Universitat Autònoma de Barcelona

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Doctoral Program in Medicine. Department of Medicine

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Analysis of Donor Lymphocyte Infusions after Allogeneic Hematopoietic Cell Transplant

(Anàlisis d'Infusions de Limfòcits de Donant post Trasplantament Al·logènic de Progenitors Hematopoètics)

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ABBREVIATIONS

Abbreviations

aGvHD	Acute Graft-versus-host disease
AL	Acute leukemia
ALL	Acute lymphoid leukemia
AlloHCT	Allogeneic hematopoietic cell transplantation
AloTPH	Trasplante alogénico de células hematopoyéticas
AML	Acute myeloid leukemia
BKV	BK-virus
BM	Bone marrow
BMA	Bone marrow aspirate
BST	Banc de Sang I Teixits
CART	Chimeric antigen receptor T-cells
CD	Cluster of differentiation
cGvHD	Chronic Graft-versus-host disease
CI	Cumulative incidence
CLA	Canine leukocyte antigen
CML	Chronic myeloid leukemia
CNI	Calcineurin inhibitors
CondR	Conditioning regimen
CTL	Cytotoxic T lymphocytes
DFS	Disease-free survival
DLI	Donor lymphocyte infusion
DLI1	First DLI
EBMT	European bone marrow transplant
EICR	Enfermedad del injerto contra el receptor
FDC	Full donor chimerism
FluMel	Fludarabine and Melfalan
GF	Graft Failure
GvHD	Graft-versus-host disease
GvT	Graft-versus-tumor

HC	Hemorrhagic cystitis
HCT2	Second AlloHCT
HD	Haploidentical donor
HLA	Human leukocyte antigen
HR	Hazard ratio
HSC	Hematopoietic Stem Cell
IL	Interleukin
ILD	Infusión de linfocitos del donante
IST	Immunosuppressive therapy
mAb	Monoclonal antibody
MC	Mixed chimerism
mHA	Minor histocompatibility antigens
MHA	Major histocompatibility antigens
MHC	Major histocompatibility complex
mMUD	Mismatched unrelated donor
MNC	Mononuclear cells
MSD	Matched sibling donor
MUD	Matched unrelated donor
MRD	Minimal residual disease
NHL	Non-Hodgkin lymphoma
NRM	Non-relapse mortality
OS	Overall survival
PB	Peripheral blood
PS	Performance status
pDLI	Preemtive DLI
prDLI	Prophylactic DLI
PTCy	Post transplant cyclophosphamide
RFS	Relapse-free survival
RIC	Reduced-intensity conditioning
SG	Supervivencia global

SLP	Supervivencia libre de progresión
TCD	T-cell depletion
TCR	T-cell replete
TBI	Total body irradiation
T_{CM}	Central memory T-cells
TCR	T-cell Receptor
TKI	Tyrosine Kinase Inhibitors
T_N	Naïve T-cells
TPH2	Segundo AloTPH
T_{REG}	Regulatory T-cells
T_{RTE}	Recent Thymic Emigrant T-cell
T_{SCM}	Stem cell memory T-cells
srGvHD	Steroid refractory GvHD
USA	United States of America

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I. ABSTRACT

1.1 Abstract

Allogeneic hematopoietic cell transplant (AlloHCT) represents a major therapeutic approach for a number of hematological malignancies. The graft-versus-leukemia effect (GvL) driven by AlloHCT can provide sustained complete responses. However, relapse remains a major complication after AlloHCT and the use of donor lymphocyte infusions (DLI) or a second AlloHCT (HCT2) are essential strategies to improve survival at this point. Several studies have reported on the effectiveness and toxicity of DLI, but response remains unpredictable and the development of graft-versus-host disease (GvHD) is a major drawback after DLI. We studied the effectiveness of DLI compared to HCT2 in 46 acute leukemia (AL) patients who relapsed after AlloHCT, and found that overall survival (OS) and progression free survival (PFS) are comparable between both strategies. Notoriously, we identified time to relapse < 6 months (p=0.007) from AlloHCT to DLI or to HCT2 a major factor with impact on OS (p=0.007) and PFS (p=0.037). Further, we studied the cell composition of 56 DLI from 36 patients who received an AlloHCT from matched sibling donors (MSD), and assessed its potential impact on the development of GvHD. We observed that a high dose of B-cells (p=0.03) and $CD27^+$B cells (p<0.01) was associated with GvHD. We identified DLI dose cut-off points for several cell populations above which GvHD was more frequent ($CD8^+T_N$>$3x10^6$ cells/Kg, $CD27^+$B-cells>$2.6x10^6$ /Kg, $CD27^+$NK>$0.35x10^6$ cells/Kg and MNC>$0.83x10^8$/Kg). Noteworthy, the proportion of $CD4^+$ Naïve T-cells (T_N) or unselected T_N were not linked with GvHD, nor any transplant or donor clinical variable. Altogether, these data might provide insight for a better understanding of the mechanistic insights of DLI.

1.2 Resumen

El trasplante alogénico de células hematopoyéticas (AloTPH) es una estrategia terapéutica mayor para de el tratamiento de neoplasias malignas hematológicas. Mediante el efecto de injerto contra leucemia que aporta el AloTPH se pueden alcanzar respuestas completas mantenidas. Sin embargo, la recaída sigue siendo la complicación más importante después del AloTPH. En este contexto, el uso de infusiones de linfocitos de donantes (ILD) o un segundo AlloHCT (TPH2) son estrategias esenciales para mejorar la supervivencia. Varios trabajos han estudiado la efectividad y la toxicidad de las DLI, pero la respuesta sigue siendo impredecible y el desarrollo de la enfermedad de injerto contra huésped (EICR) una complicación mayor post ILD. Hemos estudiado la efectividad de ILD en comparación con TPH2 en 46 pacientes diagnosticados de leucemia aguda que recayeron después de AloTPH. Hemos observado que la supervivencia global (SG) y la supervivencia libre de progresión (SLP) son comparables entre ambas estrategias. Por otro lado, identificamos un tiempo hasta la recaída <6 meses (p=0.007) del AloTPH a la DLI o al TPH2, uno de los principales factores con impacto en la SG (p=0.007) y la SLP (p = 0.037). Por otro lado, estudiamos la composición celular de 56 DLI de 36 pacientes que recibieron un AloTPH de donantes hermanos HLA idénticos, y evaluamos su potencial impacto en el desarrollo de EICR. Descubrimos que una dosis elevada de células B (p=0.03) y células B $CD27^+$ (p <0.01) se asoció con el desarrollo de EICR. También pudimos identificar puntos de corte de dosis de DLI para varias poblaciones celulares por encima de las cuales la EICR fue más frecuente (células T-naive (T_N) $CD8^+$ > $3x10^6$ células /Kg, linfocitos B $CD27^+$ > $2.6x10^6$ / Kg, células natural killer (NK) $CD27^+$ > $0.35x10^6$ células / Kg y células monocucleares (MNC) > $0.83x10^8$ / Kg). Cabe destacar que la proporción de células T_N o $CD4^+$ (T_N) no se relacionó con EICR. En conjunto, estos datos aportan información para una mejor comprensión de fisiopatología de la EICR post ILD.

II. INTRODUCTION

II. Introduction

2.1 Allogeneic Hematopoietic Cell Transplant

2.1.1 Background

Allogeneic hematopoietic cell transplantation (AlloHCT) is the only curative approach for a majority of malignant blood disorders[1]. Worldwide, a great number of institutions have adopted AlloHCT as curative treatment for malignant and non-malignant hematological malignanices. Over the last years, the number of performed AlloHCT has been increasing for many disease diagnoses[2], this is mainly due to the increase of indications for AlloHCT expand, the incorporation of non-compatible donors are incorporated into donor selection algorithms and more tolerable transplant platforms, such as reduced intensity conditionings (RIC) allow more patients to undergo AlloHCT. Hence AlloHCT remains an essential treatment for a significant number of patients.

AlloHCT was pioneered in the second half of the twentieth century. The effects of myeloablative doses of radiation after unexpected humankind incidents stimulated the interest of the scientific community on exploring this in several murine and canine experiments. Initial experimentation focused on transplanted donor bone marrow cells, initially spleen cells, after myeloablative radiation and it was observed that they protected mice from the myelotoxic effect of lethal irradiation[3]. These experiments further proved the alloreactive effect of donor cells, when the cytogenetic profile of the donor cells was identified in transplanted mice after donor cell infusion[4], indicating the engraftment of donor cells.

AlloHCT was initially developed by Professor E. Donnall Thomas, from the University of Washington. The Seattle group reported in humans on the use of marrow grafting after total body irradiation (TBI) and chemotherapy[5]. This novel approach provided full myeloablative dose of radiotherapy and allowed donor stem cells to engraft in recipients bone marrow tissue. In the initial days of transplantation, other groups also tested this approach with initially unsuccessful results. In parallel, the Seattle experimental transplantation group was animal models (mainly canine) provided more data which helped to improve transplantation in humans. Concepts such as secondary disease, at present named graft-versus-host disease (GvHD), graft failure (GF) or

peripheral blood (PB) stem cells could be ideated from canine experimentation[6]. In parallel, European colleagues discovered the canine leukocyte antigen (CLA) system and its importance in GvHD in allogeneic canine bone marrow transplant. The authors found that CLA incompatibilities lead to GvHD. But it was in 1968 when the first AlloHCT in humans was observed to provide long-term benefit[7] in an infant diagnosed with immunodeficiency. The observed benefits of AlloHCT were further tested by the Seattle group by performing AlloHCT in patients diagnosed with leukemia. From then on, the use of allogeneic transplantation has been constantly expanding to other disease and other indications.

More than fifty years have passed and AlloHCT platforms have dramatically changed and adapted to new scenarios and patients. Strategies such as the reduced-intensity conditioning (RIC) regimens, allowing more fragile and older patients to undergo AlloHCT, or the advent of T-cell depletion (TCD) have contributed on allowing more patients to undergo AlloHCT. Initially, the rational of AlloHCT was based on the possibility of delivering high dose chemotherapy to treat the hematological malignancy. But this was soon reformulated, as findings proved the graft-versus-tumor (GvT) effect. It was observed that AlloHCT with T-cell depletion associated a higher relapse rate[8], as also was observed in autologous transplants and transplants from syngeneic donors[9, 10]. Further, it was observed that patients developing GvHD associated better relapse-free survival (RFS)[11, 12] and that donor lymphocyte infusions (DLI) administered after AlloHCT for relapse provided durable remissions.

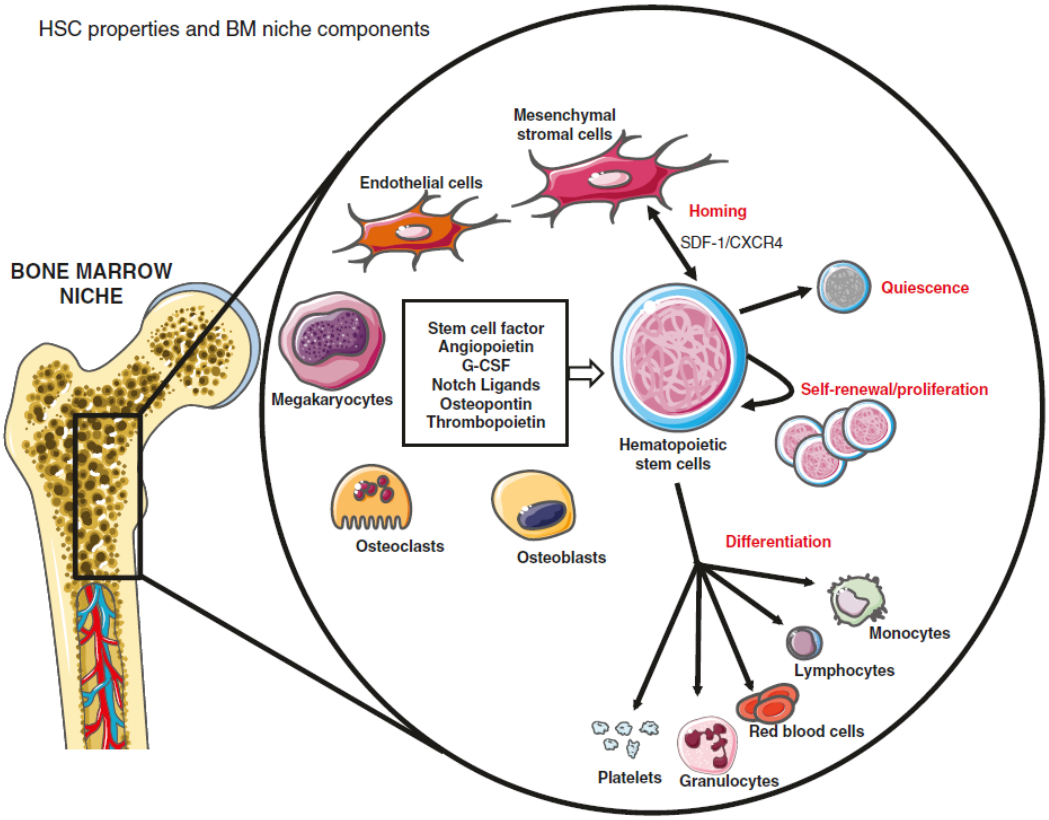
2.1.2 Scientific Fundamentals

Confirmation of the self renewal capacities of a hematopoietic stem cell (HSC) engraftment and the basis of HCT come from canine and, more recently, murine models. Few pluripotent HSC are capable of engraftment in a favorable niche. Initial murine experimentation was shown by injecting intravenously HSC in an irradiated mouse[13]. These finding have further been tested in many murine and xeno transplant models[14,15], highlighting the importance of the lack kit receptor in allowing the engraftment of HSC with no further chemotherapy[16].

2.1.3 Conditioning Regimen

Conditioning regimen (CondR) is one of the essential components of HCT. It is a group of chemotherapy, radiotherapy and biologic agents administered before AlloHCT in order to enhance engraftment. CondR also provides anti tumoral effect. Various groups have shown the influence of conditioning regimen on immune reconstitution, relapse risk or GvHD development.

Figure 1. HSC properties and its niche (from the European Bone Marrow Transplant (EBMT) Handbook, Carreras et al. 2019)[17].



Thus, the more intense the conditioning the lower is the risk of relapse and a fastest engraftment is observed[18]. Several types of conditioning regimen have been developed. Initially, as mentioned before in this manuscript, CondR were TBI-based, but this approach evolved to the use of several myelosuppresive agents linked to a lower adverse event profile. Briefly, CondR have been described as myeloablative, RIC and non-myeloablative, according to its degree of myelosuppression and engraftment dynamics.

2.1.4 Immunosuppressive Therapy

On the other hand, the successful outcomes after AlloHCT profoundly depend upon the GvHD immunosuppressive therapy (IST) administered. IST is essential as it allows the temporary tolerance of both donor and recipient's hematopoietic immune system, without which an immune attack would occur between donor and recipient. A high number of drugs with immunomodulatory and anti T-cell potential have been used as IST lines of treatment. Initial transplants were immunosuppressed with calcineurin inhibitors (CNI). Subsequently, other agents such as micophenolate mofetil or low-dose methotrexate were added to CNI and are part of standard recommendation[19]. More recently, the use of post transplant cyclophosphamide (PTCy) has allowed the incorporation of not compatible donors into AlloHCT algorithms[20].

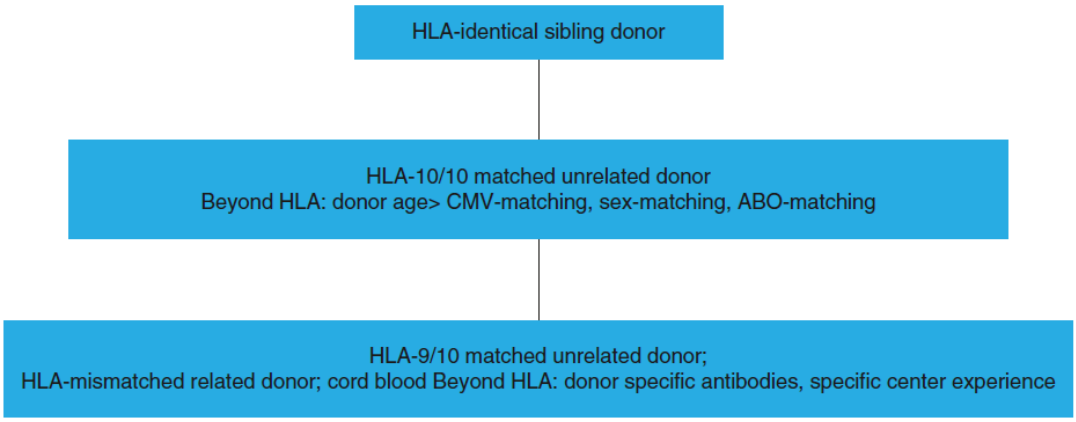
Therefore, IST is necessary after AlloHCT; nevertheless, a prolonged use of IST after AlloHCT might be associated to an increased risk of relapse as a number of patients achieve remission after IST withdrawal in hematological malignancy relapse[21,22]. This is due to the GvT effect showed in many blood disorders driven by donor T-cells, abolished by IST. Interestingly, it has been shown it may be donor $CD8^+$ T-cells specifically drivers of the GvT effect[23].

2.1.5 The role of the donor

Another factor with tremendous impact on transplant outcomes is the donor selection. As observed in canine transplant models, the human leukocyte antigen (HLA) compatibility is a major factor to be considered before transplant. HLA is encoded by a group of genes in chromosome 6 which are inherited from the mother and the father (haplotype). The most relevant HLA genes involved in AlloHCT are HLA-A, HLA-B, HLA-C (major histocompatibility complex [MHC] class I), HLA-DR, HLA-DQ and HLA-DP (MHC class 2); notoriously, not all genes retain the same immune effect in case of incompatibility.

Thus, the use of a matched sibling donor (MSD) is the first choice, followed by a matched unrelated donor (MUD) and if unavailable, mismatched unrelated donor (mMUD). Recently, retrospective studies suggest that the use of PTCy might provide comparable results with haploidentical donors (HD) in the donor selection algorithms, compared to more compatible donors.

Figure 2. Algorithm for donor selection in AlloHCT for hematological malignancies (from the European Bone Marrow Transplant (EBMT) Handbook, Carreras et al. 2019)[17].



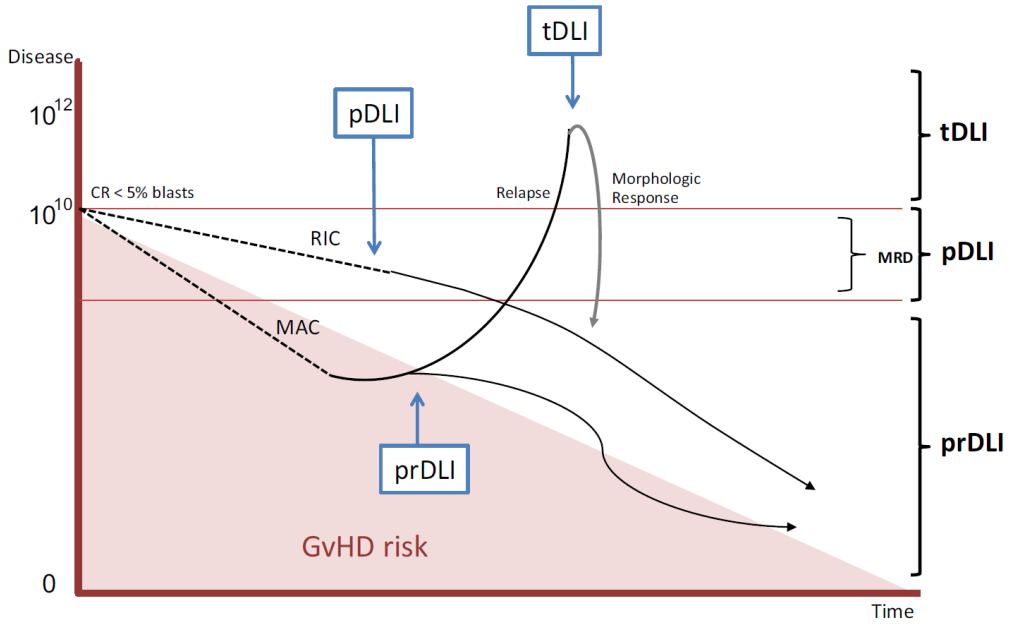
2.2 Donor Lymphocyte Infusions

2.2.1 Background

DLIs are infusions of $CD3^+$ cells from donor origin which are administered post AlloHCT after previous donor stem cell engraftment, complete or parcial. The indications for DLI are diverse. At present, DLI can be given at different scenarios: at relapse (therapeutic DLI), for mixed chimerism (MC) or minimal residual disease (MRD) (preemptive DLI, pDLI) or prophylactic (prDLI) to prevent relapse or graft failure (Figure 1). DLI were initially pioneered by the Seattle group. Weiden and colleagues[24] used canine radiation chimeras to investigate the mechanisms responsible for maintaining the stable chimeric state. Chimeras were studied 7 to 46 months after 1200 Gy TBI and transplantation of marrow from a littermate donor matched at the major histocompatibility complex (MHC). Infusions of 0.6-13.7×10^8/kg of donor PB lymphocytes were performed in two groups, a control group and a group sensitized against minor histocompatibility antigens of the chimera. None of the nine chimeras in the first group developed significant GvHD, however eight of the 12 chimeras in the second group did develop GvHD. With these results the authors postulated that the presence of an active mechanism

suppressing recognition of host antigens by the infused donor lymphocytes and development of GvHD.

Figure 3. Diagram displaying the types of DLI: tDLI (therapeutic DLI), pDLI (preemptive DLI) and prDLI (prophylactic DLI).



Later, the first works of DLI in clinical practice were published. Kolb and colleagues studied the results after infusion of donor leukocytes from the donor in transplant from bone marrow (BM)[25, 26]. In these work three patients with hematologic relapse of chronic myeloid leukemia (CML) after bone marrow transplantation were treated with interferon alpha and transfusion of viable donor buffy coat. All patients had complete hematologic and cytogenetic remission, which persisted 32 to 91 weeks after treatment. In two patients GvHD occurred and it was treated by immunosuppressive therapy. These results were proof of concept of the GvT effect that donor leukocytes retained in humans. Soon after, many groups adopted the treatment with DLI as the increasing use of RIC AlloHCT produced mixed chimeras and post transplant relapses increased.

2.2.2 Graft-versus-Host Disease after DLI

GvHD is a major complication after DLI, as it is after AlloHCT. GvHD is an allogeneic antigen recognition process in which donor T-cells react against mismatched major histocompatibility antigens (MHA) or minor histocompatibility antigens (mHA) in the recipient´s tissue[27]. GvHD has been historically divided in acute GvHD (aGvHD) and cGvHD. The physiopathology of aGvHD and cGvHD is different. aGvHD is a immune-mediated disease triggered by donor T-cells that attack recipient's tissue antigens in the skin, colon, thyroid and gut[28]. In a GvHD, main cytokines involved are IL-1 and TNFα. On the contrary, cGvHD is a substantially different disease in which autoimmunity and fibrosis are main ways of presentation. cGvHD can affect a wide range of tissues including mucosa, lung or genital tract[29] and the citokyne profile involved is extensive[30]. In the DLI setting, GvHD rates have been reported in 30-60% of patients, depending on the transplant characteristics, cell dose and DLI timing[31]. Potential factors to ameliorate GvHD after DLI have been a matter of study. Hence, strategies to ameliorate this complication after DLI have been actively developed. Among these approaches, one of the initial strategies, and most relevant, was developed by the Memorial Sloan Kettering Cancer Center transplant group (32).

2.2.3 Cell Composition of DLI and GvHD

2.2.3.1 DLI Dose Escalation

The authors ideated a dose-escalation schedule of DLI attempting to maintain the GvT while reducing the rate of GvHD. They included 22 patients diagnosed with CML who relapsed after AlloHCT: 2 in molecular relapse, 6 in cytogenetic relapse, 10 in chronic phase, and 4 in accelerated phase. Each patient received escalating doses of donor leukocytes at 4 to 33 week-intervals. Leukocyte doses were calculated as T cells per kilogram of recipient weight. There were 8 dose levels between $1x10^5$ and $5x10^8$ cells/patient`s weight. Nineteen of the 22 patients achieved remission at different dose; the dose used in the majority of patients was 1×10^7/Kg (in eight patients). Notoriously, the incidence of GvHD was correlated with the T-cell dose administered. GvHD occurred in 70% of the responders who received a T-cell dose of $\geq 5 \times 10^7$ cluster of differentiation (CD) 3^+/kg, whereas it only happened in 12% of patients treated with 1

x 10^7/Kg. These results outlined that, in some patients, GvT could be achieved with no increase of the development of GvHD. From then on, most centers adopted $CD3^+$ dose escalation protocols. These data showed the relevance of the total $CD3^+$ infused dose which, as in other studies, it has been associated with the development of GvHD after DLI. Interestingly, the total $CD3^+$ dose has not always been identified as a key factor for the development of GvHD as other authors have identified the total dose of mononuclear cells (MNCs) associated with the development of GvHD[33]. The authors investigated a cohort of 298 CML patients that received DLI, and analyzed the impact on MNCs on GvHD, progression-free survival, among other outcomes. Based on the observed outcomes, the authors suggest that a first DLI dose should not exceed 0.2x10^8 MNCs/Kg in order to avoid unacceptable rates of GvHD.

2.2.3.2 $CD8^+$ Dose

$CD8^+$ T-cells are essential in GvHD development. Fowler and colleagues studied the $CD8^+$ compartment phenotypes[34]. By performing murine transplant modeling, the authors found that different $CD8^+$ cytokine-secreting subsets had different impact on the development of GvHD, separating the role of Tc1 (interleukin (IL) 12 secreting $CD8^+$ T-cell) and Tc2 (IL-4 secreting $CD8^+$ T-cell). In line with this, the role of $CD8^+$ cells on DLI has been explored, as many groups studied the infusion of $CD8^+$-depleted DLI[35, 36]. In a paper reported by the Royal Free Hospital, 28 patients received $CD8^+$-depleted DLI (n=16 unrelated or mismatched, n=12 human leukocyte antigen-identical sibling). The median overall dose of CD4+ cells/kg was 4 x 10^6 cells/Kg. Conversion from mixed to full donor chimerism (FDC) was observed in 8 of 16 evaluable patients, and disease responses occurred in 5 of 11 patients (complete response in four). Five of 28 patients developed grade II-IV GvHD, suggesting that the development of GvHD was not purely caused by $CD8^+$ cells. In summary, the $CD8^+$-depleted DLI studies proved the feasibility of $CD8^+$ T-cell depletion, that GvHD is not only caused by circulating $CD8^+$ T-cells and that response was not purely dependent on the cytotoxic T-cell compartment. Besides GvHD, bone marrow toxicity is another complication observed after DLI, which has been reported to happen in up to 20% of patients. Interestingly, the mechanisms involved in this complication remain largely unknown although it is thought to be caused cause by effector T-cells.

2.2.3.3 Regulatory T-cells

On the contrary, regulatory T cells (T_{REG}) are T-lymphocytes, retaining a helper phenotype, that are able to modulate immune responses and control autoimmunity, and retain a strong inhibitory profile[37]. Initially described decades ago, this cell subset was shown to facilitate the engraftment of donor cells in a mismatched MHC or mHC transplant setting. In AlloHCT and DLI, donor polyclonal T_{REG} are actively being investigated as potential treatment for GvHD or in order to enhance the GvT effect by depleting the T_{REG} pool from the product. For example, attempting to increase the antitumor effect, a phase I trial studies the use of T_{REG}-depleted DLI for relapse of hematological malignancy after AlloHCT[38]. They reported twenty-one patients who received CD25/T_{REG}-depleted infusions following removal of $CD25^+$ cells using antibody-conjugated magnetic beads. At a dose 3×10^7 $CD3^+$ cells/kg, nine patients (60%) achieved or maintained responses (8 complete responses, 1 partial response), including seven with active disease at the time of infusion. Additionally, the authors found an expansion of the number of T_N and T_{CM} $CD4^+$ cells in PB, however there were no immunophenotipic findings of response. This study proved feasibility of T_{REG}-depleted DLI, but the number of patients was scarce to pull out any solid conclusion. On the contrary, donor infusions of polyclonal T_{REG} have been studied as treatment for GvHD in murine model[39]. The authors explored ways of expanding T_{REG} by the use of interleukin-2/monoclonal antibody (IL-2/mAb) complexes and by infusions of donor T_{REG}. T_{REG} DLI increased T_{REG} frequency, prevented GvHD and had a therapeutic role in established cGvHD. These data were proof of concept that the infusion of donor T_{REG} cells can contribute to treat GvHD.

2.3.3.4 $CD3^+$ Dose

There has been interest in exploring the impact of the graft composition and its relation with GvHD. In this line, the graft total $CD3^+$ dose has been associated to GvHD with unalike results between studies[40, 41], which suggests that the $CD3^+$ might not be similarly relevant in all transplant platforms. For example, the total $CD3^+$ dose was shown to impact in GvHD when administered an as an ad back DLI after a TCD AlloHCT[42], but not in a T-cell replete setting. On the other hand, the proportion of $CD8^+$ cells, within the $CD3^+$ compartment, has also been

studied in this setting, as a higher dose of $CD8^+$ cells in the graft predicted better survival[43]. There have been approaches to lower the GvHD by modifying the cell context of the graft, such as to perform a CD34+ selection[44] or to perform $CD45RA^+$ graft depletion[45]. In this phase I study, the authors reported outcomes of 35 patients undergoing AlloHCT from peripheral blood grafts, and they observed a 9% rate of chronic GvHD (cGvHD) with median follow-up 932 days. The observed cGvHD rate was lower compared to the expected in this transplant platform and these findings will need further exploration. In line with this, another group studies the risk of GvHD according to the expression of $CCR7^+$ in the cells of the graft in a cohort of 85 patients that received a PB AlloHCT[46]. The authors found that the migratory index to the C-C chemokine receptor type 7 ($CCR7^+$) ligands was higher in T-cells from donors whose recipients developed GvHD. Interestingly, patients that developed acute GvHD (aGvHD) received a higher percentage of $CD4^+CCR7^+$ T-cells, whereas chronic GvHD (cGvHD) patients were transplanted with higher percentages of CD8+CCR7+ T-cells; compared with the non-GvHD group. The $CCR7^+$ protein is expressed in the membrane of T-cells and it is involved in homing of T-cells to secondary lymphoid organs; it is expressed in naïve T-cells (T_N), stem cell memory T cells (T_{SCM}) and central memory T cells (T_{CM}).

The graft cell composition impacts on the risk of GvHD and thus in transplant outcomes. The role of the DLI cell composition besides $CD3^+$ is only partially understood. Initial studies focused on the total $CD3^+$ [33] or MNCs[34] dose, but studies in depth on the DLI populations are lacking. A flow cytometry analysis of DLI cell subsets focused on analyzing the $CD4^+$, $CD8^+$ and $CD14^+$ compartment identified that the higher proportion of $CD14^+$ cells contained in a DLI, the less likely the development of GvHD was[47]. The authors however studied a limited number of cell subsets, and did not included essential alloreactive cell subsets such as T-cells expressing $CD45RA^+$ and $CCR7^+$, it was neither studied the NK^+ cell or B-cell pool. Hence the role of $CD14^+$ is therefore uncertain in this setting. Nevertheless, findings suggest that the proportion of cell subsets might have a role in DLI. In AlloHCT murine experimentation, T_N have been shown to be the most relevant cell subset to associate[48]. Likewise, T_{CM}, a cell subset lacking the CD45RA+ membrane protein, have also been linked to the development of GvHD[49] (Figure 2), although to a lesser degree. The association of T_N and alloreactivity in murine AlloHCT models is clear; its role is unclear in the DLI clinical setting.

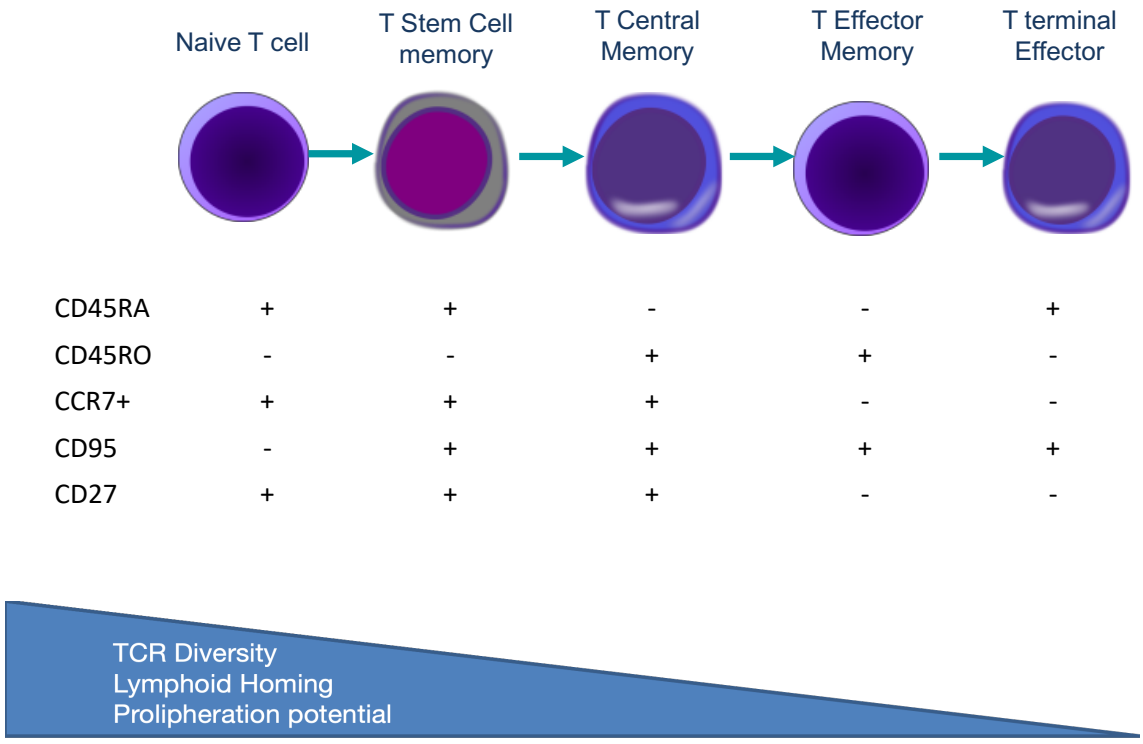


Figure 4. Diagram showing T-cell subsets according to their expression of cluster of differentiation.

2.3 Donor Lymphocyte Infusions for Relapse

Relapse after AlloHCT is terrible complication that entails poor prognosis in the majority of hematological malignancies[50, 51]. Several approaches have been undertaken at this scenario. There is not a standard therapy and the therapeutic decision depends on the physician´s choice. In this setting, the effectiveness of DLI depends on many clinical and transplant variables. DLI were initially pioneered in relapsed CML patients, hence initial clinical data is pulled out from these retrospective studies. It has been reported that up to 70% of chronic phase CML relapsing patients after AlloHCT can achieve remission with DLI[31, 52]. Initially, transplants were performed using BM as graft source, which has been shown to contain a lower number of T-cells. In these types of transplants, relapse has been reported more frequent and often the detection of BCR-ABL1 transcripts after AlloHCT is frequent.

Unfortunately, such DLI effectiveness is not observed in all diseases. In acute myeloid leukemia (AML) or acute lymphoid leukemia (ALL) relapse, the response rate has been reported not superior to 30%[53]. The observed lack of response might be due to several factors such as the highly proliferative dynamics of the disease or the AML blasts or the fact that AML can have HLA loss at relapse[54] (which can occur in up to 30% of AML relapse) allowing blasts to escape from the GvT immune surveillance provided by DLI. Hence AML and ALL relapse is a very complex clinical scenario and several strategies have been adopted, being DLI or HCT2 two major approaches at this point[43].

HCT2 provides long term survival in 30-40% of patients, and it is particularly effective in patients diagnosed with late relapse and achieving CR before HCT2[55, 56]. The counterpart of HCT2 is the non-relapse mortality (NRM) associated to this procedure.

On the other hand, DLI can be effective in 20-20% of AML relapse[57]. But responses are erratic and they have been reported more frequent if a debulky treatment before DLI is added.

Both DLI and HCT2 are chosen by physicians according to clinical variables such as the patient's clinical status, availability of the donor or the previous occurrence of GvHD. It is therefore unclear which strategy provides more benefit. The CIBMTR reported outcomes of 1231 patients who received treatment for AML relapse, including HCT2 or DLI[58]. They found that patients offered any cell therapy option associated higher overall survival compared to patients who were only treated with a non-cell therapy approach. Besides this finding, no conclusion can be pulled out from this analysis regarding the use of DLI or HCT2 in this setting.

2.4 Donor Lymphocyte Infusions and Second Allogeneic Hematopoietic Cell Transplant.

Relapse of acute leukemia (AL) after AlloHCT entails a dismal prognosis, and its approach remains challenging. The relapse rate AML and ALLafter transplantation vary between 30-50%, depending on a wide range of factors such as the transplant platform[59], the disease risk[60] or the intensity of T-cell depletion (TCD)[61], among others. Post transplant relapse associates poor survival rates[62-64]. In this scenario, reducing the tumor burden prior to further immunotherapy, name it a second AlloHCT or donor lymphocyte infusions (DLI), seems essential to improve

overall survival (OS) and disease-free survival (DFS). In line with this, patients responding to debulking therapy prior to second AlloHCT seem to associate superior outcomes[65, 57]. Further, responses have been reported higher when DLI is received for low disease burden (cytogenetic relapse) compared to hematologic relapse[66], strongly suggesting that the tumor burden or disease status is fundamental to predict outcomes.

Although no prospective comparative trials have been performed, data from retrospective studies suggests that relapsed patients treated only with chemotherapy seem to associate worse outcomes compared to patients receiving chemotherapy plus immunotherapy (DLI or second AlloHCT)[63, 65]. In this particular situation data are scarce, and physicians decide on an individualized basis according to factors such as the presence of active GvHD at relapse or the patient´s performance status (PS). In accordance with this, patients with poor PS would not generally be considered for a second AlloHCT. Further, with active GvHD at relapse, DLI are not usually offered given the risk of worsening due to GvHD. On the other hand, the absence of GvHD at relapse and prior to relapse enforces the use of DLI. Therefore, the question of who benefits most from each approach remains elusive.

We have performed a comparative of patients treated either with a second AlloHCT or DLI for relapse. We focused on AL patients treated with salvage therapy for transplant relapse prior to further enhancement of the Graft-versus-Leukemia effect (GvL). Herein, we present results for a cohort of relapsed AL patients treated to reduce the disease load, and who subsequently received a second transplantation or DLI.

2.5 Insights into DLI cell composition

DLI are a major therapeutic approach for relapse and conversion of mixed chimerism (MC) after AlloHCT[67, 53]. The effectiveness and toxicity of DLI has been a matter of study, and several variables have been identified. Of these, the total number of $CD3^+$ cells infused per recipient's weight has been identified as key for the development of GvHD, a major drawback after DLI[52]. In this context, an approach using a $CD3^+$ dose-escalation schedule was ideated in order to minimize GvHD while attempting to retain the GvT[32]. At present, DLI dose escalation protocols

are a standard, although the total $CD3^+$ dose may differ according to donor type and center policy.

Within the $CD3^+$ compartment, several T-cell subsets retain different effector and memory properties. T_N, a T-cell subset expressing $CD45RA^+$ and $CCR7^+$ in the membrane, are main drivers of alloreactivity in the AlloHCT setting[48]. In murine GvHD models, it was shown that the infusion of T_N alone could cause GvHD; nevertheless, $CD8^+$ T_{CM}, a T-cell subset lacking $CD45RA^+$, has been shown in murine studies to retain alloreactivity as well and in consequence cause GvHD[49]. However, *in vitro* modeling revealed that by coculturing monocyte-derived dendritic cells and purified $CD4^+$ T-cell subsets from healthy Human Leukocyte Antigen (HLA) identical sibling donors (MSD), the $CD4^+$ T_N compartment developed the highest proliferative response, compared to $CD4^+$ T_{CM}[68]. In the clinical setting lymphocyte subsets have been shown to impact in transplant outcomes and GvHD. In a retrospective report studying a T-cell depleted (TCD) AlloHCT cohort, a graft containing the higher number of $CD4^+$ recent thymic emigrants T-cell (T_{RTE}) was linked to higher overall survival[69]. This finding was explained by the fact that early differentiated T-cell are very important in achieving a complete T-cell repertoire. In allogeneic transplantation, there is a correlation between the degree of T-cell differentiation and alloreactivity; the less differentiated T-cells are, the more alloreactivity they seem to retain. Given these data, several strategies have been developed to minimize GvHD whilst retaining GvT and preserve the memory compartment. Interestingly, the Seattle group is studying the role of T_N depletion in the infused transplant graft, which seems to associate lower chronic GvHD (cGvHD) rates[45].

The majority of data on lymphocyte subsets in AlloHCT is focused on the proportion of cell subsets in the graft product and subsequent patient's immune reconstitution. DLI data in this regard is limited as few cell populations have been studied. However, a number of therapeutic approaches aiming to control the risk of GvHD have been taken. For instance, several groups studied the depletion of $CD8^+$ cells of DLI[35, 36]. Data pulled out from these studies suggested that $CD8^+$-depletion DLI was feasible, that $CD8^+$-depleted DLI stills retains GvT and that peripheral blood circulating $CD8^+$ cells might not be essential for GvHD development. Others adopted different strategies, such as the infusion of $CD45RA^+$-depleted $CD8^+$ DLI, with the rational of providing antiviral memory defense, while avoiding GvHD[70]. In this line, a phase I

study on T_N –depleted DLI is currently being developed and preliminary results have been reported (NCT01627275).

On the other hand, the regulatory T-cell (T_{REG}) compartment, capable of modulating the GvHD response, has also been a subject of interest in DLI. The Boston group reported a strategy based on T_{REG}-depleted DLI for relapse of hematological malignancy[38], aiming to increase the GvT effect after DLI. In the opposite way, the infusion of T_{REG} donor lymphocytes has been studied in experimental models as treatment for GvHD[71]. These approaches are early phase clinical studies and the findings will require further confirmation. Importantly, besides the T-cell compartment, it is unclear what may be the role of B-cells, NK-cells or circulating antigen presenting cells in the unfractionated DLI setting.

On top of that, the T-cell compartment experiences changes with age, sport and many other physiological factors in healthy people[72, 73]. In the AlloHCT setting, the donor characteristics are relevant for the development of GvHD and impact in patient's clinical outcomes[74]. In line with this, the assessment of the clinical and immunological status of the donor is essential, as age or previous pregnancies have been incorporated into clinical practice for AlloHCT donor selection.

Overall, seminal studies have reported on cell subsets such as T_N and T_{CM} in AlloHCT, however there is limited data regarding the cell composition of DLI in the clinical setting[47], particularly on the role of other cell subsets besides T-cells. To the best of our knowledge, a comprehensive analysis of the DLI cell subsets in the clinical setting is lacking, and therefore the impact of each cell population in GvHD remains unclear. Herein, the results of a cell subsets analysis of DLI from sibling donors are presented. We aimed to study its association with alloreactivity by means of the development of GvHD, in a fully matched Major Histocompatibility Antigen (MHA) setting.

III. HYPOTHESIS

III. Hypothesis

1. At AlloHCT relapse, donor lymphocyte infusions and HCT2 show comparable overall survival (OS) and disease-free survival (DFS).

2. The proportion and cell composition of cell subsets in a DLI impacts on the probability of developing GvHD.

IV. AIMS

IV. Aims

The overall aim of this dissertation is the study of the outcomes associated to the DLI infused within the cell therapy program of the *Banc de Sang i Teixits de Barcelona* (BST), and collaborative institutions. There are two aims linked to this thesis.

1. To study transplant and patient-related variables with impact on clinical outcomes after DLI in a selected patient population; and to compare it with HCT2 at relapse.

   Primary Endpoint

   - To analyze the 2-year OS of DLI and HCT2

   Secondary Endpoints

   - To analyze the 2-year DFS of DLI and HCT2
   - To analyze the 2-year NRM of DLI and HCT2
   - To analyze the 1-year Cumulative incidence (CI) of relapse of DLI and HCT2

2. To study the rate of GvHD according to the proportion and total cell dose of each cell subset in DLI, in a selected homogeneous population of patients treated with DLI from HLA matched donors.

   Primary Endpoint

   - To analyzed the GvHD rate at 6 and 12 months, and analyze the rate of GvHD according to each cell subset.
   - To analyze the reversion of mixed chimerism according to each cell subset.

   Secondary Endpoints

   - To analyze the OS and disease response after DLI

V. METHODOLOGY

V. Methodology

5.1 First Hypothesis

Patients from five EBMT centers were consecutively included and data retrospectively collected. Informed consent in accordance with the Declaration of Helsinki was obtained prior to undergo transplantation. Inclusion criteria were as follows: 1) post AlloHCT relapse of AL (AML or ALL), 2) morphological remission or post-chemotherapy aplasia with absence of blasts on bone marrow after salvage treatment; and 3) to receive DLI or second AlloHCT post salvage treatment. Patients who did not receive treatment for relapse were not included. Morphological remission was defined as a blast count of <5% on a bone marrow aspirate (BMA) performed at post chemotherapy peripheral blood recovery. Post chemotherapy aplasia was defined as a significant absence of the three haematopoietic cell lines observed on a BMA sample, along to pancytopenia observed on a peripheral blood sample at any point after salvage therapy. Response and disease status were assessed by BMA. Data regarding immunophenotyping, karyotyping analysis or minimal residual disease by molecular genetics at relapse were not available.

Thirty (65%) patients were diagnosed with AML and sixteen (35%) patients were diagnosed with ALL. All lymphoid leukemias were B-ALL but one, which was a pro T ALL. 4 ALL carried the BCR/ABL rearrangement. Regarding the AML cohort; one patient had myeloid sarcoma; moreover, 10 of 19 evaluable AML carried poor prognostic cytogenetic and molecular features (seven complex karyotype, two poor prognosis karyotype abnormality and one FLT3-ITD mutation). One additional AML patient had inv16. Twenty-seven (59%) patients underwent a second Allo-HCT and 19 (41%) patients received DLI. The median patient age was 38 years (4-66), 28 years (range 4-54) and 42 years (range 22-66) for the second Allo-HCT and the DLI cohorts, respectively (p=0.015). Median Time to Relapse was 285 days (range 35-3956), the median Time to Relapse of the second AlloHCT was 378 days (range 61-1508) and the median Time to Relapse for the DLI cohort was 152 days (range 35-3956) (p=0.019). The median Time from relapse to second AlloHCT was 118 days (range 30-902) and the time from relapse to DLI was 34 days (range 6-63) (p<0.001).

Myeloablative conditioning (MAC) was used in 13 (68%) of DLI patients and in 16 (59%) of second Allo-HCT patients. Nineteen (62%) out of 29 MAC transplants were based on total body irradiation based and nine were busulfan-based (missing data in one patient). RIC Transplants were all fludarabine based, in combination with either busulfan (11 patients) or melphalan (3 patients) (missing data in 3 patients. Immunosuppressive therapy (IST) was cyclosporine-based in the vast majority of transplants (85% and 78% in the second AlloHCT and DLI cohorts, respectively). Further information is shown on Table 1.1, which compares baseline patient´s characteristics, and Table 1.2 and 1.3, which describes characteristics of the second transplant, and the characteristics of the first transplant of the DLI cohort, respectively.

Table 1.1. Comparative of baseline patient´s characteristics.

	Second Allo-HCT	DLI	*p value*
Median Age; years (range)			
	28 (4-54)	42 (22-61)	*0,015*
Disease; n (%)			
AML	20 (74%)	10 (52%)	*0,112*
ALL	7 (26%)	9 (42%)	
First HCT Date; years (%)			
<2004	7 (26%)	4 (21%)	*0,492*
≥2004	20 (74%)	15 (79%)	
Median Time to Relapse; days (range)			
	378 (61-1508)	152 (35-3956)	*0,019*
Median Time from Relapse; days (range)			
	118 (30-902)	34 (6-63)	*<0.001*

(HCT: Hematopoietic Cell Transplant; DLI: Donor Lymphocyte Infusions; AML: Acute Myeloid Leukemia, ALL: Acute Lymphoblastic Leukemia).

For the analysis, day zero was set as the second AlloHCT or first DLI day, and the analysis was performed from this point. The analysis of *Time to second AlloHCT* or *Time to DLI* was performed as a dichotomic variable, dividing in 2 groups according above and below the median. For the descriptive results analysis, the clinical manifestations of acute and chronic GvHD were graded according to the Keystone 1994 consensus criteria[75] and the historical criteria[76]. If the patients were diagnosed with GvHD beyond day 100, GvHD was defined as chronic.

Table 1.2. Description of the second Allo-HCT characteristics of the second Allo-HCT cohort.

	Second Allo-HCT, n (%)
Donor / Recipient gender	
Female --> Male	8 (38%)
Other	15 (62%)
Missing	4
Stem Cell Source	
PB	16 (59%)
Other	11 (41%)
Mismatch	
Yes	3 (12%)
No	22 (88%)
Missing	2
Conditioning	
MAC	16 (59%)
RIC	11 (41%)
Immunosuppressive therapy	
CsA-based	23 (88%)
Other	3 (12%)
Missing	1
T-cell depletion	
Yes	7 (26%)
No	20 (74%)
Donor	
Related	20 (80%)
Unrelated	5 (20%)
Missing	2
Mismatches	
No mismatch	22 (88%)
Mismatch	3 (12%)
Missing	2

(HCT: Hematopoietic Cell Transplant; PB: Peripheral Blood; MAC: Myeloablative Conditioning; RIC: Reduced Intensity Conditioning; CsA: Cyclosporine; CR: Complete Remission)

Statistical Method

The Second AlloHCT cohort was compared against the DLI cohort. A chi-square test was performed to identify significant differences between groups. The following variables were considered for its prognostic value and assessed for Overall Survival (OS), Disease-Free Survival (DFS), Non-Relapse Mortality (NRM) and the Cumulative Incidence (CI) of Relapse univariate analysis: Second AlloHCT or DLI, patient´s age, disease, patients and donor´s

Table 1.3. Description of the Allo-HCT characteristics of the DLI cohort.

	DLI, n(%)
Donor / Recipient gender	
Female --> Male	7 (39%)
Other	12 (61%)
Stem Cell Source	
PB	18 (95%)
Other	1 (5%)
Mismatch	
Yes	6 (31%)
No	13 (69%)
Conditioning	
MAC	13 (68%)
RIC	6 (32%)
Immunosuppressive therapy	
CsA-based	15 (78%)
Other	4 (22%)
T-cell depletion	
Yes	7 (37%)
No	12 (63%)
Donor	
Related / Haploidentical	14 (74%) / 1
Unrelated	5 (26%)
Mismatches	
No mismatch	14 (74%)
Mismatch	5 (26%)

(HCT: Hematopoietic Cell Transplant; DLI: Donor Lymphocyte Infusions; PB: Peripheral Blood; MAC: Myeloablative Conditioning; RIC: Reduced Intensity Conditioning; CsA: Cyclosporine; CR: Complete Remission)

gender, donor type, T-cell depletion (TCD), myeloablative versus reduced intensity conditioning, source of stem cells, first AlloHCT date, disease status at first HCT, number of mismatches, *Time to Second AlloHCT* and *Time to DLI* and type of immunosuppressive therapy. A sub analysis of the DLI cohort also included: total DLI dose, total first DLI dose and DLI collection method (DLI cryopreserved vs. DLI obtained by lymphapheresis).

The probabilities of OS and DFS were calculated using Kaplan–Meier estimates. Cumulative incidence of NRM and CI Relapse were calculated to accommodate for competing risks and results were presented according to the Fine and Gray model. Log-rank and Breslow tests were used for univariate comparisons for all variables considered. Univariate analysis and multivariate analysis used the Cox proportional hazards regression model and the Analysis of

Variance (ANOVA), respectively. For multivariate analysis, we included all independent covariates with p value <0.1 in UA. The p value was set at <0.05 for statistical significance. Statistical analyses were performed with the statistical package SPSS version 17 and R software version 3.4.1.

4.2. Second Hypothesis

Data cut-off was December 2018. All patients signed informed consent according to the Helsinki rules and the study was approved by the Vall d'Hebron University Hospital ethics committee, with the code PR(AG) 369-2015. From 2000 Hematopoietic cell donors (all sibling donors) signed consent for cryopreservation of peripheral blood mononuclear cells (PBMCs) samples for research purposes. Inclusion criteria were as follows: 1) ≥ 18-year-old, 2) AlloHCT, 3) MSD; 4) treatment with DLI; and 5) signed informed consent. Exclusion criteria were as follows: 1) unrelated or mismatched related donors, 2) HCT2 prior to DLI, and 3) GvHD at DLI.

The disease diagnosis was confirmed in all patients based upon the WHO classification. Disease status, response and progression were assessed according to international guidelines and recommendations[77-80]. Acute GvHD (aGvHD) and chronic GvHD (cGvHD) were graded according to the Keystone 1994 consensus criteria[67] and the historical criteria[68], respectively. Chimerism analysis was performed by analyzing short tandem repeat sequences of DNA extracted from peripheral blood (PB) samples. Variations of chimerism <5% were considered as stable chimera. Two consecutive tests showing decreasing donor chimerism were indication for DLI.

Donor lymphocytes were obtained at the stem cell collection date (G-CSF mobilized DLI) after storing $CD3^+$ aliquots of the remaining not infused graft product (G-DLI) or by lymphapheresis of donors (L-DLI). The first scheduled L-DLI was infused fresh after lymphapheresis, and the remaining aliquots were cryopreserved and infused after thawing; whereas all G-DLI were infused after thawing. Donor lymphocytes were not manipulated *in vitro* before infusion in any patient. No patient was on immunosuppressive therapy at DLI time.

A landmark analysis was performed setting the day zero at the first lymphocyte infusion day, and the GvHD analysis was performed from that time point. HLA matching level was obtained by testing in A, B, C, DR and DQ locus in both donor and recipient. As per institutional protocol, the initial $CD3^+$ dose aimed to infuse was $1x10^7$/Kg for MSD, although physicians' choice and DLI availability were also factors that were taken into account for DLI dosing. Response after DLI was assessed at the time of best response achieved.

Flow Cytometry

Flow cytometry analysis was performed using the ten color Navios EX machine (Beckman Coulter Life Sciences, Indianapolis, Indiana, USA). For immunostaining, we used the following monoclonal antibodies (MoAbs): CD45-KRO (Beckman Coulter (BC), CD3-AAF750 (BC), CD31-PaB (BC); CD197 (CCR7)-PE (Becton Dickinson (BD), CD45RO-ECD (BC), CD4-PC5.5 (BC), CD27-PC7 (BC), CD95-APC (BD), CD8-AF700 (BC), CD45RA-FITC (BC), CD25-PE (BC), CD127-PC7 (BC), CD56-PE (BC), CD19-ECD (BC) and CD16-PC7 (BC). Briefly, cryopreserved PBMCs samples from the donors were thawed at 37ºC, suspended and washed twice with FCS 10%. Cell counting and viability were assessed with simple microscopy using tryptan blue 0.4%, aiming a viability >70%. $0.8\text{-}1x10^6$ cells/ ml were added to each tube. MoAbs were added to the mix and incubated 30'. Then, cells were washed with PBS and re suspended in 400µl of PBS for the FACS analysis. We aimed a minimum of $4x10^5$ events /tube. We analyzed the following cell populations: T-cells ($CD3^+$), $CD3^+CD4^+$, $CD3^+CD8^+$, $CD19^+CD3^-$ (B-cell), $CD27^+$B-cell, $CD16^+CD56^+CD3^-$ (NK cell), $CD27^+$NK cell, $CD16^+CD56^+CD3^+$ (NK T-cell), $CD3^+CD45RA^+CCR7^+CD31^+$ (recent thymic emigrants [T_{RTE}]), $CD3^+CD45RA^+CCR7^+$ (naïve T-cell [T_N]), $CD3^+CD45RA^+CD95^+CD27^+$ (stem cell memory T-cell [T_{SCM}]), $CD3^+CD45RA^-CCR7^+$ (central memory T-cell [T_{CM}]), $CD3^+CD45RA^-CCR7^-$ (effector memory T-cell [T_{EM}]), $CD3^+CD45RA^+CCR7^-$ (terminal effector T-cell [T_{TE}]), $CD3+CD4+CD25^{bright}CD127^{dim}$ (regulatory T-cell [T_{REG}]), $CD45RA^+$ T_{REG} (naïve T_{REG}) and mononuclear cells (MNCs) (Table 2.1). Also, we analyzed T_N and T_{CM} grouped (separately for $CD4^+$ and $CD8^+$ cells, and grouped) and the ratio $CD8^+/T_{REG}$ and conventional T-cell (T_{CONS})/T_{REG}. The absolute dose of each cell subset was analyzed with percentages obtained by the flow cytometry, and calculated using the $CD3^+$ and MNCs dosing calculated at stem cell o PBMC donor collection.

Table 2.1. Infused dose of the first DLI cell subsets.

Cell Subset	First DLI (DLI1)
CD3+ cells (x10^6 cells/Kg)	Median (range)
CD3+	10 (0.1-100)
CD4+	6.5 (0.65-70.91)
CD8+	3.4 (0.29-42.69)
NK T-cell - CD3+CD16+CD56+	0.76 (0.06-7.55)
CD4+ cells (x10^6 cells/Kg)	Median (range)
T_N - CD4+CD45RA+CCR7+	2.7 (0.1-23.4)
T_{RTE} - CD4+CD45RA+CCR7+CD31+	0.65 (0.01-6.49)
T_{SCM} - CD4+CD45RA+CCR7+CD95+	0.02 (<0.01-1.01)
T_{CM} - CD4+CD45RA-CCR7+	2.09 (0.28-34.76)
T_{EM} - CD4+CD45RA-CCR7-	2.37 (0.19-17.31)
T_{TE}- CD4+CD45RA+CCR7-	0.12 (0.01-3.47)
T_{REG}- CD4+CD127+CD25(bright)	0.41 (0.04-4.52)
Naïve T_{REG}- CD4+CD127+CD25(bright) CD45RA+	0.03 (<0.01-0.59)
CD8+ cells (x10^6 cells/Kg)	Median (range)
T_N - CD8+CD45RA+CCR7+	0.86 (0.06-12.21)
T_{CM} - CD8+CD45RA-CCR7+	0.28 (0.03-4.27)
T_{EM} - CD8+CD45RA-CCR7-	1.56 (0.03-4.27)
T_{TE}- CD8+CD45RA+CCR7-	0.83 (0.06-16.31)
B-cells (x10^6 cells/Kg)	Median (range)
CD19+	3.1 (0.19-90.80)
CD19+CD27+	0.82 (0.07-14.06)
NK cells (x10^6 cells/Kg)	Median (range)
CD16+ CD56+(dim)	1.77 (0.09-24.18)
CD16+ CD56+CD27+	0.06 (<0.01-0.76)
MNC (x10^8 cells/Kg)	Median (range)
MNC	0.39 x10^8 /Kg
Cell Subsets Combinations / Ratio	**First DLI**
(x10^6 cells/Kg)	Median (range)
CD4+ T_N + CD8+ T_N	4.05 (0.67-31.64)
CD4+ T_N + CD4+ T_{CM}	4.85 (0.46-56.47)
CD8+ T_N + CD8+ T_{CM}	1.09 (0.09-14.01)

(DLI: donor lymphocyte infusion; NK: natural killer; T_N: naïve T-cell; T_{RTE}: recent thymic emigrants; T_{SCM}: stem cell memory T-cell; T_{CM}: central memory T-cell; T_{EM}: effector memory T-cell; T_{TE}: terminal effector T-cell; T_{REG}: regulatory T-cell; MNC: mononuclear cells)

The following variables were considered for its prognostic value and assessed for overall survival (OS): patient's age, donor's age, disease diagnosis, conditioning regimen, disease status at DLI, myeloablative versus reduced intensity, the response after DLI and patient and donor´s gender (the combination female donor and male recipient) and donor and recipient's cytomegalovirus (CMV) status (CMV donor negative and recipient negative versus others, and

recipient's CMV status). For the GvHD analysis, the following clinical variables were considered and analyzed: donor´s age, donor and recipient's gender combinations, type of immunosuppressive therapy at transplant, the use of T-cell depletion at transplant, donor and recipient's cytomegalovirus status, GvHD before DLI, the time interval from AlloHCT to DLI, the DLI collection and administration method (G-DLI vs. L-DLI) and the proportion and dose of each cell subset.

Study End Points and Statistical Analysis

The primary endpoint of the study was to analyze the development of GvHD, and the relation of several cell subsets with GvHD. We also aimed to analyze the reversion of mixed chimerism (MC) and its association with the donor PBMC's cell subsets. However, due to the low statistical power (only five events), MC reversal data was reported in a descriptive manner. Secondary endpoints were overall survival (OS) and disease response after DLI. Variables were defined following the Statistical Guidelines of EBMT. OS was defined as the probability of being alive at any time. OS analysis was performed from the DLI date and was performed in all patients.

The probability of OS was calculated using Kaplan–Meier estimates[81] and the log-rank test was used for univariate comparisons for all variables assessed. Cumulative incidence functions were computed for GvHD, using death or disease progression as a competing event. Fine & Gray competing risks regression[82] were used to obtain sub-distribution hazard ratio (SHR) with 95% confidence intervals (CI). The maximally selected log-rank statistics was used to estimate the optimal cut-off in GvHD analysis. The *p value* was set at ≤0.05 for statistical significance. For multivariate analysis, all independent covariates with p value ≤0.1 in univariate analysis were included. Statistical analyses were performed with the statistical package SPSS (IBM SPSS Statistics 21, New York, NY, USA) (version 17) and R statistical software version 3.4.1 R (R studio, Boston, MA, USA).

VI. RESULTS

VI. Results

6.1 First Hypothesis

From 1995 to 2017, 46 patients were in total included. The median follow-up of the whole cohort was 273 days (range 9-7013), and the median follow up of the patients alive was 692 days (range 28-7013). The median follow up of the second AlloHCT and DLI cohorts were and 404 days (range 22-7013) and 66 days (range 9-4249), respectively. Twenty-one (46%) patients were dead at last follow up. Of them, eight (17%) died due to non-relapse mortality (NRM): six patients due to GvHD and its complications, two patients due to secondary malignancy and one patient due to pulmonary disease. Thirteen patients died after relapse or progressive disease, including the patient diagnosed with myeloid sarcoma, who was treated with DLI at relapse.

Graft-versus-Host Disease

Grade II-IV acute GvHD was diagnosed in 10 (37%) and 5 (26%) patients post second Allo-HCT and DLI, respectively. And chronic GvHD was diagnosed in 10 (4 extensive) and 3 patients after a second Allo-HCT and DLI, respectively. For the DLI patients, the median time to GvHD development was 54 days (range 7-465).

Cumulative Incidence of Relapse

The 2-year CI Relapse was 34% (SE±7%). The 2-year CI Relapse for second Allo-HCT and DLI was 24% (SE±6%) and 49% (SE±3%), respectively (Table 1.4). Univariate analysis identified DLI (p=0.093) (Figure 1.1) and a shorter Time to Relapse (p=0.022) (Figure 1.2) to higher Relapse CI. The CI Relapse multivariate analysis only confirmed a trend for higher relapse in Time to Relapse ≤ 9 months (p=0.059).

Non-Relapse Mortality

The 2-year NRM of the whole cohort was 21% (SE±7%). The 2-year NRM for second Allo-HCT and DLI was 16% (SE±6%) and 31% (SE±3%), respectively (SHR 0.6 (CI 0.15-2.39), p=0.460) (Table 1.4). The NRM univariate analysis did not identify a statistically significant variable.

Table 1.4. Univariate Analysis comparing the 2nd Allo-HCT with DLI patients.

	Second Allo-HCT	DLI	*p value*
2-year OS	60% (SE±10%)	22% (SE±12%)	*0.033*
2-year DFS	56% (SE±10%)	20% (SE±12%)	*0,097*
2-year NRM	16% (SE±6%)	31% (SE±3%)	*0,460*
2-year RI	24% (SE±6%)	49% (SE±3%)	*0.093*

(HCT: Hematopoietic Cell Transplant; DLI: Donor Lymphocyte Infusions; OS: Overall Survival; DFS: Disease-free Survival; NRM: Non-relapse mortality; RI: Relapse Incidence)

Figure 1.1. CI Relapse according to *DLI* (continuous line) and s*econd Allo-HCT* (dotted line) cohorts. (*p=0.093)*.

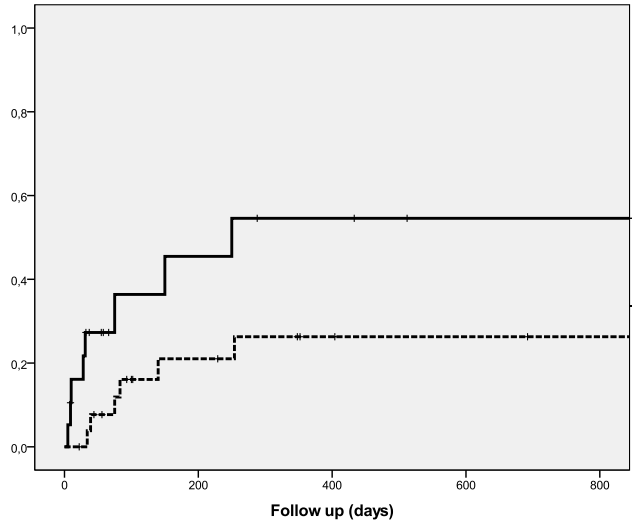
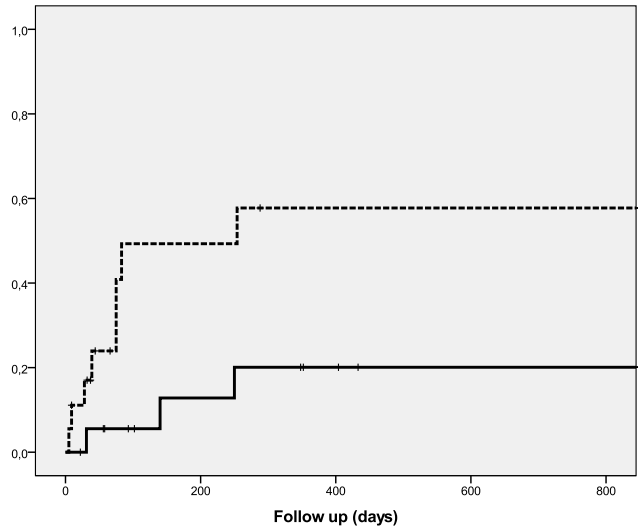


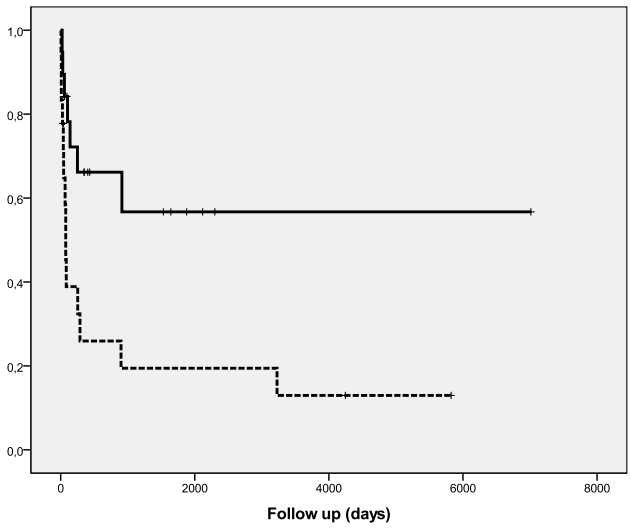
Figure 1.2. CI Relapse according to *Time to Relapse* ≥ 9 months (continuous line) and *Time to Relapse* < 9 months (dotted line) (*p=0.022)*.



Disease Free Survival

The 2-year DFS of the whole cohort was 38% (SE±8%). The 2-year DFS for second Allo-HCT and DLI was 56% (SE±10%) and 20% (SE±12%), respectively (Table 1.4). The DFS univariate analysis linked a longer Time to Relapse (p=0.008) (Figure 1.3) and Second Allo-HCT (p=0.097) to better DFS. The DFS multivariate analysis confirmed the association of Time to Relapse >9 months to better DFS (p=0.011).

Figure 1.3. DFS according to *Time to Relapse* ≥ 9 months (continuous line) and *Time to Relapse* < 9 months (dotted line) (*p=0.011).*



Overall Survival

The 2-year OS of the whole cohort was 44% (SE±9%). The 2-year OS for second Allo-HCT and DLI was 60% (SE±10%) and 22% (SE±12%), respectively (Table 1.4) (Figure 1.4). OS univariate analysis liked Second Allo-HCT (p=0.033) (Figure 1.4) and a Time to second Relapse > 9 months (p=0.013) (Figure 1.5) to better OS. OS multivariate analysis only confirmed Time to Relapse as statistically significant (p=0.033).

DLI subanalysis

The median DLI dose per patient was $5x10^7$ CD3+/Kg ($0.1\text{-}19.4x10^7$/Kg) cells, the median first DLI dose was $1.03x10^7$ CD3+/Kg ($0.1\text{-}19.4\ x10^7$/Kg) cells and the mean number of infused DLI

was 1.4/ patient. Ten (52%) patients received DLI from available cryopreserved cells, whereas the remaining 9 (42%) patients received DLI after requesting a donor´s lymphapheresis.

Figure 1.4. OS according to *DLI* (continuous line) and *second Allo-HCT* (dotted line) cohorts. (*p=0.033).*

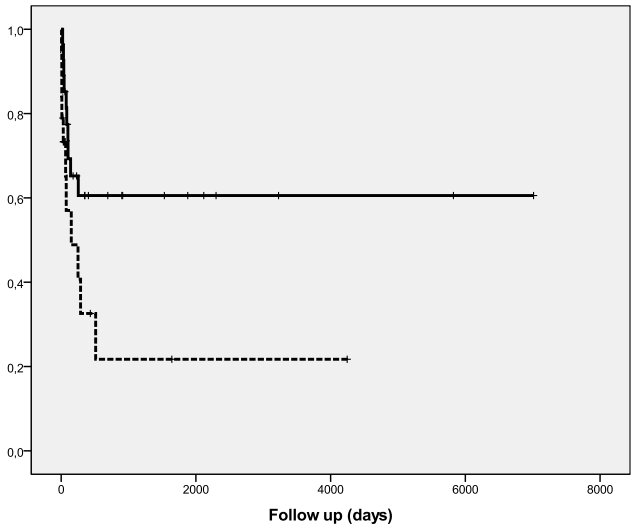
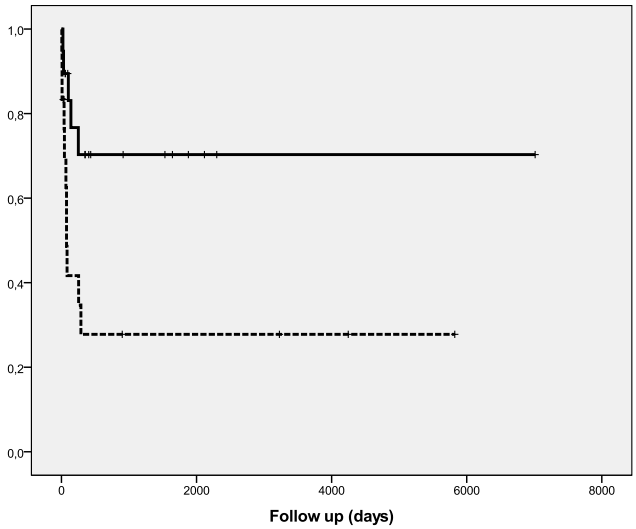


Figure 1.5. OS according to *Time to Relapse* ≥ 9 months (continuous line) and *Time to Relapse* < 9 months (dotted line) (*p=0.013).*



DLI-CI relapse univariate analysis showed the use of TCD linked to higher relapse (p=0.005), also confirmed on multivariate analysis (p=0.003). DLI-DFS univariate analysis linked the use of TCD (p=0.002) and a trend of the use of unrelated donor (UR) (p=0.071) to better DFS. DFS

multivariate analysis only confirmed the use of TCD (p<0.001). And DLI-OS univariate analysis linked the use of TCD (p=0.010) and the use of UD (log-rank, p=0.107, Breslow, (p=0.043) to better OS. OS multivariate analysis only confirmed the use TCD to higher OS (p=0.002).

6.2 Second Hypothesis

Data cut-off was December 2018. All patients signed informed consent according to the Helsinki rules and the study was approved by the Vall d'Hebron University Hospital ethics committee, with the code PR(AG) 369-2015. From 2000 Hematopoietic cell donors (all sibling donors) signed consent for cryopreservation of peripheral blood mononuclear cells (PBMCs) samples for research purposes. Inclusion criteria were as follows: 1) ≥ 18-year-old, 2) AlloHCT, 3) MSD; 4) treatment with DLI; and 5) signed informed consent. Exclusion criteria were as follows: 1) unrelated or mismatched related donors, 2) HCT2 prior to DLI, and 3) GvHD at DLI.

The disease diagnosis was confirmed in all patients based upon the WHO classification. Disease status, response and progression were assessed according to international guidelines and recommendations (77-80). Acute GvHD (aGvHD) and chronic GvHD (cGvHD) were graded according to the Keystone 1994 consensus criteria[67] and the historical criteria[68], respectively. Chimerism analysis was performed by analyzing short tandem repeat sequences of DNA extracted from peripheral blood (PB) samples. Variations of chimerism <5% were considered as stable chimera. Two consecutive tests showing decreasing donor chimerism were indication for DLI.

Donor lymphocytes were obtained at the stem cell collection date (G-CSF mobilized DLI) after storing $CD3^+$ aliquots of the remaining not infused graft product (G-DLI) or by lymphapheresis of donors (L-DLI). The first scheduled L-DLI was infused fresh after lymphapheresis, and the remaining aliquots were cryopreserved and infused after thawing; whereas all G-DLI were infused after thawing. Donor lymphocytes were not manipulated *in vitro* before infusion in any patient. No patient was on immunosuppressive therapy at DLI time.

Methods

A landmark analysis was performed setting the day zero at the first lymphocyte infusion day, and the GvHD analysis was performed from that time point. HLA matching level was obtained

by testing in A, B, C, DR and DQ locus in both donor and recipient. As per institutional protocol, the initial $CD3^+$ dose aimed to infuse was $1x10^7$/Kg for MSD, although physicians' choice and DLI availability were also factors that were taken into account for DLI dosing. Response after DLI was assessed at the time of best response achieved.

Flow Cytometry

Flow cytometry analysis was performed using the ten color Navios EX machine (Beckman Coulter Life Sciences, Indianapolis, Indiana, USA). For immunostaining, we used the following monoclonal antibodies (MoAbs): CD45-KRO (Beckman Coulter (BC), CD3-AAF750 (BC), CD31-PaB (BC); CD197 (CCR7)-PE (Becton Dickinson (BD), CD45RO-ECD (BC), CD4-PC5.5 (BC), CD27-PC7 (BC), CD95-APC (BD), CD8-AF700 (BC), CD45RA-FITC (BC), CD25-PE (BC), CD127-PC7 (BC), CD56-PE (BC), CD19-ECD (BC) and CD16-PC7 (BC). Briefly, cryopreserved PBMCs samples from the donors were thawed at 37ºC, suspended and washed twice with FCS 10%. Cell counting and viability were assessed with simple microscopy using tryptan blue 0.4%, aiming a viability >70%. $0.8\text{-}1x10^6$ cells/ ml were added to each tube. MoAbs were added to the mix and incubated 30'. Then, cells were washed with PBS and re suspended in 400µl of PBS for the FACS analysis. We aimed a minimum of $4x10^5$ events /tube. We analyzed the following cell populations: T-cells ($CD3^+$), $CD3^+CD4^+$, $CD3^+CD8^+$, $CD19^+CD3^-$ (B-cell), $CD27^+$B-cell, $CD16^+CD56^+CD3^-$ (NK cell), $CD27^+$NK cell, $CD16^+CD56^+CD3^+$ (NK T-cell), $CD3^+CD45RA^+CCR7^+CD31^+$ (recent thymic emigrants [T_{RTE}]), $CD3^+CD45RA^+CCR7^+$ (naïve T-cell [T_N]), $CD3^+CD45RA^+CD95^+CD27^+$ (stem cell memory T-cell [T_{SCM}]), $CD3^+CD45RA^-CCR7^+$ (central memory T-cell [T_{CM}]), $CD3^+CD45RA^-CCR7^-$ (effector memory T-cell [T_{EM}]), $CD3^+CD45RA^+CCR7^-$ (terminal effector T-cell [T_{TE}]), $CD3+CD4+CD25^{bright}CD127^{dim}$ (regulatory T-cell [T_{REG}]), $CD45RA^+$ T_{REG} (naïve T_{REG}) and mononuclear cells (MNCs) (Table 2.1). Also, we analyzed T_N and T_{CM} grouped (separately for $CD4^+$ and $CD8^+$ cells, and grouped) and the ratio $CD8^+/T_{REG}$ and conventional T-cell (T_{CONS})/T_{REG}. The absolute dose of each cell subset was analyzed with percentages obtained by the flow cytometry, and calculated using the $CD3^+$ and MNCs dosing calculated at stem cell o PBMC donor collection.

Table 2.1. Infused dose of the first DLI cell subsets.

Cell Subset	First DLI (DLI1)
CD3+ cells (x10^6 cells/Kg)	Median (range)
CD3+	10 (0.1-100)
CD4+	6.5 (0.65-70.91)
CD8+	3.4 (0.29-42.69)
NK T-cell - CD3+CD16+CD56+	0.76 (0.06-7.55)
CD4+ cells (x10^6 cells/Kg)	Median (range)
T_N - CD4+CD45RA+CCR7+	2.7 (0.1-23.4)
T_{RTE} - CD4+CD45RA+CCR7+CD31+	0.65 (0.01-6.49)
T_{SCM} - CD4+CD45RA+CCR7+CD95+	0.02 (<0.01-1.01)
T_{CM} - CD4+CD45RA-CCR7+	2.09 (0.28-34.76)
T_{EM} - CD4+CD45RA-CCR7-	2.37 (0.19-17.31)
T_{TE}- CD4+CD45RA+CCR7-	0.12 (0.01-3.47)
T_{REG}- CD4+CD127+CD25(bright)	0.41 (0.04-4.52)
Naïve T_{REG}- CD4+CD127+CD25(bright) CD45RA+	0.03 (<0.01-0.59)
CD8+ cells (x10^6 cells/Kg)	Median (range)
T_N - CD8+CD45RA+CCR7+	0.86 (0.06-12.21)
T_{CM} - CD8+CD45RA-CCR7+	0.28 (0.03-4.27)
T_{EM} - CD8+CD45RA-CCR7-	1.56 (0.03-4.27)
T_{TE}- CD8+CD45RA+CCR7-	0.83 (0.06-16.31)
B-cells (x10^6 cells/Kg)	Median (range)
CD19+	3.1 (0.19-90.80)
CD19+CD27+	0.82 (0.07-14.06)
NK cells (x10^6 cells/Kg)	Median (range)
CD16+ CD56+(dim)	1.77 (0.09-24.18)
CD16+ CD56+CD27+	0.06 (<0.01-0.76)
MNC (x10^8 cells/Kg)	Median (range)
MNC	0.39 x10^8 /Kg
Cell Subsets Combinations / Ratio	**First DLI**
(x10^6 cells/Kg)	Median (range)
CD4+ T_N + CD8+ T_N	4.05 (0.67-31.64)
CD4+ T_N + CD4+ T_{CM}	4.85 (0.46-56.47)
CD8+ T_N + CD8+ T_{CM}	1.09 (0.09-14.01)

(DLI: donor lymphocyte infusion; NK: natural killer; T_N: naïve T-cell; T_{RTE}: recent thymic emigrants; T_{SCM}: stem cell memory T-cell; T_{CM}: central memory T-cell; T_{EM}: effector memory T-cell; T_{TE}: terminal effector T-cell; T_{REG}: regulatory T-cell; MNC: mononuclear cells)

The following variables were considered for its prognostic value and assessed for overall survival (OS): patient's age, donor's age, disease diagnosis, conditioning regimen, disease status at DLI, myeloablative versus reduced intensity, the response after DLI and patient and donor´s gender (the combination female donor and male recipient) and donor and recipient's cytomegalovirus (CMV) status (CMV donor negative and recipient negative versus others, and

recipient's CMV status). For the GvHD analysis, the following clinical variables were considered and analyzed: donor´s age, donor and recipient's gender combinations, type of immunosuppressive therapy at transplant, the use of T-cell depletion at transplant, donor and recipient's cytomegalovirus status, GvHD before DLI, the time interval from AlloHCT to DLI, the DLI collection and administration method (G-DLI vs. L-DLI) and the proportion and dose of each cell subset.

Study End Points and Statistical Analysis

The primary endpoint of the study was to analyze the development of GvHD, and the relation of several cell subsets with GvHD. We also aimed to analyze the reversion of mixed chimerism (MC) and its association with the donor PBMC's cell subsets. However, due to the low statistical power (only five events), MC reversal data was reported in a descriptive manner. Secondary endpoints were overall survival (OS) and disease response after DLI. Variables were defined following the Statistical Guidelines of EBMT. OS was defined as the probability of being alive at any time. OS analysis was performed from the DLI date and was performed in all patients.

The probability of OS was calculated using Kaplan–Meier estimates[81] and the log-rank test was used for univariate comparisons for all variables assessed. Cumulative incidence functions were computed for GvHD, using death or disease progression as a competing event. Fine & Gray competing risks regression[82] were used to obtain sub-distribution hazard ratio (SHR) with 95% confidence intervals (CI). The maximally selected log-rank statistics was used to estimate the optimal cut-off in GvHD analysis. The *p value* was set at ≤0.05 for statistical significance. For multivariate analysis, all independent covariates with p value ≤0.1 in univariate analysis were included. Statistical analyses were performed with the statistical package SPSS (IBM SPSS Statistics 21, New York, NY, USA) (version 17) and R statistical software version 3.4.1 R (R studio, Boston, MA, USA).

Results

Patient and transplant characteristics

A total of 36 adult patients were consecutively included from four EBMT centers, all related to the same blood bank program. Transplants were performed from 2001 to 2018, with a median

follow up of the surviving patients of 42 months (range, 3-197). Most patients received a fludarabine and melphalan conditioning regimen and cyclosporine-based GvHD prophylaxis.

The patient's median PB total lymphocyte count (TLC) at DLI day and at day +30 after DLI (data available in 21 patients) was $1.2x10^9$/L (range, 0-6.49) and $1.25x10^9$/L (range, 0.37-7.95), respectively. The donor's median PB TLC at donation day (data available in 22 donors) was $3.89x10^9$/L (range, 1.9-6.4). Comprehensive data regarding patient and transplants characteristics can be checked in Table 2.2 and 2.3.

Donor lymphocyte infusions analysis

A total of 56 DLI were infused, with a median number of 1 DLI/patient (range, 1-3). Twenty-two patients (61%) received one DLI (DLI1) (median dose of first DLI $1x10^7$ CD3+ cells/Kg [range 0.1-10 $x10^7$ $CD3^+$ cells/Kg]), 8 patients (22.2%) received two DLI (median dose of the second DLI 5 $x10^7$ $CD3^+$ cells/Kg [range 1-18 $x10^7$ $CD3^+$ cells/Kg]) and 6 patients (16.7%) received three DLI (median dose of the third DLI 7.1 $x10^7$ $CD3^+$ cells/Kg [range 4.5-10. $x10^7$ $CD3^+$ cells/Kg]). The median time from AlloHCT to DLI was 253 days (range, 70-4293 day), and the median time interval from DLI to last follow up was 282 days (range, 9-5560). Eighteen patients (50%) received L-DLI and 18 patients (50%) received G-DLI. The indication of DLI was relapse in thirty-one patients (86%) whereas it was MC in five (14%) patients. The median dose of MNCs in the first DLI was 0.39 $x10^8$ /Kg [range 0.04-4 $x10^8$ MNCs/Kg]). Data regarding the dosing of each cell subset can be found in Table 2.1.

Graft-versus-host disease analysis

Fourteen patients (38%) had GvHD after DLI; all after DLI1 but one, who had limited chronic GvHD (cGvHD). The median time interval form DLI to GvHD was 76 days (95% CI: 78.8-NA). Seven patients have had GvHD before DLI; of these, only one patient was diagnosed with GvHD after DLI. As per clinical presentation, 10 patients (27%) had acute GvHD (two patients grade II-IV GvHD), whereas eight patients (22%) were diagnosed with cGvHD (six patients

Table 2.2. Disease characteristics.

Disease Diagnosis	N (%)	Disease characteristics
Myeloid Disorders	18 (50%)	
Acute Myeloid Leukemia	High Risk: 4 patients	46,XY, del (17) (p13)
		45,XY,t(3;3)(q21;q26),-7
		45,XY,-5, FLT3mut
		CK, *mll* rearrangement
	Intermediate Risk : 4 patients	NK
	Unknown Risk: 1 patient	Unknown karyotype and genetics
Chronic Myeloid Leukemia	Chronic Phase: 1 patient	46 XY (t 9;22) (q34; q11)
	AP / BP: 3 patients	t(6,9,22)(p23;q34;q11.2)
		45, XY, -7, t (9; 22) (Q34; Q11) del (6)
		45,XY,-7, t(9;22)(q34;q11), t(17;18) +mar.
Myelodisplastic Syndrome	High Risk: 2 patients	AREB II, 52,XY,+6,+8,+10,+11,+15,-18
		45,X,-X,-7,+mar
	Normal Karyotype: : 2 patients	NK: 2
Primary Myelofibrosis	1 patient	47,XX,+8/46,XX
Acute Lymphoblastic Leukemia	1 (3%)	
B-cell ALL	High Risk: 1 patient	t(9;22)
Hodgkin's Lymphoma	6 (17%)	
Non-Hodgkin's Lymphoma	4 (11%)	
	3 patients	B-NHL
	1 patient	T-NHL (HTLV1+)
Multiple Myeloma	3 (8%)	
	1 patient	Normal karyotype
	2 patients	Unknown
Chronic Lymphoid Leukemia	4 (11%)	
Chronic Lymphoid Leukemia	1 patient	del 13q14.3 y 11q22.3
	1 patient	NK
	1 patient	Unknown
Prolinfocitic T Leukemia	1 patient	

(NK: normal karyotype; mut: mutated; AP: accelerated phase; BP: blastic phase; ALL: acute lymphoblastic leukemia; NHL: non-Hodgkin's lymphoma; del: deletion).

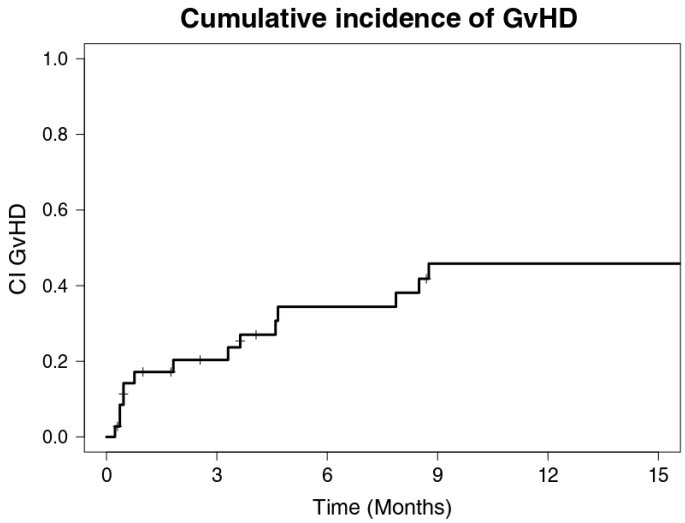
Table 2.3. Transplant characteristics.

	n (%)
Donor / Recipient gender	
Donor female → Recipient male	5 (14%)
Other	31 (86%)
Patient Age, years	
Median (range)	54 (range, 21-66)
Donor Age, years	
Median (range)	53 (range, 18-66)
Stem Cell Source	
BM	1 (3%)
PB	35 (97%)
Disease Diagnosis	
AML / ALL	10 (28%)
MDS / MPN	5 (14%)
HL / NHL / CLL	14 (39%)
CML	4 (11%)
MM	3 (8%)
HLA Mismatch	
No	36 (100%)
Yes	0 (0%)
Myeloablative Conditioning	
MAC	8 (22%)
RIC	28 (78%)
CMV	
Donor negative – Recipient negative	8 (25%)
Other	24 (75%)
Missing	4
Conditioning	
FluMel	16 (44%)
FluBu	13 (36%)
CyTBI	4 (11%)
Other	3 (9%)
Immunosuppressive therapy	
CsA / MTX	18 (50%)
CsA / MMF	8 (22%)
Other	10 (28%)
Type of Donor	
HLA Identical Sibling	36 (100%)
GvHD before DLI	
Yes	7 (20%)
No	29 (80%)
T-cell depletion	
Yes	6 (17%)
No	30 (83%)
DLI Collection Method	
G-DLI	18 (50%)
L-DLI	18 (50%)

(HCT: Hematopoietic Cell Transplant; PB: Peripheral Blood; BM: bone marrow; AML: acute myeloid leukemia; ALL: acute lymphoid leukemia; MDS: myelodisplastic syndrome; MPN: myeloproliferative disorder; HL: Hodgkin lymphoma;

NHL: non-Hodgkin lymphoma; CLL: chronic lymphocytic leukemia; CML: chronic myeloid leukemia; MM: multiple myeloma; HLA: human leukocyte antigen; MAC: Myeloablative Conditioning; RIC: Reduced Intensity Conditioning; CMV: cytomegalovirus; FluMel: fludarabine and melphalan; FluBu: fludarabine and busulfan; CyTBI: cyclophosphamide and total body irradiation; CsA: Cyclosporine; MTX: methotrexate; MMF: micophenolate mofetil; GvHD: graft-versus-host disease; DLI: Donor Lymphocyte Infusions; G-DLI: G-CSF mobilized DLI; L-DLI: lymphapheresis).

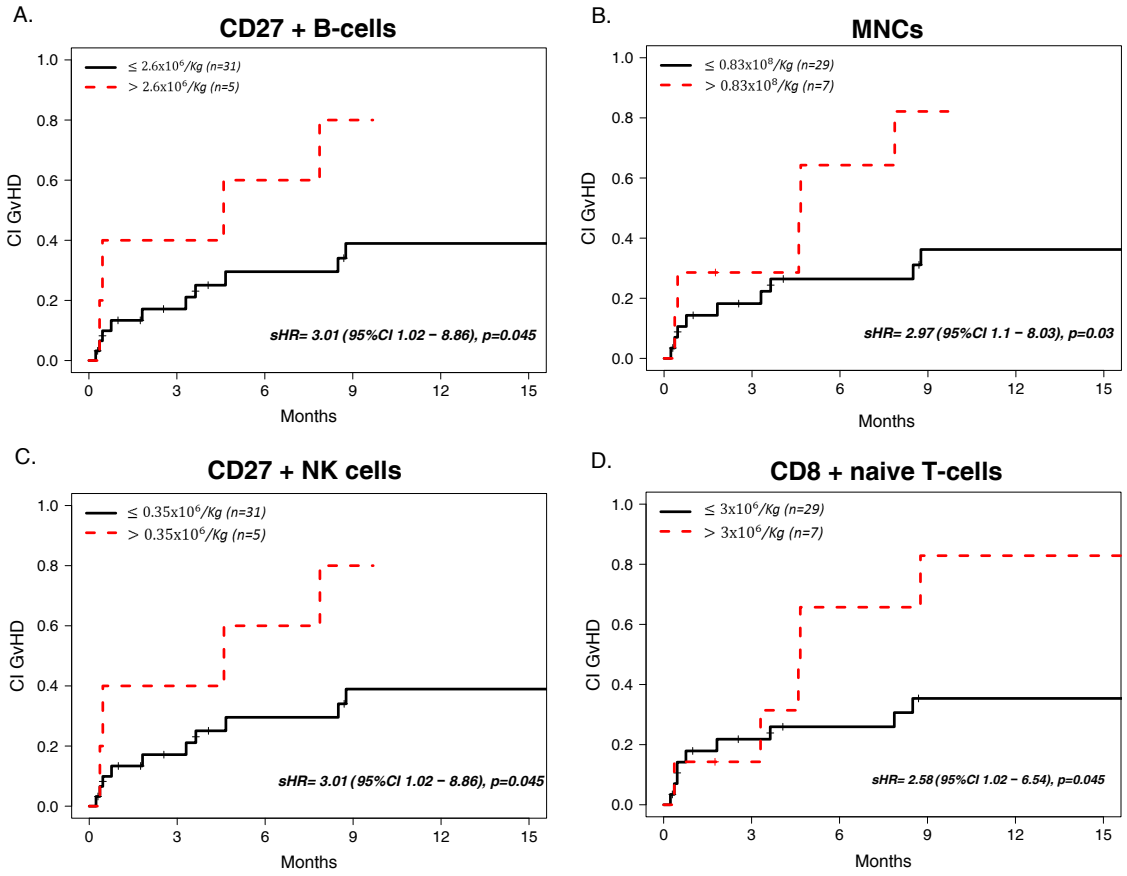
Figure 2.1. Cumulative incidence (CI) of graft-versus-host disease (GvHD).



extensive cGvHD), 4 of them following initial acute presentation. The 3-month and 1-year CI of GvHD after DLI was 21% and 46%, respectively (Figure 2.1).

The DLI1 cell subset analysis identified an association of B-cell (Hazard Ratio (HR): 1.03 [confidence interval (CI) 95%, 1-1.05], p=0.03) and $CD27^+$B-cell (HR 1.18 [CI95%, 1.06-1.33], p<0.01) with GvHD. Further, we observed a trend to higher GvHD in the following cell populations: $CD8^+T_N$ (HR 1.12 [CI95%, 1-1.27], p=0.06), MNC (HR 1.84 [CI95%, 0.94-3.61], p=0.08), NK cells (HR 1.09 [CI95%, 0.99-1.21], p=0.08) and $CD27^+$NK cells (HR 10.1 [CI95%, 1.01-102], p=0.05). Then, we identified a dose cut-off point in the cell subsets with association to or a trend to GvHD, and we found that a DLI1 with a $CD8^+$ T_N dose >$3x10^6$/Kg (p=0.045), MNC dose >$0.8x10^8$/Kg (p=0.030), $CD27^+$B-cell dose >$2.6x10^6$/Kg (p=0.030) or $CD27^+$NK cell dose >$0.35x10^6$/Kg (p=0.045) associated with GvHD (Table 2.4a, Figure 2.2). A cell dose cut off was also identified for patients with severe GvHD (grade II-IV acute GvHD or severe chronic GvHD) for the same cell populations with statistical significance (data not shown).

Figure 2.2 Cumulative incidence of graft-versus-host disease (GvHD) according to the dose cut-off point of distinct cell subsets. A: $CD27^{+}$B-cells, B: Mononuclear Cells (MNCs); C: $CD27^{+}$NK cells; D: $CD8^{+}$ Naïve T-cells.



Finally, we assessed the potential impact of transplant clinical variables with the development of GvHD and we did not identify any variable with statistically significant association (data not shown). Of interest, the DLI collection method (G-DLI vs. L-DLI) had not impact on the development of GvHD.

Response analysis

In those patients who received DLI for relapse, overall response rate (ORR) was 29% (9 of 31 patients), six patients achieved complete remission and three partial remission. Of note, eight patients responded after the first DLI, whereas only one patient responded after the second DLI. Of the nine patients that received DLI for relapse and responded, four patients (31%) had treatment before DLI (two patients received chemotherapy and two patients diagnosed with

Table 2.4a Analysis of the DLI1 cell subsets (cut-off point) and its association with GvHD.

Cell Subset	**First DLI**	**Hazard Ratio**	**GvHD**
CD3+ cells (x10^6 cells/Kg)	Cut-off point	sHR (CI95%)	*p value*
T_N - CD8+CD45RA+CCR7+	>3x10^6 /Kg	2.58 (1.02-6.54)	0.045
$CD27^+$ B cells (x10^6 cells/Kg)	Cut-off point	sHR (CI95%)	*p value*
CD19+CD27+	>2.6x10^6 /Kg	2.97 (1.1-8.03)	0.030
$CD27^+$ NK cells (x10^6 cells/Kg)	Cut-off point	sHR (CI95%)	*p value*
CD16+CD56+CD27+	>0.35x10^6 /Kg	3.01 (1.02-8.86)	0.045
MNC (x10^8 cells/Kg)	Cut-off point	sHR (CI95%)	*p value*
Total MNC	>0.83 x10^8 /Kg	2.97 (1.1-8.03)	0.030

(DLI: donor lymphocyte infusion; DLI1: first DLI; T_N: naïve T-cell; NK: natural killer; MNC: mononuclear cells).

chronic myeloid leukemia were treated with tyrosine kinase inhibitors (TKI) pre DLI). Further, five out of the nine patients who responded (41%) developed GvHD after DLI. Interestingly, only two of the six patients who received a $CD8^+$ T_N dose >3x10^6/Kg (associated with GvHD) responded after DLI. Moreover, none of the patients who received >0.35x10^6/Kg $CD27^+$NK responded after DLI, on the contrary 7 of 9 patients who responded had a higher proportion of NK cell cells. Regarding the additional treatment non responders, one CML patient was treated with TKI and 7 patients receiving chemotherapy before DLI.

Among the patients who had DLI for relapse, five patients had DLI to treat minimal residual disease (MRD), four CML patients and one MM patients. Of the CML patients, 3 of 4 had the AlloHCT for blastic phase and one for chronic phase, 2 of 4 responded to DLI. The MM patient did not respond to DLI but to subsequent therapies. On the other hand, five patients had DLI for MC, and full donor chimerism was achieved in all patients, 4 patients after receiving the first DLI and one patient after the second DLI. Two patients who had DLI for MC developed GvHD.

Finally, no statistically significant association was found between the proportion of $CD8^+$ T_N dose, MNC dose, $CD27^+$B-cell dose or $CD27^+$NK cell dose and response (Table 2.4b).

Overall survival analysis

The median OS was 53 months and the 1-year OS was 61% (95% CI, 52-70). Twenty-two patients (61%) were dead at last follow up. None of the above-mentioned clinical variable had impact on OS. Fifteen patients died due to disease progression, and seven patients died due to non-relapse mortality: three patients died due to GvHD, one patient died due to graft failure, one

Table 2.4b. Analysis of the DLI1 cell subsets (cut-off point) and its association with response.

Cell Subset	First DLI	Response (%)	Hazard Ratio	p value
CD3+ cells (x10^6 cells/Kg)	≤3x10^6 /Kg	34.5%	2.58 (1.02-6.54)	*0.39*
T_N - CD8+CD45RA+CCR7+	>3x10^6 /Kg	57.1%		
$CD27^+$ B cells (x10^6 cells/Kg)	≤2.6x10^6 /Kg	41.4%	2.97 (1.1-8.03)	*0.68*
CD19+CD27+	>2.6x10^6 /Kg	28.6%		
$CD27^+$ NK cells (x10^6 cells/Kg)	≤0.35x10^6 /Kg	45.2%	3.01 (1.02-8.86)	*0.14*
CD16+CD56+CD27+	>0.35x10^6 /Kg	0%		
MNC (x10^8 cells/Kg)	≤0.83 x10^8 /Kg	41.4%	2.97 (1.1-8.03)	*0.68*
Total MNC	>0.83 x10^8 /Kg	28.6%		

(DLI: donor lymphocyte infusion; DLI1: first DLI; T_N: naïve T-cell; NK: natural killer; MNC: mononuclear cells).

patient due to a *Pseudomonas aeruginosa* septic shock, one patient died due to secondary malignancy (breast cancer) and one due to intracerebral hemorrhage.

VII. DISCUSSION

VII. Discussion

The role of DLI has been a matter of interest in multiple studies[52, 53]. In our works, we aimed to focus on two particular scenarios within the DLI setting. Firstly, we wanted to compare the outcomes of DLI in AL patients relapsing after AlloHCT with a cohort of similar patients treated with HCT2. With this, we aimed to observe whether there was any benefit with one or another approach at this point. In this scenario physicians decide according to several clinical factors, but there is not a decision-making algorithm.

Secondly, due to the lack of consistent data on GvHD and DLI, we aimed to study in depth the role of the cell subsets and the development of GvHD after DLI. We performed a flow cytometry analysis of donor PBMCs samples cryopreserved at the BST (Barcelona) and studied potential association between a particular cell subset and GvHD. Notably, we also studied a number of clinical variables with impact on GvHD.

Despite the available approaches, the prognosis of acute leukemia patients relapsing post AlloHCT remains poor. Among patients candidate to intensive therapy, the treatment often entails the administration of chemotherapy plus a consolidation treatment. At that moment, further immunotherapy-based treatment, such as a second Transplant or DLI, is a major approach.

We describe a cohort of patients treated at relapse to reduce the tumour burden, who subsequently received a second AlloHCT or DLI. Outcomes between DLI and second AlloHCT cohorts were comparable as multivariate analysis results showed. Nevertheless, we found some differences in univariate analysis that might be worth to comment. We observed higher OS towards second AlloHCT (p=0.022); and a trend to higher DFS (p=0.097) and higher incidence of relapse (p=0.054) in the DLI cohort (see Table 1.3). The retrospective and non-randomized inclusion of patients might have influenced these results. Further, when analysing outcomes only on patients with longer Time to Relapse (>9 months), we did not find any difference on OS, DFS and CI relapse (data not shown). On the other hand, it is important to take into account that the second AlloHCT cohort included patients with a longer time interval form first AlloHCT to relapse (Time to Relapse), which might be pointing towards the fact that the second transplantation cohort included patients with less aggressive diseases. Of note, we

also calculated the time from relapse to second AlloHCT and DLI and found that it was statistically longer in the second AlloHCT group. This former result might be partially explained by the fact that the preparation of an AlloHCT entails longer time compared to DLI, particularly if DLI is already cryopreserved.

There is limited data comparing the use of DLI or second AlloHCT for haematological malignancy´s relapse[83-86], as it is not defined which approach should prevail against the other. Because it would be of utmost complexity to develop a randomized study, available data comes from retrospective studies. The CIBMTR retrospectively reported outcomes of relapsed AML patients[65]. The authors found that an approach with chemotherapy alone (comparing chemo plus DLI or chemo plus second AlloHCT) associated worse survival, reporting CR rates of 16% post chemotherapy, whereas CR rates post second AlloHCT or DLI were 44% and 37% respectively. Furthermore, median survival was inferior among patients receiving DLI comparing Second Transplant, 7 and 12 months respectively. The authors also observed a 3-year OS of 4% for early relapsing patients (within 6 months of AlloHCT). In line with this, we also found a lower OS and DFS in patients with shorter Time to Relapse, setting the cut off time point at the median value (9 months). Significantly, others have also reported the relation of early relapse with poorer outcomes for immunotherapy after AlloHCT relapse, with data in this regard being published at different time cut off points[54, 87, 88].

Interestingly, the EBMT Acute Leukemia party reported outcomes of patients receiving DLI or HCT2 for myeloid malignancy relapse after transplant[89]. This study reported a retrospective comparative of DLI and HCT2 of EBMT centers. The authors reported a cohort 418 adult patients who received a HCT2 (n = 137) or DLI (n = 281) for relapse. They found a 2-year and 5-year OS with HCT2 of 26% and 19%, respectively; and a 2-year and 5-year OS with DLI of 25% and 15%, respectively (p=0.860). These results highlight the similar results between both approaches and validate the data previously reported by us.

Furthermore, as in our study, the authors identified time from relapse (or first AlloHCT) to HCT2 or DLI, and disease status at HCT2 or DLI as main prognostic factors for survival and progression risk in relapsing patients. These factors have been identified in a significant number

of AlloHCT relapse studies and they probably highlight biological characteristics of the disease that modulate its response to therapy. Thus, for instance, diseases with early relapse might harbour molecular mutations that increase refractoriness and relapse incidence.

In the current study, the median initial lymphocyte dose of the patients receiving DLI was $1.03x10^7$ $CD3^+$/Kg. Initial doses of $1x10^7$ $CD3^+$/Kg have been linked to a GvHD rate of 21%[90], although higher GvHD rates have also been reported[91,92]. In the DLI cohort, GvHD was diagnosed in 42% of patients. To note only 3 of the 17 evaluable DLI patients developed GvHD prior to receive the DLI and the median time from AlloHCT to DLI was 179 days. Interestingly, time from transplant to DLI <2 years have been linked to the development of GvHD[93]. One of the reasons to explain the GvHD rate here reported could be the fact that most DLI transplants (63%) were T-cell replete, although there might be many factors influencing this result. GvHD could not be analysed in a time-dependent manner but the NRM UA of the DLI cohort showed comparable results between TCD and TCR transplants (data not shown, p=0.163). To the best of our knowledge, there is few data comparing the use of DLI in both *in vivo* TCD and TCR transplants[94]. In this former study only 3 of 23 patients had DLI in the TCR setting. In our study, DLI patients who received TCD conditionings associated favourable outcomes (OS, DFS and RI). The number of patients in this subgroup is small and this finding surely requires further confirmation, but we would like to speculate that in AL, perhaps, the enhancement of the T-cell compartment in TCD transplants (either pre or post relapse) might be of more benefit for lowering the relapse risk than in TCR transplants (in which the T-cell reservoir is earlier expanded), where DLI appear to provide less benefit.

Regarding the not statistically significant NRM difference between DLI and second AlloHCT cohorts, the second AlloHCT patients were younger compared to patients receiving DLI, which might be of relevance to understand why NRM was lower (although not statistically significant) in the second AlloHCT group comparing the DLI group. The selection bias of fitter patients for secondary transplantation may also partially explain these results. On the other hand, 37% of the patients of the DLI cohort received a TCD-based AlloHCT, whereas only 26% of the Second Transplant cohort had *in vivo* TCD. Patients receiving a second AlloHCT presented more GvHD than patients post DLI. The use of T-cell depletion has been associated to less GvHD and

therefore a more tolerable procedure[95] in our study 11 (41%) and 6 (32%) patients received a RIC conditioning regimen in the second AlloHCT and DLI cohort, respectively. This data might have also influenced the final results; although it has been reported that the use of a RIC as second AlloHCT conditioning do not appear to significantly improve NRM rates[96].

We acknowledge that this study carries limitations, some related to its retrospective nature and other to its design itself. There is an obvious bias regarding patient selection that received second transplant or DLI. The only way to overcome this bias would be by performing a randomized trial. In terms of GvHD, it would have been interesting to have data on the presence GvHD at the time of relapse or therapy (DLI or Second AlloHCT), since others have linked the presence of GvHD at those time points to inferior survival. On the other hand, although data regarding outcomes was mostly complete, data about the variable Time to Relapse was unavailable in 5 patients. We calculated the time from first AlloHCT to second AlloHCT and Time from first AlloHCT to DLI and found comparable outcomes to Time to Relapse (data not shown). Finally, the GvHD analysis is incomplete because there was missing data on GvHD dates; however, the development of GvHD post DLI in AL seems to have little impact on survival outcomes[97].

Overall, outcomes of HCT2 and DLI appear to be comparable in this study. Results from a prospective randomized comparative would be of great interest to discern what procedure would provide better results and less toxicity. But this possibility is rather unlikely to be developed, given that this would need to be an internacional, multicentric study. In that study variables such as the disease´s characteristics, the donor´s availability or the patient´s performance status would be of high complexity to control. Until then, retrospective trials including higher number of patients are warranted.

On the other hand, in termns of the second work, DLI are prescribed based on the total $CD3^+$ dose of each aliquot, but many cell subsets comprise this compartment. Within the $CD3^+$ compartment, we have identified that a DLI1 containing $>3x10^6$ $CD8^+T_N$ /Kg increased the probability of GvHD. Remarkably, B-cells and specifically $CD27^+$B-cells, showed a strong association to GvHD and we found a dose cut-off above which the development of GvHD was more likely. Further, MNCs and $CD27^+$NK+ cells were also linked with the development of

GvHD. There is limited available data regarding the cellular composition of DLI, as only few studies have focused on this setting[76]. Hence to the best of our knowledge, the study of this homogeneous cohort provides comprehensive and novel data.

We did not find any statistically significant association of the proportion of $CD4^+$, T_N nor $CD4^+T_N$ with the development of GvHD. However, in previous DLI studies the $CD4^+$ dose was found associate with GvHD[98] and $CD4^+T_N$ have been shown to cause alloreactivity in murine hematopoietic cell transplant models[48]. Interestingly, we found a trend of association of GvHD with a high dose of $CD8^+T_N$ in DLI1. Several clinical and experimental lines of evidence suggest that $CD8^+$ T-cells play a major role in the pathogenesis of GvHD[99]. Based on this rationale, attempts in depleting the $CD8^+$ compartment of DLI have been undertaken[36,100], as well as strategies to deplete both the $CD4^+$ and the $CD8^+$ naïve compartment from the graft[45, 100]; and more recently, a phase I trial on DLI naïve T-cell depletion has been published[101]. In this interesting study, the authors report results on a dose escalation schedule of $CD45RA^+$ depleted DLI. They prophylactically infused $CD45RA^+$ depleted DLI in 16 patients, at a median of 116 days post AlloHCT. Interestingly they did not observe dose-limiting grade III/IV acute GvHD or adverse events attributable to the DLI were observed at any dose level. One patient developed grade 2 cutaneous and intestinal cGvHD, and another patient developed moderate pulmonary cGvHD, which outline sthe feasibility of this approach. Previously, the infison of $CD45RA^+$ depleted DLI in another setting have been reported[70]. In this case $CD8^+$ enritched $CD45RA^+$ depleted DLI were infused at relapse in fifteen patients. The authors observed a 67% of response and only one case of GvHD, proving the feasibility of this approach, acknowledging that no conclusions on effectiveness can be formulated.

In the study herein presented, $CD8^+T_N$, and not $CD4^+T_N$, showed a trend to higher GvHD. There might be potential reasons to explain the association. Firstly, $CD8^+$ lymphocytes can induce GvHD by secreting interferon-γ (IFN-γ) and tumor necrosis factor-α (TNF-α), which is more relevant for Th1 responses and aGvHD. Of interest, in this study aGvHD was more frequently diagnosed. Secondly, different mechanisms to induce tolerance to $CD4^+$ and $CD8^+$ have been described. $CD8^+$ cells have been shown to be not effectively deleted extrathymically [102], whereas it has been reported that $CD4^+$ T-cells are subject to both central and peripheral

deletion[103]. These tolerance mechanisms could partially explain the lack of $CD4^+T_N$ alloreactivity found in this study. Further, in a MHC compatible setting, as in the manuscript herein presented, GvHD is mainly due to mismatches in a minor histocompatibility complex. In this setting, the peptide repertoire for MHC class I or class II is known to be subject to individual variations that are known to a lesser extent and could potentially impact in the immune response of $CD4^+$ and $CD8^+$. Lastly, it has been reported that a synergistic mechanism between $CD4^+$ and $CD8^+$, and not a particular cell subset, can drive GvHD[104]. Overall, the cohort size in this study is limited and hence these results showing a trend must be taken with caution. Data on the particular role of $CD8^+T_N$ and $CD4^+T_N$ in DLI remains unclear and will warrant further study.

Moreover, we found no association of the total $CD3^+$ dose ($CD4^+$ and $CD8^+$) and the risk of GvHD, although it is important to acknowledge that the dose of $CD3^+$ was tailored in our population which probably difficult to find the impact of total $CD3^+$ dose. $CD3^+$ dose and its relation with GvHD has been reported with unalike results; it seems to be essential for response[105], but not all studies have found relation between GvHD and $CD3^+$ dose[106], likewise the data reported in this study. Notoriously, there are some T-cell subsets that we predicted they could be protective for GvHD, such as total T_{REG}, a low $CD8^+/T_{REG}$ ratio, $CD4^+T_{REG}$ nor naïve T_{REG}; but we did not find such protective effect. T_{REG} DLIs initially developed in murine models were effective in preventing the onset of, and treating established GvHD[76]. In the former study, the $CD4^+T_{REG}$ DLI dose administered to mice was $0.5\text{-}1x10^6$ /Kg, whereas in our study the median $CD4^+T_{REG}$ dose was $0.41x10^6$ /Kg. (Table 2.3). Potential reasons to explain the lack of protective effect found may be related to the proportion of T_{REG} within the donor $CD3^+$ compartment of our donor pool[107], the total T_{REG} dose infused, or the fact that Foxp3 was not used in the flow cytometry analysis, hence an accurate measurement of T_{REG} might have not been performed. Interestingly, there are ongoing phase 1 studies on the use of T_{REG} DLI in steroid refractory GvHD patients (NCT03683498), which results will help to understand the mechanistic insights of T_{REG} in the GvHD setting.

B-cells have been reported to be a fundamental driver of cGvHD and a number of B-cell inhibition strategies have been incorporated in order to lower cGvHD rates[108]. It has been

shown that circulating pre germinal center $CD27^{+}$B-cells comprise *in vivo* activated B-cells capable of IgG production without requiring additional antigen stimulation[109], which has been linked with cGvHD in a B-cell activating factor (BAFF) dependent manner. Actually, cGvHD patients with elevated BAFF had a peripheral B-cell pool composed primarily of $CD27^{+}$B-cells. Notoriously, there is scarce data on the role of B-cells and GvHD in the DLI setting. In our study we found an association of B-cells and GvHD, particularly in the $CD27^{+}$B-cell compartment, and identified a dose cut-off point for this subset (Table 2.4). The findings herein reported open new questions on whether the dose of B-cells should be taken into consideration in DLI, as B-cells might be an essential collaborator for the development of GvHD in this setting. However, a validation of the dose cutoff findings in a larger controlled trial setting is warranted, as the dose cutoff point identified only applies to this series.

We also identified that a DLI1 containing a higher dose of MNCs was associated with GvHD. The MNC dose of the DLI1 has been previously linked to the development of GvHD[33]. Although MNC comprises a large variety of cell populations, the association of MNCs and GvHD could be partly explained by the dose of infused monocytes, which might play a role as antigen presenting cells from donor origin, actively participating in the development of GvHD. Moreover, another potential reason to explain this associacion could be that a higher MNCs dose is pointing out that the product has a higher $CD34^{+}$ content. This could reflect the potential effect of this product on enhancing a rapid immune recovery, which has been shown to associate a higher probability of developing GvHD. Our data support a previous study and the dose identified in our study is similar to that previously reported to be associated with GvHD. On the other hand, we found that a higher dose of $CD27^{+}$NK cells/Kg in DLI1 associated with GvHD. NK cells are not considered a major cause of GvHD, but the role of NK cells and GvHD is not fully understood, as these cells have also been reported to be potential cause of GvHD[110]. In a murine immunodeficiency model, it was observed that the infusion of NK cells producing IFN-γ and TNF-α was capable of induce an allogeneic immune responses[111]. Further, NK cells have been isolated in GvHD target tissues in a number of experimental approaches[112]. Most particularly in relation with the findings of this study, a specific subset of NK cells $CD27^{high}$ has been reported to entail effector functions[113], which would be in line with an increased rate of

inflammatory cytokine secretion. However, we acknowledge that with the available data, we cannot fully explain the association found in this study and these results should be further studied and confirmed.

Finally, we find relevant to mention that as others did, we focused on the study of the effect of DLI1 to avoid survival bias and because all patients but one developed GvHD after DLI1. However, we also analyzed data regarding GvHD and the cumulative dose of $CD3^+$, MNCs and all cell subsets, and we did not identify any statistical correlation with GvHD (data not shown). Furthermore, there were a number of clinical variables that we studied to rule out competing risks with the cell subset results (donor age, G-DLI v L-DLI, IST, GvHD pre DLI, donor gender), and we did not find found any association with GvHD.

We acknowledge several drawbacks in this study such as its retrospective nature and the limited number of patients. The inclusion period of patients is wide, as we included AlloHCT from 2001 onwards, but we consider that the relation of cell subsets and GvHD may not be influenced by the year of transplant. Another subject of interest is the fact that donor selection was limited to MSD. We narrowed the donor type to gain homogeneity in the cohort, but by doing this we acknowledge that we cannot extrapolate the results to the unrelated or haploidentical donor setting, or the MHA mismatch setting. Another limitation of the study is that we did not perform mixed lymphocyte reactions or cell functionality assays after cell thawing, nor T-cell polarisation and cytokine secretion in either the naïve state or after stimulation with PMA-Ionomycin assays. We acknowledge that this data would have been of great interest, and should be studied in future controlled trials.

In the work related to the second hypothesis, we report the proportion and cell dose of a number of cell subsets in DLI from MSD. Interestingly, we identified that a higher proportion of $CD27^+$B-cells might have a role in the development of GvHD in this setting and that other cell populations ($CD8^+$ T_N, $CD27^+$NK cells and MNCs) may deserve further study on GvHD. MNCs has been previously linked to GvHD in DLI studies, hence our findings further confirm those results. Most remarkable, the data regarding cell subsets herein reported is novel and it may

provide insight for the understanding of GvHD after DLI, which can be relevant in the clinical practice and in future investigational approaches.

Targeted therapies such as CART or CTL are currently being developed and gaining indications in a number of hematological diseases[114], however its use after AlloHCT needs further exploration. In this setting, CTL have been used for several years to treat oportunic infections in patients refractory to conventional treatments[115,116], but this type of cell therapy is developed differently depending on the institution, with different expansion and selection protocols. This causes that the use of TLC is limited to clinical trials and compassionate use, and many institutions lack of this therapy and. CTL are expanded T-cells, usually HLA restricted, stimulated to retain anti viral (one or various virus) or anti fungal effect. Based on this rationale, CTL associate low GvHD, which makes them a rather attractive therapy after AlloHCT. However, in real-life setting its effectiveness is difficult to predict and it is essentially focused on the treatment of viral infections and not relapse. In the 2017 American Society of Hematology meeting, Gottlieb et al. reported data on a phase I study on third party off-the-shelf CD137 selected CTL for treatment of early viral viral reactivativations after AlloHCT. The authors observed a 6-month 93% of complete response. Strategies such as the CD137 magnetic selection will increase the effectiveness of CTL in the forthcoming years.

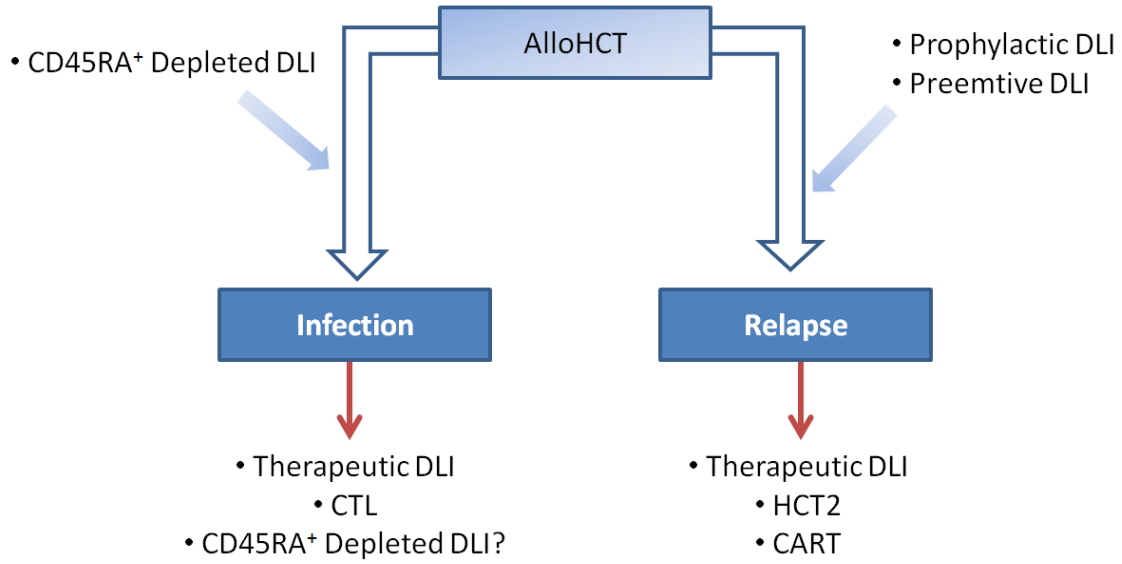
$CD45RA^{+}$ depleted DLI are another strategy with great interest in this setting[101], also carrying low GvHD rates. This approach is being studied to provide an optimal immune recovery after transplant avoiding the potential risk of GvHD link to early DLI. A pahse I study in this regard including 16 patients have been recently reported[101].

At present, the available CARTs to treat hematological malignancies target CD19; hence they are only effective against B-cell lymphomas and leukemias. CARTs can be delivered at relapse after transplant and represent a therapeutic approach at this point; nevertheless, fewer patients with B-cell lymphomas receive an AlloHCT because CART has replaced AlloHCT in relapsed NHL. On the other hand, CARTs for myeloid malignancies in ongoing development appear to be a promising approach. To date, no clear target has been identified and this treatment is in

ongoing development. Thus, besides clinical trials, this therapy does not represent an option at present in AlloHCT relapse.

On the other hand, in this set of patients relapsing after AlloHCT, unfractionated DLI represents an effective treatment for a selected patient population. However, the risk of GvHD is high and represents a drawback in this setting. In this clinical scenario, a fine tunning of the DLI's cell dose, the cell subsets, or co treatment with other drugs to mitigate its toxicity, will help to better adapt this treatment to each patient, increasing tolerability and effectiveness. A better understanding of the immunology of DLI after AlloHCT will help to more accurately develop another cell therapy approaches after AlloHCT.

Figure 5. Figure depicting available cell therapy options for refractory infection and relapse after AlloHCT.



(DLI: donor lymphocyte infusion; CTL: Cytotoxic T Lymphocytes; CART: Chimeric Antigen receptor T-cells; HCT2: Second AlloHCT).

VIII. CONCLUSIONS

VIII. Conclusions

Unfractionated DLIs remain a major therapeutic approach after allogeneic hematopoietic cell transplant. New cell therapy-based strategies in ongoing development seem to be promising in terms of disease treatment and tolerability. However, the quick availability and accessibility of DLI, and the well-established dosing and timely protocols, place DLI in many therapeutic algorithms at relapse.

First conclusion:

A proportion of AlloHCT patients can benefit from DLI at AL relapse. After a careful patient selection, DLI outcomes have been shown to be comparable with HCT2. Probably, DLI are less effective in high-burden early relapse, but an initial control of the disease and a subsequent DLI can salvage a significant number of patients. Thus, those patients that are diagnosed with late relapse and that achieve any response with therapy would be the best candidates for DLI.

Second conclusion:

GvHD is still a major drawback after DLI affecting up to 30-50% of patients, depending on several factors such as the HLA compatibility. Clinical factors impact on the development of GvHD, but the cell composition of a DLI do as well. In this scenario, besides $CD3^+$ cells, other cell subsets such as MNCs and B-cells, more specifically $CD27^+$B-cells, may have a role in GvHD development after DLI. The control and measurement of the cell composition of DLI might be useful in controlling GvHD, that is the case of MNCs (previously associated with GvHD after DLI) and T_N, which warrant further exploration. Prospective exploration of the impact of the cell subsets might provide knowledge of the mechanistic insights of GvHD after DLI and help improving the toxicity.

IX. FUTURE DIRECTIONS

IX. Future Directions

Post transplant cellular therapy has dramatically evolved over the last decades. There is a general trend to develop target therapies that directly retain the effect to the target, which is linked to lower adverse events. Unfractionated DLI represent a widely used therapeutic approach in the AlloHCT setting. However, its effect associates with toxicity, which needs further improvement. For instance, DLI have been used to enhance the immune recovery after AlloHCT, particularly in the TCD AlloHCT setting. According to the results herein presented, the forthcoming steps might be focused toward a prospective interventional study of DLI controlling the dose and proportion of a particular cell dose population. At present, the monoclonal antibody anti $CD45RA^{+}$ (microBeads, human, Miltenyi) is available for experimental and clinical use and its used to perform a removal of the T-cell naïve compartment from a hematopoietric product. Notoriosuly, at our institution we perform a significant number of AlloHCT using $CD34^{+}$ selection as GvHD prophylaxis in patients diagnosed with hematological malignancies, particularly AML and MDS. These patients suffer from severe opportunistic viral complications due to the absence of memory immunity against microbial pathogens in the graft. By infusing $CD45RA^{-}$ lymphocytes in this setting, we might provide immune competence against these pathogens with no increment of GvHD. This approach is currently being undertaken by several groups prophylactically and therapeutically, with promising results. Interestingly, this approach has not been tested in a $CD34^{+}$ selection transplant platform. This is interesting because in the $CD34^{+}$ selection transplant platform used by us there is no post transplant immunosupression, and hence the tolerance (GvHD development) and effectiveness of $CD45RA^{-}$ DLI is unclear in this setting. Hence, we aim to perform a prospective phase I trial on the infusion of $CD45RA^{+}$-depleted DLI in patients suffering from a suboptimal immune reconstitution.

On the other side, in the work herein presented, it was observed that $CD27^{+}$ B-cells associated with GvHD. This is a rather provocative finding that will require further validation, and it would be necessary to test this in a controlled setting. For this, one approach could be to perform a phase I B-cell dose escalation DLI study. DLI could be negatively $CD19^{+}$ depleted, and B-cell could be administered along with T-cells in a dose escalation manner. At present, B-cell depletion can be perfomed within a Good Manufacture Practice environment and the products can be delivered into clinical setting.

Further, another subject to look at is the role of T_{REG} in a DLI. Policlonal T_{REG} are currently being investigated to treat GvHD. Previosu data suggests that T_{REG} DLI are feasible, tolerable, and might provide effect against GvHD. Hoevere, there is room for improvement as the effectivenees of this cell product is yet to be improved. Some groups perform a T_{REG} DLI infusion by performing a positive T_{REG} selection of the product form other cells, others perfom a T_{REG} activation after cell expansion; and furthermore, combinations of T_{REG} DLI with inmusosuppresive agents have also been explored. All in all, the most appropriate methodoly to obtain an effective T_{REG} DLI remains unclear. To achieve an immunologically active T_{REG} compartment it might be necessary to adopt some strategies such as: *in vitro* expansion*, in vitro* activation or *in vivo* expansion. Regarding this latter approach, there are potential drug candidates that have been proven to expand in vitro the T_{REG} compartment. In line with this drugs such as rapamycin, IL-2 or more recently published, dexamethasone, seem to be important for T_{REG} expansion and effectiveness in murine autoimmune and allergic inflammation models, in which the action of the dexamethasone was dependent on T_{REG} compartment via the Dex-miR-342-Rictor axis[117]. Therefore, based on these scenarios, I would propose a clinical trial on the infusion of polyclonal T_{REG} for steroid refractory (srGvHD) GvHD. Response to steroids of GvHD is around 50%, hence there is an unmet need in the treatment of this complication after AlloHCT. We would set a strategy to expand *in vivo* the T_{REG} repertoire based on the administration of IL-2 and/or sirolimus, or considering co treatment with dexamethasone in a phase I trial of steroid refractory GvHD treatment in patients that failed ≥2 lines of treatment.

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X. Bibliography

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XI. ANNEX

XI. Annex

In this section, I include the peer-reviewed manuscripts that studied both hypothesis of this thesis. Annex 5.1 and 5.2 summarize the main findings of this research. Furthermore, over the period of producing the herein presented dissertation, in parallel, we published a review on the use of DLI for myeloid malignancy relapse after AlloHCT, also included in this section (annex 5.3).

11.1 Annex 1

Analysis of relapse after transplantation in acute leukemia: A comparative on second allogeneic hematopoietic cell transplantation and donor lymphocyte infusions. Guillermo Ortí, Jaime Sanz, Irene García-Cadenas, Isabel Sánchez-Ortega, Laura Alonso, Maria José Jiménez, Luisa Sisinni, Carmen Azqueta, Olga Salamero, Isabel Badell, Christelle Ferra, Cristina Diaz de Heredia, Rocio Parody, Miguel Angel Sanz, Jorge Sierra, Jose Luis Piñana, Sergi Querol, David Valcárcel. *Experimental Hematology 2018; 62:24–32. doi: 10.1016/j.exphem.2018.03.002.*

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11.2 Annex 2

Analysis of Cell Subsets in Donor Lymphocyte Infusions from HLA Identical Sibling Donors after Allogeneic Hematopoietic Cell Transplant. Guillermo Ortí, Carles Palacio-Garcia, Irene García-Cadenas, Isabel Sanchez-Ortega, María José Jimenez, Carmen Azqueta, Guillermo Villacampa, Christelle Ferrà, Rocio Parody, Rodrigo Martino, Francesc Bosch, Sergi Querol, David Valcárcel. *Biol Blood Marrow Transplant. 2020 Sep 25:S1083-8791(20)30593-0. doi: 10.1016/j.bbmt.2020.09.024.*

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11.3 Annex 3

Donor lymphocyte infusions in AML and MDS: Enhancing the graft-versus-leukemia effect. Guillermo Ortí, Pere Barba, Laura Fox, Olga Salamero, Francesc Bosch, David Valcárcel. *Exp Hematol. 2017 Apr;48:1-11. doi: 10.1016/j.exphem.2016.12.004.*

We published a review on DLI for myeloid malignancies as a request from the International Society for Experimental Hematology. In this review we focused on exploring the up-to-date data on DLI for myeloid malignancies (particularly acute myeloid leukemia and myelodisplastic syndromes). We also included the up-to-date data on HCT2 for myeloid malignancies. Likewise, DLI, HCT2 is a major approach for relapse of such diseases after AlloHCT. We reviewed the data regarding the use of DLI and HCT2 for AML and MDS. We also described the reported factors with influence on DLI outcomes. Further, given the high impact of extramedullary relapse in AML, we described the data regarding the use of DLI in this setting and we outlined the poor responses that are generally observed in this complication. We also reviewed potential factors with impact on GvHD and response.

Experimental Hematology. 2017 Apr;48:1-11. doi: 10.1016/j.exphem.2016.12.004.

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