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CICLO XXXI

Outcome and immune reconstitution of children and young adults with acute leukemia after alfa/beta T-cell and B-cell depleted HLA-Haploidentical transplantation

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Abstract

Allogeneic hematopoietic stem cell transplantation (HSCT) from an HLA-haploidentical relative (haplo-HSCT) is a suitable option for children/young adults with acute leukemia (AL) either relapsed or at high-risk of treatment failure and in urgent need of an allograft. A novel method of graft manipulation based on the selective, negative depletion of αβ T and B cells has been recently developed.

In the present study, enrolled and analyzed are 111 children with AL, with a median age of 10 years (range 0.9-22.2) transplanted between September 2011 and May 2018. Eighty-two (74%) and 29 (26%) patients had acute lymphoblastic leukemia (ALL) or acute myeloid leukemia (AML), respectively; all children were transplanted in complete morphological remission and received a fully myeloablative preparative regimen. The donor was mainly chosen according to immunological criteria, giving priority to NK-cell alloreactivity, KIR B haplotype, higher B-content score and size of NK alloreactive subset. They received Anti-Thymocyte Globulin (ATG) prior to HSCT to prevent GvHD and no patient was given any post-transplant pharmacological GvHD prophylaxis. With a median follow-up 47 months (range: 2 months – 7.7 years), the 5-year probability of overall survival was above 70% for both AML and ALL patients. The cumulative incidence of grade I-II acute GvHD was 25% (95% confidence interval, CI, 17-33), with skin GvHD being the most frequent organ involved, and no patient developed grade III/IV aGvHD. Four out of 91 patients at risk developed chronic GvHD, in all cases of limited severity, with a cumulative incidence of 5%. Six patients died for transplant-related complications, this resulting into a 5-year cumulative incidence of transplant-related mortality (TRM) of 6% (95% CI, 2-11) while the 5-year cumulative incidence of relapse was 24% (95% CI, 16-33) at a median time of 186 days (range 60-1012) after transplantation. The 5-year probability of LFS in children with ALL and AML was 69%

(95% CI, 57-79) and 73% (95% CI, 52-86), respectively., and the use of total body irradiation (TBI) during the preparative regimen was associated with better patient's outcome, since it protected against the risk of leukemia recurrence [18% (95% CI, 10-28) vs. 45% (95% CI, 22-66) in patients who did or did not receive TBI, respectively, $p<0.01$]. The median CD3+ cell count on day +90, +180 and +360 were 247, 659 and 1380/mcl, respectively.

This study confirms that αβ T- and B-cell depleted haplo-HSCT is an effective option for patients in need of an urgent allograft and lacking an HLA-identical donor. While TRM is impressively low, the main cause of treatment failure is leukemia recurrence, whose incidence could be lowered by the use of TBI during the conditioning regimen. The remarkably low risk of chronic GvHD renders the approach attractive also in terms of patient's quality of life.

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Allogeneic Stem Cell Transplantation (HSCT)

Allogeneic marrow transplantation is a potentially curative therapy for a variety of hematologic malignancies due to two separate components: chemo/radiotherapy administered before the transplant (conditioning regimen), and the presence of immunocompetent cells in the graft , capable of inducing a "graft-versus-malignancy effect " also known as GvL **“**[1].

Only a small percentage of patients has an HLA identical family donor. For the majority of patients (approximately 70%) who lack an HLA-identical sibling, alternative donors include matched unrelated donors and cord blood. The chance of finding an unrelated donor (UD) in the international voluntary donor registries , is limited by (a) frequency of the HLA phenotype and (b) the time required to identify the appropriate donor for patients with a high risk disease. Recent data from the National Marrow Donor Program donor registry showed that the probability of finding an 8/8 matched adult donor is 51% for Caucasians[6]. The event-free survival of adults undergoing an UD transplant ranges between 20% and 50% and refers only to patients who actually undergo the transplant, without taking into account those who do not find a donor or those who do find a donor but cannot be grafted based on medical reasons[7,8]. Because of these limitations, the proportion of UD transplants /year (12.000 in 2009) compared to UD search activations/year (44.000 in 2009) is less than 1/3[9].

Umbilical cord blood offers the advantage of easy procurement, no risks for donors, reduced risk of transmitting infections, immediate availability and less stringent criteria for HLA matching[10,11]. However, disparity between patients body weight and CB cell

content, particularly when associated with a two-antigen HLA mismatch, increases the risk of graft failure and delays hematopoietic reconstitution[12,13].

In recent years, Haploidentical hematopoietic stem cell transplantation (haplo-HSCT) has become a valuable and effective treatment option for patients with malignant hematological disorders who lack a suitable HLA matched donor or for whom a HSCT is urgently required [1-3]. There are at least two advantages for using HLA – haploidentical family donors, as compared to unrelated donor. First, haploidentical transplantation offers an immediate source of hematopoietic stem cells for almost all patients. Second, donors can be identified promptly , within a clinically useful time frame.

Until the early 1990s, haplo-HSCT was associated with a high incidence of graft rejection in T-cell–depleted transplants and severe graft-vs-host disease (GVHD) in unmanipulated transplants because of the high frequency of T cells that recognized major class I or II HLA disparities between donor and recipient [3-5]. To overcome these problems, two approaches were developed: a megadose of T-cell–depleted hematopoietic progenitor cells without any post-transplant immunosuppression [4,6,8,9] and unmanipulated grafts with innovative pharmacological immunosuppression for GVHD prophylaxis [3,5,10-12].

Unmanipulated Haplo-HSCT

Crossing the histoincompatibility barrier in HSCT is today feasible without *ex vivo* T-cell depletion. Two major approaches have been so far used: the GIAC-based strategy and the posttransplant CY-based protocol.

The "GIAC" Strategy. This modality is based on the following four elements: (G) donor treatment with recombinant granulocyte colony-stimulating factor (rhG-CSF); (I), intensified immunologic suppression; (A), (ATG; (C), combination of PBPCs and bone marrow cells. In the original study, Huang et al [8,5] reported the results in 171 patients

who had received a myeloablative conditioning and intensive posttransplant immunosuppression that included ATG, cyclosporine, methotrexate, mycophenolate mofetil, and anti-CD25 antibody (basiliximab). All patients achieved sustained, full donor chimerism. The 2-year incidence of opportunistic infections was 40%. In their most recent update including 250 acute leukemia patients, a total of 120 occurrences of opportunistic infections were recorded in 106 patients during the duration of follow-up [86,87]. The median time for an opportunistic infection to develop was 280 days (range, 5-1120) after transplantation. At 3 years after transplantation, the cumulative incidence of opportunistic infections was 49.1%. The cumulative incidence of grade III-IV aGVHD was 13.4%, the incidence of cGvHD and extensive cGVHD at 2 years was 54% and 22.6%, respectively. Even though a higher disease-free survival was achieved –partly due to inclusion of standard and good risk patients - the concern remains that a higher incidence of GVHD is associated usually with a higher treatment-related mortality and higher cost of care for these patients.

Consistent with their previous work, the Benjing group showed that high-dose ATG was associated with delayed recoveries of CD19+ B cells, CD3+ T cells, and CD4+ T cells during the first month after haploHSCT [8]. Furthermore, they also showed that high-dose ATG delayed the recoveries of CD4+, CD4+CD45RA+, and CD4+CD45RO+ T cells for 2 months, delayed the recovery of CD4−CD8− T cells for 6 months, and delayed the recovery of CD8+CD28+ T cells for 12 months after transplantation. The persistent delay in CD4−CD8− T cell recovery was closely related to an increased risk of EBV infection post-haploHSCT. The study showed that the schedule based on 6 mg/kg ATG was associated with a faster recovery of T cell subsets and a lower incidence of EBV infection compared to the schedule of 10 mg/kg ATG.

Using the Peking-based strategy, Di Bartolomeo et al [9] yielded promising results in 80 acute leukemia patients (median age of 37 years, range, 5-71). A myeloablative conditioning (MAC) regimen was used in 64 (80%) patients and a reduced intensity conditioning (RIC) in the other 16 (20%). They achieved a 91% engraftment rate, with a median of 21 days (range, 12-38) for absolute neutrophil count and 28 days (range, 14-185) for platelets. The cumulative incidences of grade 2-4 aGVHD and cGVHD was 24% and 17%, respectively. Twenty-seven patients (34%), 13 in the standard-risk group and 14 in the high-risk group, respectively, died from transplantation related complications at a median time of 76 days (range, 6-369). TRM was 32% at 6 months and 36% at 1 and 3 years. The 3-year probability of OS for all patients was 45% (54% for standard-risk group and 33% for high-risk group (P=06).

Arcese et al. [89] have recently updated the results of 97 patients who received a unique conditioning regimen, even though with different intensity according to age and comorbidity (TBF-MAC=68; TBF-RIC=29), before the infusion of an unmanipulated G-CSF-primed BM from a haploidentical donor. Regardless of the conditioning regimen, the GvHD prophylaxis was identical for all the patients and included five drugs: ATG, CSA, MTX, MMF and the anti-CD25 monoclonal antibody (basiliximab). Neutrophil and platelet engraftment rates were 94% and 84%, respectively. The cumulative incidence of grade II-IV acute and extensive chronic GvHD was 31% and 12%, respectively. Overall, 31 patients (32%) died of transplant-related complications at a median of 76 days (range 9–527). The infections were the main cause of NRM accounting for 48% of all deaths. At 1 and 5 years, NRM was 31% and 34%, respectively.

Post transplant Cyclophosphamide

Alternatively, unmanipulated T replete grafts can be performed and high dose post-transplant cyclophosphamide (PT-CY) is used to eliminate rapidly dividing donor T cells generated by the HLA mismatch graft, thus controlling GvHD.

A series of preclinical studies have shown that cyclophosphamide administered a few days after transplantation of skin or spleen cell, prolongs graft survival and reduce the risk of Graft Versus Host Disease (GvHD) [20 -22]. Luznik et al. have shown that in a murine haploidentical transplantation, conditioning with fludarabine and low-dose TBI, associated with post transplantation cyclophosphamide (g +3), is able to produce stable engraftment of donor cells with a low risk of GvHD[23,24] . The rationale for these results is that alloreactive donor T lymphocytes (responsible for GvHD) are activated immediately after the infusion and then are particularly sensitive to cytotoxic activity of cyclophosphamide while sparing T cells that no react. These cells may provide the transplants recipient with immunity to infection in the short term and immune reconstitution in the long term[24]. In addition, other potential mechanisms of cyclophosphamide are the deletion of clones reactive to the intra-thymic T, and the development of suppressor T lymphocytes[25]. The hematologic toxicity is not relevant because of the resistance of stem cells with cyclophosphamide, linked to the high intracellular concentration of aldehyde dehydrogenase[26].

The first demonstration of efficacy of this approach was published by the group of J. Hopkins in Baltimore who introduced the use of high-dose cyclophosphamide immediately after allogeneic Haplo-HSCT, preceded by an NMA conditioning, using unmanipulated bone marrow as stem cells source, for advanced patients[27],with encouraging results. They demonstrated the feasibility and non-inferiority of post-transplant cyclophosphamide in different cohorts of patients affected by haematological diseases[28].

Based on these results, a study from the same group was published using high dose of Cy as sole prophylaxis of GVHD after myeloablative HLA matched related or unrelated donor BMT. Transplanted patients were 117 and the most common diagnosis were acute myeloid leukaemia (58%) and 68/117 patients (58%) were not in remission at time of transplantation. Sustained engraftment of donor cells occurred in 114 patients (98%)[29]. The initial results of this study were recently updated[30]. The OS and EFS for all patients at 2 year after transplantation were 55 and 39% respectively. The cumulative incidence of relapse for patients transplanted in remission was 26% at 2 years. AML/MDS patients who were not in complete remission at the time of the transplantation had a worse EFS than patients in complete remission but the difference was not statistically significant (p=0,26), although the presence of circulating blasts in patients with active disease was associated with significant poorer outcome compared to patients in complete remission (p= 0.01).

One major remaining problem was the relapse of the underlying disease, especially for patients transplanted with chemorefractory and/or active hematological malignancies[2]. In the first series of advanced patients transplanted from haploidentical donor the cumulative incidence of relapse at 1 year was 51%[3,4]. Recent data data showed an actuarial LFS at 1 year is 29% for patients transplanted in advanced disease phase[13].

A retrospectical study was recently published by Genoa group assessing a similar outcome between HSCT from HLA-identical siblings (n=176), matched unrelated donors (n=43), mismatched unrelated donors (n=43), umbilical cord blood (n=105), and Haplo-HSCT (n=92) in terms of OS, EFS, and NRM. Our study showed significant lower incidence of aGvHD and cGvHD for Haplo patients.

T-cell depleted Haplo-HSCT

In general, TCD techniques can be classified as *in vitro* if the stem cell manipulation is performed exclusively *ex vivo*, normally by column adsorption. In contrast, *in vivo* techniques are based on a partial or complete depletion of donor lymphocytes in the patient after transplanting the stem cell product using ATG or alemtuzumab.

While *in vivo* T-cell depletion is largerly used nowadays to refine GvHD prophylaxis strategies, Haplo-HSCT started to become successful in the 1990s, when Aversa et al. exploited the principle of a megadose T cell depleted HaploHSCT in patients with acute leukemia and showed that an extensive ex vivo T-cell depletion followed by the infusion of a mega-dose of immune-selected CD34+ cells prevents both graft rejection and GvHD even in the absence of post-transplant immunosuppression [8,9]. This approach, studied mainly by the Perugia group, led to promising leukemia free survival (LFS) rates in adult with acute leukemias[17,18], refining through the last decade conditioning regimen and graft selection to allow a stable hematopoietic engraftment across major HLA barrier.

This type of graft mainly relies on NK cells, since they are the first lymphocyte subset that reconstitutes the patients. A better outcome of the transplanted patients has been associated with donor NK alloreactivity, by means of KIR/KIR-L mismatch in graft versus host (GvH) direction. Indeed, donor-derived alloreactive NK cells could play a crucial role in the eradication of leukemia blast (GvL effect) and in the clearance of residual recipient DCs and T lymphocytes, thus preventing GvHD and graft rejection, respectively [10]. Notably, the differential expression of activating ligands on hematopoietic and not hematopoietic tissues may provide an additional explanation for the observed GVL effect in the absence of GVHD [25-30].

NK Cells

Human NK cells are a subset of PB lymphocytes defined by the expression of CD56 or CD16 and the absence of the T-cell receptor (CD3)[15]. They recognize and kill transformed cell lines in an MHC-unrestricted fashion and play a critical role in the innate immune response. Several studies demonstrated that NK function, which is distinct from the MHC-restricted cytolytic activity of T cells, may be relevant for the immune control of tumor development and growth [2,16]. Although NK cell killing is MHC-unrestricted, NK cells display a number of activating and inhibitory receptors that ligate HLA-class I molecules to modulate the immune response[17].

The discovery of HLA-class I specific inhibitory receptors and various activating receptors, as well as their ligands, provided the basis for understanding the molecular mechanism of NK cell activation and function.

In peripheral blood two different NK cell subsets can be identified on the basis of surface density of CD56 expression, the majority being CD56dim while the minority (5-15%) is CD56bright. CD56dim NK cells are CD16+ KIR+/- CD94+ (associated with either NKG2A or NKG2C), and predominantly mediate cytotoxicity responses. Conversely, CD56bright NK cells are CD16- KIR- CD94/NKG2A+, and produce high levels of proinflammatory cytokines. Several evidences suggest that CD56bright NK cells are precursors of CD56dim, and CD57 expression marks terminally differentiated cells[8] . The two subsets display a different pattern of chemokine receptors, CD56bright are characterized by CCR7, CCR5 while CD56dim by CX3CR1. Notably, human mature NK cells can change their surface antigen expression profile upon stimulation by target interaction and/or cytokines.

NK cell receptors that recognize antigens at the HLA-A, -B, or -C loci are members of the immunoglobulin super family and have been known as killer immunoglobulin receptors or KIRs[22]. Engagement of these NK cell receptors results in stimulation or

inhibition of NK cell effector function, which ultimately depends on the net effect of activating and inhibitory receptors. The KIR family of genes is characterized by a high degree of polymorphism and includes both inhibitory (iKIR, including KIR2DL, KIR3DL) and activating receptors (aKIR, including KIR2DS, KIR3DS).

On the basis of their gene content, two groups of KIR haplotypes (referred to as A and B) have been defined. The A haplotypes have an identical KIR gene content, mainly iKIRs that can vary by allelic polymorphism. The B haplotypes differ one from another in terms of gene content, being more variable, and including several aKIR genes. Two KIR haplotypes combine to form KIR genotypes, A/A or B/x (i.e. either A/B or B/B)[21]. In addition to KIR, other receptors recognizing HLA class I exist, as the inhibitory CD94/NKG2A and activating CD94/NKG2C recognizing HLA-E molecules. NK cells are also equipped with activating receptors, including NCR (NKp46, NKp30 and NKp44), NKG2D and DNAM-1, whose ligands are mainly stress-inducible molecules. This great array of activating and inhibitory receptors finely regulates NK cell function. The NK cell receptor repertoire is primarily determined by KIR genotype, which is extremely variable in terms of number and identity of KIR gene content, it is clonally distributed and selected in a way that each NK cell expresses at least one inhibitory receptor for self HLA (3).Thus, in an autologous setting, licensed NK cells can only lyse target cells that have lost or express low levels of HLA class I molecules. Moreover, licensed NK cells are potentially capable of killing allogeneic cells (i.e. alloreactive). While inhibitory interactions predominate when NK cells encounter normal autologous cells, tumor cells can be susceptible to lysis through a mechanism of “missing self recognition”, because they down-regulate HLA-class I molecules, and/or “induced self recognition”, because they up-regulate ligands for activating receptors. Pende et al documented that both events could be observed in leukemia blasts that could be killed

by NK cells; in particular, CD155 and CD112 were over-expressed as compared to the normal counterpart and DNAM-1 was involved in the lysis [6].

Alloreactive NK cells express only iKIRs that do not recognize any of the HLA class I molecules (KIR-L) expressed by allogenic target cells (KIR/KIR-L mismatch). Remarkably, clinical and experimental data from HSCT revealed that the presence of a KIR/KIR-L mismatch in the GvH direction correlates with a more favorable clinical outcome [25-27]. The presence of alloreactive NK cells can be predicted by the analysis of the donor KIR gene profile and by the HLA class I typing of both donor and recipient. The actual presence and size of the alloreactive NK subset can be assessed by cytofluorimetric analysis using appropriate combinations of anti-KIR mAb. [28] Several studies have demonstrated that patients transplanted from donors characterized by a B/x genotype and a B content value ≥2, have a better clinical outcome (12), suggesting that an higher expression of KIR-activating subset could be associated with a more effective NK activation to exert GvL

Recently, data from haplo-HSCT suggest that NK alloreactivity may significantly impact on tumor cell killing[25-27]. In fact, these studies show that AML patients transplanted with KIR/KIR-L mismatched are significantly protected against leukemia relapse. In addition, preclinical and clinical investigations demonstrated that alloreactive NK cells play the main role as anti-leukemia effector cells and they exert their cytotoxic activity within 4-5 days [26-27] . In particular, high risk AML patients transplanted from NK allloreactive donors had a relapse rate of 0% compared to KIR-ligand matched patients who had a relapse rate of 75% [27]

Unfortunately, in T-depleted haplo-HSCT, the first emergence of fully functional, KIR+ alloreactive NK cells from HSC may require at least 6-8 weeks and, thus, the benefit offered by their anti-leukemia effect is relatively delayed [11]. Moreover, although primary

engraftment and low GvHD rate were achieved, the extensive T-cell depletion caused a slow post-transplant immune recovery leading to many opportunistic infections and likely decreased GvL effect.

Post-transplant immunological reconstitution and infections

Whereas the use of reduced intensity conditioning (RIC), infusion of mega doses of CD34+ cells, and graft manipulations such as selective T cell depletion were helpful to achieve engraftment with lower rates of GvHD and toxicity, delayed immune reconstitution and infectious complications remain outstanding issues for haplo-HSCT and are important causes for morbidity and mortality [3,7,10,12,13]. In the early post-transplant period, neutropenia is the principal risk factor for infections while, once engrafted, the capacity to mount an adaptive immune response to pathogens is a key factor for protecting from severe and recurrent infectious complications

Reconstitution of the T-cell pool after HSCT is achieved both through peripheral expansion of naïve and memory T-cells[14], and de novo differentiation from hematopoietic stem cells in the thymus [15]. T-cells originating from peripheral expansion would most likely have a more limited TCR repertoire. They could also, at least in theory, be more allo-reactive, not having gone through the process of negative selection in the recipient. In adults, due to the decay in thymus function, post-grafting immune recovery depends for months on expansion of the mature T cells infused with the graft. Naıve T cells are produced months after transplantation because conditioning induced tissue damage prevents T cell homing to peripheral lymphoid tissues, where T cell memory is generated and maintained [17]. Furthermore, the post-HSCT adaptive immune response is influenced by the strategy used to prevent GvHD [3,6,13]. In unmanipulated haplo-HSCT, peripheral T-cell expansion is antagonized by the immune suppressive therapy for GVHD prophylaxis. In T cell depleted haplo-HSCT the T-cell repertoire is

very narrow since the number of T lymphocytes in the graft has to be particularly low to prevent GvHD, and anti-thymocyte globulin (ATG) in the conditioning exerts an additional in vivo T-cell depletion [13,18]. Even in the absence of pharmacologic agents, GVHD itself is known to have deleterious effects on immune function and can cause profound lymphoid hypoplasia, B cell defects and damage to thymic stroma, resulting in impaired T cell development [19]. Thus, the immune recovery is slow and patients tend to remain susceptible to opportunistic infections for several months after HSCT.

T cell depleted HaploHSCT.

As the Achilles heel of T cell depleted haploHSCT was linked to the paucity of T lymphocytes in the graft, over the past decade, various strategies of adoptive donor T-cell immunotherapy have been investigated to improve immune recovery and reduce non-relapse mortality (NRM) from infectious complications.

Infusion of Pathogen-Specific T Cells. Some groups have focused on adoptive transfer of pathogen-specific T lymphocytes against CMV, aspergillus, adenovirus and EBV. In the original study by Perruccio et al, [61] large numbers of donor pathogen-specific T-cell clones were generated, then screened individually for alloreactivity against recipient cells, deleted of those cross-reacting against recipient alloantigens, and infused soon after haplo-HSC. Infusion of Aspergillus-specific type-1 CD4+ clones controlled Aspergillus antigenemia and helped to clear invasive aspergillosis in 9 of 10 patients. Similarly, infusion of CMV-specific CD4+ clones largely prevented CMV reactivation and reduced CMV mortality. Since clearance of virally infected cells is mediated by specific CD8+ cytotoxic cells, the infused CD4+ cells might have conditioned APCs to stimulate the CMV-specific CD8+ T cells transferred with the graft, thus promoting their clonal expansion. In fact, unlike non-infused control patients, CMV-specific CD8+ cells were detected shortly after infusing CMV-specific CD4+ clones. Among patients

receiving T-cell therapy, total CD4+ and CD8+ T-cell counts were significantly higher. The successful transfer of immunity to Aspergillus and CMV did not trigger neither acute nor chronic GvHD [61].

An alternative to pathogen-specific therapy is adoptive T-cell immunotherapy, which provides large numbers of wide repertoire cells, mirroring the physiologic immune system. The key challenge is to infuse sufficient T cells without causing GVHD. Strategies include broad repertoire T cells depleted of alloreactive T lymphocytes or engineered with a suicide gene.

Ex Vivo Photodepletion of Alloreactive Donor T Cells. Photodynamic purging appears to be an effective strategy for selectively depleting donor alloantigen-specific T cells, thus preventing GvHD and preserving the T cell anti-leukemia function. In a mixed lymphocyte reaction, alloantigen-stimulated T cells uptake 4,5-dibromorhodamine methyl ester (TH9402), a compound that is structurally similar to rhodamine [62]. The study by Perruccio et al, [63] investigated a range of parameters, and combinations thereof, with the aim of achieving optimal T cell allodepletion and preservation of pathogen-specific responses. The remarkable drop in frequency of alloreactive T cells is expected to allow safe infusion of relatively large numbers of T cells across histocompatibility barriers for adoptive transfer of donor immunity. Patients up to age 62 years with high-risk hematologic malignancies were enrolled in a phase-1 dose escalating study [64]. All patients engrafted rapidly and no severe acute GVHD occurred in the absence of immune suppressors. Higher doses were associated with lower TRM and improved survival. This effect was mainly attributed to a decrease in infectious complications and low relapse rates. These findings led to the initiation of a multicenter international phase II clinical trial and, at interim analysis, patients receiving $2x10^6$/kg photodepleted CD3+

T cells did not have severe GVHD and demonstrate a high overall survival (69% at 12 months after HSCT) [10].

Infusion of T Cells Engineered to Express Suicide Genes. Polyclonal T cells were engineered to express suicide genes, eg, the herpes simplex thymidine kinase (HSV-TK) gene, to guarantee engineered cell lysis if they triggered GvHD [65-68]. Ciceri et al reported the results in a cohort of 50 high-risk leukemia patients enrolled in a phase I–II, multicentre, non-randomised trial [67]. Overall, there were 196 infectious events (median four events per patient, range 0-14), 161 of which occurred with 130 days. In immune reconstituted patients, progressive normalization of antiviral responses was associated with a decline in the number of infectious events, while patients who failed immune reconstruction continued to have frequent infectious complications. After 130 days, median peaks in blood titres of CMV antigen were 0 nuclei per 10^5 peripheral blood mononuclear cells (PBMC) (range 0–20) in immune reconstituted patients and 21 nuclei per 10^5 PBMC (range 14–58) in patients without immune reconstitution ($p<0{\cdot}0155$); and median length of antiviral treatment was 0 days (range 0–44) in immune reconstituted patients and 47 days (range 33–105) in patients without immune reconstitution ($p<0{\cdot}0052$). The conditional benefit of immune reconstitution obtained by TK-cell infusion was assessed by the cumulative incidence of non-relapse mortality for patients alive 100 days after transplant; non relapse mortality was 14% (infectious mortality 9%) in TK-treated immune-reconstituted patients and 60% in non-immune-reconstituted patients. A randomized phase III trial to address the role of HSV-TK donor lymphocyte addbacks for recipients of haplo-HSCT is ongoing at present.

Other researchers devised an inducible T-cell safety switch based on the fusion of human caspase 9 to a modified human FK-binding protein, allowing conditional dimerization and cell suicide following administration of the small molecule dimerizing drug AP1903

[69]. Since preliminary interesting results, the Rome group has recently launched a phase I/II study enrolling children with either malignant or nonmalignant disorders who will receive TCR-αβ/B cell depleted HaploSCT, followed by the infusion of titrated numbers of iC9 T cells on day 14±4. These iC9-modified T cells can contribute to T cell immune reconstitution after T cell depleted HaploSCT and are eliminated by the administration of AP1903, if aGVHD occurs [70].

Regulatory T Cells. More recently, a pioneer experience of the Perugia group has clearly demonstrated that naturally occurring Tregs harvested from healthy donors efficiently control the alloreactivity of large numbers of otherwise lethal, conventional T cells [71-73]. Using this strategy, there was a rapid, sustained increase in peripheral blood T-cell subpopulations. A wide T-cell repertoire developed rapidly. Naïve and memory T-cell subsets increased significantly over the first year after transplantation, demonstrating sustained immune recovery over time. B-cell reconstitution was rapid and sustained and immunoglobulin serum levels normalized within 3 months. Compared with standard haplo-HSCT, specific CD4+ and CD8+ for opportunistic pathogens such as Aspergillus fumigatus, Candida albicans, CMV, ADV, HSV, and toxoplasma emerged significantly earlier, fewer episodes of CMV reactivation occurred, and no patient developed CMV disease. Nevertheless, 8 of the 13 non-relapse deaths were due to infections: adenoviral infection (n=2), bacterial sepsis (n=1), toxoplasmosis (n=1), fungal pneumonia (n=3) or central nervous system aspergillosis (n=1).

Selective T cell depletion. Other attempts to improve post-transplant immune recovery focused on improving graft content by shifting from CD34-positive selection to negative selection of PBPCs so as to include other immune cells [10,74]. Selective T-cell removal means depletion of a given subset from the whole T-cell population. The aim is to reduce the incidence of GvHD while preserving other beneficial cell functions carried out by

the residual T-cell subsets. In an innovative approach, Handgretinger's group in Tubingen depleted the leukapheresis product of only TCR αβ+ T cells, thus retaining large numbers of effector cells such as TCRγδ+ T cells and NK cells[75,76]. TcRγδ+ T cells combine conventional adaptive features with direct, rapid responses against sterile stresses and many pathogens. They participated in the anti-CMV response in the early period of post-transplant immune recovery. They are not expected to initiate GVHD, because they do not recognize specific processed peptide antigens as presented on major histocompatibility complex (MHC) molecules. First clinical results of these new T-depletion strategies are encouraging and interestingly none of the studies reported a significantly increased incidence of infections, even using MAC regimens[75-78]. This could be partially explained by the high number of γδ T cells in donor's graft. Indeed, γδ T cells are considered as a bridge between adaptive and innate immunity. γδ T cells receptors detect unconventional antigens such as phosphorylated microbial metabolites and lipids, non-classical MHC-I molecules and unprocessed proteins[79]. They are concentrated within epithelial and mucosal surfaces to maintain epidermal integrity of the skin and intestinal epithelium[80]. It has been hypothesized that tissue-specific antigens are recognized by γδ T-cells resulting in immune responses protecting potential sites of pathogen entry into the body[8].

In two cohorts of children transplanted either in Tubingen [75,76] or in Roma [77,78], no post-transplant GVHD prophylaxis was given. Engraftment was very rapid in all patients. Few had acute grade I-II GVHD, and none developed chronic GVHD. Immune reconstitution was fast. Our group [77] prospectively assessed functional and phenotypic characteristics of γδ T lymphocytes up to 7 months after haplo-HSCT depleted of αβ+ T cells and CD19+ B cells in 27 children with either malignant (n=15) or nonmalignant disorders. Notably, in patients that experienced CMV reactivation they observed a

significant expansion of Vδ1 T-cell subset; these subsets display a cytotoxic phenotype and degranulate when challenged with primary acute myeloid and lymphoid leukemia blasts. These results have been recently confirmed in 23 children with non-malignant disorders[82]. The cumulative incidence of grade 1 to 2 acute GVHD was 13.1%. None of the 21 patients at risk developed chronic GVHD. The 2-year DFS was 91%. Two died of infectious complications (one CMV-related pneumonia and one disseminated adenovirus infection) 120 and 116 days after HSCT, respectively. Overall, 9 children experienced viral infections and/or reactivations, the cumulative incidence of CMV and adenovirus infection being 38%. Nevertheless, the cumulative incidence of TRM was 9%.

Perko et al [83] recently investigated immunological reconstitution of 102 pediatric patients with acute leukemia who underwent HSCT in first complete remission, focusing on potential role of γδ T-cells. They found that γδ T cell recovering during the first year after HSCT correlated with a reduced incidence of infection. Indeed, patients with an elevated number of γδ T cell experienced only viral infection, while low/normal γδ T cell group had viral, bacterial and fungal infections; cumulative incidence of bacterial infection was 0% vs 26.4%, respectively. Enhanced γδ T cell recovery resulted in higher EFS rate at 1 year. Possible reason to explain these results could include faster reconstitution of intestinal mucosa integrity, or prompt anti-infective function of γδ T cell, and possibly a better balance within gut microbiota.

All these recent experiences confirm that current T cell-depleted HSCT strategies (either Treg/Tcon immuno-therapy or αβ T cell depletion) offer the unique opportunity to harness both natural and adaptive immunity to control leukemia relapse and infections in the absence of GvHD.

Patients and Methods

This is a single arm, prospective study conducted by Department of Pediatric Oncohematology and Bone Marrow Transplant, IRCCS Bambino Gesù Pediatric Hospital, Rome.

We analysed 111 patients who underwent allogeneic T-alpha/beta/CD19 depleted HSCT from an HLA-Haploidentical donor between September, 2011 and May 2018 (median follow up 47 months, range 2-50) were enrolled in this study. Informed written consent was provided according to the Declaration of Helsinki.

Original diagnosis were Acute myeloid Leukemia (AML, N 29) or Acute Lymphoblastic Leukemia (ALL, N 82). Twelve patients with ALL and 13 patients with AML had recurrent molecular/cytogenetic mutation. Of ALL patients, 19 patients were in fist complete remission (CR1) at time of transplantation (17%), 51 (46%) patients were in second complete remission (CR2) and 12 patients were in third or later complete remission at HSCT (11%); 4 patients had undergone prior HSCT. Besides, 20 AML patients were in CR1 (18%) and 9 patients underwent HSCT in CR2; one patient had undergone previous HSCT.

All patients received fully myleoablative conditioning regimen, TBI-based or busulfan-based. All conditioning regimen are detailed below

Treatment Plan (1)

Day -7,-6,-5	Total body irradiation (TBI) 12 Gy (total dose 200 cGy x 2 x 3 days).
Day -4,-3	Thiotepa 5 mg/kg x 2 days
Day -2,	Melfalan 140 mg/m^2
Day -4,-3,-2	Rabbit ATG Neovii 4 mg/kg x 3 days
Day -1	Rituximab 200 mg/m^2
Day 0	T-alpha/beta/CD19 depleted graft

Treatment Plan (2)

Day -10,-9,-8	Total body irradiation (TBI) 12 Gy (total dose 200 cGy x 2 x 3 days).
Day -7	Thiotepa 5 mg/kg x 2/day (total 10 mg/kg)
Day -6,-5,-4,-3,	Fludarabine 40 mg/m^2/day x4 days
Day -4,-3,-2	Rabbit ATG Neovii 4 mg/kg x 3 days
Day -1	Rituximab 200 mg/m^2
Day 0	T-alpha/beta/CD19 depleted graft

Treatment Plan (3)

Day -7,-6,-5,-4	Busulfan 0,8-1,2 mg/kg x 4 /day x 4 days Fludarabine 30 mg/m^2 x 4 days
Day -3	Fludarabine 30 mg/m^2
Day -2,	Thiotepa 5 mg/kg x 2 /day
Day -4,-3,-2	Rabbit ATG Neovii 4 mg/kg x 3 days
Day -1	Rituximab 200 mg/m^2
Day 0	T-alpha/beta/CD19 depleted graft

Treatment Plan (4)

Day -8,-7,-6,-5	Busulfan 0,8-1,2 mg/kg x 4 /day x 4 days
Day -4, -3	Cyclophosphamide 60 mg/kg x 2 days
Day -2,	Melfalan 140 mg/m^2
Day -4,-3,-2	Rabbit ATG Neovii 4 mg/kg x 3 days
Day -1	Rituximab 200 mg/m^2
Day 0	T-alpha/beta/CD19 depleted graft

Anti-T lymphocyte globulin was administered at a dose of 12 mg/Kg from day -5 to -3 for preventing graft rejection and graft-versus-host disease (GvHD). Moreover, to reduce the risk of EBV-related post-transplant lymphoproliferative disorder (PTLD), on day -1, patients received rituximab (200 mg/m^2) for *in vivo* depletion of both donor and recipient B cells.

Stem Cell Source

Peripheral blood stem cells were collected from donor at day -1, then incubated overnight and manipulated on day 0.

Donor mobilization and graft manipulation procedures have been following common standard practice guidelines as already published . Briefly, donors received granulocyte-colony stimulating factor for 4 days at 12 mg/kg body weight in 2 divided doses to induce peripheral mobilization of CD34+ hematopoietic progenitors. Apheresis was performed on day 5 after start of mobilization. When on day 4 the CD341 cell count was lower than 40/mL and/or the predicted apheresis yields was $<12.0 \times 10^6$ CD34+ HSC/kg recipient's body weight, according to a previously reported formula, Plerixafor (Mozobil) was given at 0.24 mg/kg with the aim of boosting mobilization of hematopoietic stem/progenitor cells. Plerixafor was usually given at midnight, 9 hours prior to collection on day 5. Large-volume apheresis was performed with the Spectra Optia Cell Separator (Terumo BCT, Leuven, Belgium). Manipulations were performed in a closed system. Clinical grade reagents, disposable kits, and instrumentation were from Miltenyi Biotec (Bergisch Gladbach, Germany). Procedures were performed with the fully automated CliniMACS device in a laminar-flow hood, located in a clean room certified for sterile manipulations

Supportive Care

Antimicrobial prophylaxis was started during the conditioning regimen and consisted of acyclovir 10 mg/kg 3 times a day, piperacillin/tazobactam 100 mg/kg 3 times a day, cotrimoxazole 5 mg/kg twice daily until day -2, and lyposomal amphotericin B 3 mg/kg twice a week until day +60

Clinical Follow up

Patients were clinically monitored after HSCT to check early and late clinical complications or viral reactivations until time of last follow up or death due to relapse or other causes. Biweekly cytomegalovirus (CMV) and adenovirus (ADV) monitoring by PCR was started on day -7, until day þ100 and weekly until day þ180.Weekly Epstein-Barr virus (EBV) and HHV6 monitoring by PCR was started on day þ15, as described by Coppoletta et al. Sore swab for respiratory viruses RT-PCR and blood adenovirus and HHV-6 RT-PCR detection were run according to clinical symptoms or suspect.

Donor selection

The donor was mainly chosen according to immunological criteria, giving priority to NK-cell alloreactivity, evaluated according to the killer immunoglobulin-like receptor (KIR)/KIR-Ligand mismatch in graft-versus-host direction model, KIR B haplotype, higher B-content score and size of NK alloreactive subset.

HLA typing

Complete high-resolution, allele-level *HLA-A*, *HLA-B*, *HLA-C*, *HLA-DRB1*, *HLA-DRB5*, *HLA-DQA1*, *HLA-DQB1*, and *HLA-DPB1* typing data were obtained from Histocompatibility Laboratory of IRCCS Bambino Gesù Pediatric Hospital, Rome

Analysis of KIR-L in donor/recipient pairs

Donor and patient high resolution HLA class I alleles were analyzed for KIR-ligand presence using the web site http://www.ebi.ac.uk/ipd/kir/ligand.html, obtaining also the information if there was any mismatching in the GvH direction, essential to define the donor as potentially NK alloreactive versus the patient. We implemented the information obtained from this web site with the knowledge from the literature concerning KIR

recognition. Regarding C1 epitope, in addition to HLA-C alleles carrying Asn^{80}, we included also B*46:01 and B*73:01, demonstrated to be recognized by KIR2DL2/L3 (Moesta, JI 2008?). Regarding Bw4 epitope, we included $Bw4^+$ HLA-A alleles (e.g. A*23, A*24, A*32), while we excluded HLA-B*13:01 and –B*13:02 from $Bw4^+$ HLA-B, taking into consideration the documented recognition by KIR3DL1 (Foley, Blood 2008).

Analysis of donors KIR genotype

DNA of the tested samples were extracted using QIAamp DNA Blood Mini kit (QIAGEN, GmbH, Germany). The KIR genes profiles were performed using Olerup SSP-PCR KIR genotyping kit (GenoVision, Saltsjoebaden, Sweden) following the manufacture's instruction. This protocol has been already successfully used and allows detection of the presence/absence of all KIR genes (Falco JI 2010). The results obtained were utilized to assess the type of KIR genotype (A/A or B/x), and the B content value (through the analysis of the centromeric and telomeric regions), as previously described (Cooley Blood 2010).

Phenotypic analysis of donor NK cells

Peripheral blood mononuclear cells (PBMC) were obtained after ficoll gradient separation of heparinized blood samples. Phenotype of donor NK cells, gating $CD3^-$ $CD56^+$ cells in PBMC, was evaluated in multi-color immunofluorescence analysis using appropriate combinations of the various anti-KIR, anti-NKG2A and anti-NKG2C mAbs, as already described (Pende Blood 2009). This allowed to discriminate aKIR versus iKIR expressing cells; in particular, the combination of EB6 (anti-KIR2DL1/S1) with 143211 (anti-KIR2DL1) allowed to detect $KIR2DS1^+$ ($EB6^+143211^-$) cells. Staining with DX9 (anti-KIR3DL1) was highly informative especially in donors carrying KIR3DL1*004 allele, known to be retained in the cytoplasm, to understand if another

surface expressed allele was also expressed. Staining the iKIR specific for the KIR-L mismatch and the aKIR, conjugated with one fluorochrome (e.g. PE), while the other iKIRs (i.e. specific for patients KIR-L) together with NKG2A, conjugated with another fluorochrome (e.g. FITC), allowed to detect the size of the alloreactive subset (e.g. % of $PE^{+}/FITC^{-}$ cells).

Definitions

NK alloreactivity

Donors were defined as NK alloreactive if expressed a KIR-L which was missing in the recipient, and their KIR gene profile included the iKIR specific for the mismatched KIR-L, thus demonstrating a KIR/KIR-L mismatch in GvH direction. This genetically defined NK alloreactivity was implemented by the cytofluorimetric analysis of NK cells, documenting the surface expression of the iKIR of interest and the size of the alloreactive subset (i.e. cells expressing only this iKIR as HLA-specific inhibitory receptor)

B-content

Donors were divided in two groups according to the number of KIR B gene motifs: donors with a total number of KIR-associated domains of 2 or greater versus donors with a total B-content score of 0-1. According to Cooley et al. KIR-B content >=2 seems to be associated to better outcome in AL patients.

Primary graft failure

Primary graft failure was defined as <5% donor chimerism at day + 30

Engraftment

Time to neutrophil engraftment was defined as time from haplo-HSCT to the first of 3 consecutive days with an absolute neutrophil count >0.5 x 10^{9}/L, whereas time to

platelet engraftment was defined as time from transplantation to the first of 7 consecutive days with an unsupported platelet count >20 x 10^9/L.

Blood stream infection (BSI)

Pre-engraftment BSI was defined as the isolation of a bacterial or fungal pathogen from at least 1 blood culture, or 2 consecutive blood cultures for coagulase negative staphylococci (CoNS), corynebacteria, and other common skin contaminants

CMV reactivation

CMV reactivation was defined as RT-PCR CMVDNA >1000 copies/mmc with RT-PCR method. ***Other Viral infection***

We considered symptomatic upper or lower respiratory tract infections which had at least one sore swab positive for RT-PCR detection of respiratory viruses, as well as adenovirus (ADV)-DNA, HHV6-DNA and EBV-DNA RT-PCR blood detection (if > 1000copies/mmc).

Acute and chronic GVHD

Incidence, organ involvement and maximum grade of acute GvHD by day +100 post-transplant graded according to the Glucksberg scale (Appendix N. 2). Incidence, organ involvement and severity of chronic GvHD by 1year post-transplant graded according to Shulman scale (Appendix N. 3).

First-line therapy of GvHD was methylprednisolone up to 2 mg/kg/day; second-line therapy included extracorporeal photopheresis, monoclonal antibodies, MMF, as per institutional protocols.

Non relapse mortality

Non relapse mortality (NRM) was defined as death due to any other cause than progression of malignancy after allogeneic stem cell transplantation.

Overall survival

Overall Survival (OS) was defined as the time between transplantation and date of death due to any cause or the last date the patient was known to be alive (censored observation).

Leukemia free survival

Disease free survival (DFS) was defined as the time between transplantation and date of relapse or date of death due to NRM.

Statistical analysis

Quantitative variables were reported as median value and range, whereas categorical variables were expressed as absolute value and percentage. Dicothomious variables were compared using Chi-square Test or, whereas necessary, Fisher's exact test, whereas the Mann-Whitney rank sum test or the Student t test was used for continuous variables, as appropriate. Rejection, engraftment, acute and chronic GVHD, OS, LFS, NRM, and relapse incidence were estimated from the date of transplantation to the date of an event or last follow-up. Probabilities of OS, LFS, and EFS were calculated according to the Kaplan and Meier method.24 Engraftment, acute GVHD and chronic GVHD,NRM, and relapse were calculated as cumulative incidence curves in order to adjust the estimates for competing risks.25 All results were expressed as probability or cumulative incidence (%) and 95% confidence interval (95% CI). The significance of differences between survival probabilities was estimated by the log-rank test (Mantel-Cox), whereas Gray's test was used to assess, in univariable analyses, differences between cumulative incidences. Multivariable analysis was performed using the Cox proportional hazard regression model.

Results

Engraftment and early complications

Patients were infused with allogeneic PBSC at day 0, with a median value of CD34+ of 14.4 x 10^6/kg (range 6-40.4); median T alpha/beta were 0.04 x 10^6/kg, T gamma/delta 7.5 x 10^6/kg (range 0.002-0.099), NK cells 32.2 x 10^6/kg (range 2-146.1), respectively.

Primary sustained engraftment was achieved in 109 out of 111 evaluable patients.

Two patients did not engraft; 1 patient was successfully rescued through haplo-HSCT from the other parent, whereas the other died of disseminated adenovirus infection, despite receiving a second allograft from the same donor with engraftment and hematopoietic recovery. This patient was 1 of the 2 with donor-specific alloantibodies.

Median time to PMN and PLT engraftment was respectively 13 days (9-22) and 11 days (8-20).

Overall outcomes

86 out of 111 patients were alive at time of last follow up (range 2- 92 months). Median age at diagnosis was 6.7 years old (range 0.4-22), while median age at transplant was 10 years (range 0.9-22.2). Median donor age was 41 years old (range 21-56).

Overall survival was 72,2%, with a median follow up of 47 months (range 2-92); Leukemia free survival was 70.2 % at 5 years. **(Fig.1)** Moreover, 23 patients relapsed with a median interval from transplant to hematological relapse of 186 days (range 60-1012), being cumulative incidence of relapse 24% (95% CI, 16-33).

Overall incidence of 5 years-NRM was 6% (6/111). One patient died of multi-organ failure, while 5 out of 6 patients died of infection (1 ARDS due to systemic infection caused by P.Aeruginosa, 2 idiopathic pneumonia, 1 pulmonary hemorrhage, 1 viral pneumonia due to adenovirus). **(Fig.2)**

aGvHD and cGvHD

Overall incidence of acute GvHD (aGvHD) grade I-II was 25%; remarkably, no patient experienced grade III-IV aGvHD and skin was the only organ involved in all but one child who had gut involvement. Nine patients and 19 patients experienced Grade I and II aGvHD, respectively.

Overall incidence of chronic GvHD (cGvHD) was 5%, with 4 patients experiencing limited severity cGvHD; no patient developed moderate/severe cGvHD. **(fig. 3-4)**

Disease related variables

Patients characteristics are shown in Table 1.

The 5-year probability of LFS in children with ALL and AML was 69% (95% CI, 57-79) and 73% (95% CI, 52-86), respectively **(Figure 5).** Use of total body irradiation (TBI) during the preparative regimen was associated with better patient's outcome **(Figure 6)**, since it protected against the risk of leukemia recurrence [18% (95% CI, 10-28) vs. 45% (95% CI, 22-66) in patients who did or did not receive TBI, respectively, $p<0.01$]. The correlation between use of TBI and better outcome remained significant in multivariable analysis, with a hazard ratio of 0.35 (95% CI, 0.16- 0.78, $p=0.01$) for LFS.

Infectious complications

Forty-one out of 111 patients experienced CMV reactivation, with a median time interval from Haplo-HSCT of 23 days (range 4-158), with a cumulative incidence of 30.4% (95% CI 15.7-42.5). None of the patient developed CMV disease; nor CMV serostatus nor CMV reactivation did affect OS and DFS. CMV serostatus in donor/recipient pairs was CMV +/+ in 81 cases, CMV +/- in 7, CMV -/+ in 14, CMV -/- in 7 cases, respectively.

Adenoviremia was observed in 16 out of 111 patients, with a cumulative incidence of 19.5% (95% CI 7.2-30.2); median time to adenovirus reactivation was 34 days (range 1-

121) and one patient experienced disseminated uncontrolled ADV-disease which eventually caused his death.

HHV6 reactivation was seen in 16 out of 111 patients, and 2 patients experienced EBV reactivation. Moreover, 66 patients developed at least one viral infection, 8 patients experienced upper respiratory tract viral infections other than CMV and ADV, with 9 patients experiencing both CMV and other viral infection.

Two patients experienced invasive pulmonary aspergillosis, one of whom was a fatal complication.

Five patients developed BSI, all caused by Gram negative bacteria; 3 of them were pre-engraftment BSI. One patient died of ARDS caused by *P.Aeruginosa ESBL-producer* after post-engraftment BSI.

NK alloreactivity and KIR genotype

Donor's characteristics according to NK alloreactivity, KIR-genotype and KIR 2DS1 are reported in Table 2. Of 111 patients who were studied for NK alloreactivity, 31 patients were alloreactive versus 60 non-NK alloreactive patients. LFS and OS did not significantly changed between the two groups. Relapse rates were also similar and incidence of aGvHD and cGvHD did not differ between the 2 groups.

Discussion

T- and B-cell depleted Haplo-HSCT has been considered for decades a risky treatment option, since the use of extensive T-cell depletion was associated with extremely delayed immune reconstitution and a high risk of developing opportunistic infections even years after HSCT. The intriguing success rate of this platform relied on NK cell alloreactivity, which supplied T-cell function in the early post-engraftment period. Unfortunately, NK cell activation, even if effective in controlling leukemia relapse, was not sufficiently helpful in post-HSCT acquisition of immune adaptive response, leading to harmless weapons against viral reactivation or fungal infections. In the last decade, several tecniques have been refined in order to add T-lymphocytes to the graft to enhance adaptive immunity, leading to the selective depletion of alfa/beta T-cells, sparing gamma/delta T-cells who are not responsible for GvHD and can contribute to early adaptive immune response.

Gamma/delta T-cells combine conventional adaptive features with rapid, innate-like responses that place them in the initiation phase of immune reactions. In addition, gamma/delta T-cells recognize tumor cells without recourse to the classical major histocompatibility complex (MHC) presentation, with rare exceptions. Among circulating gamma/delta T cells, there is a major subset bearing Vd2 chain, always associated with Vg9 (ie,Vg9Vd2), and a minor subset bearing Vd1chain. Vg9Vd2 cells recognize nonpeptide phosphoantigens and kill a wide variety of tumor cells including acute myeloid leukemia (AML) blasts, lymphoma cells, and putative cancer stem cells. Aminobisphosphonates, such as zoledronic acid (ZOL), activate and expand Vg9Vd2 T cells in vitro and sensitize tumor target cells to Vd2-mediated lysis, their use thus representing an attracting approach for immunotherapeutic strategies against cancer. Vd1 cells reside within epithelial tissues, especially at sites of cytomegalovirus (CMV)

replication, and exert potent cytotoxic effects against acute lymphoblastic leukemia (ALL) or AML cells, chronic lymphocytic leukemia cells, and primary multiple myeloma cells. Overall, gamma/delta T lymphocytes are important effector cells, especially in situations where the function of adaptive immunity is impaired.

Based on these findings, we studied long-term outcome results of AL patients after alpha-beta/CD19 depleted haplo-HSCT. We observed a high rate of engraftment (98%) with a low incidence of both acute and chronic GVHD, which contributed to the reduced risk of NRM. Remarkably, none of patients had either grade 3-4 or gut/liver acute GVHD and all cases of chronic GVHD were of limited severity. One could argue that low incidence of GvHD, due to the absence of alpha/beta T-cells in the graft, could lead to higher rate of NRM compared to conventional grafts, but this was not observed. In fact, although T cells displaying the ab TCR are responsible for GVHD, T cells carrying the gamma/delta receptor chains have no alloreactive capacity, but contribute an important anti-infectious activity. It is conceivable that the high number of gamma/delta T cells adoptively transferred with the graft in our patients may have contributed to prevent disease recurrence and severe infections. Also donor-derived, mature NK cells, lost in the procedure of positive selection of CD34+ cells and spared in our ab T-cell–depleted graft, exhibit a graft-versus-leukemia (GVL) effect and participate in the control of opportunistic infections, including HCMV. In previous studies, we documented that in haplo-HSCT recipients given positively selected CD34+ cells, 8 weeks after transplantation are needed to detect mature KIR+ NK cells, and this gap in reconstitution may favor early leukemia relapse in the case of high residual tumor burden and/or rapidly proliferating blasts. Through the approach of selective alpha/beta T- and B-cell depletion, the recipient immediately benefits from high numbers of donor mature NK cells that can fully display their activity, because the recipient is not exposed to the

effect of pharmacological prophylaxis of GVHD, which can impair differentiation/expansion of this lymphocyte subset. Altogether, the infusion of cells belonging to innate immunity, together with that of high numbers of committed hematopoietic progenitors and monocyte/ dendritic cells (in particular, in patients whose donor was mobilized with granulocyte-colony stimulating factor and plerixafor) may have contributed to the low risk of NRM, which we found to be comparable to that observed after transplantation from an HLA-compatible donor, either a sibling, or a UD. We did not document any favorable influence of NK alloreactivity and of donor KIR B haplotype reported in other studies mainly based on infusion of CD34+ cells, likely because the NK-mediated GVL effect was partially obscured by other cells present in the graft, including gamma/delta T cells.

Recently, nonprospective studies enrolling smaller cohorts of patients with shorter follow-up, analyzing the outcome of children given alpha/beta T- and B-cell–depleted haplo-HSCT, have been published. Maschan et al analyzed the outcome of children with high-risk AML, who received transplantation from UD (n=20) and haploidentical donors (n=13) after this graft manipulation. Twenty-eight patients were given posttransplantation pharmacological immune suppression, including tacrolimus until day 130 and methotrexate in 21 patients, tacrolimus in 5, methotrexate in 2, whereas 5 patients did not receive prophylaxis. Notably, recipients of haploidentical grafts more commonly developed isolated skin GVHD, whereas gastrointestinal involvement was more common in UD HSCT. Cumulative incidence of relapse at 2 years in the 13 haplo-HSCT recipients was 40% (95% CI: 20-80), whereas the LFS probability was 59% (95% CI: 31-87). Lang et al recently published the retrospective analysis of immune recovery in a cohort of 41 pediatric patients, with AL, myelodysplastic syndrome, and nonmalignant diseases (n=55), who received alpha/beta T and B-cell–depleted allografts

from a haploidentical relative after reduced-toxicity regimens. Grade 3-4 acute GVHD occurred in 15% of patients; with a median follow-up of 1.6 years, 21 of the 41 patients were alive and relapse was the major cause of death (n=17). Also in this cohort of patients, disease recurrence was the main cause of treatment failure, with a 24% CI of relapse. The lower incidence of relapse in our patients can be attributed, at least partly, to the use of fully myeloablative conditioning regimens and to the lack of posttransplantation GVHD prophylaxis, potentially able to impair the innate immunity-mediated GVL effect. Support to the former interpretation is given by the observation that a better outcome was observed when we used conditioning regimens including TBI, which, although more toxic in the long term for children, displays potent antileukemia activity potentially compensating for the lack of ab T-cell–mediated GVL effect. In addition, we hypothesize that the accurate identification and determination of alloreactive NK cells, as well as a refined analysis of the main activating NK receptors, allowed selecting donors with high antileukemia activity, thus contributing to reduce the risk of relapse.

A large, retrospective multicenter comparative study analyzing outcome of 98 alpha/beta T- and B-cell depleted Haplo-HSCT, 127 HSCT from MUD and 118 from mismatched unrelated donors (MMUD)has been published. All these AL patients were transplanted between 2010 and 2015 in 13 italian centers in morphological remission, after myeloablative conditioning regimen. Graft failure occurred in 2% each of UD-HSCT and αβhaplo-HSCT group. In MUD vs MMUD-HSCT recipients, the cumulative incidence (CI) of grade II-IV and grade III-IV acute GvHD was 35% vs 44% and 6% vs 18%, as compared to 16% and 0% in αβhaplo-HSCT recipients (P<0.001). Eight (6%) MUD, 32 (28%) MMUD and 9 (9%) αβhaplo-HSCT patients died from transplant-related complications. With a median follow-up of 3.3 years, the 5-year probability of

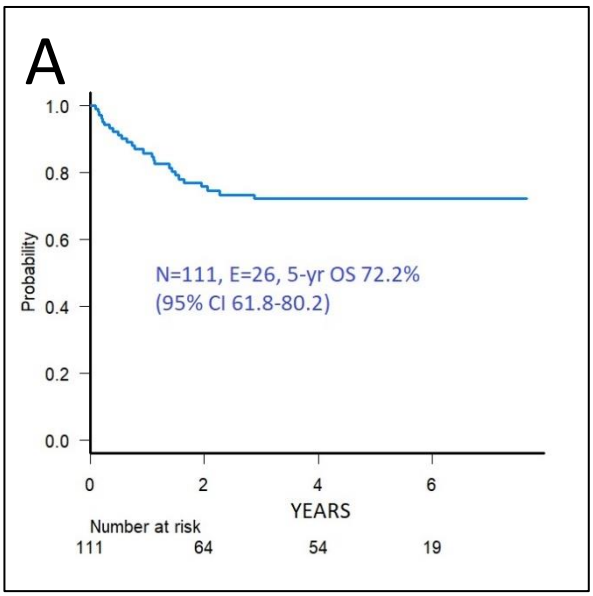
leukemia-free survival in the 3 groups was 67%, 55% and 62% respectively. In the three groups, chronic GvHD-free/relapse-free (GRFS) probability of survival was 61%, 34% and 58%, respectively (P<0.001). When compared to patients given MMUD-HSCT, αβhaplo-HSCT recipients had a lower CI of NRM and a better GFRS (P<0.001)

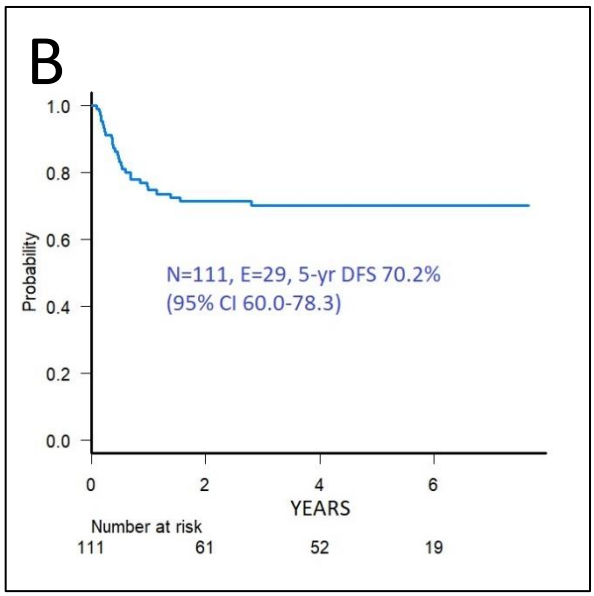
These data confirm that αβhaplo-HSCT is an effective treatment option for patients with AL in need of transplantation, especially when an allele-matched UD is not available.

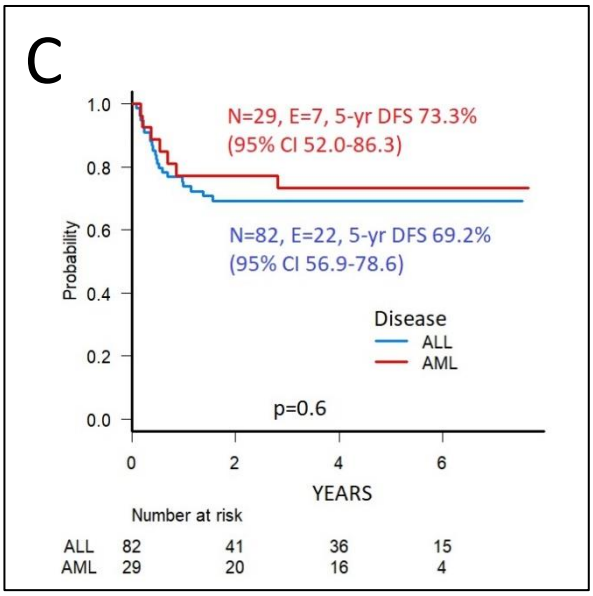
In the last few years, alternative platforms, such as that based on posttransplantation infusion of cyclophosphamide, have been developed. Although largely used in adults, few studies have been published on the use of this approach for modulating alloreactivity in AL children. Although certainly cheaper than the ab T- and B-cell depletion, the use of posttransplantation cyclophosphamide seems to be associated with a risk of leukemia recurrence higher than that observed in our cohort. Future studies will further clarify the relative advantages and limitations of these 2 different haplo-HSCT platforms.

Conclusion

In summary, our data indicate that, through more refined approaches of graft manipulation, haplo-HSCT offers the opportunity to transplant virtually every child in need of an allograft, with an expected outcome comparable to that obtained when the donor is a HLAmatched sibling or an allelic-matched volunteer.







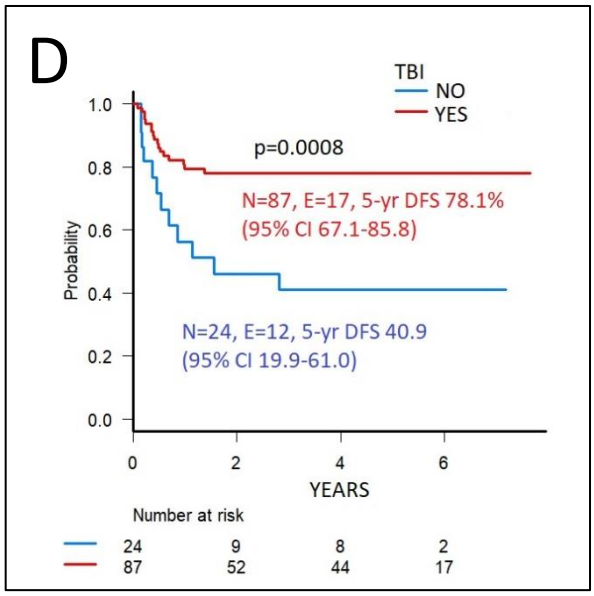


Fig.1 A &1B OS and DFS. 1C DFS according to disease type, 1D DFS according to use of TBI or not

Patients	(n = 111)
Sex	
Male	78 (70%)
Female	33 (30%)
Median (range) age at diagnosis, y	6.7 (0.4-22)
ALL phenotype	
BCP	67 (82%)
T	15 (18%)
ALL recurrent molecular lesions	
t(4;11)(AF4/MLL)	3
t(9;22)(BCR/ABL)	2
SIL-TAL	1
t(12;21)(TEL/AML1)	5
Hypodiploid	1
AML recurrent molecular/cytogenetic lesions	
MLL rearranged	4
FLT3-ITD	2
Complex karyotype	3
inv(16) (MYH11-CBFB)	2
7-	1
Other	1
Disease status at transplantation	
ALL	
CR1	19 (17%)
CR2	51 (46%)
≥CR3	12 (11%)
Previous HSCT	4 (4%)
AML	
CR1	20 (18%)
CR2	9 (8%)
Previous HSCT	1 (1%)
Conditioning regimen§	
TBI+TT+Flu	55 (50%)
TBI+TT+L-PAM	29 (26%)
TT+Bu+Flu	11 (10%)
Bu+Cy+L-PAM	9 (8%)
Others	6 (6%)
CMV serology (donor/recipient)	
Neg/Neg	7 (6%)
Neg/Pos	7 (6%)
Pos/Neg	14 (13%)
Pos/Pos	83 (75%)
Donor characteristics	
Age, y	41 (21-56)
Type of donor	
Mother	61 (55%)
Father	40 (45%)
Sex mismatch	65 (59%)
Female donor → Male recipient	45/65 (69%)
Cell dose infused, median (range)	
$CD34^+$ cells × 10^6/kg	14.4 (6-40.44)
$\alpha\beta^+$ T cells × 10^6/kg	0.04 (0.002-0.099)
$\gamma\delta^+$ T cells × 10^6/kg	7.56 (0.86-56.7)
NK cells × 10^6/kg	32.2 (2-146.1)

Table 1: Patients characteristics

References

1. Moretta A, Bottino C, Vitale M, Pende D, Biassoni R, Mingari MC, Moretta L. Receptors for HLA class-I molecules in human natural killer cells. Annu Rev Immunol. 1996; 14:619-48. Pubmed PMID:8717527

2. Parham P. MHC class I molecules and KIRs in human history, health and survival. Nat Rev Immunol. 2005; 5:201-14. PMID: 15719024

3. Elliott JM, Yokoyama WM. Unifying concepts of MHC-dependent natural killer cell education. Trends Immunol. 2011; 32(8):364-72. PMID: 21752715

4. Moretta A, Bottino C, Vitale M, Pende D, Cantoni C, Mingari MC, Biassoni R, Moretta L. Activating receptors and coreceptors involved in human natural killer cell-mediated cytolysis. Annu Rev Immunol. 2001;19:197-223. PMID11244035

5. Vivier E, Raulet DH, Moretta A, Caligiuri MA, Zitvogel L, Lanier LL, Yokoyama WM, Ugolini S. Innate or adaptive immunity? The example of natural killer cells. Science. 2011; 331(6013):44-9. PMID:21212348

6. Pende D, Spaggiari GM, Marcenaro S, Martini S, Rivera P, Capobianco A, Falco M, Lanino E, Pierri I, Zambello R, Bacigalupo A, Mingari MC, Moretta A, Moretta L. Analysis of the receptor-ligand interactions in the natural killer-mediated lysis of freshly isolated myeloid or lymphoblastic leukemias: evidence for the involvement of the Poliovirus receptor (CD155) and Nectin-2 (CD112). Blood. 2005;105(5):2066-73. PMID:15536144

7. Yu J, Freud AG, Caligiuri MA. Location and cellular stages of natural killer cell development. Trends Immunol. 2013; 34(12):573-82. PMID:24055329

8. Björkström NK, Riese P, Heuts F, Andersson S, Fauriat C, Ivarsson MA, Björklund AT, Flodström-Tullberg M, Michaëlsson J, Rottenberg ME, Guzmán CA, Ljunggren HG, Malmberg KJ. Expression patterns of NKG2A, KIR, and CD57 define a process of CD56dim NK-cell differentiation uncoupled from NK-cell education. Blood. 2010; 116(19):3853-64. PMID: 20696944

9. Aversa F, Tabilio A, Terenzi A, Velardi A, Falzetti F, Giannoni C, Iacucci R, Zei T, Martelli MP, Gambelunghe C, et al. Successful engraftment of T-cell-depleted haploidentical "three-loci" incompatible transplants in leukemia patients by addition of recombinant human granulocyte colony-stimulating factor-mobilized peripheral blood progenitor cells to bone marrow inoculum. Blood. 1994; 84(11):3948-55. PMID: 7524753

10. Ruggeri L, Capanni M, Urbani E, Perruccio K, Shlomchik WD, Tosti A, Posati S, Rogaia D, Frassoni F, Aversa F, Martelli MF, Velardi A. Effectiveness of donor natural killer cell alloreactivity in mismatched hematopoietic transplants. Science. 2002 Mar 15;295(5562):2097-100. PMID: 11896281

11. Pende D, Marcenaro S, Falco M, Martini S, Bernardo ME, Montagna D, Romeo E, Cognet C, Martinetti M, Maccario R, Mingari MC, Vivier E, Moretta L, Locatelli F, Moretta A. Anti-leukemia activity of alloreactive NK cells in KIR ligand-mismatched haploidentical HSCT for pediatric patients: evaluation of the functional role of activating KIR and redefinition of inhibitory KIR specificity. Blood. 2009; 113(13):3119-29. PMID: 18945967

12. Cooley S, Weisdorf DJ, Guethlein LA, Klein JP, Wang T, Le CT, Marsh SG, Geraghty D, Spellman S, Haagenson MD, Ladner M, Trachtenberg E, Parham P, Miller

JS. Donor selection for natural killer cell receptor genes leads to superior survival after unrelated transplantation for acute myelogenous leukemia. Blood. 2010; 116(14):2411-9. PMID: 20581313
13. Cooley S. et al. J. Immunol. 2014, 192:4592-4600.
14. Oevermann L, et al. Blood. 2014, 124:2744-2747.
15. Cooper MA, Fehniger TA, Caligiuri MA. The biol- ogy of human natural killer-cell subsets. Trends Immunol. 2001;22:633-640.
16. Freud AG, Yokohama A, Becknell B, et al. Evi- dence for discrete stages of human natural killer cell differentiation in vivo. J Exp Med. 2006;203: 1033-1043.
17. Chklovskaia E, Nowbakht P, Nissen C, Gratwohl A, Bargetzi M, Wodnar-Filipowicz A. Reconstitu- tion of dendritic and natural killer-cell subsets af- ter allogeneic stem cell transplantation: effects of endogenous flt3 ligand. Blood. 2004;103:3860- 3868.
18. Jacobs R, Stoll M, Stratmann G, Leo R, Link H, Schmidt RE. CD16- CD56 natural killer cells after bone marrow transplantation. Blood. 1992; 79:3239-3244.
19. Shilling HG, McQueen KL, Cheng NW, Shizuru JA, Negrin RS, Parham P. Reconstitution of NK cell receptor repertoire following HLA-matched hematopoietic cell transplantation. Blood. 2003; 101:3730-3740
20. Davies SM, Ruggieri L, DeFor T, et al. Evaluation of KIR ligand incompatibility in mismatched unre- lated donor hematopoietic transplants: killer im- munoglobulin-like receptor. Blood. 2002;100: 3825-3827
21. Farag SS, Bacigalupo A, Eapen M, et al. The ef- fect of KIR ligand incompatibility on the outcome of unrelated donor transplantation: a report from the center for international blood and marrow transplant research, the European blood and marrow transplant registry, and the Dutch registry. Biol Blood Marrow Transplant. 2006;12:876-884.
22. Bornhuser M, Schwerdtfeger R, Martin H, Frank KH, Theuser C, Ehninger G. Role of KIR ligand incompatibility in hematopoietic stem cell trans- plantation using unrelated donors. Blood. 2004; 103:2860-2861; author reply 2862.
23. Giebel S, Locatelli F, Lamparelli T, et al. Survival advantage with KIR ligand incompatibility in he- matopoietic stem cell transplantation from unre- lated donors. Blood. 2003;102:814-81
24. Ganguly S, Fuchs EJ, Radojcic V, Luznik L. Critical Role of CD4+Foxp3+ T cells in GVHD Prevention with High-dose Posttransplantation Cyclophosphamide (Cy). Blood. 2010; 116
25. Galy A, et al. Human T,B. natural killer, and dendritic cells arise from a common bone marrow progenitor cell subset. Immunity. 1995; 3(4):459–737
26. Ferlazzo G, et al. The abundant NK cells in human secondary lymphoid tissues require activation to express killer cell Ig-like receptors and become cytolytic. J Immunol. 2004; 172(3):1455–62
27. Symons HJ, Leffell MS, Rossiter ND, Zahurak M, Jones RJ, Fuchs EJ. Improved survival withinhibitory killer immunoglobulin receptor (KIR) gene mismatches and KIR haplotype B donors after nonmyeloablative, HLA-haploidentical bone marrow transplantation. Biol Blood Marrow Transplant. 2010; 16:533–42.
28. Rauch A, Laird R, McKinnon E, Telenti A, et al. Influence of inhibitory killer immunoglobulin-like receptors and their HLA-C ligands on resolving hepatitis C virus infection. Tissue Antigens. 2007;69:237-240
29. Alter G, Martin MP, Teigen N, et al. Differential natural killer cell mediated inhibition of HIV-1 replication based on distinct KIR/HLA subtypes. J Exp Med. 2007;204:3027-3036

30. Miller JS, Soignier Y, Panoskaltsis-Mortari A, et al. Successful adoptive transfer and in vivo expansion of human haploidentical NK cells in patients with cancer.Blood. 2005;105:3051-3057
31. Choi I1, Yoon SR2, Park SY2, Kim H et al,. Donor-derived natural killer cells infused after human leukocyte antigen-haploidentical hematopoietic cell transplantation: a dose-escalation study. Biol Blood Marrow Transplant. 2014 May;20(5):696-704. doi: 10.1016/j.bbmt.2014.01.031. Epub 2014 Feb 11
32. .Passweg JR, Tichelli A, Meyer-Monard S, et al. Purified donor NK- lymphocyte infusion to consolidate engraftment after haploidentical stem cell transplantation. Leukemia 2004;18:1835-1838
33. Curti A, Ruggeri L, D'Addio A, et al. Successful transfer of alloreactive haploidentical KIR ligand-mismatched natural killer cells after infusion in elderly high risk acute myeloid leukemia patients. Blood. 2011;118: 3273-3279

34. A Perillo, L Pierelli, A Battaglia, MG Salerno, S Rutella, E Cortesi, A Fattorossi, L De Rosa, F Ferrau, M Lalle, G Leone, S Mancuso and G Scambia, Administration of low-dose interleukin-2 plus G-CSF/EPO early after autologous PBSC transplantation: effects on immune recovery and NK activity in a prospective study in women with breast and ovarian cancer. Bone Marrow Transplant, 2002. 30(9): p. 571-578.
35. J Storek, T Gillespy, 3rd, H Lu, A Joseph, MA Dawson, M Gough, J Morris, RC Hackman, PA Horn, GE Sale, RG Andrews, DG Maloney and HP Kiem, Interleukin-7 improves CD4 T-cell reconstitution after autologous CD34 cell transplantation in monkeys. Blood, 2003. 101(10): p. 4209-4218.
36. SJ Lin, PJ Cheng, DC Yan, PT Lee and HS Hsaio, Effect of interleukin-15 on alloreactivity in umbilical cord blood. Transpl Immunol, 2006. 16(2): p. 112-116.
37. XJ Huang, DH Liu, KY Liu, LP Xu, H Chen, W Han, YH Chen, XH Zhang and DP Lu, Treatment of acute leukemia with unmanipulated HLA-mismatched/haploidentical blood and bone marrow transplantation. Biol Blood Marrow Transplant, 2009. 15(2): p. 257-265.
38. P Di Bartolomeo, S Santarone, G De Angelis, A Picardi, L Cudillo, R Cerretti, G Adorno, S Angelini, M Andreani, L De Felice, MC Rapanotti, L Sarmati, P Bavaro, G Papalinetti, M Di Nicola, F Papola, M Montanari, A Nagler and W Arcese, Haploidentical, unmanipulated, G-CSF-primed bone marrow transplantation for patients with high-risk hematologic malignancies. Blood, 2013. 121(5): p. 849-857.
39. F Ciceri, M Bregni and J Peccatori, Innovative platforms for haploidentical stem cell transplantation: the role of unmanipulated donor graft. J Cancer, 2011. 2: p. 339-340.
40. J Peccatori, D Clerici, A Forcina, M Bemardi, R Crocchiolo, C Messina, M Noviello, S Mastaglio, F Giglio, S Malato, MTL Stanghelllini, S Marktel, A Assanelli, M Battaglia, A Ferraro, S Rossini, ME Bernardo, A Bondanza, MG Roncarolo, C Bonini, F Locatelli and F Ciceri, In-vivo T-regs generation by rapamycin-mycophenolate-ATG as a new platform for GvHD prophylaxis in T-cell repleted unmanipulated haploidentical peripheral stem cell transplantation: results in 59 patients. Bone Marrow Transplantation, 2010. 45: p. S3-S4.
41. L von Reyn Cream, WC Ehmann, WB Rybka and DF Claxton, Sirolimus in unmanipulated haploidentical cell transplantation. Bone Marrow Transplant, 2008. 42(11): p. 765-766.
42. AH Owens, Jr. and GW Santos, The effect of cytotoxic drugs on graft-versus-host disease in mice. Transplantation, 1971. 11(4): p. 378-382.

43. GW Santos, PJ Tutschka, R Brookmeyer, R Saral, WE Beschorner, WB Bias, HG Braine, WH Burns, ER Farmer, AD Hess, H Kaizer, D Mellits, LL Sensenbrenner, R Suart and AM Yeager, Cyclosporine plus methylprednisolone versus cyclophosphamide plus methylprednisolone as prophylaxis for graft-versus-host disease: a randomized double-blind study in patient undergoing allogenic marrow transplantation. Clin Transplant, 1987. 1: p. 21.
44. RJ Jones, JP Barber, MS Vala, MI Collector, SH Kaufmann, SM Ludeman, OM Colvin and J Hilton, Assessment of aldehyde dehydrogenase in viable cells. Blood, 1995. 85(10): p. 2742-2746.
45. TB Prigozhina, O Gurevitch, J Zhu and S Slavin, Permanent and specific transplantation tolerance induced by a nonmyeloablative treatment to a wide variety of allogeneic tissues: I. Induction of tolerance by a short course of total lymphoid irradiation and selective elimination of the donor-specific host lymphocytes. Transplantation, 1997. 63(10): p. 1394-1399.
46. L Luznik, PV O'Donnell, HJ Symons, AR Chen, MS Leffell, M Zahurak, TA Gooley, S Piantadosi, M Kaup, RF Ambinder, CA Huff, W Matsui, J Bolanos-Meade, I Borrello, JD Powell, E Harrington, S Warnock, M Flowers, RA Brodsky, BM Sandmaier, RF Storb, RJ Jones and EJ Fuchs, HLA-haploidentical bone marrow transplantation for hematologic malignancies using nonmyeloablative conditioning and high-dose, posttransplantation cyclophosphamide. Biol Blood Marrow Transplant, 2008. 14(6): p. 641-650.
47. A Munchel, C Kesserwan, HJ Symons, L Luznik, YL Kasamon, RJ Jones and EJ Fuchs, Nonmyeloablative, HLA-haploidentical bone marrow transplantation with high dose, post-transplantation cyclophosphamide. Pediatr Rep, 2011. 3 Suppl 2: p. e15.
48. KJ Lafferty and AJ Cunningham, A new analysis of allogeneic interactions. Aust J Exp Biol Med Sci, 1975. 53(1): p. 27-42.
49. EC Guinan, VA Boussiotis, D Neuberg, LL Brennan, N Hirano, LM Nadler and JG Gribben, Transplantation of anergic histoincompatible bone marrow allografts. N Engl J Med, 1999. 340(22): p. 1704-1714.
50. JK Davies, CM Barbon, A Voskertchian, LM Nadler and EC Guinan, Ex vivo alloanergization with belatacept: a strategy to selectively modulate alloresponses after transplantation. Cell Transplant, 2012.
51. RG Miller, An immunological suppressor cell inactivating cytotoxic T-lymphocyte precursor cells recognizing it. Nature, 1980. 287(5782): p. 544-546.
52. N Rachamim, J Gan, H Segall, R Krauthgamer, H Marcus, A Berrebi, M Martelli and Y Reisner, Tolerance induction by "megadose" hematopoietic transplants: donor-type human CD34 stem cells induce potent specific reduction of host anti-donor cytotoxic T lymphocyte precursors in mixed lymphocyte culture. Transplantation, 1998. 65(10): p. 1386-1393.
53. H Gur, R Krauthgamer, A Berrebi, T Klein, A Nagler, A Tabilio, MF Martelli and Y Reisner, Tolerance induction by megadose hematopoietic progenitor cells: expansion of veto cells by short-term culture of purified human CD34(+) cells. Blood, 2002. 99(11): p. 4174-4181.
54. H Gur, R Krauthgamer, E Bachar-Lustig, H Katchman, R Arbel-Goren, A Berrebi, T Klein, A Nagler, A Tabilio, MF Martelli and Y Reisner, Immune regulatory activity of CD34+ progenitor cells: evidence for a deletion-based mechanism mediated by TNF-alpha. Blood, 2005. 105(6): p. 2585-2593.

55. MV Dhodapkar, RM Steinman, J Krasovsky, C Munz and N Bhardwaj, Antigen-specific inhibition of effector T cell function in humans after injection of immature dendritic cells. J Exp Med, 2001. 193(2): p. 233-238.
56. L Zangi, YZ Klionsky, L Yarimi, E Bachar-Lustig, Y Eidelstein, E Shezen, D Hagin, Y Ito, T Takai, S Reich-Zeliger, A Lask, O Milstein, S Jung, V Shinder and Y Reisner, Deletion of cognate CD8 T cells by immature dendritic cells: a novel role for perforin, granzyme A, TREM-1, and TLR7. Blood, 2012. 120(8): p. 1647-1657.
57. A Moretta, C Bottino, D Pende, G Tripodi, G Tambussi, O Viale, A Orengo, M Barbaresi, A Merli, E Ciccone and et al., Identification of four subsets of human CD3-CD16+ natural killer (NK) cells by the expression of clonally distributed functional surface molecules: correlation between subset assignment of NK clones and ability to mediate specific alloantigen recognition. J Exp Med, 1990. 172(6): p. 1589-1598.
58. L Moretta, F Locatelli, D Pende, E Marcenaro, MC Mingari and A Moretta, Killer Ig-like receptor-mediated control of natural killer cell alloreactivity in haploidentical hematopoietic stem cell transplantation. Blood, 2011. 117(3): p. 764-771.
59. A Moretta, F Locatelli and L Moretta, Human NK cells: from HLA class I-specific killer Ig-like receptors to the therapy of acute leukemias. Immunol Rev, 2008. 224: p. 58-69.
60. L Ruggeri, A Mancusi, M Capanni, E Urbani, A Carotti, T Aloisi, M Stern, D Pende, K Perruccio, E Burchielli, F Topini, E Bianchi, F Aversa, MF Martelli and A Velardi, Donor natural killer cell allorecognition of missing self in haploidentical hematopoietic transplantation for acute myeloid leukemia: challenging its predictive value. Blood, 2007. 110(1): p. 433-440.
61. P Lang, M Pfeiffer, HM Teltschik, P Schlegel, T Feuchtinger, M Ebinger, T Klingebiel, P Bader, PG Schlegel, J Beck, J Greil and R Handgretinger, Natural killer cell activity influences outcome after T cell depleted stem cell transplantation from matched unrelated and haploidentical donors. Best Pract Res Clin Haematol, 2011. 24(3): p. 403-411.
62. T Feuchtinger, M Pfeiffer, A Pfaffle, HM Teltschik, D Wernet, M Schumm, R Lotfi, R Handgretinger and P Lang, Cytolytic activity of NK cell clones against acute childhood precursor-B-cell leukaemia is influenced by HLA class I expression on blasts and the differential KIR phenotype of NK clones. Bone Marrow Transplant, 2009. 43(11): p. 875-881.
63. W Leung, R Iyengar, V Turner, P Lang, P Bader, P Conn, D Niethammer and R Handgretinger, Determinants of antileukemia effects of allogeneic NK cells. J Immunol, 2004. 172(1): p. 644-650.
64. JS Miller, S Cooley, P Parham, SS Farag, MR Verneris, KL McQueen, LA Guethlein, EA Trachtenberg, M Haagenson, MM Horowitz, JP Klein and DJ Weisdorf, Missing KIR ligands are associated with less relapse and increased graft-versus-host disease (GVHD) following unrelated donor allogeneic HCT. Blood, 2007. 109(11): p. 5058-5061.
65. P Parham, MHC class I molecules and KIRs in human history, health and survival. Nat Rev Immunol, 2005. 5(3): p. 201-214.
66. S Cooley, E Trachtenberg, TL Bergemann, K Saeteurn, J Klein, CT Le, SG Marsh, LA Guethlein, P Parham, JS Miller and DJ Weisdorf, Donors with group B KIR haplotypes improve relapse-free survival after unrelated hematopoietic cell transplantation for acute myelogenous leukemia. Blood, 2009. 113(3): p. 726-732.
67. S Cooley, DJ Weisdorf, LA Guethlein, JP Klein, T Wang, CT Le, SG Marsh, D Geraghty, S Spellman, MD Haagenson, M Ladner, E Trachtenberg, P Parham and JS

Miller, Donor selection for natural killer cell receptor genes leads to superior survival after unrelated transplantation for acute myelogenous leukemia. Blood, 2010. 116(14): p. 2411-2419.
68. HJ Symons, MS Leffell, ND Rossiter, M Zahurak, RJ Jones and EJ Fuchs, Improved survival with inhibitory killer immunoglobulin receptor (KIR) gene mismatches and KIR haplotype B donors after nonmyeloablative, HLA-haploidentical bone marrow transplantation. Biol Blood Marrow Transplant, 2010. 16(4): p. 533-542.
69. M Stern, L Ruggeri, A Mancusi, ME Bernardo, C de Angelis, C Bucher, F Locatelli, F Aversa and A Velardi, Survival after T cell-depleted haploidentical stem cell transplantation is improved using the mother as donor. Blood, 2008. 112(7): p. 2990-2995.
70. MA Cooper, JM Elliott, PA Keyel, L Yang, JA Carrero and WM Yokoyama, Cytokine-induced memory-like natural killer cells. Proc Natl Acad Sci U S A, 2009. 106(6): p. 1915-1919.
71. R Romee, SE Schneider, JW Leong, JM Chase, CR Keppel, RP Sullivan, MA Cooper and TA Fehniger, Cytokine activation induces human memory-like NK cells. Blood, 2012. 120(24): p. 4751-4760.
72. A Moffett and C Loke, Immunology of placentation in eutherian mammals. Nat Rev Immunol, 2006. 6(8): p. 584-594.
73. B Fu, X Li, R Sun, X Tong, B Ling, Z Tian and H Wei, Natural killer cells promote immune tolerance by regulating inflammatory TH17 cells at the human maternal-fetal interface. Proc Natl Acad Sci U S A, 2012.
74. K Perruccio, A Tosti, E Burchielli, F Topini, L Ruggeri, A Carotti, M Capanni, E Urbani, A Mancusi, F Aversa, MF Martelli, L Romani and A Velardi, Transferring functional immune responses to pathogens after haploidentical hematopoietic transplantation. Blood, 2005. 106(13): p. 4397-4406.
75. P Comoli, S Basso, M Zecca, D Pagliara, F Baldanti, ME Bernardo, W Barberi, A Moretta, M Labirio, M Paulli, M Furione, R Maccario and F Locatelli, Preemptive therapy of EBV-related lymphoproliferative disease after pediatric haploidentical stem cell transplantation. Am J Transplant, 2007. 7(6): p. 1648-1655.
76. P Comoli, MW Schilham, S Basso, T van Vreeswijk, ME Bernardo, R Maccario, MJ van Tol, F Locatelli and LA Veltrop-Duits, T-cell lines specific for peptides of adenovirus hexon protein and devoid of alloreactivity against recipient cells can be obtained from HLA-haploidentical donors. J Immunother, 2008. 31(6): p. 529-536.
77. F Ciceri, C Bonini, MT Stanghellini, A Bondanza, C Traversari, M Salomoni, L Turchetto, S Colombi, M Bernardi, J Peccatori, A Pescarollo, P Servida, Z Magnani, SK Perna, V Valtolina, F Crippa, L Callegaro, E Spoldi, R Crocchiolo, K Fleischhauer, M Ponzoni, L Vago, S Rossini, A Santoro, E Todisco, J Apperley, E Olavarria, S Slavin, EM Weissinger, A Ganser, M Stadler, E Yannaki, A Fassas, A Anagnostopoulos, M Bregni, CG Stampino, P Bruzzi and C Bordignon, Infusion of suicide-gene-engineered donor lymphocytes after family haploidentical haemopoietic stem-cell transplantation for leukaemia (the TK007 trial): a non-randomised phase I-II study. Lancet Oncol, 2009. 10(5): p. 489-500.
78. L Vago, G Oliveira, A Bondanza, M Noviello, C Soldati, D Ghio, I Brigida, R Greco, MT Lupo Stanghellini, J Peccatori, S Fracchia, M Del Fiacco, C Traversari, A Aiuti, A Del Maschio, C Bordignon, F Ciceri and C Bonini, T-cell suicide gene therapy prompts thymic renewal in adults after hematopoietic stem cell transplantation. Blood, 2012. 120(9): p. 1820-1830.

79. A Di Stasi, SK Tey, G Dotti, Y Fujita, A Kennedy-Nasser, C Martinez, K Straathof, E Liu, AG Durett, B Grilley, H Liu, CR Cruz, B Savoldo, AP Gee, J Schindler, RA Krance, HE Heslop, DM Spencer, CM Rooney and MK Brenner, Inducible apoptosis as a safety switch for adoptive cell therapy. N Engl J Med, 2011. 365(18): p. 1673-1683.
80. S Mielke, R Nunes, K Rezvani, VS Fellowes, A Venne, SR Solomon, Y Fan, E Gostick, DA Price, C Scotto, EJ Read and AJ Barrett, A clinical-scale selective allodepletion approach for the treatment of HLA-mismatched and matched donor-recipient pairs using expanded T lymphocytes as antigen-presenting cells and a TH9402-based photodepletion technique. Blood, 2008. 111(8): p. 4392-4402.
81. JP Bastien, G Krosl, C Therien, M Rashkovan, C Scotto, S Cohen, DS Allan, D Hogge, RM Egeler, C Perreault and DC Roy, Photodepletion differentially affects CD4+ Tregs versus CD4+ effector T cells from patients with chronic graft-versus-host disease. Blood, 2010. 116(23): p. 4859-4869.
82. DC Roy, M Guerin, RS Boumedine, S Lachance, S Cohen, G Sauvageau, T Kiss, L Busque, J Morin, MC Guertin, A Velardi, K Rezvani, S Mielke, M Egeler, AJ Barrett, C Perreault and J Roy, Reduction in Incidence of Severe Infections by Transplantation of High Doses of Haploidentical T Cells Selectively Depleted of Alloreactive Units. Blood, 2011. 118(21): p. 1301-1301.
83. M Edinger, P Hoffmann, J Ermann, K Drago, CG Fathman, S Strober and RS Negrin, CD4+CD25+ regulatory T cells preserve graft-versus-tumor activity while inhibiting graft-versus-host disease after bone marrow transplantation. Nat Med, 2003. 9(9): p. 1144-1150.
84. VH Nguyen, S Shashidhar, DS Chang, L Ho, N Kambham, M Bachmann, JM Brown and RS Negrin, The impact of regulatory T cells on T-cell immunity following hematopoietic cell transplantation. Blood, 2008. 111(2): p. 945-953.
85. M Di Ianni, F Falzetti, A Carotti, A Terenzi, L Ruggeri, P Antonio, V de Fazio, V Zoi, MS Massei, Y Reisner, A Velardi, F Aversa and MF Martelli, Adoptive Immunotherapy with Tregs and Tcons Ensures Low TRM and a Low Incidence of Post Transplant Leukaemia Relapse After HLA Haploidentical Transplants for Acute Leukemia. Blood, 2011. 118(21): p. 76-76.
86. RJ Berenson, EJ Shpall, K Auditore-Hargreaves, S Heimfeld, C Jacobs and MS Krieger, Transplantation of CD34+ hematopoietic progenitor cells. Cancer Invest, 1996. 14(6): p. 589-596.
87. R Handgretinger, P Lang, M Schumm, G Taylor, S Neu, E Koscielnak, D Niethammer and T Klingebiel, Isolation and transplantation of autologous peripheral CD34+ progenitor cells highly purified by magnetic-activated cell sorting. Bone Marrow Transplant, 1998. 21(10): p. 987-993.
88. M Schumm, P Lang, G Taylor, S Kuci, T Klingebiel, HJ Buhring, A Geiselhart, D Niethammer and R Handgretinger, Isolation of highly purified autologous and allogeneic peripheral CD34+ cells using the CliniMACS device. J Hematother, 1999. 8(2): p. 209-218.
89. PR Gordon, T Leimig, I Mueller, A Babarin-Dorner, MA Holladay, J Houston, G Kerst, T Geiger and R Handgretinger, A large-scale method for T cell depletion: towards graft engineering of mobilized peripheral blood stem cells. Bone Marrow Transplant, 2002. 30(2): p. 69-74.
90. GA Hale, KA Kasow, K Gan, E Horwitz, JP Woodard, U Yusuf, R Barfield, WH Leung, DK Srivastava and R Handgretinger, Haploidentical stem cell transplantation with CD3 depleted mobilized peripheral blood stem cell grafts for children with hematologic malignancies. Blood, 2005. 106(11): p. 816A-816A.

91. GA Hale, KA Kasow, R Madden, U Yusuf, E Horwitz, R Barfield, J Woodard, WH Leung, K Srivastava and R Handgretinger, Mismatched family member donor transplantation for patients with refractory hematologic malignancies: Long-term followup of a prospective clinical trial. Blood, 2006. 108(11): p. 895A-895A.
92. X Chen, GA Hale, R Barfield, E Benaim, WH Leung, J Knowles, EM Horwitz, P Woodard, K Kasow, U Yusuf, FG Behm, RT Hayden, SA Shurtleff, V Turner, DK Srivastava and R Handgretinger, Rapid immune reconstitution after a reduced-intensity conditioning regimen and a CD3-depleted haploidentical stem cell graft for paediatric refractory haematological malignancies. Br J Haematol, 2006. 135(4): p. 524-532.
93. EJ Gerritsen, ED Stam, J Hermans, H van den Berg, A Haraldsson, MJ van Tol, RL van den Bergh, JL Waaijer, AC Kroes, PM Kluin and JM Vossen, Risk factors for developing EBV-related B cell lymphoproliferative disorders (BLPD) after non-HLA-identical BMT in children. Bone Marrow Transplant, 1996. 18(2): p. 377-382.
94. RC Barfield, M Otto, J Houston, M Holladay, T Geiger, J Martin, T Leimig, P Gordon, X Chen and R Handgretinger, A one-step large-scale method for T- and B-cell depletion of mobilized PBSC for allogeneic transplantation. Cytotherapy, 2004. 6(1): p. 1-6.
95. R Handgretinger, X Chen, M Pfeiffer, I Mueller, T Feuchtinger, GA Hale and P Lang, Feasibility and outcome of reduced-intensity conditioning in haploidentical transplantation. Ann N Y Acad Sci, 2007. 1106: p. 279-289.
96. P Lang, I Mueller, J Greil, P Bader, M Schumm, M Pfeiffer, W Hoelle, T Klingebiel, F Heinzelmann, C Belka, PG Schlegel, B Kremens, W Woessmann and R Handgretinger, Retransplantation with stem cells from mismatched related donors after graft rejection in pediatric patients. Blood Cells Mol Dis, 2008. 40(1): p. 33-39.
97. MM Pfeiffer, T Feuchtinger, HM Teltschik, M Schumm, I Muller, R Handgretinger and P Lang, Reconstitution of natural killer cell receptors influences natural killer activity and relapse rate after haploidentical transplantation of T- and B-cell depleted grafts in children. Haematologica, 2010. 95(8): p. 1381-1388.
98. P Bader, J Soerensen, A Jarisch, E Ponstingl, T Krenn, J Faber, M Durken, H Reinhardt, A Willasch, R Esser, H Bonig, U Koehl and T Klingebiel, Rapid immune recovery and low TRM in haploidentical stem cell transplantation in children and adolescence using CD3/CD19-depleted stem cells. Best Pract Res Clin Haematol, 2011. 24(3): p. 331-337.
99. J Palma, L Salas, F Carrion, C Sotomayor, P Catalan, C Paris, V Turner, H Jorquera, R Handgretinger and GK Rivera, Haploidentical stem cell transplantation for children with high-risk leukemia. Pediatr Blood Cancer, 2012. 59(5): p. 895-901.
100. G Dufort, S Pisano, A Incoronato, M Castiglioni, M Carracedo, C Pages, E Simon, S Zuccolo, R Barcelona, R Mezzano, A Tiscornia, F Lemos, F Morosini, M Schelotto, H Giordano, E Carreto, M Bengoechea, B Boggia, I Rodriguez, L Guerrero, A Dabezies and L Castillo, Feasibility and outcome of haploidentical SCT in pediatric high-risk hematologic malignancies and Fanconi anemia in Uruguay. Bone Marrow Transplant, 2012. 47(5): p. 663-668.
101. A Perez-Martinez, M Gonzalez-Vicent, J Valentin, E Aleo, A Lassaletta, J Sevilla, JL Vicario, M Ramirez and MA Diaz, Early evaluation of immune reconstitution following allogeneic CD3/CD19-depleted grafts from alternative donors in childhood acute leukemia. Bone Marrow Transplant, 2012. 47(11): p. 1419-1427.

APPENDIX 1 - ACUTE GVHD CLASSIFICATION

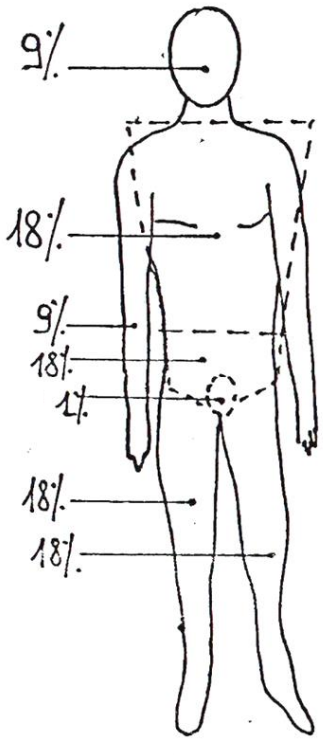
Acute GVHD staging (Consensus Conference grading).

STAGE	SKIN	LIVER	INTESTINAL TRACT
0	No rash	Total bilirubin: < 2 mg/dl	Diarrhea < 500 ml/day
1	Maculopapular rash < 25% of BS §	Total bilirubin: 2-3 mg/dl	Diarrhea: 500-1000 ml/day
2	Maculopapular rash 25-50% of BS §	Total bilirubin: 3-6 mg/dl	Diarrhea: 1000-1500 ml/day
3	Generalized erythroderma	Total bilirubin: 6-15 mg/dl	Diarrhea: > 1500 ml/die
4	Generalized erythroderma with bollous formation and desquamation	Total bilirubin : >15 mg/dl	Severe abdominal pain ± ileus

Acute GVHD grading (Przepiorka et al. Bone Marrow Transplant. 1995)

GRADE	SKIN	LIVER	INTESTINAL TRACT	PERFORMANCE STATUS
I	1-2	0	0	0
II	3 e/o	1 e/o	1	1
III	fino a 3 e	2-3 oppure	2-3	2
IV	4	4	4	3-4

§ BODY SURFACE AREA, BSA %:



APPENDIX 2 -CHRONIC GVHD CLASSIFICATION

Shulman Classification for Chronic GvHD

Limited chronic GVHD

Either or both

- Generalized skin involvement, or
- Hepatic dysfunction due to chronic GVHD

Extensive chronic GVHD

Either:

- Generalized skin involvement, or
- Localized skin involvement and/or hepatic dysfunction due to chronic GVHD

Plus:

- Liver histology showing chronic aggressive hepatitis, bridging necrosis, or cirrhosis, or
- Involvement of eye (Schirmer´s test with less than 5 mm wetting), or
- Involvement of minor salivary glands or oral mucosa demonstrated on labial biopsy, or
- Involvement of any other target organ