown by the European Group for Blood and Marrow Transplantation (EBMT) survey.[37]

There has been no direct comparison of selective T-cell depleted with unmanipulated haplo-mismatched grafts. This may prove difficult also in the future, as centers tend to develop their own strategy. The current trend seems to suggest use of selective T-cell depletion in the pediatric setting and post-transplant CY in adults.

Haplo-transplants remain an alternative donor procedure and should be regarded as such. These patients are exposed to a high rate of blood stream infections, invasive fungal disease, and viral infections, which may all prove lethal. Acute and chronic GvHD seem less frequent, as compared to unrelated donor grafts. At any rate, HAPLO grafts, whether T-cell depleted or unmanipulated, should be performed in experienced centers, best in the context of prospective clinical trials.

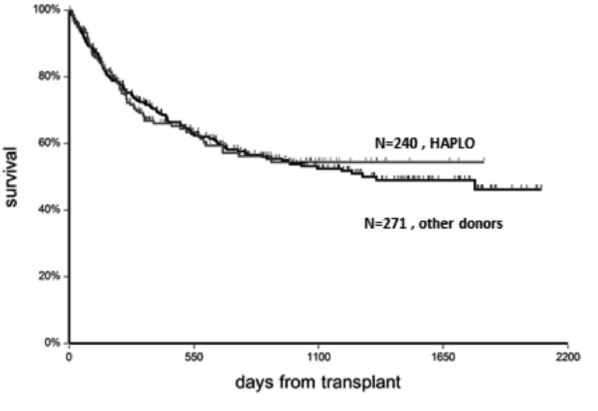

Figure 2. Overall survival of patients receiving a haplo-mismatched family graft compared with transplants from other donors in the San Martino Transplant Unit, Genoa, Italy, in the period 2007-2014. No difference in survival was observed.

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