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DETECTION OF AN IMMUNOLOGICAL RESPONSE AGAINST TEL/AML1 FUSION PROTEIN

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Premise

In the present PhD thesis I will present my activity during my PhD studies.
The report will focus on two main projects I carried out during the time I spent at Tübingen University Hospital (Germany) for a period of training in research.
The former is a laboratory research project, aiming to investigate the possible immunological responses against peptidic antigens.
The latter is a clinical research project with the purpose to evaluate safety and efficacy of CD34 positively selected stem cell boosts for the treatment of poor graft function after hematopoietic stem cell transplantation (HSCT).
I will also report on another clinical research project I was involved in, performed at Padova University Hospital and focusing on the evaluation of the role of extracorporeal photochemotherapy as treatment for acute graft-versus-host disease after paediatric HSCT. Results of such project were object of publications I co-authored.
All this works reflect my specific interest in the biology of stem cell transplantation, in its different aspects. This was also the main topic of my clinical activity as physician, which I carried out at the Paediatric Haematology and Oncology Department of Padova University Hospital.

Detection of an immunological response against TEL/AML1 fusion protein.

1. Abstract

Immunotherapy represents a therapeutic option for subgroups of paediatric patients with leukaemia who, despite the impressing advances of the last decades in the field, still show a poor prognosis because of high risk-disease or relapse.

A deeper understanding of how the immune system physiologically recognizes and eradicates tumour cells is mandatory.

Peptidic antigens are of great interest in the field of immunotherapy because they could be used as vaccines to boost immunity.

TEL/AML1 mutant protein, whose sequence is known, is the result of a balanced t(12;21) translocation which generates a fusion gene. Peptides can be artificially synthetized from TEL/AML1 fusion protein and their HLA-binding capacity and immunogenicity can be predicted through bioinformatic tools.

This project aimed to investigate whether the excellent prognosis showed by patients who suffer from a B-lineage ALL harbouring the TEL/AML1 mutation could be related to an immune response against peptidic antigens derived from the TEL/AML1 mutant protein.

For such purpose, 8 priming experiments were performed with healthy donors' leucocytes. Six experiments were carried out according to a dendritic cells-mediated protocol, whereas two experiments were performed according to a beads-mediated protocol. Successfully primed lymphocytes (identified by mean of intracellular cytokines production) were selected through flow cytometric sorting and single-cell seeded in order to get T-cell clones. This was possible in 3 out of 8 priming experiments. Growing T-cell clones were tested after stimulation with peptides (or through tetramer staining) but they did not show enough specificity.

We also tried to show an immune response against fusion peptides in peripheral blood leucocytes of patients who survived a TEL/AML1 positive B-lineage ALL, through exposure to peptides and a short course stimulation with cytokines.

We tested 22 patients, but unfortunately we weren't able to show an answer against fusion peptides in any of them.

Possible reasons might be the lack of specificity of the activation markers we used to identify reactive cells, the not enough restrictive gates we used for sorting, the fact that the HLA super type B*07 (for which the restricted peptides had the best prediction score) was underrepresented in our patients' cohort.
We suggest to perform further experiments using new activation markers, such as CD25 or PD-L1, or different techniques to identify reactive cells (such as Elispot), to use more restrictive gates for sorting and to exploit the beads priming protocol.
In order to sample such lymphocyte populations (i.e. antigen specific T-cells) with an extremely low frequency, a possibility may be collect repeatedly blood samples from the same patient at different time points.
Further studies are warranted to test the hypothesis of an autologous, spontaneously arising immune response against TEL/AML1 fusion peptides as reason for the good prognosis of TEL/AML1 positive leukaemia.
Another possible approach in order to validate fusion peptides might be to test them in a situation of HLA B*07 mismatch between lymphocytes and APCs. The clinical counterpart could be the generation of reactive T-cell clones, cloning of their TCR and its transduction in the patient's or donor's lymphocytes, the latter in the perspective of a post-hematopoietic stem cell transplantation adoptive immunotherapy.

1. *Sommario*

L'immunoterapia costituisce un'opzione terapeutica per alcuni sottogruppi di pazienti con leucemia dell'età pediatrica i quali, nonostante i notevoli progressi degli ultimi decenni, ancora non mostrano una prognosi soddisfacente perché affetti da malattia ad alto rischio oppure da ricaduta.
Una comprensione più profonda di come il sistema immunitario fisiologicamente riconosce ed elimina le cellule tumorali è essenziale.
Gli antigeni peptidici sono di grande interesse nel settore dell'immunoterapia perché possono essere utilizzati come vaccini per potenziare l'immunità.
La proteina mutante TEL/AML1, la cui sequenza è nota, è il risultato di una traslocazione bilanciata t(12;21) che genera un gene di fusione. Dalla proteina di fusione TEL/AML1 si possono sintetizzare artificialmente peptidi, la cui capacità di legare le molecole HLA ed immunogenicità si può prevedere attraverso strumenti bioinformatici.

Questo progetto ha l'obiettivo di indagare se l'eccellente prognosi dei pazienti affetti da leucemia linfoblastica di linea B con la mutazione TEL/AML1 possa essere correlata ad una risposta immunologica nei confronti di peptide di fusion derivati dalla proteina mutante TEL/AML1.

A tale scopo, sono stati realizzati 8 esperimenti di priming con leucociti di donatori sani. Sei sono stati realizzati secondo un protocollo mediato da cellule dendritiche, mentre altri due esperimenti sono stati condotti secondo un protocollo mediato da beads. I linfociti responsivi al processo di priming (identificati mediante la produzione intracellulare di citochine) sono stati selezionati mediante sorting citofluorimetrico e coltivati a singola cellula in modo da ottenete cloni T-cellulari. Ciò è stato possibile in 3 esprimenti su 8. I cloni T-cellulari con evidenza di crescita sono stati testati dopo re-stimolazione con i peptidi (o mediante tetramer-staining) ma non hanno dimostrato sufficiente specificità-

Abbiamo inoltre provato a dimostrare una risposta immunologica nei confronti dei peptidi di fusione nei leucociti (da sangue periferico) di pazienti con leucemia linfoblastica di linea B TEL/AML1 positiva in remissione, mediante esposizione ai peptidi e una breve stimolazione con citochine. Sono stati testati 22 pazienti, ma purtroppo non è stato possibile evidenziare una risposta nei confronti dei peptidi di fusione in nessuno di loro.

*Possibili spiegazioni potrebbero essere la mancanza di specificità dei marcatori di attivazione che sono stati utilizzati per identificare le cellule reattive, i gate non sufficientemente restrittivi utilizzati per il sorting, il fatto che il supertipo HLA B*07 (i peptidi B*07 ristretti avevano il migliore score predittivo) era sotto-rappresentato nella coorte di pazienti presa in esame.*

Ci riproponiamo di realizzare ulteriori esperimenti utilizzando nuovi marcatori di attivazione, come CD25 o PD-L1, oppure differenti tecniche per identificare le cellule reattive (come l'Elispot), di usare gates più restrittivi per il sorting e di utilizzare esclusivamente il protocollo mediato da beads per il priming.

Per riuscire a includere nel campione popolazioni linfocitarie (cellule T antigene-specifiche) la cui frequenza è estremamente bassa, una possibilità potrebbe essere eseguire prelievi ematici ripetuti nel tempo nello stesso paziente.

Sono necessari ulteriori studi per testare l'ipotesi di una risposta immune autologa, spontanea, nei confronti dei peptidi di fusione TEL/AML1 come spiegazione della buona prognosi della leucemia linfoblastica di linea B TEL/AML1 positiva.

*Un altro possibile approccio per validare i peptidi di fusione potrebbe essere quello di testarli in una situazione di HLA B*07 mismatch tra linfociti ed APCs. La ricaduta clinica potrebbe*

essere la generazione di cloni T-cellulari dalle cellule reattive al priming, il clonaggio del loro TCR e la sua transduzione nei linfociti del paziente o del suo donatore, in quest'ultimo caso nella prospettiva di un'immunoterapia adottiva post-trapianto di cellule staminali ematopoietiche.

2. Introduction

Paediatric malignancies have features, such as overall prevalence, distribution into subtypes and age groups, biological hallmarks and behaviour, response to treatment, which distinguish them from adult tumours.

Acute leukaemias are the commonest paediatric neoplasms, representing nearly 31% of all malignancies in children < 15 years of age. Among them, Acute Lymphatic Leukaemias (ALLs) represent the largest group. Nearly 85% of lymphoblastic Leukaemias are of B-lineage, the remaining 15% are of T-lineage, a small proportion co-exhibit B and T lineage features.[1]

Since the late sixties, several polichemo- and radiotherapy protocols have been developed for ALL diagnose and treatment, each based on positive acquisitions of the previous ones. Thanks to this constant evolution, overall free survival has increased, for standard risk patients, from less than 10% to about 90% nowadays.[2]

Clinical and translational research efforts are focusing at the moment on specific disease subgroups which still show unsatisfactory response rates, i.e. high risk disease, refractory disease, relapses. For such patients a conventional therapeutic approach may not be sufficient for disease control and different options, including hematopoietic stem cell transplantation and other immunotherapeutic strategies, are warranted. [2,3]

Immunotherapy of tumours is a fast-developing research field, which aims to reproduce, or enhance, physiological patterns of recognition and elimination of tumour cells by the individual immune system.

Many acquisitions have already been translated into clinical practice: i.e. monoclonal antibodies, adoptive cellular therapies and cancer vaccinations.

However, the impact of survival of such approaches in paediatric cancers remains limited. This is due to technical issues which are still to be addressed (especially in the field of cancer vaccination) and to the lack of studies in paediatric patients.[3] Hence, a deeper understanding of physiological and naturally occurring processes is mandatory.

The presence of TEL/AML1 rearrangement, resulting from a balanced t(12;21)(p12:q22) is one of the commonest genetic abnormalities in children with B-ALL, and its frequency in patients treated according to the main ALL protocols worldwide ranges from 18% (in Europe, Associazione Italiana di Emato-Oncologia Pediatrica, AIEOP, and Berlin-Frankfurt-Münster, BFM, data) [4] to 25% (in the USA, Dana-Farber data).[5]

The TEL/AML1 fusion gene product is a constitutively expressed transcription factor, whose action leads to the down regulation of genes involved into cell differentiation and promotes leukemogenesis.[6]

Since the earliest studies, it has been shown that the presence of this such mutation associates with a good outcome in terms of response to treatment, Overall Survival (OS) and Event-Free-Survival (EFS).

On the basis of these findings, in the latest protocols children harbouring this such mutation are stratified into the standard risk group and are potential candidates to receive a less intense chemotherapy regimen.

The cellular and molecular basis of this favourable effect is not yet completely understood. It has been postulated that the expression of the TEL/AML1 fusion transcript may influence sensitivity to drugs as Etoposide, Doxorubicin, Dexamethasone and L-Asparaginase.[7,8]

So far, there is no data about the ability of the TEL/AML1 fusion protein to elicit a long lasting immune response. The individuation and characterization of tumour-specific antigens is a field of clinically-oriented immunology which aims to the realization of cancer-vaccines.

A T-cell response against mutated antigens has already been identified and characterized for different oncoproteins in many cancer types, such as p21 ras in pancreatic, colorectal and follicular thyroid cancer[9,10,11] N ras mutations in melanoma[12], BCR/ABL in chronic myelogenous leukemia[13,14] Mutated peptides have already been used in cancer vaccination.[15]

Scope of the present work is to investigate the role of TEL/AML1 mutant protein as leukaemia-specific immunogenic antigen.

3. State of the art

In this section, some general key concepts for the understanding of the present study will be pointed out and the choice of specific procedures and materials will be justified.

3.1 Pediatric B- lineage ALL

Acute leukaemia is a blood cancer characterized by malignant proliferation of abnormal (i.e. with an aberrant phenotype and biological behaviour) hematopoietic precursors. The term “acute” refers to the fast development of the process, which if untreated can lead to death in a short time.

Acute leukaemias can affect both adult and paediatric patients, and are classified according to the involved cell line (myeloid vs lymphatic).

Paediatric malignancies are peculiar according to prevalence, distribution into subtypes and age groups, biological hallmarks and behaviour, response to treatment.[1]

Acute leukaemias account for nearly 31% of all malignancies in children < 15 years of age, thus being the commonest paediatric neoplasms. Acute Lymphatic Leukaemia (ALLs) represents the largest group, among which 85% of cases are of B-lineage. Patients with ALL are stratified, for treatment purposes, according to several criteria, including age at diagnosis, baseline white cell blood count, biological features of the blasts (i.e. DNA index, the presence of specific chromosomal and molecular rearrangements) central nervous system (SNC) involvement at diagnosis and, above all, response to the early treatment phases.[2,16, 17]

As for biological features, several translocations have been identified, which associate with a poorer or better prognosis, among them the t(12;21) translocation and the subsequent TEL/AML1 rearrangement.

3.2 TEL/AML1 rearrangement.

TEL/AML1 rearrangement is the consequence of a balanced t(12;21)(p12:q22) translocation and is one of the commonest genetic abnormalities in children with B-ALL (reported frequency ranging from 18% to 25%). [4,5,6]

3.2.1 TEL gene

TEL gene is located on chromosome 12 at p12, and it is also called ETV6. Together with AML1 it is one of the genes most frequently rearranged in human leukaemias. It is made up of eight exons (for a total of 39,825 bp) and its protein product is a transcriptional factor, member of the Ets family, which regulates the expression of many other genes implicated in cellular development and response to external stimuli: its function is essential for normal haematopoiesis. TEL protein has an evolutionary conserved Ets domain at the C-terminal extremity which binds to DNA and is involved in interactions with other proteins, while at the N-terminal extremity it has a helix–loop–helix (HLH) domain with a region rich in proline residues, responsible for stabile homotypic oligomerization and transcriptional activation.

3.2.2 AML1 gene

AML1 gene, also named RUNX1 or CBFA2 (261,537 bp of length) is located on chromosome 21 at q22 and is expressed in cells of all hematopoietic lineages. It often participates in rearrangements with other genes detected in myeloid neoplasms, such as t(8;21)(q22;q22)-AML1/ETO, t(3;21)(q26;q22)-AML1/EVI1, AML1/EAP, AML1/MDS1.

It codes for a transcriptional factor as well, whose affinity for DNA increases after heterodimerisation with the CBF protein forming a complex called human core-binding factor (CBF). This complex plays a critical role in the expression of genes implicated in haematopoiesis, like the granulocyte colony stimulating factor, interleukin 3, T cell receptor δ and myeloperoxidase (MPO) genes. AML1 protein has at the N-terminal extremity a domain highly homologous to the Drosophila RUNT domain, responsible for the interaction with DNA and CBF, and the transactivation domain (TA) at the C-terminal extremity.

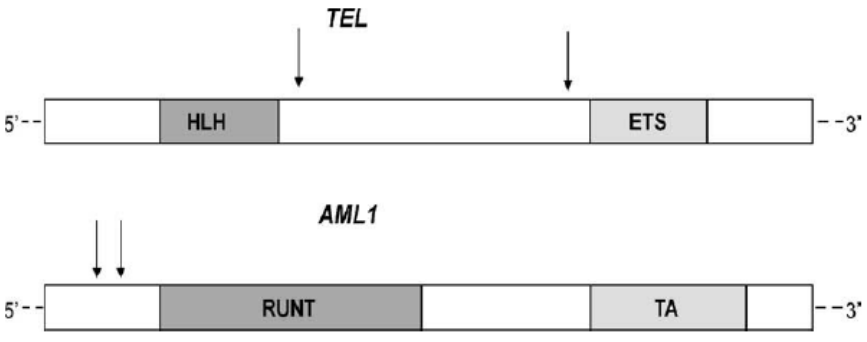


Fig. 3.1 Structures of the TEL and AML1 genes. HLH= helix-loop-helix domain, ETS= Ets domain, RUNT= RUNT domain, TA= transactivation domain. The arrows represent the t(12;21) (p12;q22) breakpoints. Figure in[18].

3.2.3 TEL/AML1 fusion transcript and protein

Breakpoints of the TEL gene are most often observed between exons 5 and 6, while genomic breakpoints on the AML1 gene can occur either in intron 1 (between exons 1 and 2) or in intron 2.

As a result, the fusion product is made up of the 5´part of TEL with nearly all the AML1 gene, including its RUNT and TA domains, and its expression is under control of the TEL promotor. As a consequence of the translocation, two alternative fusion transcripts may be generated: a longer transcript may result from the joining of exon 5 of TEL to the second exon of AML1; the alternative splicing can cause the skipping of AML1 exon 2 giving rise to a shorter transcript. These two transcripts may be both expressed in the same patient: this is the commonest situation. Alternatively, if the breakpoint in AML1 gene occurs in intron 2, the TEL exon 5 joins to the third but not to the second exon of AML1, generating only the shorter transcript: this happens less frequently. The proteins derived from the two different fusion transcript have, however, the same structure, which includes the HLH domain of TEL and RUNT and the TA domain from AML1, and is essential for its function.[18]

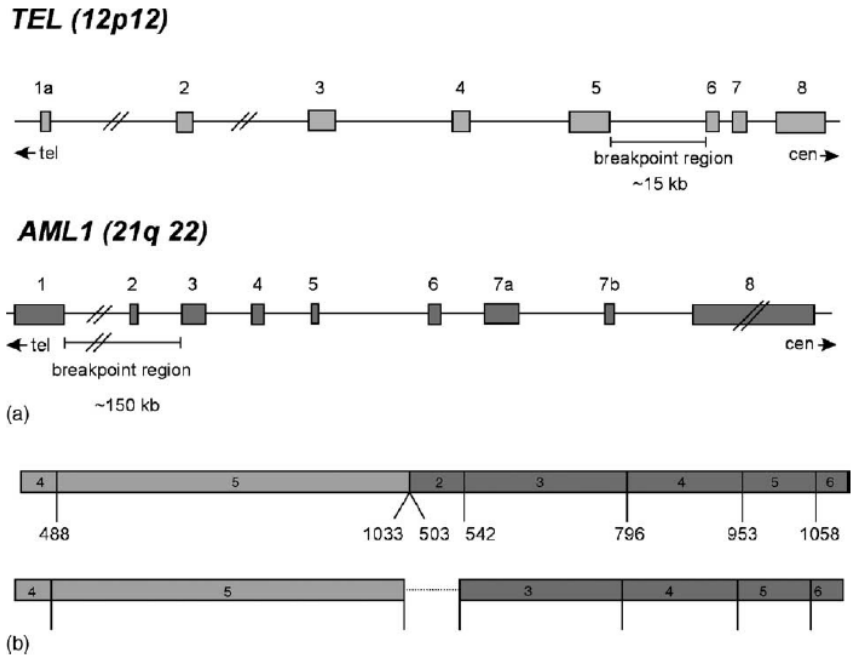


Fig. 3.2 a) Exonic-intronic sequence of the TEL and AML1 genes (exon number and breakpoints are reported). b) Diagram of the TEL/AML1alternative transcripts. The numbers indicate the single nucleotides. (Figure in[18]).

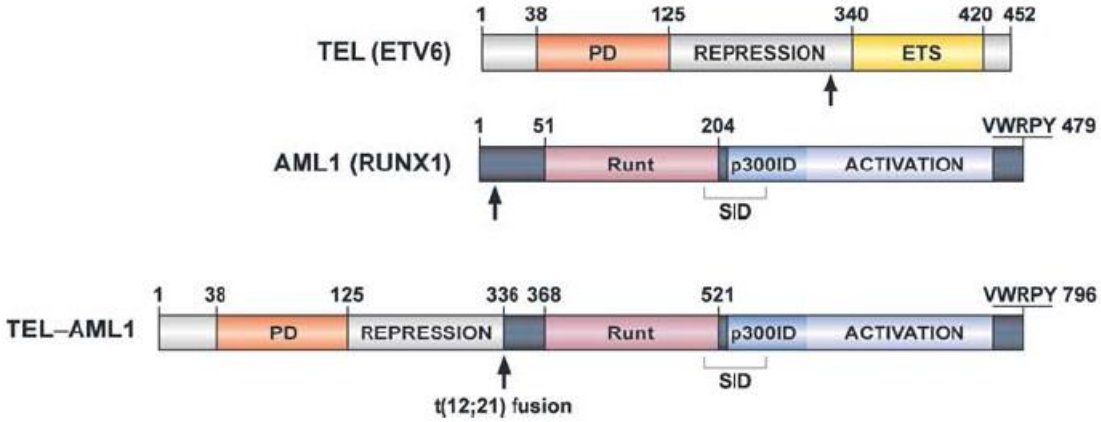


Fig. **3.3** A schematic representation of the full-length AML1, TEL and TEL-AML1 proteins. The RUNT DBD (RUNT), mSin3A interaction domain (SID, bracket), a region reported to interact with the p300 HAT (p300 ID) and transcriptional activation (activation) in AML1 are as indicated. Carboxy-terminal VWRPY motif that can bind Groucho-related corepressors is also indicated. Different functional regions in the TEL protein such as oligomerisation pointed domain (PD), central repression domain (repression) and ETS DBD are as indicated. Arrows indicate fusion points between TEL and AML1 sequences due to t(12;21). Amino acids that border the key functional domains are numbered with number 1 corresponding to the first methionine (Figure in [6]).

Both TEL and AML1 normal protein products bind to protein-corepressors (such as N-CoR or mSin3) through their HLH and TA domains, and thereby regulate/repress the transcription of other genes. The fusion protein TEL/AML1 has increased affinity with corepressors, and is able to act on normal AML1 target genes as well as on normal TEL target genes, trough HLH mediated heterodimerisation, thereby disrupting TEL function. The final effect is the repression of genes whose role is crucial for the normal maturation of hematopoietic precursor cells.

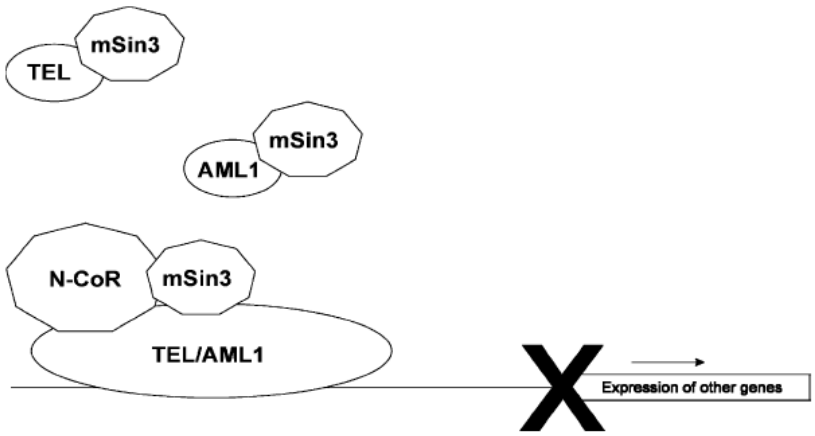


Fig. 3.4 Schematic representation of how the heterodimeric fusion protein could influence the expression of other genes. Figure in [16].

The occurrence of TEL/AML1 rearrangement is a precocious event in life (it may arise prenatally), but it is not sufficient for leukemogenesis. Many studies on concordance between twins, in vitro and animal models, have shown that additional genetic anomalies are required to induce leukaemia, according to the “double hit” cancer genesis model.[6]

The majority of patients with TEL/AML1 positive leukaemia have additional chromosomal abnormalities apart from a typical t(12;21) (p12;q22), the most frequent of which are: deletion of 12p [del(12)(p12)] , which brings to the wild-type TEL gene loss of function, and supplementary copies of AML1 gene.[18]

The t(12;21)(p12;q22) translocation can be identified by means of FISH analysis (classical cytogenetic is not sensitive enough), while the TEL/AML1 fusion gene can be detected through RQ-PCR and the transcript analysed trough RT-PCR. These techniques allow not only the individuation of the anomaly at diagnosis, but also individualized monitoring during therapy and in case of relapse, since each patient has his own, unique (clonotypic) genomic breakpoints.[18]

It is well known that the presence of such translocations in paediatric B-lineage acute leukaemias associates with good outcomes in terms of response to treatment, EFS and OS. This finding was pointed out by the first, retrospective case series in the nineties, and confirmed by prospective, multicentre studies a few years later. The AIEOP-BFM European Cooperative Group reported an incidence of the t(12;21)(p12;q22) of 18.9% in a cohort of 334 paediatric patients treated with uniform modalities, and showed a significantly better EFS (90.1%) in children harbouring the rearrangement (18.9% of total), which however was not an independent positive prognostic factor.[4] Data from the Dana-Farber Cancer Institute, regarding a cohort of 491 children treated in the US according to the DFCI 95-01 protocol for ALL, showed a frequency of TEL/AML1 rearrangement of 26%, a significantly higher OS (97%) in patients with the translocation, and a not significantly different EFS (89% vs 80%). This underlies the finding that TEL/AML1 patients may experience relapse, but they can be successfully re-treated.[5]

t(12;21) is encountered at relapse with a frequency ranging from 8.9% to 21.9%: this value is higher as expected, given the good reported outcomes. Such patients however have been shown to relapse later than TEL/AML1 negative patients, thus they may not have been included in follow-up studies with short time of observation. This finding has not yet been elucidated, but some authors postulated that high incidence of TEL/AML1 at relapse might be the consequence of a new genetic alteration, probably induced by the preceding treatment and the relapse may represent actually a second malignancy.[19]

On the basis of these findings, in the latest ALL protocols children harbouring TEL/AML1 mutation are stratified into the standard risk group and randomized to receive a less intense chemotherapy regimen.

The molecular mechanism through which the presence of the rearrangement exerts its positive effect on patients' outcome, is still unclear. It has been shown by in vitro studies that TEL/AML1 positive lymphoblasts have increased sensitivity to drugs as Etoposide, Doxorubicin, Dexamethasone and L-Asparaginase[7,8], but the correlation between these findings and the genetic and molecular anomaly itself has not yet been elucidated.

The TEL/AML1 fusion protein can theoretically generate peptidic fragments of various lengths by enzymatic digestion, which can be presented on the cell surface and act as immunogenic antigens. However, this hypothesis is complex to test, because several factors of influence have to be taken into account, for instance generation modalities and immunogenicity of TEL/AML1-derived peptides, as well as their interaction with MHC molecules on the cell surface.

To date, only one study explored the capacity of peptides derived from the TEL/AML1 fusion protein to act as antigens, that may induce an immune response.[20]

To understand how tumour-specific antigens can be identified in order to study immune responses, it is worthy to briefly mention what peptidomics is and how it could help for such purposes.

3.3 Peptidomics

Peptidomics is a field of proteomics, i.e. the discipline which studies proteins according to their structure, function, activity and interactions with other molecules systematically.

Peptidomics is the study of small peptides, which are constantly generated by protein digestion in the cells and presented on the cell surface by MHC molecules. Characterization of these MHC ligands is particularly appealing in the field of clinical immunology because they can be used as markers of specific diseases, since they can be presented in a disease-restricted fashion or they could be differentially presented in healthy versus disease-affected cells from a quantitative point of view.

Different possible approaches have been developed to identify tumour-associated epitopes, which can be roughly grouped into "direct" methods, which focus on pre-existing peptide-specific T-cell or B-cell clones and study their gene expression and antibody production (i.e. complementary DNA (cDNA) expression cloning or serological analysis of recombinant

cDNA expression libraries (SEREX)) and “reverse” methods, which start from antigens actually presented on the tumour cells surface.[21]

Peptides displayed on MHC molecules on the cell surface can be isolated from specific tissues or cell lines through several methods.[22] For immunotherapy purpose, specific tumour cells, or biological fluids, are used as a source and the bulk of enriched peptides is called the “tumour peptidome”. Peptidomes are then analysed through Mass-spectrometry based methods (see beyond).

The repertoire of peptides that can be presented by MHC molecules is characterized by a high degree of complexity, which can be better understood reviewing some features of the physiological antigen presentation process.

The portion of an antigen which is effectively recognized by cells and molecules of the immune system is called epitope. Epitopes can be peptidic or non-peptidic. A peptide epitope is a series of amino acids of a protein, which may present in continuity (linear) or as a surface patch (conformational). Epitopes recognized by B-lymphocytes are both linear and conformational, while epitopes recognized by T-cells are linear. Linear epitopes of length between 8 and 11 amino acids are presented to T-cells in the context of MHC class I molecules, while longer epitopes (length 13-17) are presented in the context of MHC class II molecules on the surface of APCs.

Antigenic proteins (mainly endogenous) which give rise to MHC class I restricted epitopes are cleaved by the proteasomes (large multi-enzymatic complexes) in the cytoplasm of cells. This process generates peptides retaining the correct C terminal extremity (i.e. containing hydrophobic or basic amino acids), which is essential for binding to MHC I molecules. Such peptides are then transported to the endoplasmic reticulum (ER) by the so-called “transporters associated with antigen processing” (TAP). TAPs have higher affinity for some specific peptides, namely peptides of length between 8 and 12 amino acids and which possess specific amino acidic residues in specific positions. In ER peptides are trimmed again, mostly at the N-terminal extremity, to generate fragments of appropriate length for interaction with MHC molecules. The binding process involves aminoacids in specific positions within the peptidic sequence (mainly position 2 and 9 at C-terminus), the so called “anchor-aminoacids”. Only MHC-peptide complexes are stable and can be translocated and presented on cell surface for recognition by CD8+ cytotoxic T-lymphocytes. The pathway of MHC class II restricted epitope generation and processing exhibits some key differences. The source of peptides usually are endocytosed proteins, which are degraded in endocytic vesicles (endosomes) by lytic enzymes. MHC class II molecules are synthetized within the

ER and the peptide-binding cleft is occupied by the so-called invariant chain, which prevents peptides generated in the ER to enter the cleft itself. Class II molecules are then transported in vesicles towards the cell surface. Endosomes merge themselves with empty MHC class II molecules-containing vesicles. The invariant chain is degraded by proteolytic enzymes to a 24-amino acid peptide, the so-called CLIP (class II-associated invariant chain peptide), which is then replaced by digested peptides because of higher affinity. The final complex MHC class II molecule-peptide is then presented for recognition by the T-cell receptors of CD4+ T-helper cells.[23, 24]

MHC molecules are highly polymorphic. Genes encoding for MHC in humans (also named HLA, human leucocyte antigens) are located on chromosome 6, at six loci for class I (HLA-A, HLA-B, HLA-C, HLA-E, HLA-F, and HLA-G) and at five loci for class II (HLA-DP, HLA-DQ, HLA-DR, HLA-DM, and HLA-DO). The number of possible alleles for each locus varies from as low as a single allele each, for HLA-DRB2, HLA-DRB8, and HLA-DRB9 to as high as 4242 alleles for HLA-B as reported in the current version of the IMGT/HLA database [as on April, 2016].

Despite this high degree of polymorphism, different MHC molecules share similar peptide-binding specificities, and have been grouped together to form the so called “MHC supertypes”. Molecules belonging to the same supertype have sequential and structural similarities, similar peptide binding domains, amino acid pattern in the binding pocket, amino acid binding preferences, etc. and thus tend to bind to overlapping peptide repertoires.

So far nine major MHC class I supertypes, (HLA-A1, A2, A3, A24, B7, B27, B44, B58, B62) and twelve MHC class II supertypes (five DRs, three DQs, and four DPs) have been identified.[24]

Given the astonishingly amount of peptides which can be generated in a cell and the polymorphism of HLA molecules, the possible MHC-peptide combinations may seem unlimited; however, the number of potential epitopes that can be generated from the proteome of a cell is restricted by MHC peptide binding motifs/specificities.

A completely new and peculiar research field, the so called “ligandomics”, aims to directly identify the MHC ligands. Through the knowledge of the naturally presented peptides, HLA-binding motifs could be defined, which allow a reverse approach, namely the possibility to predict which sequences within a protein contain an HLA-binding motif and could be presented for T-cell recognition. For such purpose, different approaches have been ideated and improved over time: amphipathicity based methods, motif-based methods, quantitative matrix based methods, methods based on Artificial neural networks, Support vector

machines, QSAR and docking simulations. A great impulse came in more recent years from immunoinformatics developments.

A detailed description of each method is beyond the objectives of this work; however motif-based methods will be briefly described given their relevance to the present study.

Peptides contain specific amino acid residues which allow the binding to MHC molecules: these are called “anchor residues”. The sequence of anchor residues within a protein is called “motif”.

Motif-based methods use the knowledge of specific MHC-binding motifs to predict the sequence of an epitope, i.e. to define a peptide as a potential epitope.

Different algorithms and databases have been developed over time: basically, they all look for MHC-binding motifs within a protein sequence, and give as output a list of peptides (derived from the protein itself) which can act as epitopes with different likeness according to the density of anchor residues they contain. Some of them are not only a prediction tool, but also databases of already reported epitope sequences. Some of the most widely used epitope prediction informatics tools are EpiMer, OptiMer, SYFPEITHI, netMHC, TEPITOPE and TEPITOPEpan.[24]

Immunogenicity of peptides is always studied, and scored, in relation with a certain MHC molecule: this means that specificity refers to the complex MHC-peptide, and not to the peptide alone.

This is why potential epitopes are identified in association with HLA alleles, within supertypes, which are frequent in the general population and for which information about natural ligands are available.

The HLA-B7 supertype includes the B*0702, B*0703, B*0705, B*3501-03, B*4201, B*5101, B*5102, B*5103, B*5301, B*5401, B*1508, B*5501-02, B*5601, B*6701 and B*7801 alleles.[25,26]

The molecule HLA B*0702, which alone accounts for more than 95% of HLA B07 supertype, has an high frequency in Caucasian population, reaching a value of more than 17% in some countries like Ireland.[27]

For this reason, there is growing interest in identifying, synthetizing and validating peptides which are HLA B*07 restricted.[26]

In the present study, two HLA B*07 restricted fusion peptides were available to be tested.

3.4 T-cell priming

In order to experimentally verify the immunogenicity of TEL/AML1-derived peptides it appears mandatory to try to reproduce what occurs in physiological circumstances, i.e. the generation of clones of peptide-specific T-lymphocytes from naïve T-cells, also called T-cell priming. This may be seen as an “education” process in which T-cells become able to recognize a specific antigen and to mount an immune response against it. This immune response is expected to be long-lasting, with the possibility to re-trigger it by subsequent exposure to the antigen.

There are different ways to perform it, either cell mediated or antibody-coated solid supports (beads) mediated.

In the present study, the technique of dendritic cell mediated T-cell priming was mainly used and will be briefly presented.[28] In the last experiments, we used the Beads Priming protocol, which has the advantage of a shorter duration but the limitation of being suitable only to Class I peptides priming.

A detailed description of the techniques used will be performed in chapter 4. However, it is worthy to point out some key features of mutual interactions of T-cells and dendritic cells.

Dendritic cells (DC) are antigen presenting cells (APCs) particularly suitable to activate naïve T-cells, due to their capacity to present peptide-MHC complexes and to an adequate expression of costimulatory molecules such as CD40, CD80, CD86.

The term DCs refers actually to a variety of cell types which are thought to arise from adult bone marrow precursors (with the exception of Langerhans cells in the skin).

The two main DC subpopulations characterized so far are the so called classical DCs (cDCs) and plasmocytoid DCs (pDCs).

Briefly, cDCs are generated mainly from myeloid precursors in the bone marrow and migrate in lymphoid organs and peripheral tissues where they maturate and reside, capturing self-tissue antigens and presenting them to T cells. Among DCs subpopulations, they are the most efficient as APCs. When they come in contact with microbes or cytokines, activation occurs and pDCs upregulate costimulatory molecules, secrete cytokines and concentrate themselves into lymph nodes.

pDCs also develop in the bone marrow from the same progenitor of cDCs, but they circulate in peripheral blood and are rarely found in lymphoid organs. Their morphology resembles the one of plasmacells (as the name says) and they have poor antigen-presenting capacity. They acquire the morphology and function of DCs only after activation, and their role consists in secreting IFNγ during viral infections. There is a certain degree of plasticity

among DCs subsets and between monocytes and DCs: under specific conditions, like inflammation, both monocytes and pDCs can differentiate into cDCs.[23]
The process can be artificially reproduced: DCs can be generated *ex-vivo* from circulating monocytes under the influence of GM-CSF and IL-4 (as it was done in the present study).
For the full maturation of DCs, exposure to tumour necrosis factor-alpha (TNF-α) or CD40 is also required.
Differentiation, upon activation, implies the decline of the antigen processing capacity and the increase of antigen presenting capacity (mirrored by the increase of CD80 and CD83 surface expression).
The majority of DCs physiologically found in tissues (cDCs) are immature and exhibit a great antigen-uptake capacity: this means, for instance, that only picomolar and nanomolar quantities of antigens are required for efficient endocytosis instead of micromolar as it is for other APCs.
After antigen uptake, the antigen-processing ability decreases and antigen-loaded DCs start to migrate into secondary lymphoid organs, where they interact with naïve CD4+ and CD8+ T-cells presenting MHC/peptide complexes: this process includes maturation steps and functional changes occurring in both cell types.

3.5 Antigen-specific T-cell identification

The final common goal of experiments with patients and healthy donors´ cells is to identify and isolate antigen-specific T-cells. For this purpose, FACS-based methods are widely used. Antigen-specific T cells are identified by expression of activation markers or cytokine production upon antigen stimulation. Surface markers can be used also for cell sorting.
In the present work, IL-2, TNFα, IFNγ and CD154 were used as intracellular markers.
IL-2 is a cytokine produced by T-cells itself with a key role in T-cells homeostasis, since it promotes survival, proliferation and differentiation of antigen-activated T-cells and enhances survival and function of regulatory T-cells.
TNFα, although its principal cell source are macrophages, is produced upon activation also by T cells (CD4+), B cells and NK cells, and has a strong pro-inflammatory function.
IFNγ is produced mainly by CD4+ T-cells and it promotes further differentiation towards Th1 subset. Moreover, it acts on macrophages, B-cells and it stimulates further MHC class II and costimulatory molecules expression on APCs.[29]

CD154 (or CD40L) is a member of the TNF receptors superfamily, is expressed by activated helper T-cells and trough binding to his ligand CD40 (constitutively expressed on B-cells) mediates T-cell dependent B-cell activation.[23]

We also used surface activation markers, namely OX-40 and 4-1BB.

OX-40 (or CD134) is also a TNF receptors superfamily member, is expressed on activated CD4+ cells and acts as costimulatory molecule on T-cells itself, promoting survival, proliferation and further differentiation, either towards CD4+ and CD8+ effector phenotype or Treg phenotype.

4-1BB (or CD137) is expressed on activated T cells and on a variety of cells of the immune system and, through interaction with its physiological ligand (4-1BBL) enhances the expression of anti-apoptotic genes. Both OX-40 and 4-1BB, given their key-role in preserving and enhancing activity of antigen-specific T cells, are also promising targets for cancer immunotherapy.[29]

3.6 T-cell cloning

T-cell clones are populations of T-lymphocytes which derive from the same progenitor. There are different techniques to obtain clones from PBMCs, which are a highly heterogeneous population. Obtaining antigen-specific T-cell clones is a complex and time-consuming procedure, because the frequency of cells of interest is low. It comprises a first step of primary culture to enrich in antigen-specific cells (which in our case was DC priming) and then (after testing) a selection of cells of interest. For cell selection, a possibility is the limiting dilution technique, based as the name says on the serial dilution of cultures until in a given volume is theoretical present a single cell.

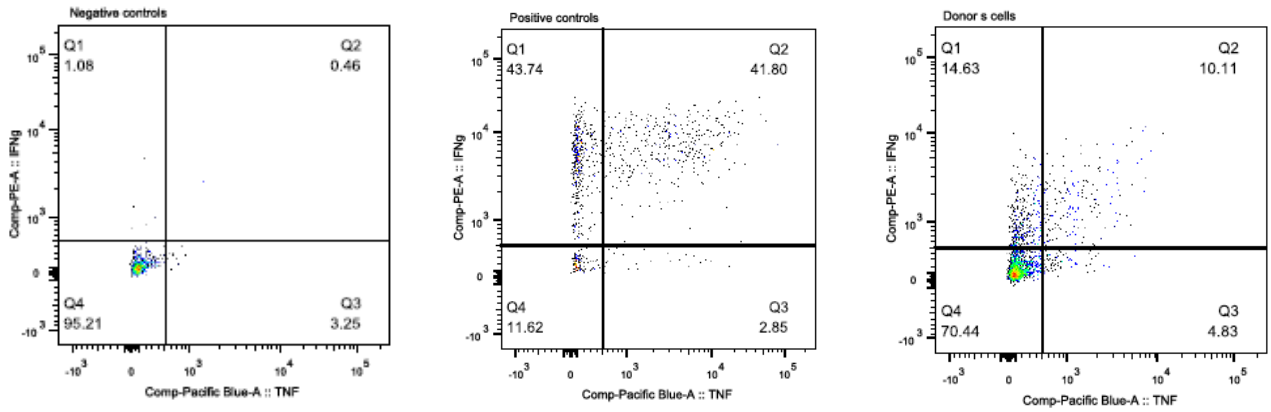
This method has been used by others for cloning of TEL/AML1 peptides responsive T-cells.[20] It has many limitations due for instance to inter-operators variability in cell counting or to the unequal distribution of T-cells within the diluted volume (because they may form clumps).[30]

Another possibility is the automated FACS sorting, which uses the expression of a given surface marker by the cells of interest as a criterion to electrostatic deviate and separate them. After FACS sorting it is theoretically possible to obtain either a single cell culture (which will give rise to a clone), or a polyclonal cell culture. For the study purpose, we used an automated FACS sorting method aiming to obtain a single cell culture.

3.7 Own previous work on priming experiments

Professor´s P. Lang research staff performed several DC priming experiments with healthy donors´ samples in order to validate artificially synthetized TEL/AML1 fusion protein-derived peptides (either HLA class I and class II restricted). 22 healthy donors were tested, and a T-cell priming and expansion was identified in two of them. The response was detected after testing through intracellular cytokine staining (ICS) and FACS analysis, as shown below.

a)



b)

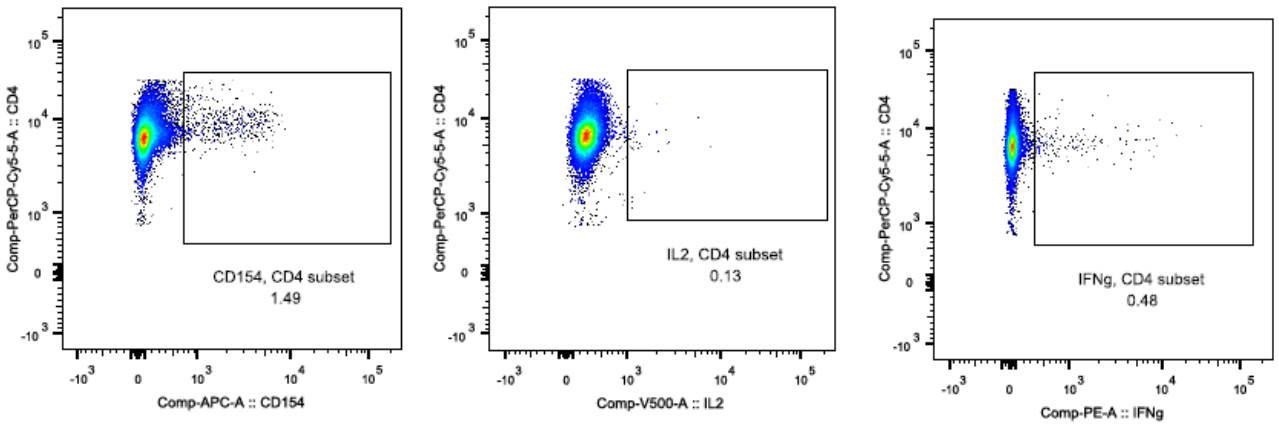


Fig. 3.5

a) Peripheral blood mononuclear cells were primed with a TEL/AML derived, HLA class I restricted peptide. In figure are shown, respectively, a negative control (cells incubated overnight with DMSO), a positive control (cells stimulated and incubated overnight with PMA/Ionomycin) and the peptide-stimulated cells. The day after, an intracellular cytokine staining for TNFα and IFNγ was performed. A CD8 restricted response was observed.

b) Peripheral blood mononuclear cells were primed with a TEL/AML derived, HLA class II restricted peptide. An intracellular cytokine staining for TNFα, IIFNγ, IL-2 and CD154 was performed. A CD4 restricted response was observed.

These observations prompted to confirm such findings in a larger cohort of donors, in order to get T-cell clones which could be functionally tested. We also speculated, and tried to detect, a similar behaviour of human T cells in vivo in patients who were “physiologically” exposed to the TEL/AML1 protein, i.e. patients who suffered, and successfully overcome, TEL/AML1 positive B-lineage ALL.

4. Objectives

Main objective of this study is to investigate whether the good outcome of TEL/AML1 positive B-lineage paediatric ALL is related to a long-lasting immunological response against peptides derived from the fusion protein.

In other words, we aimed to prove that fusion peptides are naturally presented by specific HLA molecules, or group of molecules (namely HLA-B*0702, B*0703, HLA-DR1, DR2, DR3, DR4) and the peptide-MHC complex can be recognized by T-lymphocytes and activate their TCR.

To verify such hypothesis, peripheral blood leukocytes of patients with TEL/AML1 positive B-ALL, either off-therapy or in non-intense chemotherapy (i.e. maintenance) have been tested, through co-incubation with TEL/AML1 fusion protein-derived peptides and short stimulation. After that, we have looked for an expansion of activated T-cells, that we postulated to be peptide-specific.

In order to reproduce the phenome in vitro, we challenged healthy donors´ lymphocytes with the fusion protein-derived peptides and co-cultured them with autologous dendritic cells, according to the so-called T-cell priming technique.

T-cell priming is already in use in cancer immunotherapy, in the field of anti-tumoral peptide vaccination, but it has never extensively applied to the study of immunological responses against paediatric leukaemias carrying well known genetic anomalies giving rise to mutant proteins.

We aimed to show that T-cell priming using fusion peptides is feasible and suitable to generation of T-cell clones.

In the case of successful generation of T-cell cloning with good specificity, another objective was to functionally characterize and test them, in the perspective of a possible use of the peptides in the enhancement of spontaneously arising immunity against the fusion protein (in autologous setting) or the use of clones in post-hematopoietic stem cell transplantation adoptive immunotherapy.

5. Patients and methods

Healthy donors

Fresh leucocyte cones, obtained from healthy donors´ whole blood in the occasion of a planned blood donation, after purification through leucocyte depletion filters, were kindly provided by the Transfusion Medicine Department of Tübingen University Hospital.
7 donors (all males) were tested for a total of 8 experiments (one donor was tested twice), from October 2015 to August 2016. Mean age at donation was 50,42 years (range 45,08-54,83).
A class I HLA typing was available for all tested donors: among them, 4 were HLA B*07 positive.
All experiments were started on the same day of blood collection.
PBMCs not used for the experiments were cryopreserved and stored in liquid nitrogen.

Patients

Patients were recruited by the study staff during routine outpatient clinic controls; legal representatives, or the patients themselves if age > 18 years, gave informed consent to blood sampling. An age-adapted informed consent form was also gave to and signed by patient of appropriate age. Blood samples were drawn during already planned routine check-ups; therefore, no additional clinical-biochemical evaluation and venipuncture were necessary.
Inclusion criteria were:

- Age > 1 year and < 25 years;
- Patients with B-lineage, TEL/AML1 ALL, enrolled in the Cooperative Protocol AIEOP-BFM 2009 in Germany and in Padova (Italy) University Hospital, off-therapy or on maintenance Phase (M phase) at the time of blood sampling
- Absence of relapse
- Patients who did not underwent hematopoietic stem cell transplantation.

Ten (10) patients were recruited at the Paediatric Haematology and Oncology Department of Tübingen University Hospital, 12 patients were recruited at the Paediatric Haematology and Oncology Department of Padova University Hospital.
Mean age at ALL diagnosis was 4,35 years (2,43-8,7); mean age at study recruitment (blood sampling) was 7,45 years (3,3-16,72); mean time between diagnosis and study recruitment was 3,1 years (0,55-9,07).

After sampling, PBMCS were isolated from fresh blood (collected in tubes with heparin as anticoagulant) through Ficoll-Hypaque density centrifugation method (see thereafter) and cryopreserved until they were analysed. Since all the experiments were performed in the Children Hospital´s Lab of Tübingen University, samples collected in Padova were stored at -80°C and shipped on dry-ice.

General methods

Peripheral blood mononucleated cells (PBMCs) isolation

PBMCs where isolated through Ficoll-Hypaque density centrifugation method.

All the procedure was carried out under sterile conditions. After transferring it into a vial of appropriate size, the blood product was first diluted with Dulbecco´s phosphate buffered saline (PBS, Sigma, München, Germany) in the proportion of at least 1:1 for whole blood, or brought to 125 mL for buffy-coat or leucocyte cones.

15 mL of Ficoll Hypaque solution (Biochrom, Berlin) were placed into one or more 50 mL Falcon tube and the diluted blood sample was gently and slowly layered in the proportion of 30 mL for every 15 mL of Ficoll solution, so that two clearly distinct layers could be appreciated.

The tubes were centrifuged at room temperature for 30 minutes at 800 rpm.

A little quantity of supernatant was removed, then the leucocyte layer (visible as a “ring”) was removed and transferred into a clean Falcon tube and brought to the volume of 50 ml with PBS.

The tubes were centrifugated (room temperature, 10 min, 500 rpm), resuspended with PBS, eventually merged, brought to the final volume of 50 mL and centrifugated again (room temperature, 10 min, 400 rpm). After resuspending the cells, a sample for cell count was taken and the tube was centrifugated (room temperature, 10 minutes, 250 rpm). Cells were counted using the Neubauer counting chamber method. An aliquot of 10 ul of cell suspension was diluted in 90 mL PBS or erythrocyte lysis solution, 10 ul of the mix were diluted with Tryptan Blue (Sigma Aldrich, Steinheim, Germany) and transferred thanks to capillarity as a film on the space between the Neubauer chamber (Assistant, Germany) and the cover glass. The chamber was then examined under an optical microscope and the number of bright (living) cells pro square (big squares on the grind, surface 1 mm^2) was counted. The obtained value was then divided for the number

of squares and multiplied for the dilution factor (10^4), for the number of additional dilutions (1:10 and 1:2, total 20) and for the volume of the cell suspension.
After this last washing step, the cells were either resuspended in the appropriate cell culture medium and used for experiments, or frozen.

Cell freezing

All the procedure was conducted under sterile conditions. Cells from culture were previously washed with their own medium at least twice, counted and then centrifuged, cells isolated with Ficoll-Hypaque method were directly frozen after the last washing step (see above).
The freezing medium consisted on Fetal Bovine Serum (FBS, Gibco, Life Technologies)) with 10% of DMSO (Roth, Karlsruhe, Germany).
Pellets were resuspended in an appropriate quantity of freezing medium and then transferred into marked cryo-vials in aliquots of at least $5*10^6$ cells and 1 mL volume.
The cryo-vials were placed into controlled-rate freezing containers (either with or without isopropyl alcohol), stored for at least 24 hours at -80°C and then transferred into liquid nitrogen.

Cell thawing

All the procedure was conducted under sterile conditions. Cryo-vials, either from dry-ice or normal ice, were quickly immerged into a pre-warmed 37°C thermostatic water bath, until the content was almost thawed (and a little amount of ice was still visible in the middle), then 1 mL of cold thawing medium (IMDM, Lonza, Verviers, Belgium + 3 ug/mL DNase + 1% Penicillin/Streptomycin, Biochrom, Berlin) was added drop by drop and the cells resuspended. The whole content of the cryo-vial was then transferred into a 15 mL Falcon already filled with cold thawing medium to a final volume of 10 mL. The Falcon tube was then centrifuged (+4°C, 7 minutes, 350 rpm) and the pellet resuspended in cold thawing medium. An aliquot was taken for cell count and the Falcon centrifuged again (under the same conditions). The cells were then resuspended with the appropriate volume of pre-warmed own cell culture medium.

Specific protocols

***In vitro* Priming with dendritic cells**

Glossary:

DC = dendritic cell

PBLs = peripheral blood lymphocytes (cells obtained after adherence step, monocyte-depleted)

PBMCs = Peripheral blood mononuclear cells (unmanipulated cells, obtained after Fycoll, include lymphocytes and monocytes).

Overview:

Week 1: Cells isolation, Adherence step, PBL separation and culture, PBMCs freezing, DCs generation.

Week 2: Maturation and "Priming" of DCs + autologous PBLs

(FACS Control)

Week 3-7: Restimulation with autologous, peptide-bearing, irradiated PBMCs

Week 8: evaluation (Intracellular cytokine staining of the generated cells)

Materials:

- DC-Medium: RPMI 1640 (Biochrom, F1215) + 1% L-Glutamin (Biochrom, Berlin) + 10 mM (5 ml) Hepes (Biochrom, L1613, [1 M]) + 1% Penicillin-Streptomycin (Biochrom, Berlin) + 10 % human AB-Serum (Invitrogen, 34005100)
- PBS
- Cytokines: IL-1β (CellGro, 1411-010), IL-2 Proleukin ®, Novartis, Basel, Switzerland, stock concentration 20000 UI/mL), IL-4 (Peprotech, 200-04), IL-6 (CellGro, 1404-010), IL-7 (CellGro, 1406-010), GM-CSF (Leukine), TNFα, PGE2 (Sigma-Aldrich, P6532)
- 24-Well cell-culture plate (Corning Incorporated, Corning, NY, USA)
- T 75 and T 175 cell culture flasks (Greiner bio-one, Frickenhausen, Germany)
- Peptides: HLA B*07 restricted (sequence MPIGRIAECIL and YPQCFMRDCRL), non-HLA B*07 restricted (sequences: HAMPIGRIAECILGMNPS, PSYPQCFMRDCRLLWDYV, HAMPIGRIADASTSRRFT, GMNPSRDVHDCRLLWDYV), stock concentration 1 mg/mL.

Procedure

Day 1:

- PBMCs were obtained from the blood products (leukocyte cones) according to the Ficoll-Hypaque density centrifugation method (see above).
- The pellet at the end was resuspended in 20 mL of DC-Medium and cells were counted.
- 2.1*10^8 cells were taken out for culture, diluted in 70 mL of DC medium and plated into two T 175 cell culture flasks (concentration of 3*106/cells/mL, 35 mL per flask).
- PBMCs in the two T 175 were incubated at 37°C for 1-2 hours, lying.
- In the meanwhile, the remaining PBMCs were frozen, in aliquots of 10^8 cells and 2,5-5*10^6 cells (for cell-freezing protocol, see above).
- After incubation, the supernatant in the two T 175 was removed, centrifuged, in 15 mL DC Medium + IL-7 (at the concentration of 5 ng/mL) resuspended and transferred into a T 75 cell culture flask.
- The two T 175 (containing the adherent cells) were rinsed with PBS (which was then eliminated) and 35 mL per flask of DC medium with 40 ng/mL of IL-4 and 100 ng/mL of GM-CSF were added.

The flasks were kept in the incubator for a week; in case the medium in the PBLs T 75 flask became quickly yellow, 5 mL of DC medium were added.

Day 8:

- The same quantities of DC medium (35 mL per-flask) were pre-warmed and cytokines were added at the following concentration:

Cytokine [Stock concentration]	→	Final concentration	
IL-4 [10 µg/ml]	→	40 ng/ml	(1:250)
GM-CSF [100 µg/ml]	→	100 ng/ml	(1:1000)
IL-1β [10 µg/ml]	→	10 ng/ml	(1:1000)
IL-6 [10 µg/ml]	→	10 ng/ml	(1:1000)
TNF-α [10 µg/ml]	→	10 ng/ml	(1:1000)
PGE2 [1 mg/ml]	→	1 µg/ml	(1:1000)

- The supernatant was removed from the two T 175 and 20 mL per-flask of DC Medium with cytokines was added.

- Supernatant was centrifuged (400 rpm, room temperature, 6 minutes) and the supernatant was removed.
- Each pellet was resuspended in 15 mL of the remaining DC-Medium with cytokines and given to the DCs in the T 175. The flasks were then placed back in the incubator for 24-48 hours.

Day 9/10:

- The two T 175 flasks were gently beat in order to detach the cells.
- The supernatant was transferred into a 50 mL Falcon tube.
- The bottom of the flasks was rinsed twice with PBS (7,5 mL); PBS was also added to the Falcons.
- The Falcon were centrifuged (400 rpm, room temperature, 6 minutes) and 10 mL of fresh PBS were added to the two T 175, which were temporarily placed back in the incubator (in case more cells were needed afterwards).
- The supernatant was resuspended in DC-Medium and cells (DCs) were counted. If not enough for the experiment, the two T 175 could be rinsed again twice with PBS, which was collected and then centrifuged. The pellet was then added to the already obtained DCs. An aliquot for FACS control was taken (at least $1.5*10^6$ cells).
- DCs were prepared for priming: $0.5*10^6$ cells/well were needed. According to the number of peptides to test, and to the number of wells per-peptide, aliquots (one for each peptide) were made in 15 mL Falcons, centrifuged and all brought to 1 mL volume. 25 uL of peptide were added to each Falcon, and all the tubes were incubated for two hours (slightly screwed cap).
- During DCs´incubation, PBLs were counted and the concentration adjusted to $5*10^6$ cells/mL; they were then plated on a 24 wells cell culture plate (1 mL per well).
- After incubation, DCs in the falcons were brought to the correct end volume with DC medium and then plated (1 mL per well) to the 24 wells plate with PBLs.
- The plate was placed back in the incubator and left there for a week; in case the medium became quickly yellow, 1 mL of supernatant was carefully removed and 1 mL of fresh DC medium was added.
- The aliquot for FACS control was also centrifuged, resuspended in 600 uL of DC medium and then plated in three aliquots of 200 uL on a 96 wells cell culture plate, for staining and cytometric analysis the same day or within a few days.

Day 16 or 17:

- The same day or one day before autologous PBMCs were thawed (for procedure, see above) and washed twice with DC-Medium.
- Cells were counted and $1*10^6$ per well were taken out, subdivided into aliquots in Falcon tubes (one aliquot for each peptide) and all the aliquots were centrifuged and brought to the volume of 1 mL with DC-Medium
- 25 uL of corresponding peptide were added to each Falcon
- Falcons were kept in incubation (slightly screwed cap) for two hours and in the meantime 1 mL of supernatant was carefully removed from each well of the 24 wells plate.
- After incubation, the Falcons were irradiated (30 Gy), brought to the appropriate end volume with DC-Medium and then pipetted to the DCs in the 24 wells plate.
- 20 U/mL of IL-2 were added to each well, starting from day 1 after restimulation and then every 2-3 days (only once if in the following weeks the evaluation with ICS was planned).

In case the medium became quickly yellow (every day or every two days) wells were splitted 1:1.

The restimulation was then repeated weekly with the same modalities for a total of 5 restimulations.

<u>DCs FACS Control: Staining</u>

<u>Materials</u>:

Antibodies	Dilution
CD80 FITC (Biolegend, San Diego, USA)	1:10
CD83 APC (Biolegend)	1:50
CD86 PE (Biolegend)	1:50
CD3 PerCP (Biolegend)	1:40
CD14 Pacific Blue (Biolegend)	1:50
Anti-isotype antibodies	Dilution
IgG1 (Biolegend)	1:12,5
IgG1 (Biolegend)	1:50
IgG2b (Biolegend)	1:50
IgG1 (Biolegend)	1:40
IgG2A (Biolegend)	1:50

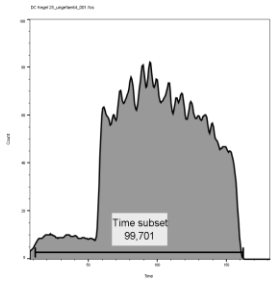
FACS-Buffer: PBS + 2% FBS+ 2mM EDTA (Sigma Aldrich) + 0,02% Na Azid (Hospital Pharmacy Department, Tübingen)
FACS-Fix (In case the FACS analysis was not performed on the same day): FACS-Buffer + 1% Formaldehyde (Stock concentration 37%).
Compensation Beads: AbC anti-mouse bead kit (Molecular probes, Thermo Fisher Scientific, Waltham, MA USA), ArC™ Amine Reactive Compensation Bead Kit ((Molecular probes, Thermo Fisher Scientific, Waltham, MA USA).

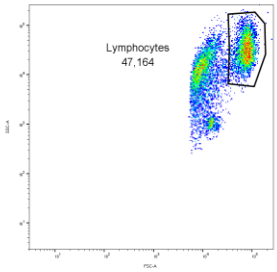
All the procedure was carried out on ice, under non-sterile conditions.
Cells were centrifuged (1800 rpm, 2 min, RT) and the supernatant was removed quickly reversing the plate. The wells were washed twice with FACS-Buffer (centrifugation 1800 rpm, 2 min, RT) and then 50 uL of the antibodies mix were given to each well (1 well = sample, mix with primary antibodies, 2 well= isotype control, mix with anti-isotype antibodies, 3 well= unstained cells, FACS-buffer).
Cells were incubated for 30 minutes at 4°C, in dark
Cells were washed twice with FACS-buffer and then resuspended in 200 uL of FACS-buffer or FACS-Fix and transferred into a labeled FACS tube.
For each experiment compensation tubes were prepared.
For negative controls, 1 drop of negative beads (anti-mouse negative and live-death negative) was diluted on a FACS tube with 200 uL of FACS Buffer.
For positive controls, 10 ul of positive beads (anti-mouse positive and live-death positive) were incubated with each of the antibodies at the same dilution used for the staining mix and diluted with 190 uL of FACS Buffer.

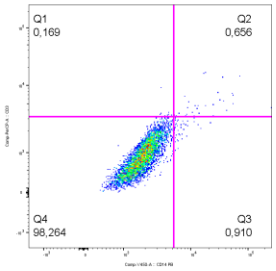
FACS analysis

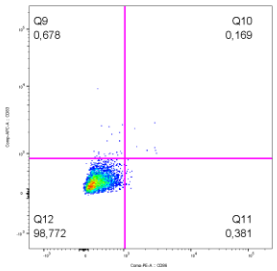
Flow-cytometry analysis was performed using a BD FACSCanto II Cytometer. BD FACSDiva software was used for acquisition, and FlowJo software was used for data analysis.
Cells of interest were first selected on the basis of size and morphology (FSC-A and SSC), then the expression of lineage specific markers (CD3 and CD14, for T cells and monocytes, respectively) and activation markers (CD80, CD83 and CD86) was analyzed. Positivity of CD80, CD83 and CD86 defined the mature (activated) DC phenotype.
An example of gating strategy is showed in Fig. 4.1.

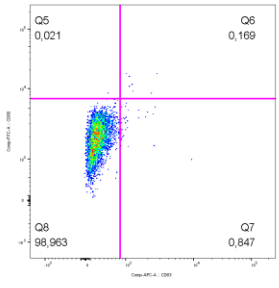
a) Unstained control



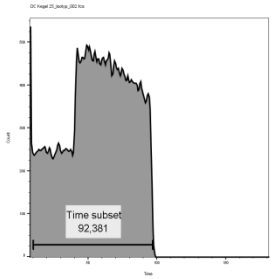


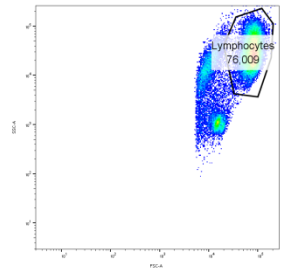


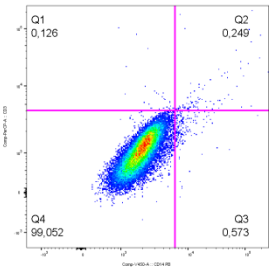


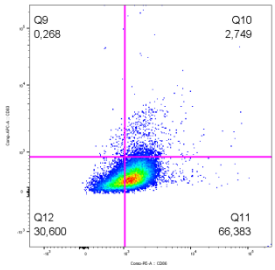


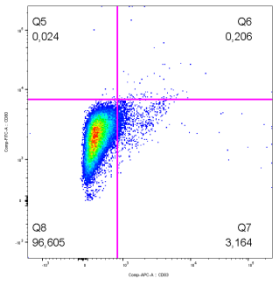
b) Isotype control











c) Sample

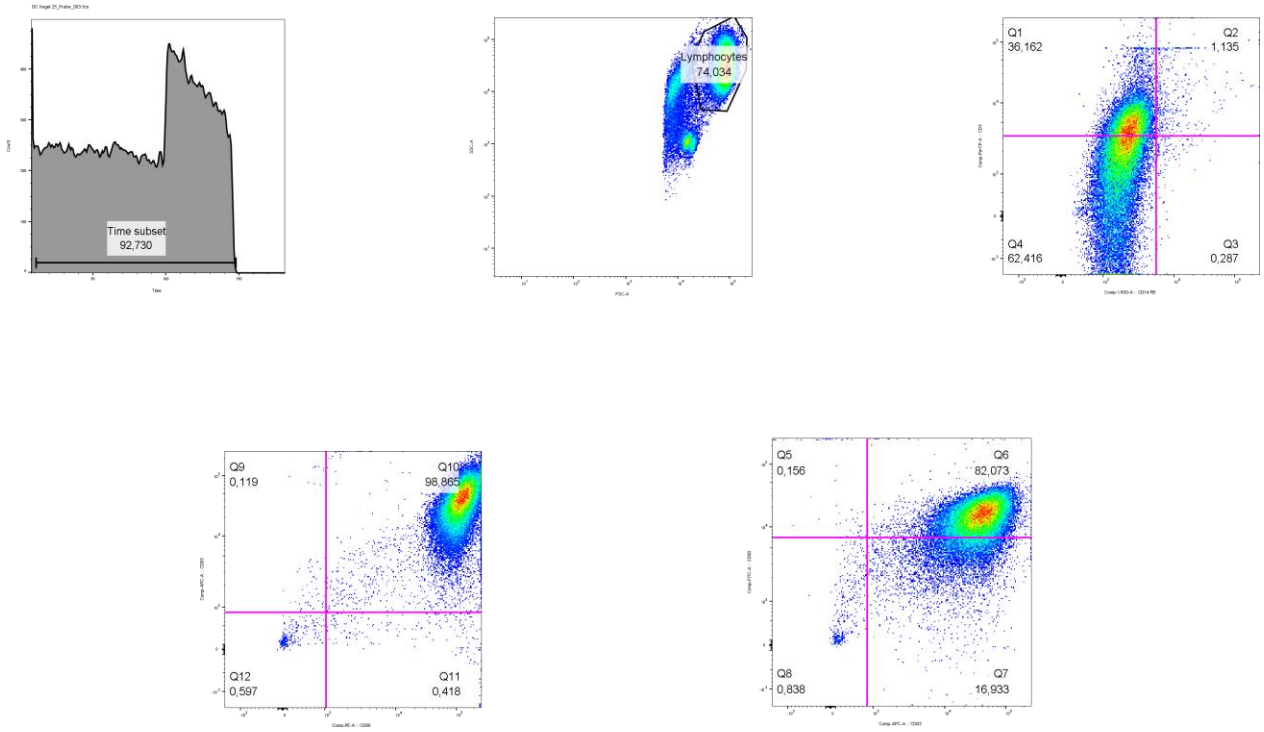


Fig. 4.1. Gating strategy for the dendritic cells FACS control.

Intracellular cytokine staining (ICS)

Overview:

Day 1 (late afternoon): cell preparation and overnight stimulation

Day 2: cells staining (sequential washing and incubation with antibody mixes passages, including cell permeabilization for intracellular staining) and FACS analysis

Stimulation

Materials:

- DC medium (see above)
- T-cell medium
- Peptides (see above)
- For negative controls: DMSO (Roth, Stock concentration: 10% DMSO in sterile water)
- For positive controls: Phorbol myristate acetate (PMA, Sigma, München, Germany), stock concentration 100 ug/mL and Ionomycin (Sigma), stock concentration 1 mM.
- Brefeldin A (Sigma), stock concentration 5 mg/mL.

Procedure

- Cells in the 24 wells plates were resuspended and aliquots of 200 uL were taken out and transferred into an appropriately labeled 96 well plate (3 aliquots per well, one for peptide-stimulation, one for positive control and one for negative control). The removed volume was replaced with an equal amount of fresh DC medium with IL-2 (20 U/mL).
- Two 50 uL aliquots were taken out from two positive-controls wells and transferred into two separate wells (for unstimulated stained and unstimulated unstained controls, respectively).
- Plates were shortly centrifuged (1800 rpm, 2 min, RT) and 100 uL of supernatant were gently discarded from each well.
- Different stimulation mixes were prepared: one for each peptide, one for positive controls (PMA + Ionomycin) and one for negative controls (DMSO).

Peptide Mix:

Volume to be given: 50 µl/well

MHC-class-I Peptides (HLA-A, HLA-B, or HLA-C) were brought to the final concentration of 1 µg/ml

MHC-class-II Peptides (HLA-DR, HLA-DQ, HLA-DP) were brought to the final concentration of 10 µg/ml

The amount of peptide was calculated according to the following formulas:

MHC I: n° of wells to be stimulated x 1,1 x 200 µl x 1 µg/ml / 1000 µg/ml

MHC II: n° of wells to be stimulated x 1,1 x 200 µl x 10 µg/ml / 1000 µg/ml

Medium volume: (n° of wells to be stimulated x 1,1 x 50 µl) – Peptide volume

DMSO Mix:

Volume to be given: 50 µl/well

The DMSO-controls 'mix was prepared in the same way as the corresponding Peptide Mixes (MHC-I or MHC-II), i.e. the identical volumes of peptides and medium were pipetted.

PMA/Ionomycin Mix:

Volume to be given: 50 µl/well

PMA was brought to the end concentration of 50 ng/ml; Ionomycin was brought to the final concentration of 1 µM.

Volumes were calculated according to the following formulas

PMA: n° of wells to be stimulated x 1,1 x 200 µl x 50 ng/ml / 100000 ng/ml

Iono: n° of wells to be stimulated x 1,1 x 200 µl x 1 µM / 1000 µM

Medium volume:

(n° of wells to be stimulated x 1,1 x 50 µl) – (Volume PMA + Volume Iono)

Brefeldin A was diluted with DC medium and brought to the final concentration of 10 ug/mL

- Finally the appropriate mixes were pipetted into the wells. Each well contained thus 100 uL of cell suspension, 50 uL of stimulation mix (either peptide or DMSO or PMA/Iono mix) and 50 uL of Brefeldin mix (for the unstimulated controls, stimulation mix was replaced with DC medium) for a final volume of 200 uL/well. The plates were then incubated overnight for 14-16 hours.

<u>Staining</u>

Materials:

PBSE (PBS-EDTA): PBS + 2mM EDTA (Sigma, stock concentration 0,5 M)

FACS-Buffer (see above)

FACS-Buffer + “Flebo G” : 500 mL PBS + 1% FBS +0,01% Na Azide (stock concentration 10%) + 250 uL “Flebo G” (Human IgG, Privigen, stock concentration 100 mg/mL)

Permwash: PBS + 0.1% Saponin S-7900 + 0.5% BSA A-3059 + 0.02% Na azide

Cytofix/Cytoperm (BD, San Diego, CA, USA)

FACS-Fix (see above)

APC-eFluor 780 (e-Bioscience, San Diego, CA, USA)

Antibodies:	Dilution
CD56 Pe-Cy7 (Biolegend, San Diego, USA)	1:75
CD4 PerCp (Biolegend)	1:100
CD8 FITC (Biolegend)	1:50
IFNγ PE (Biolegend)	1:200
TNFα Pacific Blue (Biolegend)	1:200
CD154 APC (Biolegend)	1:200
IL-2 BV510 (Biolegend)	1:50

Compensation beads: the same materials and the same procedure reported in the “DCs FACS control” section were used.

Procedure:

All the staining was carried out on ice/at -4°C, under non-sterile conditions.

- Plates with stimulated cells were cooled down at -4°C while all the reagents were prepared.
- Live-death mix was prepared (2095,8 uL PBSE, 4.2 uL eFluor 780)
- Plates were centrifuged (1800 rpm, 2 min, RT), supernatant was discarded
- Plates were washed twice with PBSE (centrifuged at 1800 rpm, 2 min, RT)
- After the last washing step, cells were resuspended with 50 uL/well of live-death mix (except the unstained control) and incubated at -4°C, in darkness, for 20 minutes.
- In the meantime, the extracellular staining mix was prepared: appropriate volumes of CD56 Pe-Cy7, CD4 PerCp and CD8 FITC were diluted in FACS-Buffer + Flebo G in order to reach a final volume of 50 uL/well.
- After the end of incubation time, 150 uL of FACS Buffer were added to each well and plates were centrifuged (1800 rpm, 2 min, RT).
- Supernatant was discarded and plates were washed once with FACS Buffer (centrifugation 1800 rpm, 2 min, RT).
- Cells in each well were resuspended in 50 uL of extracellular staining mix (except the unstained control) and incubated at -4°C, in darkness, for 20 minutes.
- After the end of incubation time, 150 uL of FACS Buffer were added to each well and plates were centrifuged (1800 rpm, 2 min, RT).
- Supernatant was discarded and plates were washed once with FACS Buffer (centrifugation 1800 rpm, 2 min, RT).
- Cells in each well were resuspended in 100 uL of Cytofix/Cytoperm solution (except the unstained control) and incubated at -4°C, in darkness, for 20 minutes.
- In the meantime, the intracellular staining mix was prepared: appropriate volumes of IFNγ PE, TNFα Pacific Blue, CD154 APC and IL-2 BV510 were diluted in Permwash in order to reach a final volume of 50 uL/well.
- After the end of incubation time, 100 uL of Permwash were added to each well and plates were centrifuged (1800 rpm, 2 min, RT).
- Supernatant was discarded and plates were washed once with Permwash (centrifugation 1800 rpm, 2 min, RT).
- Cells in each well were resuspended in 50 uL of intracellular staining mix (except the unstained control) and incubated at -4°C, in darkness, for 20 minutes.
- In the meantime, FACS-Fix solution was prepared (optional passage, FACS-Buffer + 1% Formaldehyde, final volume 200 uL/well).

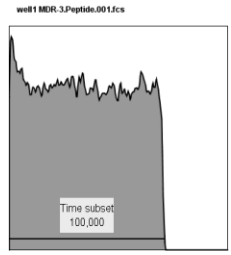
- After incubation, 150 uL of Permwash were added to each well and plates were centrifuged (1800 rpm, 2 min, RT).
- Supernatant was discarded and plates were washed twice with Permwash (centrifugation 1800 rpm, 2 min, RT).
- Cells were resuspended in 200 uL/well of FACS Buffer/FACS Fix, transferred into appropriately labeled FACS tubes and stored at -4°C until FACS analysis was performed.

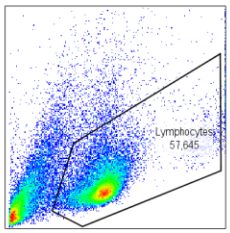
Note:
After the first experiments, we decided to test a new antibody combination in order to better detect antigen-specific T-cells. Namely, two new antibodies, targeting OX-40 and 4-1BB, replaced the anti-CD56 and anti IFNγ antibodies, being conjugated with PE and PECy7 respectively (both from Biolegend). After opportune titration, we used a concentration of 1:20 for both antibodies.
Titration experiments also showed that 4-1BB worked better if no Brefeldin was added to the cell suspension, and thereafter, at least for sorting, we carried out only the extracellular staining phase of the protocol (the target antigens of the new antibodies are both extracellular) without using Brefeldin A in the stimulation.

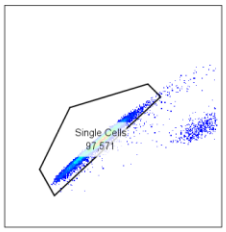
FACS Analysis

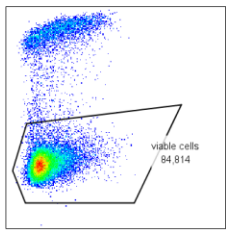
Lymphocytes were selected on the basis of FSC and SSC. Single cells were then selected on the basis of FSC-A and FSC-H, and viable cells were identified on the basis of SSC-A and low expression of eFluor780. NK cells (CD56+) were then excluded and the expression of each single intracellular cytokine was evaluated separately on CD4+ and CD8+ subsets. An example of the used sequential gating strategy is reported in figure 4.2.

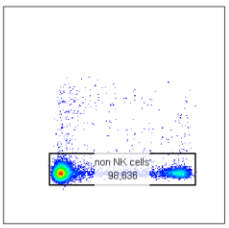
a) Peptide-stimulated cells

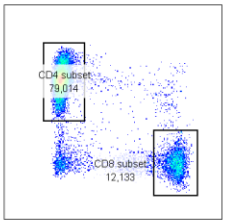


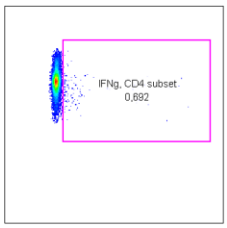


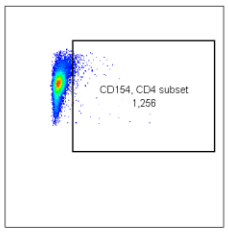


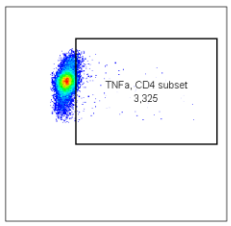


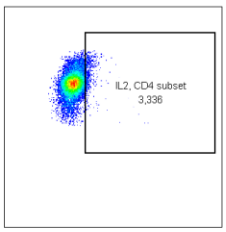


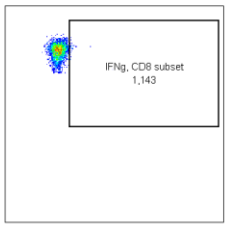


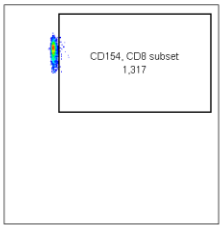


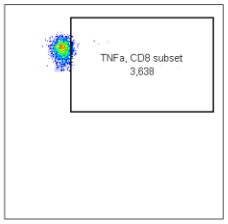


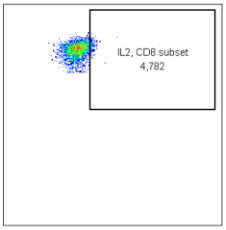




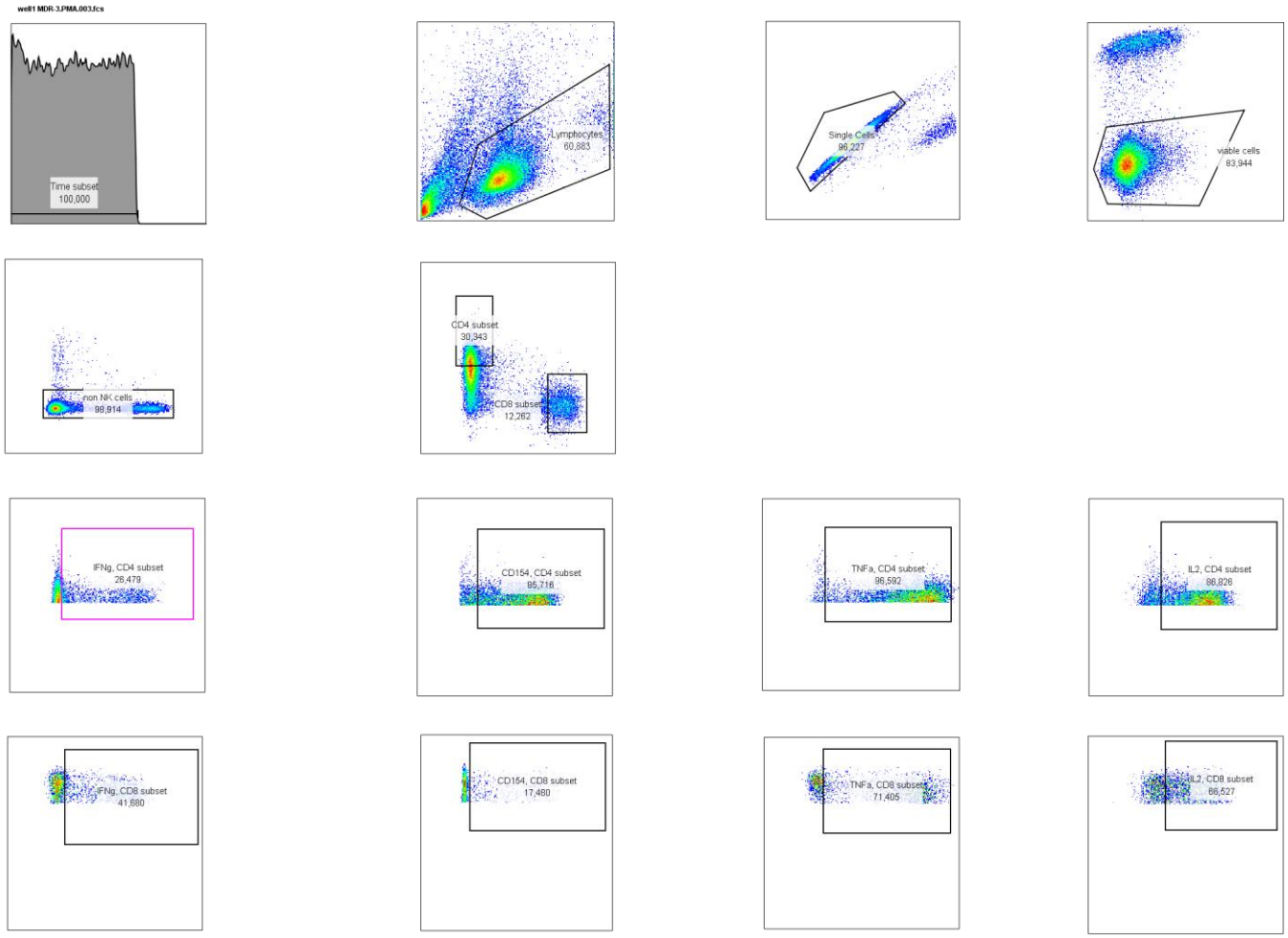








b) Positive Control (PMA stimulated cells)



c) Negative Control (DMSO stimulated cells)

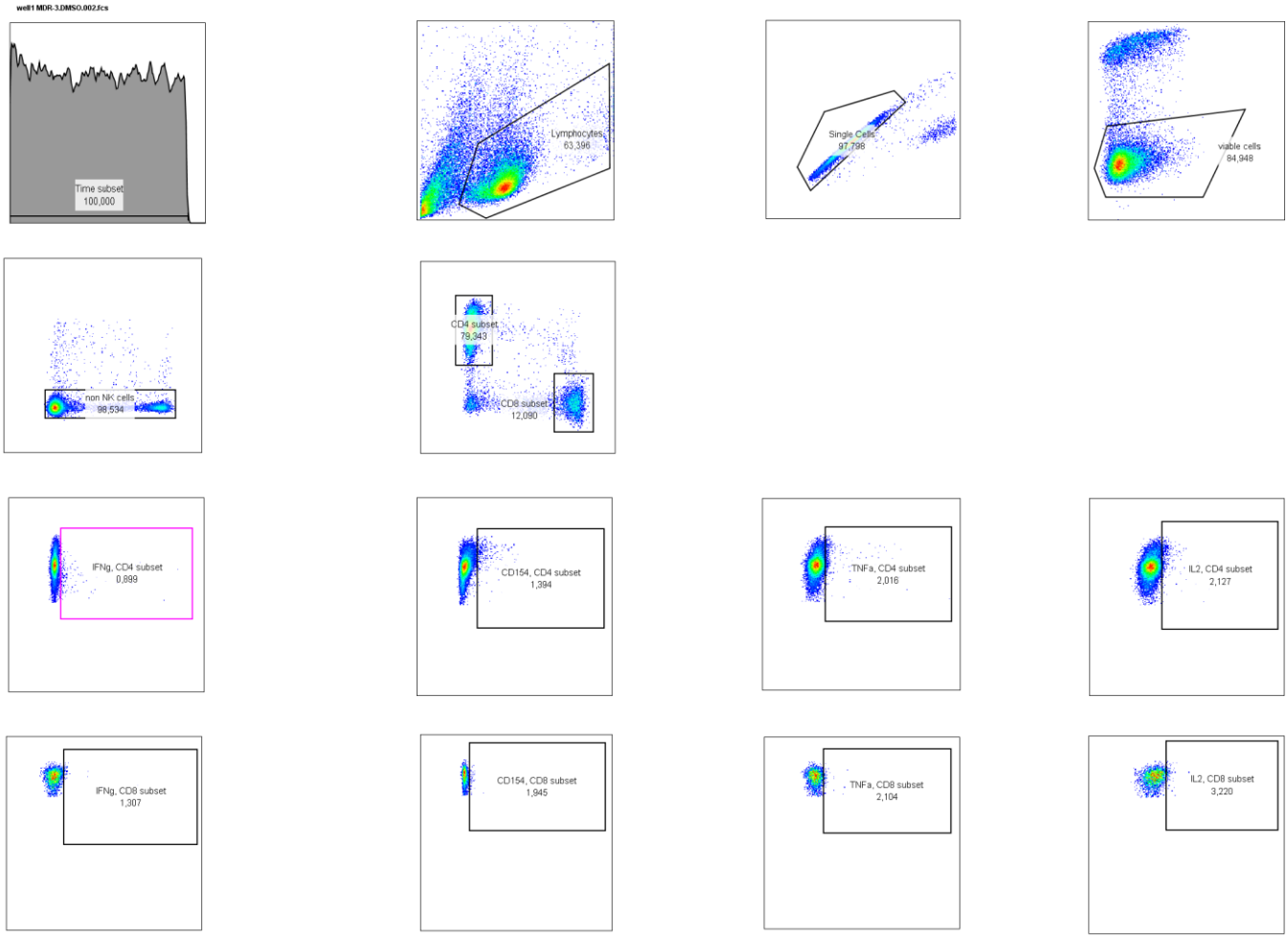


Figure 4.2 An example of ICS gating strategy.

Beads Priming Protocol

Overview:

Day 1: PBMCs isolation

Day 2: CD8+ T-cells isolation

Day 3: Loading of beads and 1st stimulation with beads

Day 5 or 6: Il-2 and medium change

Day 10: 2nd stimulation with beads

Day 12 or 13: Il-2 and medium change

Day 17: 3rd stimulation with beads

Day 19 or 20: Il-2 and medium change

Day 28 or 29: Multimerstaining

Materials:

- T-cell Medium (TCM): IMDM with L-Glutamin (Lonza Verviers, Belgium), 10% Human Serum (Invitrogen, or kindly provided by the Transfusion Medicine Department of Tübingen University Hospital), 50 µM beta-ME (), 1% Penicillin-Streptomycin (Biochrom, Berlin).
- MACS buffer: PBS + 0,5% BSA (Bovine Serum Albumin, Sigma) + 2mM EDTA, stored and used at 4°C and sterile filtered
- 96 wells round bottom plates (Costar)
- T 75 and T 175 cell culture flasks (Greiner bio-one, Frickenhausen, Germany)
- CD8 Easy Sep selection kit (Stem Cell Technologies, Vancouver, Canada) or CD8 selection kit by Miltenyi Biotech, Bergisch-Gladbach, Germany
- Cytokines: IL-2 (Proleukin ®, Novartis, Basel, Switzerland, stock concentration 20000 UI/mL), IL-7 (CellGro, 1406-010, stock concentration 10 ug/mL), IL-12 (Promokine, Heidelberg, Germany, stock concentration 10 ug/mL)
- DNase (Roche, Basel, Switzerland, stock concentration 10 mg/mL).
- Streptavidin coated Microspheres (Bangs Laboratories, Inc, Hirschberg an der Bergstrasse Germany, Mean diameter 5,61 um, binding capacity at least 0,06 ug biotin-FITC/mg microspheres)
- Monomer (peptide MPIGRIAECIL, kindly provided by the Immunology Department of Tübingen University), stock concentration of 2 mg/mL
- Anti-CD28 biotinylated antibody (kindly provided by the Immunology Department of Tübingen University), variable stock concentration.

Procedure:

All the procedure was conducted under sterile conditions.

Day 1

PBMCs were isolated from whole blood or Buffy coats through the Ficoll-Hypaque density centrifugation method (see above).

Cells were counted and then cultured overnight with TCM at a concentration of 1-2*10^8 PBMCs in 20 mL in a T 75 (standing) or at a maximal concentration of 10^8 PBMCs splitted into two T175 (lying) with 25 mL of T cell medium each, with 2,5 ng/mL IL-7 and 10 U/mL IL-2.

Day 2

PBLs were harvested from cell flasks into 50 mL tubes and flasks were rinsed firmly with PBS to collect all loosely attached PBLs. Cells were then centrifuged (1300 rpm, 7 minutes, RT) and counted.

CD8 positive cells were isolated either through the Easy Sep Human CD8 positive selection Kit II (Stem Cell Technologies, Cologne, Germany) or the Miltenyi CD8+ T cell isolation kit (Miltenyi Biotech, Bergisch Gladbach, Germany), according to manifacturers´instructions, and then cultured overnight with TCM with 2,5 ng/mL IL-7, 10 U/mL IL-2 and 2 uL DNase, at a concentration of 1-2*108 cells in 20 mL medium in a T 75 (standing).

Day 3

Loading of Beads

(all steps on ice and sterile)

1) Beads ‘washing
    - For each stimulation 200000 beads/well were needed, and there should be enough beads for 4 stimulation (3 according to the protocol + 1 extra as reserve). The stock concentration of the beads was 100000 beads/uL.
    - The amount of needed beads was calculated according to the formula: [number of wells * 1,1 * (Beads/well + 30% loss)]/ bead stock concentration→ number of wells *1,1 *(1040000)/100000.
    - The respective amount of beads was washed in a 15 mL Falcon tube by filling up to 15 mL with MACS buffer.
    - Beads were centrifuged (10 minutes, 2500 rpm, 4°C)
    - This washing step was repeated
    - Beads were resuspended in MACS buffer (100 uL/well + 200 uL extra buffer).
2) MHC Molecules dilution

- For each stimulation 200 ng monomer/well were needed, and there should be enough monomer for 4 stimulation (3 according to the protocol + 1 extra as reserve). The stock concentration of the monomer was 2 mg/mL.
- The volume of needed monomer was calculated according to the formula: number of wells * 1,1 * 50 uL + 200 uL loss.
- The monomer was then diluted to 200 ng/50 uL (desired concentration) in MACS buffer.

3) Anti CD28 biotinylated antibody dilution
   - For each stimulation 600 ng antibody/well were needed, and there should be enough monomer for 4 stimulation (3 according to the protocol + 1 extra as reserve). The stock concentration of the antibody was batch-dependent.
   - The volume of needed antibody was calculated according to the formula: number of wells * 1,1 * 50 uL + 200 uL loss.
   - The antibody was diluted to 600 ng/50 uL (desired final concentration) in MACS buffer.
4) Loading of beads
   - 96 wells plates (Costar) were used
   - 100 uL of diluted beads and 50 uL of respective diluted monomer were added to each well, mixed, and incubated for 30 minutes at room temperature while shaking.
   - 50 uL of diluted anti CD28 biotinylated antibody were added to each well, mixed, and the plate was incubated for other 30 minutes at room temperature while shaking.
   - The plate was centrifuged (2 minutes, 2500 rpm, 4°C) and then sterile flicked
   - 150 uL/well of MACS buffer were added and resuspended once
   - The plate was once more centrifuged (2 minutes, 2500 rpm, 4°C) and then sterile flicked; the washing step was repeated for a total of 4 times.
   - After the last washing step, the beads were diluted in 200 uL MACS buffer/well, enveloped with aluminum foil and stored at 4°C (beads stock plate).
5) Cell plating
   - CD8 T cells were harvested in a 50 mL Falcon tube and flasks were rinsed with PBS

- Cells were centrifuged (1300 rpm, 8 minutes, RT), resuspended in PBS, counted, centrifuged again (1300 rpm, 8 minutes, RT) and diluted with TCM to a concentration of 10^6/100 uL.
- Cells were plated in the 96 wells plate (only the inner wells were used, and the outer were filled with PBS).
- The plate was then incubated at 37°C.

6) Cells stimulation
   - The beads stock plate was resuspended.
   - 50 uL were taken out, transferred into a new plate and 100 uL of cold TCM without human serum were added.
   - The plate was centrifuged at 2500 rpm for 2 minutes and washed once more with 150 uL of cold TCM without human serum.
   - After this washing step, 100 uL/well of warm TCM with 5 ng/mL of IL-12 (final concentration, calculated in a final volume of 200 uL/well) were added.
   - Beads were resuspended and transferred to the T-cell plate. T-cells and beads were mixed by carefully pipetting up and down twice, and the plate was incubated at 37°C.

Day 5 or 6.

- 100 uL/well of supernatant were removed
- 100 uL/well of fresh TCM + 40 U/mL of IL-2 (final concentration) were added.

This passage was repeated on day 12 (13) and 19 (20).

Day 10 and 17 (Cells restimulation)

The passage 6) of day 3 was repeated, exactly in the same way, on day 10 and 17.

Tetramerstaining

Materials:

- FACS Buffer (see above)
- PBS-EDTA: 500 mL PBS, 2mM EDTA
- Tetramer-staining Buffer (TSB): 50% PBS, 50% FBS (Fetal Bovine Serum, Gibco Thermo Fischer Scientific, Waltham, MA, USA), 2 mM EDTA.
- Aqua live

- Tetramer (prepared through polymerization of monomers of the peptide MPIGRIAECIL, kindly provided by the Immunology Department of Tübingen University)

Procedure

All passages were carried out on ice, and under sterile condition if the staining had the purpose to sort thereafter the cells and use them for further experiments.

All the centrifugation steps were carried out at 4°C, 1800 rpm, 2 minutes.

Preparation

- About 0,5-1*106 cells pro-well were transferred into a 96 wells plate (round bottom), the plate was centrifuged and the supernatant discarded.

Live/death staining

- Cells were resuspended in 150 uL/well of PBS-EDTA, centrifuged and the supernatant was discarded
- The washing step was repeated
- The live/death staining mix was previously prepared: Aqua live/dead 1:400 or 1:200 was diluted in PBS-EDTA.
- The cell pellets were resuspended in 50 uL/well of live/death staining mix and incubated for 20 minutes at 4°C in dark.

Tetramer Staining

- 150 uL of PBS-EDTA were added to each well
- The plate was centrifuged and the supernatant discarded
- The tetramer staining mix was previously prepared: 2,5-5 uL/mL of tetramer were dluted in TSB, vortexed and centrifuged for 5 minutes at °4C at 13000 rpm. In case that frozen tetramer was used, 1 uL was diluted in 64 uL TBS to obtain a concentration of 5 ug/mL, in case of fresh tetramer, 1 uL was diluted in 96 uL TBS).
- The cell pellets were resuspended in 50 uL/well of tetramer staining mix and incubated for 30 minutes at 4°C in dark.

Extracellular staining

- 150 uL of FACS Buffer were added to each well
- The plate was centrifuged and the supernatant discarded

- The extracellular staining mix was previously prepared (the antibodies were diluted at the desired concentration in FACS Buffer)
- The cell pellets were resuspended in 50 uL/well of extracellular staining mix and incubated for 20 minutes at 4°C in dark.
- 150 uL of FACS Buffer were added to each well
- The plate was centrifuged and the supernatant discarded
- The washing step was repeated
- The cells were resuspended in 200 uL FACS buffer and transferred into FACS tubes, in case the FACS analysis took place the same day. In case the analysis was postponed, cells were fixed

Cell fixation

- After the last washing step, cells were resuspended in 100 uL/well of FACS-fix (FACS buffer + 1% Formaldehyde) and incubated 20 minutes at 4°C in darkness.
- 100 uL/well of FACS Buffer were added, the plate was centrifuged and the supernatant discarded.
- The washing step with FACS Buffer was repeated
- Cells were resuspended in 200 uL/well of FACS Buffer and stored, for a maximum of 3 days, at 4°C in darkness.

N.B. **Tetramer preparation**:

Materials

25 uL of monomer of the peptide of interest at the concentration of 2 mg/mL (kindly provided by the Immunology Department of Tübingen University, stored at -80°C)
Streptavidin R-phycoerythrin conjugate (SAPE) (Molecular Probes, Eugene, Oregon, USA, stock concentration 1 mg/mL).

Procedure

7,8 uL of Streptavidin were added to the monomer every 20 minutes for a total of 10 times, under continuous rotation at °4C, and then stored at 4°C until needed.

T-cell stimulation protocol

Materials:

T-cell Medium: IMDM with L-Glutamine (Lonza Verviers, Belgium), 10% Human Serum (Invitrogen, or kindly provided by the Transfusion Medicine Department of Tübingen University Hospital), 50 µM beta-ME (), 1% Penicillin-Streptomycin (Biochrom, Berlin).

Thawing medium (see above).

IL-2 (Proleukin ®, Novartis, Basel, Switzerland, stock concentration 20000 UI/mL)

IL-7 (Peprotech, Rocky Hill, NJ, USA, stock concentration 10 ug/mL)

Procedure:

All the procedure was carried on under sterile conditions.

Day 0, late afternoon:

- Cells were thawed (according to the above reported protocol)
- 2,5-3x10^6 cells/well in 250 uL of T-cell medium (10*10^6/ml) were plated on a 48-well cell culture plate. In case of small amounts of cells, a 96 wells plate (round bottom) could also be used (400000-800000 cells/well in 100 uL of medium).
- Plates were placed in the incubator tilted at 45°.

Final volume/well: 250 uL or 100 uL.

Day 1, morning

- 250 uL of T-cell medium and 1 µg/ml (concentration in the final volume) MHC class I Peptides und 10 µg/ml (concentration in the final volume) class II Peptides. 50 uL of T-cell medium and peptides with the same end-concentration in case a 96 wells plate was used.

Final volume/well: 500 uL or 150 uL.

Day 2:

- 250 uL (50 uL for the 96 wells/plates) with IL-2 10U/mL (concentration in the final volume) and IL-7 10 ng/mL (concentration in the final volume) were added to each well.

Final volume/well: 750 uL or 200 uL.

Day 5:

- 250 uL (50 uL for the 96 wells/plates) with IL-2 10U/mL (concentration in the final volume) and IL-7 10 ng/mL (concentration in the final volume) were added to each well.

Final volume/well: 1 mL or 250 uL.

Day 7:

- 250 uL (50 uL for the 96 wells/plates) of medium were removed from each well and replaced with an equal volume of fresh T-cell medium with IL-2 10U/mL

(concentration in the final volume) and IL-7 10 ng/mL (concentration in the final volume). In case of high metabolic turnover (medium quickly yellow) 500 uL (100 uL for the 96 wells plates) of medium were taken out and replaced or wells were splitted 1:1.

Final volume/well: 1 mL or 250 uL.

Day 9:

- 250 uL (50 uL for the 96 wells/plates) of medium were removed from each well and replaced with an equal volume of fresh T-cell medium with IL-2 10U/mL (concentration in the final volume) and IL-7 10 ng/mL (concentration in the final volume). In case of high metabolic turnover (medium quickly yellow) 500 uL (100 uL for the 96 wells plates) of medium were taken out and replaced and not splitted anymore.

Final volume/well: 1 mL or 250 uL.

Day 10-11:

- 250 or 500 uL (50 or 100 uL for the 96 wells/plates) of medium were removed from each well and replaced with an equal volume of fresh T-cell medium without cytokines.

Final volume/well: 1 mL or 250 uL.

Day 12:

- Cells were prepared and overnight stimulated, according to the ICS protocol

Day 13:

- Cells were stained according to the ICS protocol and FACS analysis was performed.

T-cell clones generation

Overview:

Day 1: Plates' preparation

Day 2: Cell staining and sorting

Day 8: First medium change, then every 3-4 days

Day 14: Cell feeding, then every 2 weeks.

Materials

- MACS Buffer (see above), sterile filtered
- T cell Medium (TCM; see above)

- IL-2 (Proleukin ®, Novartis, Basel, Switzerland, stock concentration 20000 UI/mL)
- PHA-L (Sigma-Aldrich, stock concentration 1 mg/mL, diluted in PBS).
- LG2-EBV cell line, cultured for at least one week before sorting in its own medium (IMDM + 10% FBS+ 1% Penicillin-Streptomycin, Biochrom, Berlin) at a density of about 2-3*10^6 cells/mL in standing T 75 cell culture flasks (non-adherent cells).

Procedure

Day 1

The sorting was carried out in 96 wells plates(round bottom), containing 150 uL of TCM with irradiated feeder cells and IL-2 (150 U/mL) and PHA-L (1 ug/mL). Pro clone were prepared 2-5 plates à 1 cell/well.

As feeder cells were used:

- 0,15*10^6/well Human fresh PBMCs of three different donors, obtained from whole blood or Buffy-coats through the Ficoll-Hypaque density centrifugation method, irradiated (60 Gy) and washed 2-3 times with TCM
- 0,015*10^6/well LG2-EBV cells, irradiated (200 Gy), and washed 2-3 times with TCM.

Day 2

The cells to be sorted were stained under sterile condition according to the chosen sorting strategy (Tetramer staining or surface marker staining or both). The stained cells, together with compensation beads and the plates were then sorted using the BD FACS Aria instrument (BD Biosciences, San Diego, CA, USA), aiming to seed a single cell/well.

Day 8 (7 days after sorting)

50 uL pro-well of T cell Medium and IL-2 at the final concentration of 150 U/mL were added to the plates, and thereafter every 3-4 days.

Day 15 (14 days after sorting)

Cell were with the same modalities of day 1 (same amounts of cell, cytokines and medium), thus 1:2 splitted. Thereafter, cells were periodically screened for contamination and growth, and fed every 14 days until the they could be tested (at least 4 weeks after sorting).

HLA B*07 testing

A cohort of 7 patients, for which enough material was available, was tested for the presence of HLA-B*07 allele by mean of low resolution molecular typing (SSO/SSP method, HLA-

Ready Gene B Low 055 kit used, performed by the Transfusion Medicine Department of Tübingen University Hospital).

DNA was extracted from the frozen available material (peripheral PBMCs or bone marrow blasts) using the innuPREP DNA Mini kit ® (Analytik Jena, Jena, Germany), according to the manufacturer instructions.

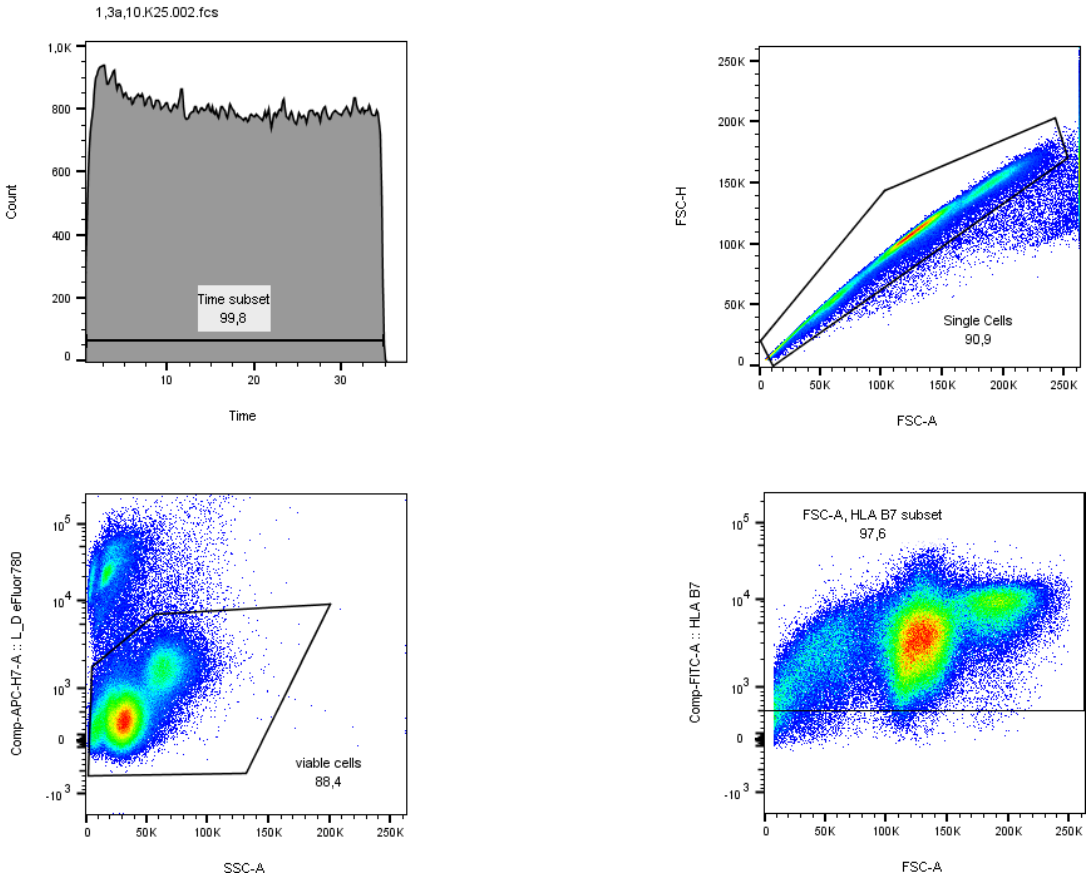
A cohort of 12 patients was tested for the expression of the HLA B*07 antigen by mean of flow cytometry. Not stimulated, in culture PBMCs (one or two days after thawing) were stained with the monoclonal mouse Anti-human B7-FITC Antibody (Bio Rad, München, Germany).

Cells were also stained with eFluor 780 (Live-death staining) according to the above described staining protocol for surface markers.

PBMCs of a donor with known positivity were always co-stained and used as positive control.

The gating strategy and a comparison between a positive and a negative control is shown in the figure below:

a)



b)

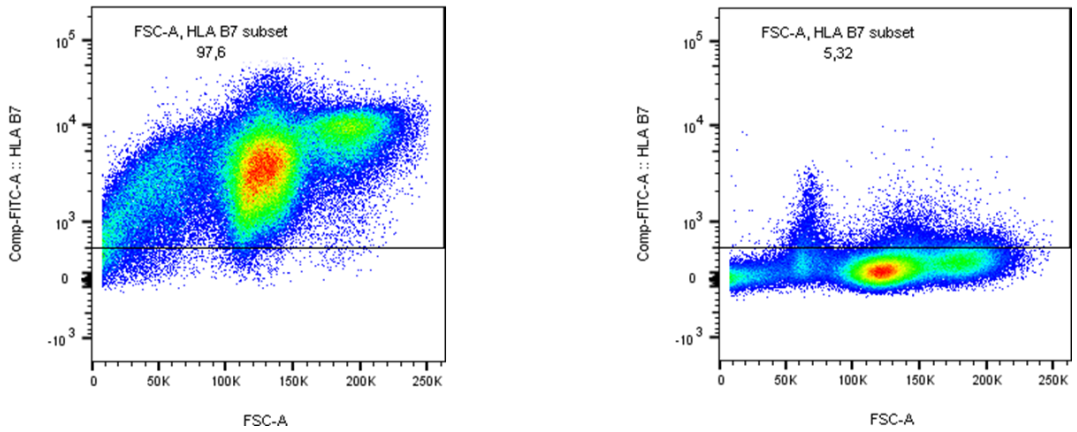


Fig. 4.3. HLA B*07 flow-cytometric typing. a): gating strategy; b) positive (left) and negative (right) control.

6. Results

6.1 Priming experiments with healthy donors

General considerations

Of the 8 Priming experiments, 6 were carried out according to the DCs Priming Protocol, two according to the Beads Priming Protocol.

The mean peripheral blood mononucleated cell (PBMC) content obtained after isolation from the blood products was $15,81*10^8$ cells (range 32,1-3,28).

DC Priming Experiments

Efficacy of DC generation

A flow-cytometry control aiming to estimate the number of obtained dendritic cells was performed for every experiment approximately 2 weeks after the beginning.

Modalities and gating strategy are described above.

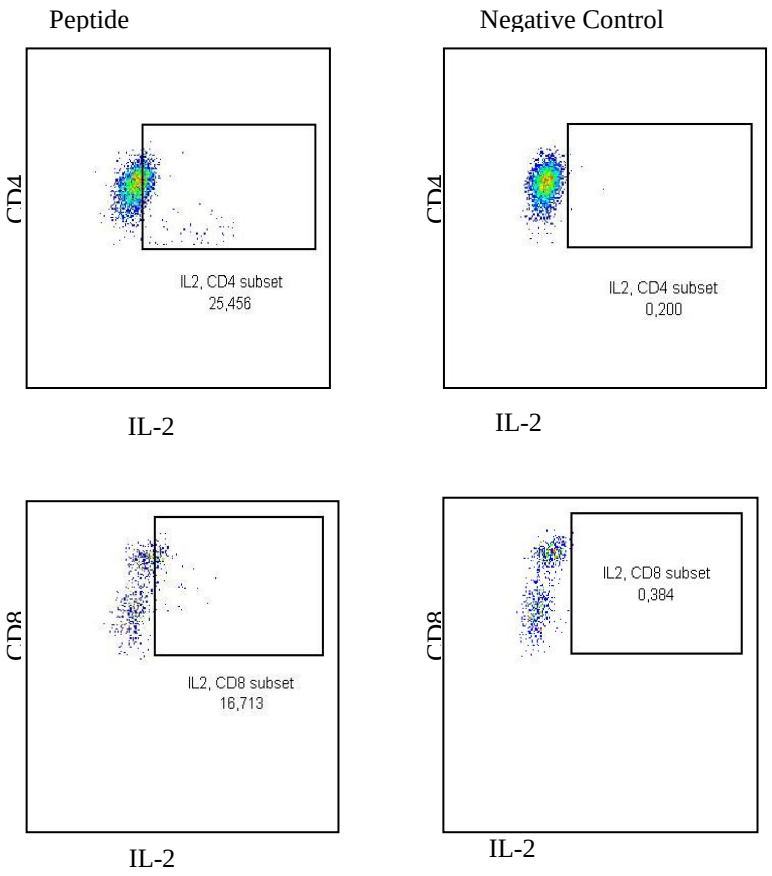
The median percentage of cells of interest, gated on the base of FSC and SSC, was 68,86% (4,77-99,98%). Among them, the median percentage of non-T-lymphocytes and non-monocytes (CD3-CD14- cells) was 79,85% (3,81-98,84%); the median percentage of CD83 and CD86 double-positive cells was 50,59% (29,12-98,87%); the median percentage of CD80 and CD83 double positives was 29,13% (18,13-82,07%).

Successful primings

Two of the 6 experiments showed T-cell activation after priming.

In detail, the first donor showed a CD4+ and CD8+ T-cell response to a class I peptide (MPIGRIAECIL) consisting in the intracellular production of IL-2 and TNFα (Fig. 6.1 a) and b)); and a CD4+ T-cell response to a class II peptide (HAMPIGRIADASTSRRFT), consisting in the intracellular production of cytokines/markers of activation, especially TNFα (Fig. 6.2), the second donor showed a CD4+ T-cell response to the same class II peptide (HAMPIGRIADASTSRRFT), through the production of TNFα, CD154 and IFNγ (Fig. 6.3)

a)


Peptide
Negative Control
CD4
IL2, CD4 subset
25,456
IL2, CD4 subset
0,200
IL-2
IL-2
CD8
IL2, CD8 subset
16,713
IL2, CD8 subset
0,384
IL-2
IL-2

b)

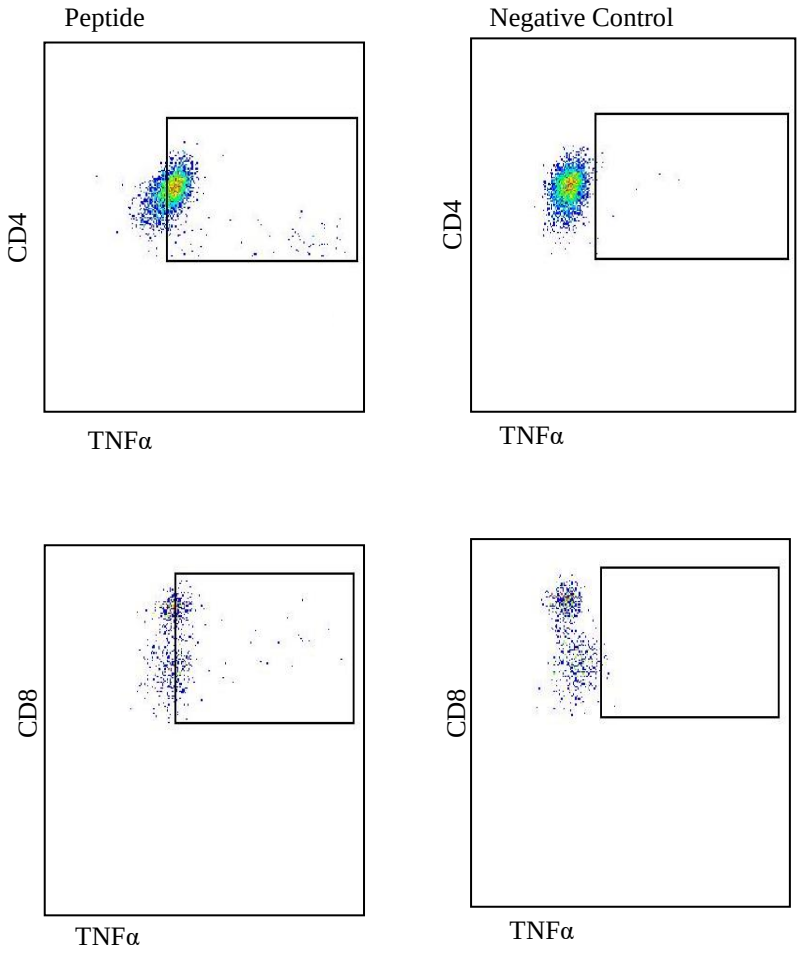

Peptide
Negative Control
CD4
CD4
TNFα
TNFα
CD8
CD8
TNFα
TNFα

Fig. 6.1 Dendritic cells-mediated priming, first donor (A). Reactive cells against the class I restricted peptide MPIGRIAECIL. The CD4 and CD8 response was more evident on the production of IL-2 (a)) and TNFα (b)). The negative control is always showed for comparison.

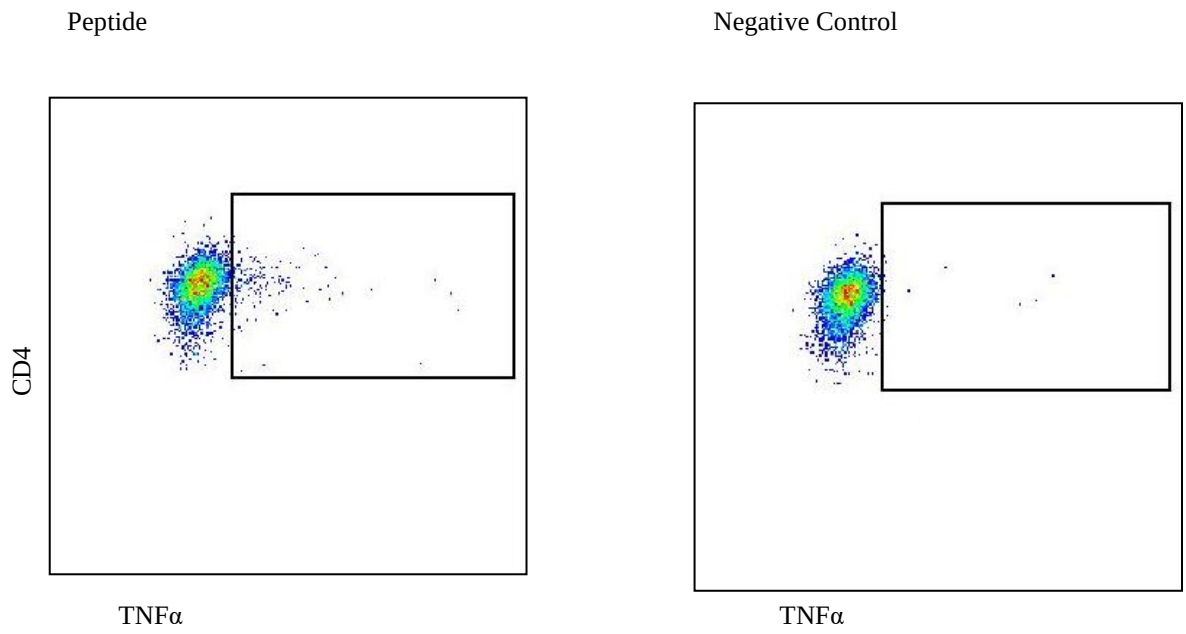
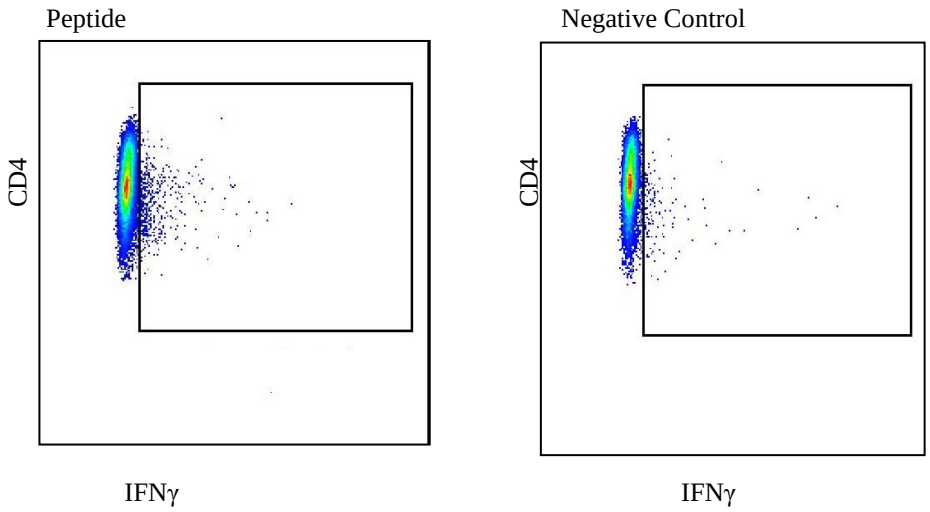
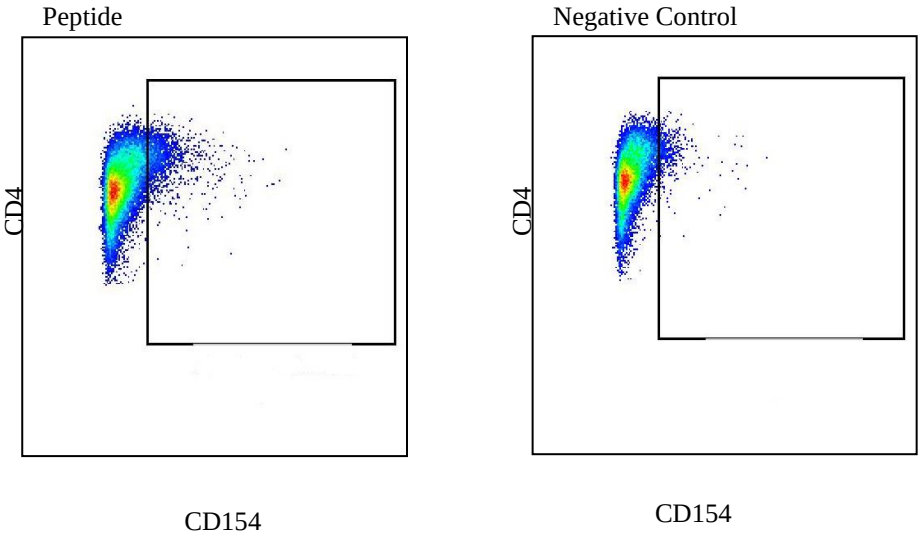


Fig. 6.2. Dendritic cells-mediated priming, first donor (A). Reactive cells against the class II restricted peptide HAMPIGRIADASTSRRFT. The CD4 response was more evident on the production of TNFα. The negative control is showed for comparison.

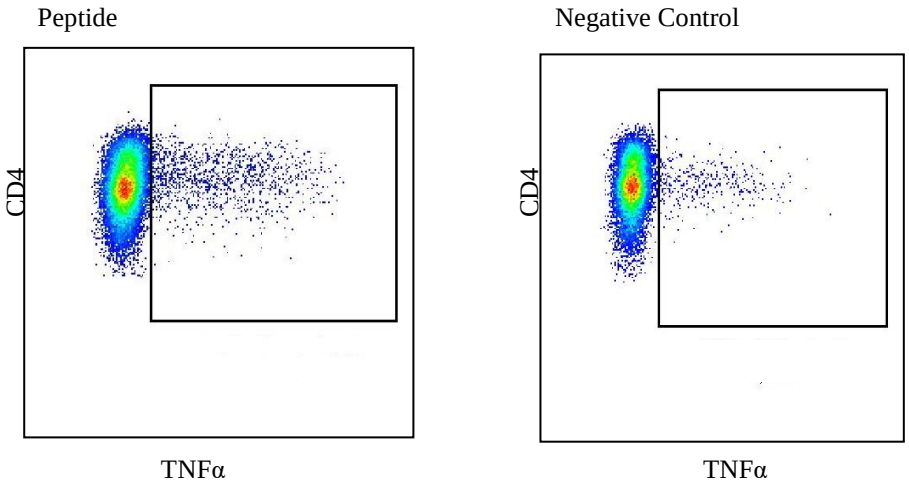
a)


Peptide
Negative Control
CD4
CD4
IFNγ
IFNγ

b)


Peptide
Negative Control
CD4
CD4
CD154
CD154

c)


Peptide
Negative Control
CD4
CD4
TNFα
TNFα

d)

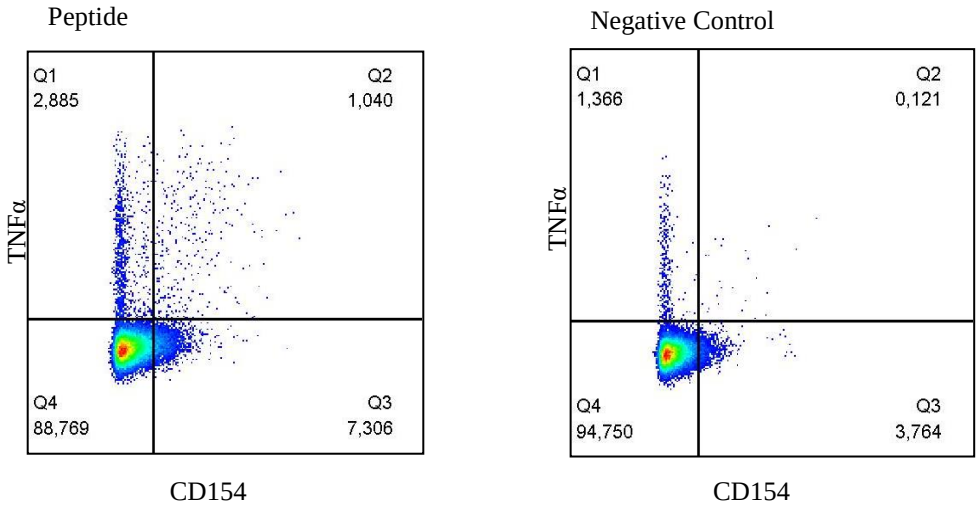


Fig. 6.3. Dendritic cells-mediated priming, second donor (B). Reactive cells against the class II restricted peptide HAMPIGRIADASTSRRFT. The CD4 response was more evident on the production of IFNγ (a), CD154 (b) and TNFα (c), CD154 and TNFα double positives are showed in d. The negative control is showed for comparison.

T-cell cloning

The generation of T-cell clones from the reactive cells was attempted.

As described above, a flow-cytometry sorting method with the aim to obtain a *single cell culture* was used.

Cells were re-stimulated (according to the ICS protocol) the day before staining and sorting. For CD4+ T-cells sorting, the marker OX-40, together with CD4, was chosen for cell identification, while for CD8+ T-cell sorting, a combination of extracellular staining with CD8 and 4-1BB and tetramer-staining (with the tetramerized MPIGRIAECIL peptide) was performed to identify the reactive cells.

Notably, in the case of the first donor, cells to be sorted were previously frozen and thawed the day before sorting (210000 and 375000 cells pro-peptide were obtained, respectively), while in the case of the second donor they were kept in culture and additionally restimulated according to the DCs priming protocol 3 more times before sorting (cultured for a total of about 3 months). The cell number at the beginning was $1,48*10^6$.

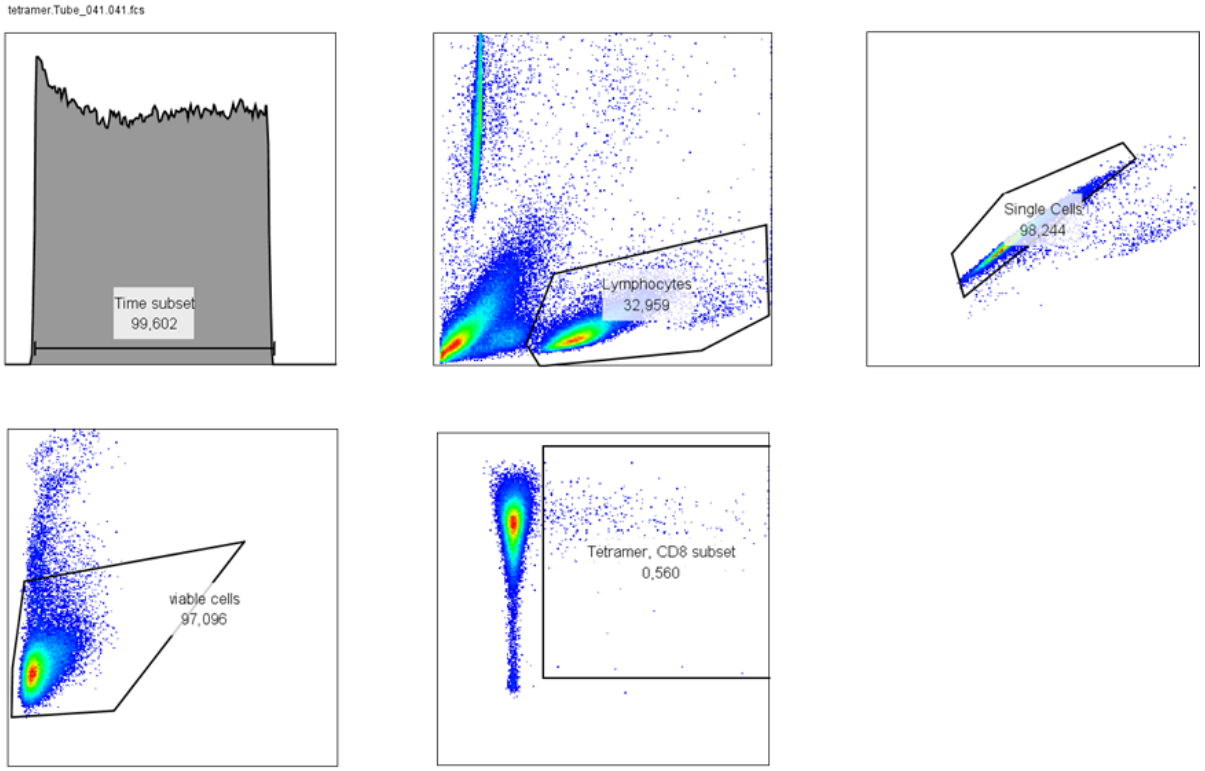
Cloning was successfully performed: two 96 wells plates at 1 cell/well (34 effective wells) were obtained for the first donor and 3 plates at 1 cell/well (165 effective wells) were obtained for the second donor.

Cells were kept in culture and periodically fed (according to protocol) for a total of almost 2 months. Visual inspection was performed regularly. A total of 36 clones which showed microscopic evidence of growth were selected for testing.
A restimulation with the corresponding peptides, and with peptides against which no reaction was previously showed as negative controls, was performed the day before staining.
Testing was performed twice, the first time looking at the expression of surface OX-40 (for CD4+ T-cells) and 4-1BB (for CD8+ T-cells) and the second time looking at the intracellular production of cytokines (according to the standard ICS protocol for stimulation and staining), but unfortunately, no specific activation of T-cell clones was observed, because no difference in the percentage of activated cells between cells of interest and negative controls could be showed.
Hence, cells could not be used for functional testing and were not further cultured.

Beads Priming experiment

Two priming experiments were performed according to the Beads Priming Protocol (see above). After PBMCs isolation, CD8+ cells were immunomagnetically positively selected, in one case through the Easy Sep Kit, and in the other case trough the Miltenyi Biotech MACS. The CD8+ T-cell recovery was 12,9% and 17,8% respectively.
At the end of the first experiment, a positive answer in a few wells (two) was observed, as it is showed in figure 6.4.

a)



b)

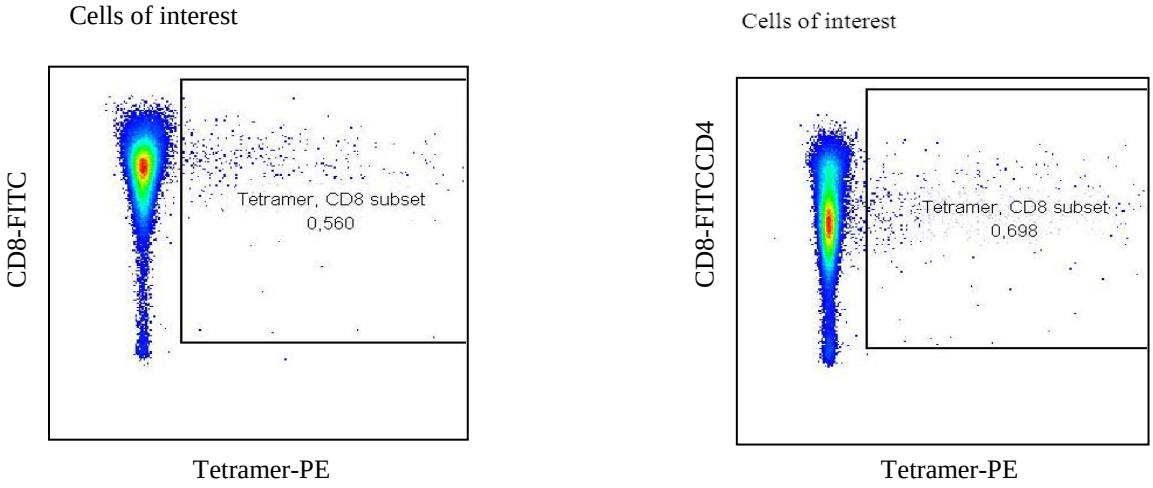


Fig. 6.4. a) Gating strategy (notably, cells were not stained for CD4 since they were CD8 positively pre-selected). PE positive CD8+ T lymphocytes were identified as reactive cells. b) The two reactive wells, compared with a negative control.

Unfortunately, it was impossible to sort those cells, even if the staining for sorting was performed 3 days later under identical condition. Actually no positive cells to sort (which means PE stained) were detectable anymore.
At the end of the second experiment, an answer was shown in a few wells (a total of 4, see figure 5.5) and such cells were successfully sorted after tetramer staining.

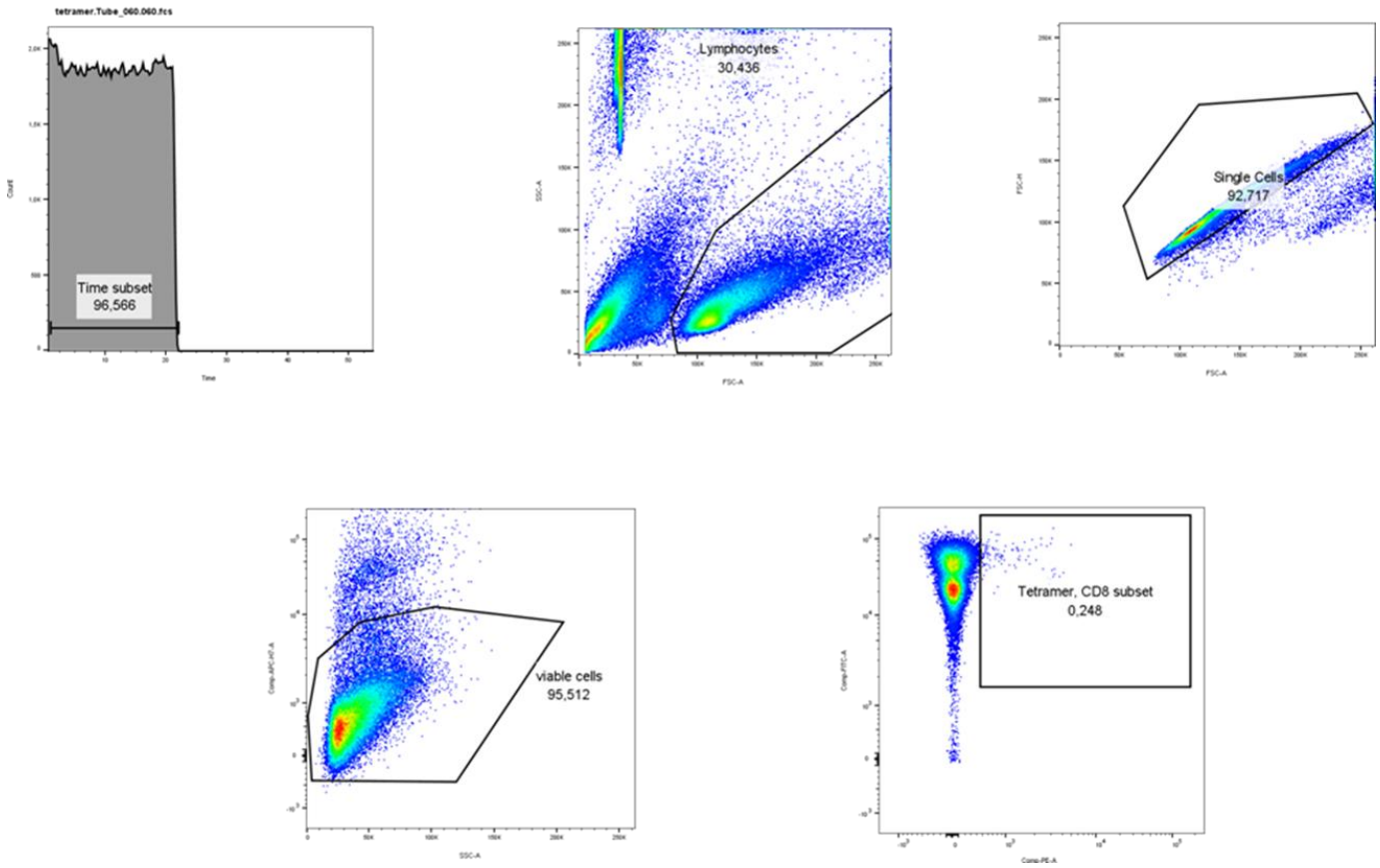


Fig. 6.5. Reactive well after Beads priming. Only the well with the higher number of reactive cells is shown.

Twelve clones showed microscopic evidence of growth after 4 weeks of culture and were tested through another tetramer staining, but unfortunately they showed no evidence of specificity (Fig. 6.6).

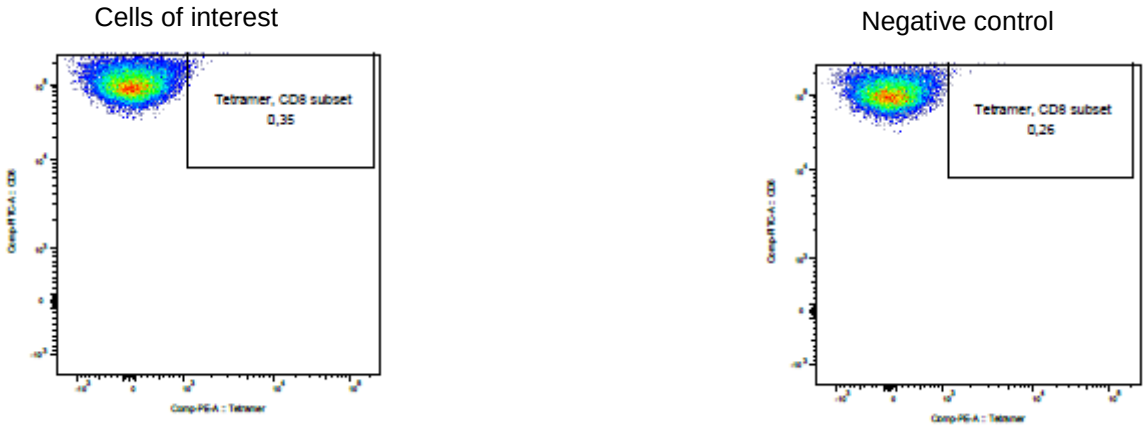


Fig. 6.6. Results of clones' testing. On the left, a clonal population stained with the MPIGRIAECIL tetramer is shown; on the right, the same population is stained with another tetramer built with another non-cross reactive peptide. There is no difference in the percentage of reactive cells (no PE positive cells) in the two samples.

6.2 Immunomonitoring of patients

The mean peripheral blood mononucleated cells (PBMC) content obtained after isolation from blood samples was $12,83*10^6$ cells (range 3-25,45).

The mean cell count after thawing of frozen samples was $6,47*10^6$ cells (range 0,42-9,45).

Among the cohort of patients who were tested by mean of RT-PCR for HLA-B*07 allele, only one resulted positive. None of the patients tested through flow-cytometry resulted positive. For two patients, there was not enough material to perform an HLA-B*07 test, with any method.

In none of the patient a T-cell activation in response to any of the fusion peptides was observed.

7. Discussion

According to the initial specific objectives, only the first goal of this project has been achieved, i.e. we were able to show that T-cell priming against fusion peptides is feasible.
We used sequentially two different priming methods: dendritic cells mediated and beads mediated priming.
The latter method showed clear advantages: it is faster, it allows a greater contact APC-lymphocytes (proportion 1: 5 vs 1:10 for dendritic cells mediated protocol) and a more controlled exposure to peptides. The only limitations are that only one peptide at once could be tested and only CD8+ response can be evaluated, since only one MHC class I-peptide monomer was available.
A T-cell activation was detected in 2 out of 6 priming experiments with dendritic cells, and in 2 out of 2 experiments with the beads protocol.
Although the frequency of reactive cells was extremely low (as one can see from the pictures in the result section), the answer we observed was reproducible since we were able to sort such cells.
We were not able to successfully accomplish the subsequent goal, which was the generation of T-cell clones specific for the fusion peptides, since the T-cell population we cultivated did not confirm the initial specificity when re-tested.
The process of T-cell cloning is made up of different phases, which could be summarized in sorting, culture and evaluation.
As possible explanations for T-cell cloning's failure, we hypothesize a problem in sorting. It may be that the used gate was too permissive, so that cells which were not truly reactive, were selected, seeded and occasionally grew.
In the two successful dendritic cell priming experiments, we used for sorting two activation markers (OX-40 and 4-1BB) which differed from the markers we used in the first ICS to evaluate the response (intracellular cytokines). This choice was due to the necessity of using the selected cells for further experiments (OX-40 and 4-1BB are surface markers), but it may be that the reliability of the two different kind of markers in identifying peptide-specific T-cells was not the same.
Moreover, T-cell priming as well as T-cell cloning process are based on long and complex cell culture protocols. This implicates that many variables could have affected the T-cell activation status, that was our main outcome indicator: environmental conditions, the

effective amount of peptides and cytokines to which T-cells were exposed, quality of medias and feeder cells, timing chosen for testing, operator's experience.

Target populations of the present study were T-cells with a single peptide specificity, supposed to be naïve in the healthy donors. It is intuitive that such populations are extremely rare in peripheral blood, and although we had a big number of cells in the beginning, the probability to randomly include them in the blood sample was very low. Moreover, given that one could successfully have sampled such populations, it is not always possible (especially with the dendritic cells protocol) to create the adequate conditions, in term of peptide exposure, to activate and expand them.

As for the third specific objective, the demonstration of a clear and specific T-cell activation in response to fusion peptides in patients with B-lineage TEL/AML1 positive leukaemia was also not possible.

An important issue to explain such finding may be the underrepresentation of HLA B*07 in our patients' cohort.

In our study, most patients lacked the prerequisite for presentation of the peptides with the best prediction score, i.e. the expression of HLA B*07. Even in the only B*07 patient, however, no response was detected.

We could not demonstrate an autologous T-cell activation against any of fusion peptides, and it is uncertain whether it may be related to the low affinity and specificity of the non-HLA B*07 restricted peptides, to the method we chose for T-cell stimulation and response detection, or to technical issues (culture conditions etc.).

<u>Suggestions to improve the results of the study.</u>

As for T-cell priming, we suggest to switch to beads priming method. The use of such protocol is limited by the availability of monomers, and its systematic exploitation implies greater costs and efforts, but in our (although limited) experience it showed clear advantages in term of shorter duration and efficacy.

As for the T-cell cloning, we suggest to use restrictive gates to select the reactive cells.

As for patients' immunomonitoring, repeated blood sampling in the same patients, at different time points, could be an option to increase the pool of antigen specific T-cell subsets and to augment the probability of randomly sample fusion peptides-reactive clones. Such approach could theoretically also allow the detection of a temporal pattern of T-cell activation.

Another hint to overcome the difficulties we faced could be switching to other activation markers (such as CD25, or PD1-L1[31]) or to another response detection method (such as Elispot).

There is growing evidence that HLA B*07 restriction is crucial for peptide presentation and recognition by T-cells. This is true for viral peptides, solid-tumours associated peptides, and also leukaemia associated peptides.[32,33,34]

The elegant study of Jahn et al.[34] shows how autologous cytotoxic T lymphocytes (CTLs) can be generated through transduction of an exogenous TCR, cloned from a T-cell clone reactive against a HLA B*07-CD22-derived peptide complex. Such paper however, like all the studies on this subject, investigates immunogenicity of the complex HLA molecule-leukaemia associated peptide in an allogenic setting, i.e. in a situation of HLA mismatch ($B*07^{neg}$ effector cells and $B*07^{pos}$ target cells).

This implies that CTLs recognize the HLA-peptide complex in a specific fashion, more than the peptide itself, and that a certain degree of alloreactivity is involved in such recognition, even if it is not entirely responsible.[32]

This is in contrast with one of the premises of our study (we wanted to show an autologous, spontaneous immunological response against fusion peptides in patients, and used autologous APC for T-cell priming, with non-completely satisfactory results) but may be a suggestion for the future direction of the study and opens the way to possible clinical applications (CTLs from the patient himself or from the HSCT donor or third part donors as adoptive immunotherapy) other than peptidic vaccines.

However, further experiments are warranted to verify (or to abandon) the hypothesis of an autologous, spontaneous, in vivo T-cell response against TEL/AML1 fusion peptides as a possible explanation of the good patients' outcome.

8. Conclusion

In this laboratory research project, we tried to investigate whether the good prognosis of patients with TEL/AML1 positive B-lineage ALL is related to an immune response against peptidic antigens derived from the mutant TEL/AML1 protein.

We tried to artificially reproduce the phenomenon with healthy donor's lymphocytes, through exposure to the fusion peptides in the so called *in vitro* priming. Our purpose, in case of success of the procedure, was to generate T-cell clones from the reactive cells to test functionally against TEL/AML1 positive lymphoblasts or peptide-loaded cells.

We also planned to look for such immune response in donors naturally exposed to peptides, i.e. patients who had had a TEL/AML1 positive B-ALL.

We accomplished only the first of the study's objectives, i.e. we showed that a T-cell priming against TEL/AML1 mutant protein fusion peptides is feasible. We were not able to generate T-cell clones from primed T-cells and we could not show a T-cell activation against fusion peptides in patients who have had a TEL/AML1 positive B-cell leukaemia.

We tried to explain our not completely satisfactory results with some technical issues, such as problems in the identification of reactive cells or in the sampling of low frequency T-cell populations, and suggested some corrective measures which could be applied to future experiments.

We think that the principle of the exploitation of fusion peptides derived from mutant proteins as immunotargets is of value and deserves further investigations. Given that a spontaneously arising autologous T-cell response against fusion peptides has yet to be denied, an alternative possible approach to continue and ameliorate our study might be T-cell priming with a HLA B*07 mismatch between effector cells and APCs, in order to exploit alloreactivity to augment the priming efficacy. This opens the way to a different perspective for clinical application, other than peptidic vaccines realization, i.e. TCR cloning of reactive cells and its transduction on autologous or heterologous T-cells.

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CD34+ selected stem cell boosts can improve poor graft function after allogeneic stem cell transplantation in paediatric patients

1. Abstract

Poor graft function (PGF) is a possible complication after hematopoietic stem cell transplantation (HSCT). Administration of stem cell boosts (SCBs) from the original donor represents one of the available therapeutic options. We performed a retrospective study on 50 paediatric patients with PGF who received a total of 61 boosts with CD34+ selected peripheral blood stem cells (PBSC) after transplantation from matched unrelated (n = 25) or mismatched related (n = 25) donors, aiming to evaluate safety and efficacy of the procedure and its possible impact on survival. Within 8 weeks after stem cell boosting, we observed a significant increase in median neutrophil counts (600 *vs* 1516/mm^3, p <0.05) and a decrease in erythrocytes and thrombocytes transfusion requirement (median frequencies 1 and 7 *vs* 0, p< 0.0001 and < 0.001), and a resolution of one or two of their initial cytopenias in 78.8% of the patients, whereas 36.5% of them had a complete haematological response. We also were able to show that median lymphocyte counts for CD3+, CD3CD4+, CD19 and CD56+ increased 8.31fold, 14.16fold, 22.34fold and 1.6fold, respectively. An enhancing effect on maturation of committed lymphoid precursors already present in the host may be hypothesized to explain that finding.

The rate of *de novo* acute GvHD grade I–III was 6% and resolved completely after treatment. No GvHD IV or chronic GvHD occurred. Patients who responded to SCB displayed a trend toward better overall survival (OS) (p= 0.07). In conclusion, administration of CD34+ selected SCBs from alternative donors is a safe and effective procedure. Further studies are warranted to better define the impact on immune reconstitution and survival.

2. Introduction

Many malignant and non-malignant diseases can nowadays be cured with hematopoietic stem cell transplantation (HSCT) from matched unrelated or full haplotype mismatched related donors.

A possible complication of HSCT is Poor Graft Function (PGF), defined as at least bilinear severe cytopenia, and/or transfusion requirement, which occurs in a situation of full donor

chimerism (thus differentiating from graft rejection) in absence of disease relapse and severe acute or chronic Graft-versus-Host Disease (aGvHD or cGvHD). It is a relevant problem since frequencies from 1% to 27% have been reported and its occurrence is associated with a high mortality rate due to severe infections and bleeding.[1, 2, 3]

The probability to develop PGF is influenced by several factors, such as underlying disease, donor type, degree of HLA-matching, ABO incompatibility, intensity of conditioning, stem cell dose and source, prior GvHD, infections, exposure to myelotoxic drugs and others.[1]

Available treatment options for PGF are use of growth factors such as G-CSF, erythropoietin and thrombopoietin,[4,5] retransplantation[6] and stem cell boost (SCB) infusion.[1, 3, 7]

The use of SCBs carries the risk of GvHD induction: options to avoid it are to administer another pharmacological immune suppression after boosting [7] or to perform ex vivo T cell depletion of the SCBs.

Options for ex vivo T-depletion include CD133+ or CD34+ positive selection of donor PBSCs and, as recently reported, the use of CD3+ or TcR αβ T-cell depletion.[8]

We performed a study on a single centre experience with CD34+ selected SCB from alternative donors (matched unrelated and mismatched, haploidentical related) for PGF treatment over a 15 year-period.

Endpoints were the evaluation of hematopoietic recovery, incidence of GvHD and survival analysis of the patients receiving SCB.

3. Methods

Patients

Clinical data of 50 patients, who underwent HSCT between 1999 and 2015 at Tübingen University Children´s Hospital were analysed.

Median age at transplantation was 11.8 years (0.33-38.4), median body weight at HSCT was 33 kg (3.7-89.1). The most common underlying diseases were ALL (46%), AML (22%) and, as for non-oncological disease, SAA (10%). Twelve patients (29%) with malignant disease were in non-remission (NR) at HSCT. 50% received a graft from a matched-unrelated donor (MUD), 50% received a mismatched-related (MMRD) HSCT either from the mother (22%), the father (24%) and a sibling (4%).

The myeloablative conditioning regimens were based either on total body irradiation (TBI, 6 fractions of 2 Gy each, n =19, 38%) or busulfan (16 mg/kg for age > 3 years and 20 mg/kg for age < 3 years, n =6.12%), Melphalan (140 mg/m^2, n= 17, 34%) with specific modifications according to patients' pre-treatment.

Serotherapy was carried out with rabbit antithimocyte globulins (either ATG F, Fresenius Kabi AG, Bad Homburg, Germany, or ATG, Genzyme, Cambridge, Massachussets, USA) or with mouse anti-CD3 antibody, Orthoclone OKT3, Janssen-Cilag, Raritan New Jersey, USA (7 patients). 5 patients (9.2%) had rejected their grafts and received an immunoablative, TLI based reconditioning regimen and stem cell grafts from a second haploidentical donor as previously described.[9]

Definition of Poor graft function

PGF was defined as 1) at least bilinear cytopenia (Hb < 8 g/dL, neutrophil count < 1000/mm3, platelet count < 20000/mm3) and/or 2) dependence on transfusion, with full-donor chimerism and absence of disease relapse and grade III-IV aGvHD.

Thirty-four out of 61 SCB (56%) were received because of trilinear cytopenia; 25 (41%) because of bilinear cytopenia; 2 (3%) because of monolinear involvement.

Boost Infusion

A total of 61 SCB were given to 50 patients. Eight patients received more than 1 boost (range 2 to 4). Stem cells were isolated from the original donor through leukapheresis. PBSC were mobilized by administration of 10 µg/kg of G-CSF daily for 5 days and were harvested by a single leukapheresis procedure. CD34$^+$ stem cells were selected by using the automated CLINIMACS device (Miltenyi Biotec Bergisch Gladbach, Germany). All the patients had a full-donor chimerism prior to SCB. They received either freshly isolated (54%) and cryoconserved (46%) stem cells. No conditioning was performed prior and no immunosuppressive therapy was administered post SCB infusion.

Chimerism

Hematopoietic chimerism characterization was performed on peripheral blood leucocytes through short tandem repetitions (STR) PCR with fluorescently marked primers and fragment length analysis through capillary electrophoresis.[10]

Response evaluation

As for descriptive statistical analysis, the neutrophil absolute count and number of infused blood products (erythrocyte/red blood cell and thrombocyte concentrates) per patient before each boost (time point 0, TP0) were compared with the corresponding values 4 weeks (TP1) and 8 weeks (TP2) after SCB. By "independence from transfusions" we meant that the

patients did not receive any blood product within 4 weeks prior to our investigation time points. Moreover, an analysis of lymphocyte subsets recovery was performed: the absolute counts of CD3, CD4, CD8. CD19 and CD56 positive cells, detected by flow cytometry analysis of whole blood at TP0, TP1 and TP2 were compared for each patient. Lineage specific recovery, or resolution of cytopenia, was defined as Neutrophil count ≥ 1000/mm3 for granulocytopenia, no administration of erythrocytes transfusion in the preceding 4 weeks before TP1 and/or TP2 for anaemia and no administration of platelets concentrates in the preceding 4 weeks before TP1 and/or TP2 for thrombocytopenia.

As for univariate and survival analysis, in case of SCB administered because of trilinear and bilinear involvement a complete response (CR) was defined as a trilinear or bilinear recovery, respectively, a partial response (PR) was defined as bilinear or monolinear recovery, respectively, and non-response (NR) meant no resolution of any of the cytopenias, at each time point. To ensure that patients had reached a sustained independence from transfusions, they were observed for an additional 4 weeks' interval after TP2. For statistical tests, the probability of any response (either CR or PR) was compared with NR.

The definition of CR and PR could be applied only to SCB given for a at least a bilinear cytopenia. For patient with monolinear involvement, only CR and NR where applicable.

Eligibility criteria for evaluation where the availability of blood cell counts and transfusions data at each time point.

GvHD

GvHD was defined according to Glucksberg criteria.[11]

Statistical analysis

For descriptive statistics, continuous variables were analysed using the Mann-Whitney test or Wilcoxon test for ranks. The chosen significance level was $\alpha = 0.05$.

Categorical variables were compared using chi-square test or Fisher´s exact test.

Kaplan-Meier´s method was used to estimate overall survival (OS). Day of HSCT was considered as day 0.

Relapse, lethal infections and death due to other causes were defined as events.

Survival curves were compared through log-rank test. When calculating the cumulative incidence (CI) of non-relapse mortality, the competing risk was relapse-related mortality.

All analyses were performed with Graph Pad Prism Statistical software (version 7).

4. Results

Composition and time point of stem cell boost infusions

Median time between HSCT and infusion of SCBs was 94 days (13-519). Grafts contained a median number of 3.15 x 10^6 CD34+ cells/kg body weight (range 0.71-27.9x10^6) with a median number of 2417/kg (range 100-23630) residual CD3+ T cells.

Lineage specific haematological recovery

Neutrophils

The course of neutrophil counts after infusion of SCBs is shown in figure 1a. Median values were 600/mm^3 at TP 0 and significantly increased to 1595/mm^3 and 1516/mm^3 at TP1 and TP2, respectively ($p < 0.001$ and $p < 0.05$). SCBs resulted in an increase of neutrophils count after 4 and 8 weeks in 74% and 64% of the procedures.

Transfusion-requiring anaemia.

Transfusion requirement significantly decreased from median value of 4 at TP0 to 3 and 1 after 4 and 8 weeks (figure 1b, <0.0001 in both cases). The decrease occurred in 79.2% (TP1) and 80.3% (TP2) of the procedures. Moreover, 24.5% and 47% resulted in independence from transfusion until TP1 and TP2, respectively. The response was permanent in all cases.

Transfusion-requiring thrombocytopenia.

Transfusion requirement significantly decreased from a median value of 7 at TP0 to 3 and 0 after 4 and 8 weeks (figure 1c, $p < 0.0001$ and < 0.001). The decrease occurred in 73.6% (TP1) and 86.3% (TP2) of the procedures.

Moreover, 21% and 58.8% resulted in independence from transfusion until TP1 an TP2, respectively. The response was permanent in all but one patient for the following 4 weeks.

Probability of haematological recovery according to number of involved lineages

Patients wo had two lines involved achieved a complete (bilinear) recovery in 54.5% of the procedures. whereas patients with initial trilinear involvement had a complete (trilinear) recovery in 25%. However, this difference remained not significant ($p = 0.136$). The subgroup of patients with monolinear involvement, was too small to draw conclusions ($n = 2$, Table 2)

Recovery of Lymphocyte subsets

CD3+and CD19+ lymphocyte showed a constant and significant increase from a median value of 19.32/mm^3 and 3.49/mm^3 at TP0 to 66.99/mm^3 and 39.32/mm^3 at TP1 ($p < 0.01$ for both) and 160.61/mm^3 and 78/mm^3 at TP2 ($p < 0.001$ for both), respectively. NK cells rose significantly until TP1 and then reached a plateau (median values: 130.05/mm^3 at TP0, 198.62/mm^3 at TP1, 217.61/mm^3 at TP2 ($p < 0.01$).

In detail, within eight weeks, lymphocytes showed a 2.96fold increase of the median count, CD3+ cells an 8.31fold increase, CD3+4+ cells a 14.16fold increase, CD3+8+ cells a 9.45fold increase, CD19+ cells a 22.34fold increase, and CD56+ cells a 1.6fold increase. Taken together, rising counts of CD3, CD19 and CD56+ cells occurred after 84.6%, 82% and 94.8% of the procedures, respectively.

Interestingly, we did not observe any significant increase in the lymphocyte subsets in the 4 weeks preceding SCB administration. This supports our assumption that the improvement in lymphopoiesis was due to the SCBs and not an already ongoing process.

Response assessment

Haematological response was evaluated according to definitions reported in the methods section. At TP1, either a CR or PR was observed in 66% of the procedures. At TP2, the percentage of responses reached 78.8% and became more profound since the rate of complete response increased from 12.5% to 36.5%. Non-response was observed in 34% of procedures at TP1and in 21.2% of procedures at TP2 (figure 3).

Response-influencing factors

Gender, age, underlying diagnosis (malignant *versus* non-malignant disease), remission status prior to HSCT (for patients with malignant disease), type of conditioning regimen (TBI-based *versus* non TBI-based), CD34+ cell dose (< vs > different cut-off values, namely 2, 3, 3.1, 3.2, 3.5, 4, 5, 6, 6.5, 7, 8, 9, 10, 15 $x10^6$ cells/kg bw) did not influence the probability of achieving any response at TP1 and TP2.

Influence of CD34+ cell dose

As for patients who showed a response (either at TP1 and TP2, or both, n = 62) the interquartile interval for CD34+ cell dose was 2.08-6.35 x 10^6/kg with a median value of 3.30 x 10^6/kg (range 0,71-27,9).

Patients who did not show any response, neither at TP1 nor at TP2 (n = 5), received a median CD34+ cell dose of 2.51 x 10^6 cells/kg (range 1.0-3.23, interquartile interval 1.69-3.15; figure 3a).

There was no significant difference in the infused CD34+ cell dose between responders and non-responders (p = 0.39, Mann-Whitney test).

Figure 3b shows the differential increase in neutrophil counts (defined as absolute neutrophil count at TP2 – absolute neutrophil count prior to SCB) as a function of given CD34+ cell dose. A trend line indicates that escalating CD34+ cell doses initially lead to a stronger improvement in neutrophil counts. However, this effect occurred only until a certain CD34+ cell dose; for higher values, no further remarkable increase in neutrophil counts could be observed. Although regression analysis did not lead us to a satisfactory description of the relationship between the two variables, we could identify a threshold for CD34+ cell dose for which a stronger effect size (neutrophil increase) occurred. Mann-Whitney tests for different cut-off values showed significance for values above 3.25 x 10^6 cells/kg.

GvHD

In 3 out of 50 patients (6%) de novo grade I-III aGvHD developed, while in 1 patient (2%) a pre-existing GvHD worsened after SCB. We observed a complete resolution of all cases of aGvHD, and no case of cGvHD developed.

Survival

Median follow-up after SCB was 413 days (range 4-5724). Median follow-up after HSCT was 630 days (range 52-5740).

Of the 50 patients, 29 died (58%), 21 survived (42%).

Death was related to relapse in 28% of patients (n = 14), in 24% (n = 12) it was due to non-relapse mortality (infections, n = 9; post-HSCT undefined cardiomyopathy, n = 1; multi-organ failure, n = 2). In 3 patients, the cause of death was unknown. Among infections, 4/9 had a proven viral aetiology, 3/9 had bacterial sepsis, and 1 patient had a fungal infection. In one case the pathogen could not be identified.

The current 5-years overall survival (OS) rate of the entire cohort is 38.67%.

We observed a trend towards better OS for patients who had a complete response to SCBs at TP2 compared to non-responders (p = 0.07), while no significant differences were observed for event-free survival (EFS) and cumulative incidence of non-relapse mortality (NRM). However, the subgroup of non-responders was too small to draw conclusions.

5. Discussion

In the present study, we focused on 50 mostly paediatric patients who underwent HSCT from matched unrelated or mismatched related donors and received CD34+ positive selected SCBs because of poor graft function. We aimed to analyse whether SCB administration from the original donor, without prior conditioning and without subsequent immunosuppression is safe in terms of GvHD induction and effective in enhancing myeloid recovery. Moreover, we analysed the impact of SCB on lymphocyte recovery and on patients´ survival.

Lineage specific recovery of all 3 myeloid cell lines could be improved. Our analysis showed a significant increase of neutrophil counts and a significant decrease in transfusion requirement for erythrocytes or platelets in 64%, 80.3% and 86.3% of the procedures, respectively.

Although no linear correlation between infused CD34+ cell dose and differential increase of neutrophils was detected we could identify a threshold for which a stronger effect size occurred: infusion of values above 3.25 x 10^6/kg CD34+ cells resulted in an optimal increase of neutrophils. For higher values, no further remarkable increase could be observed.

Moreover, we evaluated the probability of the overall haematological response and factors of influence.

The observed response rates (78.8% of CR or PR 8 weeks after SCB) are comparable with the reported values for adults, although response assessment modalities and duration of haematological follow up vary considerably among the existing studies, making a comparison difficult. In detail, Stasia reports an overall response rate of 83%[1], Klyuchinov of 81% 4 weeks after SCB[12], Askaa of 72% at +200 days[13]. None of the investigated factors (age at HSCT, gender, underlying diagnosis, remission status at HSCT, kind of conditioning regimen, infused CD34+ cell dose) had an impact on the probability of achieving a complete or partial haematological response.

Although the CD34+cell dose was found to influence the differential increase in neutrophil counts we could not demonstrate an effect in this context.

Notably, in the present study we analysed only matched unrelated or haploidentical stem cell donors, in the same proportion, with the complete absence of matched sibling donors. This underlies a higher degree of donor-recipient HLA disparity, with a higher risk of GvHD when compared with the entire population of transplanted patients. Despite this fact, the observed *de novo* aGvHD grade I-III incidence was extremely low (6%) when compared to

already published reports including also matched sibling donors (Stasia, 15% grade II, Klycuchnikov 17% grade II-IV, Larocca 70% grade I-II, 0% grade III-IV, Askaa 22% grade I-IV), and no cGvHD occurred, although no further immunosuppressive therapy was administered. This may be explained by the profound T cell depletion, resulting in a median number of only 2,417 residual CD3+ cells/kg, which was lower than the values reported by the mentioned authors.

Interestingly, the administration of SCB had a profound impact on lymphocyte recovery: we were able to show a significant increase in the absolute counts of all the main lymphocyte subsets, namely T lymphocytes (either CD4+ and CD8+), B lymphocytes, and NK cells. This is, to our knowledge, a previously never described finding. It is uncertain whether the observed immune recovery is due to proliferation and differentiation of lymphoid precursors from the CD34+ stem cells infused with the boost or, more likely, to a sort of “immune enhancing effect” of the stem cells on committed or partially differentiated lymphoid precursors already present in the recipient. The latter hypothesis is more consistent with the timing of our observations: at least three months are usually needed for lymphopoiesis and maturation.[15] A similar immune enhancing effect is observed after transplantation with TcRαβ depleted grafts compared to grafts containing solely hematopoietic stem cells.[16] Obviously the SCB itself not only enhances the haematopoiesis but produces such an immune enhancing effect to lymphoproliferation and maturation. This increase comprises the different lymphoid subsets, so it could not be restricted to improvement of a specific microenvironment, e.g. the thymus, but must be mediated by a profound stimulus of lymphoproliferation. Conceivably a rise of cytokines such as IL-2, IL-12 or IL-15 is correlated to the increase of the different lymphoid subsets. This increase is well known after allogenic SCT and is mainly contributed to the cytokine storm after conditioning therapy,[17] but CD34+ stem cell boosting itself might be able to enhance the production of those cytokines and thereby the lymphoproliferation.

We observed a trend for a better overall survival for patients who had a complete response to SCBs compared to patients who did not respond (fig. 5). The high rate of relapse-related mortality in our cohort may have altered the composition of the evaluable cohort, preventing us to reach significance.

Post-HSCT stem cell boosting strategies other than CD34+ positive selection are currently under evaluation, i.e. CD3/CD19 and TcRαβ depletion, which allow to retain important mature T cell populations, such as TcRγδ and NK cells, known to play a role in underlying disease´s and infections´ control. There are few reports about the use of such techniques,[8]

and the indication for SCB is slightly different (poor immune reconstitution rather than PGF) but, together with our own findings, they suggest additional interactions between stem cells and already committed populations of precursors, which deserve further investigation.

The present study has clear limitations due to its retrospective nature. Nevertheless, it demonstrates that CD34+ positive selected stem cell boosting is a reasonable and remarkably safe treatment option for patients with poor graft function, allowing the avoidance of any immunosuppression. Further studies are warranted to precisely evaluate the impact on immune reconstitution and on survival.

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Tables

Table 1: Patients´ characteristics

Patients	n = 50	
Age		11.87 (0.33-38.98)
Gender	m	33 (66%)
	f	17 (34%)
Weight		33 (3.7-89.1)
Diagnoses	ALL	23 (46%)
	AML	11 (22%)
	MDS	1 (2%)
	CML	1 (2%)
	NHL	1 (2%)
	CMML	1 (2%)
	Solid Tumours	3 (6%)
	SAA	5 (10)%)
	Osteopetrosis	2 (4%)
	Thalassemia	1 (2%)
	WAS	1 (2%)
Donor	MUD	25 (50%)
	MMRD	
	Mother	11 (22%)
	Father	12 (24%)
	Sibling	2 (4%)
Remission status at HSCT (patients with malignant disease, n=41)		
	CR1	9 (22%)
	CR2	14 (34.2%)
	CR>2	6 (14.6%)
	NR	12 (29.2%)
Conditioning(prior to HSCT)		
	TBI-based	19/50 (38)
	Busulfan-based	6/50 (12%)
	Melphalan-based	17/50 (34%)

ALL= Acute Lymphatic Leukemia; AML= Acute Myeloid Leukemia; MDS= Myelodisplastic Syndrome; CML= Chronic Myeloid Leukemia; NHL= Non-Hodgkin Lymphoma; CMML= Chronic myelo-monocytic Leukemia; NBL= Neuroblastoma; SAA= Severe Aplastic Anemia; SCID= Severe Combined Immunodeficiency; WAS= Wiskott-Aldrich Syndrome; MUD= Matched Unrelated Donor; MMRD mismatched related donor; CR= complete remission; NR= non remission

*evaluable patients

Table 2: Probability of hematological recovery after boosting according to number of involved lineages.

			Trilinear recovery	Bilinear recovery	Monolinear recovery	NR
TP2	Initial involvement	Trilinear (n=28)	7 (25%)	9 (32.2%)	7 (25%)	5 (17.8%)
		Bilinear (n=22)		12 (54,5%)	6 (27,3%)	4 (18.2%)
		Monolinear (n=2)			0	2 (100%)

Absolute number and percentage of procedures which resulted in a trilinear, bilinear, monolinear recovery or non-recovery (NR), according to the initial number of involved cell lineages. At TP2 52 procedures in 39 patients were evaluable (11 pat. died, no data in 5 pat.).

Figure Legends

Figure 1: Lineage Specific Recovery after stem cell boosting. a) Increase in neutrophil count (all evaluable stem cell boosts, n = 59) b) decrease in red blood cell transfusions (only SCBs given for initial transfusion requirement, n = 53, c) decrease in thrombocyte transfusions (only evaluable SCBs administered for initial thrombocyte transfusion requirement, n = 53). Significance is shown as a continuous straight line, *= p < .05, **= p < .05 and > .001, ***= p < .001 and > .0001, ****= p < .0001). TP0 = SCB administration; TP1 = 4 weeks after SCB; TP2 = 8 weeks after SCB.

Figure 2: Recovery of Lymphocyte subsets (logarithmic increase of the absolute count). Significance is showed as a continuous straight line, *= p < .05, **= p < .05 and > .001, ***= p < .001 and > .0001.

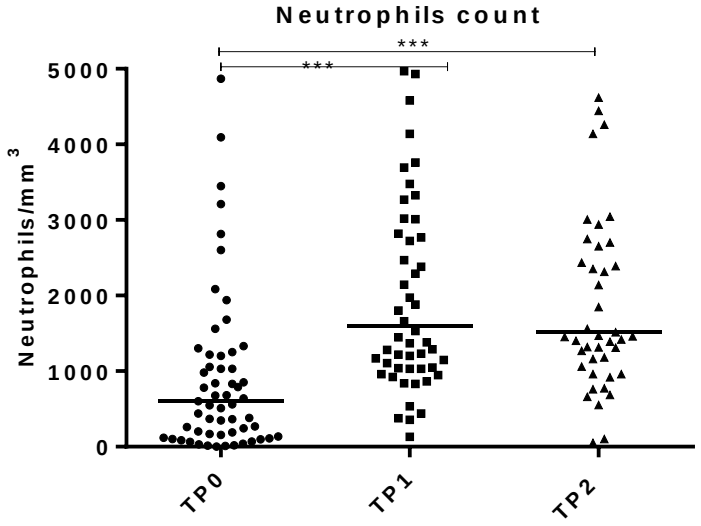
Figure 3: Evaluation of response. Percentages of complete (CR), partial (PR) non response (NR) and any response in evaluable patients are shown.

Figure 4: Impact of CD34+ cell dose. a) Comparison between infused CD34+ cell doses (2.078-6.353*10^6/kg) in patients who achieved a response either at TP1 and TP2 and non-responding patients. Median, 1st and 3rd quartile are shown. b) Increase of neutrophils (log (absolute neutrophil count at TP2 – absolute neutrophil count prior to SCB)) as a function of infused CD34+ cell dose (the line describes a hypothetical trend).

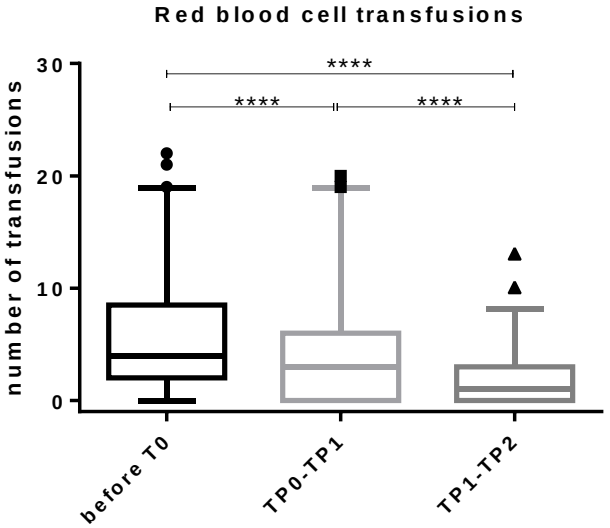
Figure 5: a) Overall survival (OS) of the entire cohort). b) Overall survival (OS) according to the response at TP2. CR= complete response, NR= non-response. *p*=0,07. Survival proportions are shown.

Figure 1.

a)


Neutrophils count
***
***
5000
4000
3000
2000
1000
0
Neutrophils/mm3
TP0
TP1
TP2

b)


Red blood cell transfusions
****
****
****
30
20
10
0
number of transfusions
before T0
TP0-TP1
TP1-TP2

c)

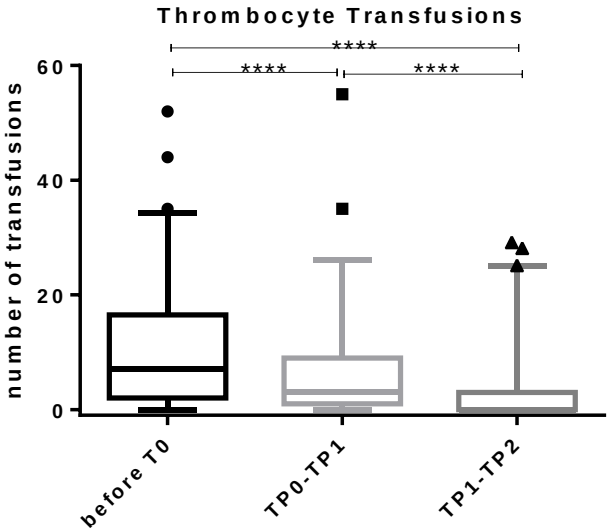

Thrombocyte Transfusions
****
****
****
60
40
20
0
number of transfusions
before T0
TP0-TP1
TP1-TP2

Figure 2.

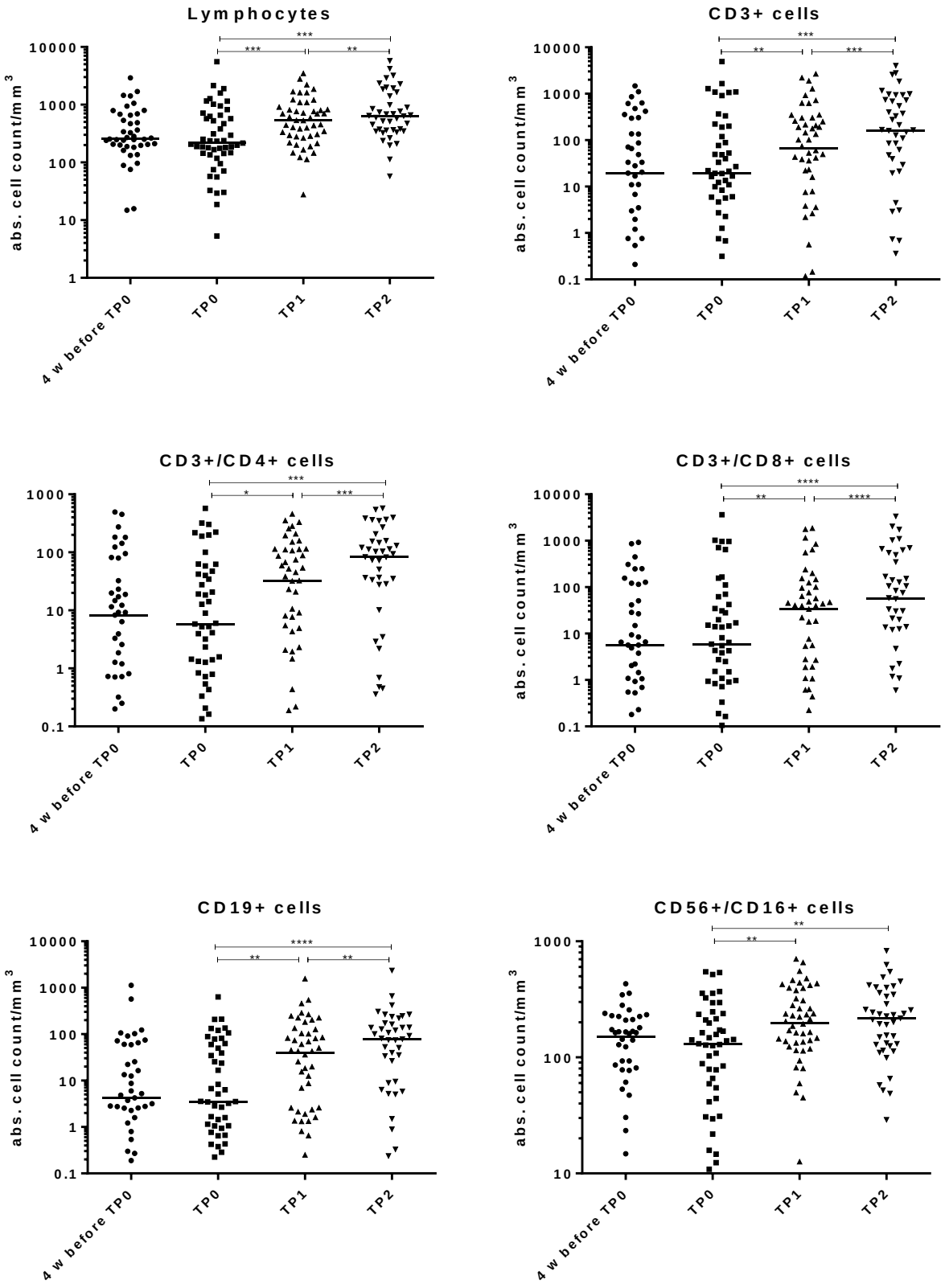

Lymphocytes
CD3+ cells
CD3+/CD4+ cells
CD3+/CD8+ cells
CD19+ cells
CD56+/CD16+ cells
abs. cell count/mm3
4 w before TP0
TP0
TP1
TP2

Figure 3.

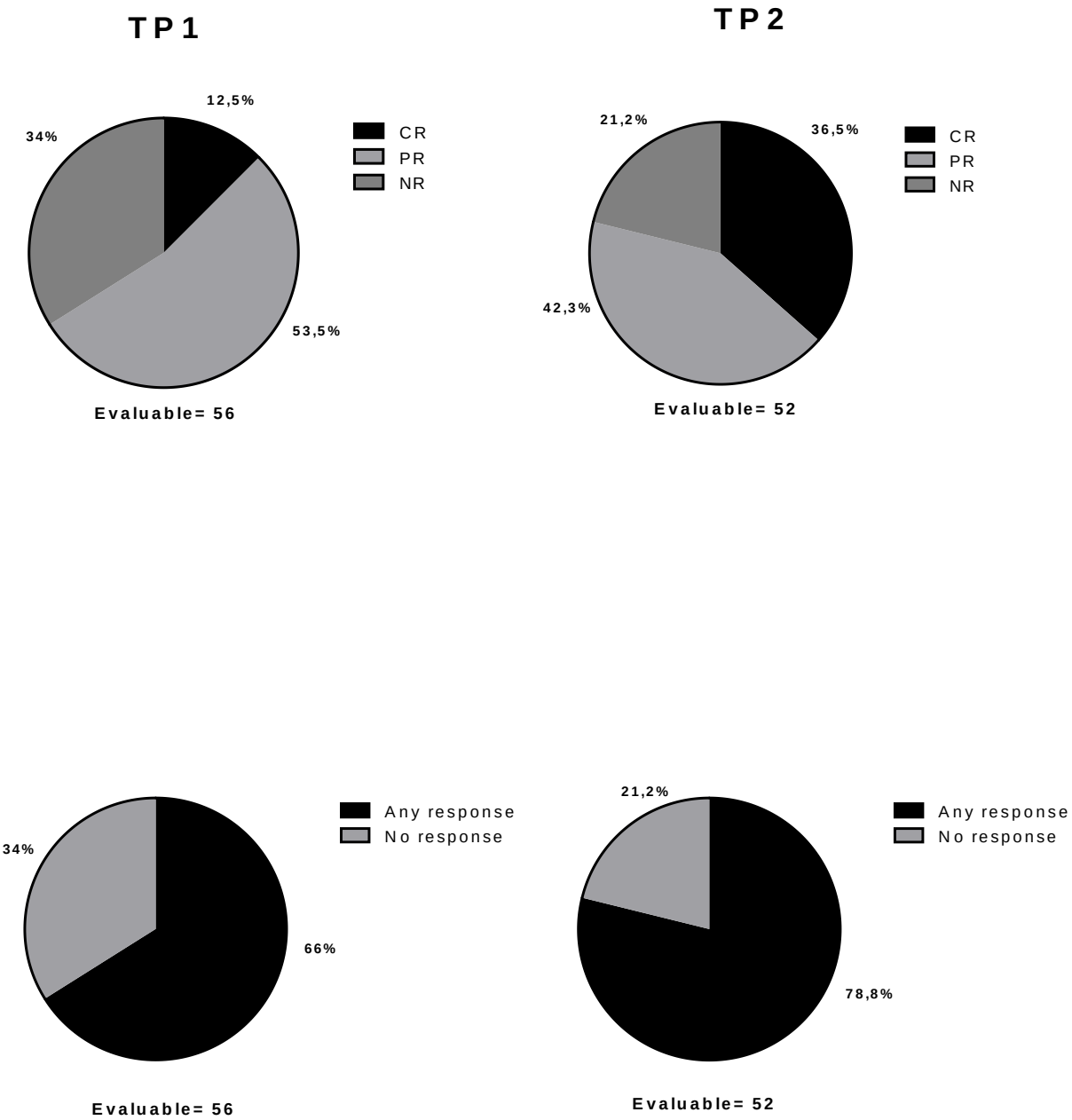
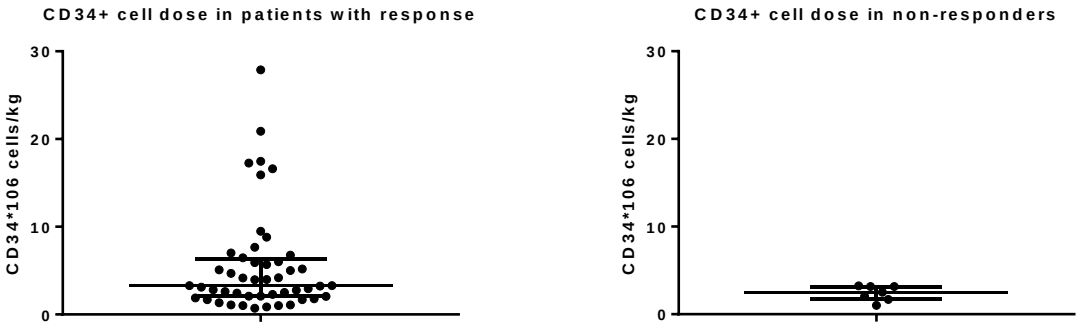

TP 1
12,5%
34%
53,5%
CR
PR
NR
Evaluable= 56
TP 2
21,2%
36,5%
42,3%
CR
PR
NR
Evaluable= 52
34%
66%
Any response
No response
Evaluable= 56
21,2%
78,8%
Any response
No response
Evaluable= 52

Figure 4.

a)


CD34+ cell dose in patients with response
CD34+ cell dose in non-responders
CD34*106 cells/kg
30
20
10
0

b)

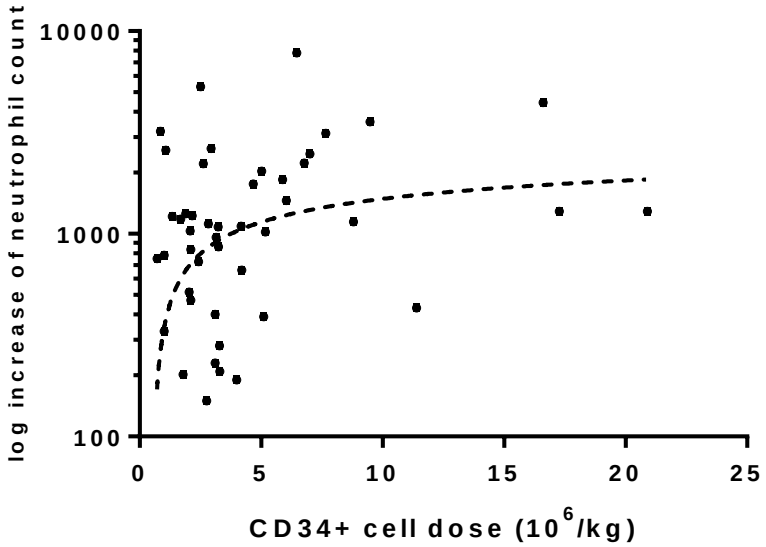
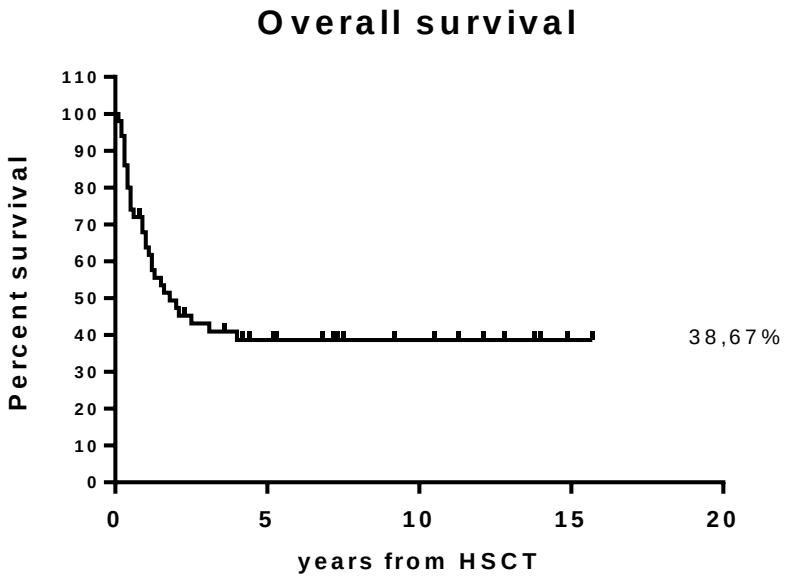
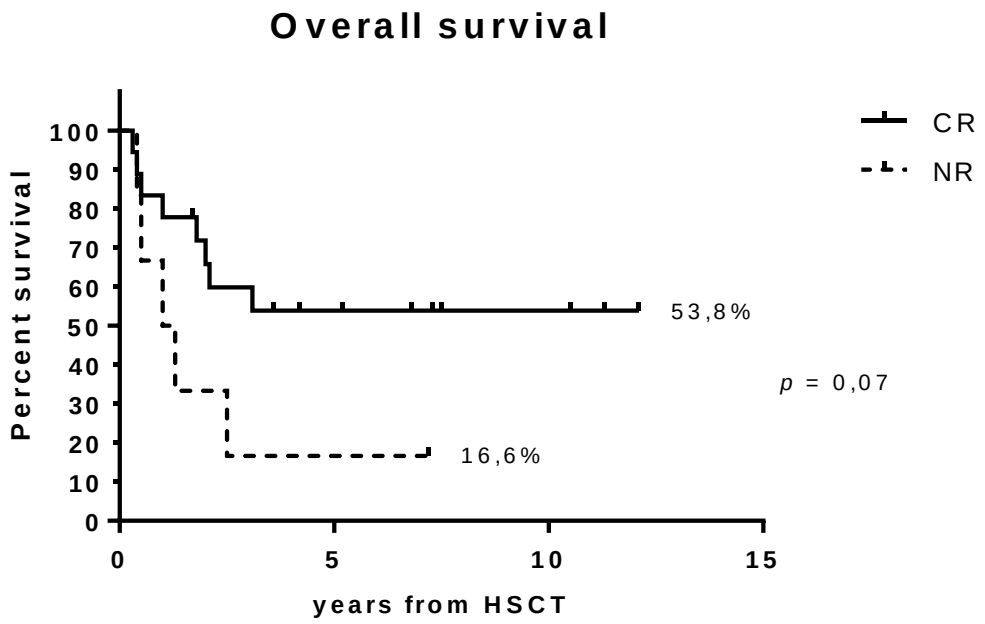

log increase of neutrophil count
10000
1000
100
0
5
10
15
20
25
CD34+ cell dose (10^6/kg)

Figure 5.

a)


Overall survival
Percent survival
110
100
90
80
70
60
50
40
30
20
10
0
0
5
10
15
20
years from HSCT
38,67%

b)


Overall survival
Percent survival
100
90
80
70
60
50
40
30
20
10
0
0
5
10
15
years from HSCT
CR
NR
53,8%
p = 0,07
16,6%

Treatment of Acute Graft-versus-host disease in childhood with extracorporeal Photochemotherapy/Photopheresis: the Padova experience.

1. Abstract

Systemic steroids are the well-established first line therapy for acute graft-versus-host disease (aGvHD), while there is no validated treatment for patients with aGvHD who fail to respond to corticosteroids. Among the proposed options, extracorporeal photochemotherapy (ECP) shows increasing evidence of efficacy, although no large randomized clinical trials have been conducted. We investigated a cohort of 72 paediatric patients treated with ECP between 1997 and 2013 at a single Institution, aiming to determine the success of the procedure and its impact on survival and the risk of relapse of primary disease. Main indication for ECP was steroid-dependency (n = 42, 58,3%), followed by steroid-resistance (n = 21, 29%). In 9 patients, ECP was performed because of contraindications to steroid treatment. 72% of patients had a complete response to ECP, 11% a partial response and 17% showed no response. Transplant related mortality (TRM) at day +180 was 4% for the entire cohort, with a significant difference between responders and non-responders to ECP (3% vs 20%, p = 0.0004). 5-years Overall Survival (OS) significantly differed among responders and non-responders (78% vs 30%, p = .0004), while the 5-years' time to progression (TTP) of primary disease was no significantly influenced by response to ECP. The 5-years PFS was significantly higher in responders to ECP (79% vs 30%, p = .0007). Our data show that ECP is effective and has a favourable impact on TRM, while it does not modify the probability of the underlying disease's relapse.

2. Introduction

Indications for hematopoietic stem cell transplantation (HSCT) have been extended in the last decades and the number of procedures per year significantly increased. Many important acquisitions in the field of donor selection, drug therapy for conditioning regimens and immunosuppression, supportive care have been achieved and led to a better survival of patients. In spite of these advances, aGvHD is the leading cause of TRM, and especially steroid dependent or steroid refractory disease is a concern. [1, 2] Recommendations of scientific societies, such as the guidelines published by the joint working group of the British

Committee for Standards in Haematology and the British Society for Bone Marrow Transplantation, consider the use of mycophenolate mofetil (MMF), anti-TNF antibodies, mammalian target of rapamycin inhibitors, IL-2 receptor antibodies, and extracorporeal photochemotherapy/photopheresis (ECP) for second line treatment of aGvHD.[3] However, no single agent has showed to significantly ameliorate survival of patients with steroid refractory or dependent disease, [4,5] who still show worse OS and higher TRM compared to responders to steroids. [6,7,8]

ECP is a non-pharmacological treatment for GvHD, which consists in collection of peripheral blood leucocytes, their exposure to UV-A light in presence of 8-methyl-psoralene and re-infusion to the patient. Such procedure induces apoptosis in treated cells (including T-lymphocytes), their phagocytosis by APCs and antigen presentation to the remaining lymphocytes. The subsequent cellular interactions, through mechanisms which are not yet fully understood, results in induction of tolerance against alloantigens, modulating cytokine secretion, antibody production and effector cells function.[9-13]

There is growing evidence of ECP efficacy for treatment of aGvHD,[14-17] also in the paediatric setting.[18]

ECP use is highly widespread in Italy, and best practice recommendations have been elaborated by a joint committee of the main scientific societies operating in the field (Italian Society of Haemapheresis and Cell Manipulation and the Italian Group of Bone Marrow Transplantation)[19], aiming to standardize treatment modalities. In fact, the majority of the already published studies on the subject deal with retrospective data and indications, treatment schedules, response assessment criteria differed significantly among centres.

We performed a retrospective analysis of 72 paediatric patients with aGvHD who underwent ECP treatment with uniform modalities. Endpoints of the study were evaluation of response rate to ECP, and assessment of its impact on TRM, OS, progression-free survival (PFS), and time to progression (TTP) of primary disease.

3. Methods

Patients

72 consecutive paediatric patients (44 males, 28 females, 15 of them previously reported[20]) underwent ECP from January 1997 to June 2013, at the HSCT Unit of Padova University Hospital. Clinical characteristics are summarized in Table 1. Median age at ECP was 8.3

years (range, .9-20.3), median body weight was 25 kg (range 7-98). Fifteen children weighted less than 15 kg. The last follow-up was June 2014.

Indications for ECP were: steroid resistance (21 patients, 29%), defined as a progression or no improvement in aGVHD after at least 3 days or 7 days on methylprednisolone (MP) ≥ 2 mg/kg body weight, respectively (SR group); 21 patients for steroid-dependency (SD), defined as a flare-up of aGVHD during the tapering of MP; and requirement for reduction of pharmacological IS or contraindications to IS therapy for viral reactivations, systemic mycoses, or intolerable side effects (30 patients, group with infectious complications [IC]). In particular, 9 of 30 patients in the IC group (IC-A group) underwent ECP without steroids as a first-line therapy because of contraindications. The other 21 patients (IC-B group) had SD aGVHD and organ toxicities or infectious complications which prompted to a reduction or avoidance of steroid therapy.

Microbiological surveillance

In our practice, surveillance for viral and fungal infections is routinely performed during the first 100 days after HSCT in all patients and includes EBV-DNA, CMV-DNA, ADV-DNA, human herpesvirus-6 DNA, BKVDNA, JC-DNA, and galactomannan antigen monitoring in peripheral blood once or twice weekly, CMV-DNA, BKV-DNA, JCV-DNA monitoring in urine once a week.

Blood, urine, and stool cultures, nasal and throat swabs and nasopharyngeal aspirates are weekly performed. Other microbiological investigations are performed upon clinical symptoms. Viral reactivations are detected by PCR positivity for EBV-DNA (cut-off: 1000 copies/mL), CMV-DNA (cut-off: 1000 copies/ mL), and ADV-DNA in qualitative test. Clinical systemic fungal infections were defined proved or probable according to European Organization for Research and Treatment of Cancer criteria.[21] The cut-off for the galactomannan index was set at .5 (Enzyme Immuno Assay (E.I.A.) method).

GVHD Prophylaxis

Immunoprophylaxis included ciclosporin A (CsA) administration for 6 months in HSCT from HLA-identical sibling, short-term methotrexate, rabbit antithymocyte globulin (ATG) and CsA for 12 months in unrelated HSCT, CsA and ATG for unrelated cord blood HSCT.

In haploidentical setting, immune prophylaxis included ex vivo depletion of αβ T cells and CD19+ B cells and ATG administration; no other IS therapy was given after HSCT.

Informed consent was obtained from patients' parents, as well as from the patients themselves when possible, and the use of ECP was approved by the local ethical committee.

aGVHD Evaluation

aGVHD was defined and graded according to Glucksberg criteria.[22] Histological confirmation was obtained whenever clinically indicated.

Eligibility Criteria for ECP

Indications to ECP treatment were as follows: children with SR aGVHD (n = 21); children with SD aGVHD (n = 21); patients with aGVHD in whom IS therapy was contraindicated or who required a rapid decrease of IS therapy for increasing EBV viral load, CMV reactivation in 2 subsequent samples, systemic fungal infections, intolerable side effects (n = 30).

A condtion of complete haematological remission and full donor chimeras, a white blood cell (WBC) count > 1 *10^9/L, and no concomitant treatment with either ATG or monoclonal antibodies were required.

ECP procedure and technical issues

ECP was performed using either the "in-line" technique (UVAR Photopheresis Instrument, Therakos, Exton, PA, 19 out of 72 patients) and the "off-line" technique (Cobe Spectra, BCT Terumo, Lakewood, CO, 53 out of 72 children). Technical details have already been published.[19] The "off-line" technique was introduced in 2003 to treat low weight children.

For patients weighing less than 15 kg, priming of the leukapheresis circuit with irradiated and leuko-depleted red blood cells (regardless of baseline haemoglobin level) was performed, as recommended in the Italian Society of Haemapheresis and Cell Manipulation-Italian Group of Bone Marrow Transplantation indications.[19,23] Pre-ECP haemoglobin levels were maintained between 10 g/dL and 12 g/dL. The cell product was treated with 8-MOP and diluted to a final concentration of 20 mg/100 mL to 34 mg/100 mL, according to the specific procedure (in-line technique, 34 mg/100 mL; off-line technique, 20 mg/100 mL).

Vascular access

In all patients, a 7 to 9 French Hickman double-lumen central catheter was systematically used for the procedure. To provide adequate flow rates, i.e., 1 to 2 mL/kg/minute, anticoagulation with urokinase 10,000 U for 2 hours as lock-therapy was performed on the day of the procedure.

Product's characteristics ("off-line" technique)

The leukapheresis product contained a median of WBC of $19.4*10^3$/uL (range 10.7-$70.1*10^3$) and a median of mononuclear cells (MNC) of 80.5% (range, 50%-90%). The median number of WBC reinfused to the patients was $2970*10^6$ (range,1150-10,$420*10^6$), whereas the median number of MNC reinfused to the patients was $2794*10^6$ (range, 782.3-$9805.4*10^6$).

Treatment schedule

ECP was performed twice weekly for the first month, every 2 weeks during the second and third month, and then monthly for at least 3 more months. Progressive tapering and discontinuation of ECP were decided upon evaluation of individual response. Any concomitant IS therapy was initially maintained, then modified or discontinued according to the clinical response.

Response Criteria to ECP

ECP response criteria were previously reported.[20] All patients were enrolled for ECP before day +100 and response was evaluated at day +28, day +56, and at the end of treatment.

Complete response (CR) was defined as the resolution of all signs of aGVHD and partial response (PR) as at least a 50% improvement in the clinical signs.

In the latter case, given the complexity of assessing response, we defined PR for each organ as follows: for the skin, at least a 50% reduction in the body surface area affected; for the GI tract and liver a 50% reduction in the volume of diarrhoea or value of bilirubin.

Any worsening of organ involvement, as well as the appearance of new signs or symptoms of GVHD, was defined as progressive disease (PD). Patients with stable or PD were considered NR.

Statistical Analysis

Patients' characteristics were compared using the chi-squared or Fisher's exact test (as appropriate) in case of qualitative variables, or the Mann-Whitney test in case of continuous variables. TRM was calculated from the date of HSCT to day +180 and to the last follow-up, considering as event any non-relapse cause of death. OS was calculated from the date of HSCT to the date of death from any cause, or to the last follow-up. PFS was calculated from

the date of HSCT to the date of relapse of underlying disease or death for any cause or to the last follow-up. TTP was calculated from the date of HSCT to the date of relapse of primary disease or to the last follow up. Cumulative incidences (CI) of relapse of underlying disease were estimated

in the competing risk model, considering death from any cause or cGVHD as the competing events. Survival analysis was performed using Kaplan-Meier method with 95% confidence interval. Standard error (SE) for each survival and incidence rate is given. Differences between groups were compared using the log-rank test and the Gray's test. All reported P values were 2-sided, and statistical significance was set at a = .05 (SAS Institute, Cary, NC; release 8.2).[24]

4. Results

aGVHD

Seventy-two consecutive patients with aGVHD underwent ECP at a median time of 46 days (range 13-91) after HSCT and 22 days (range 4-81) from aGVHD diagnosis. Sixty-four patients (88%) had skin involvement (grade IV, n = 13; grade III, n = 20; grade II, n = 21; grade I, n = 10). Fifty-five patients (76,3%) had gastrointestinal (GI) aGVHD (grade IV, n = 8; grade III, n = 2; grade II, n = 18; grade I, n = 27). Twelve patients (16,6%) had liver involvement (grade III, n = 3; grade II, n = 4; grade I, n = 5). Seventeen patients (23,6%) had isolated skin involvement and 7 (9.7%) presented isolated GI involvement, whereas 36 patients (50%) had combined skin and GI aGVHD, 1 patient had combined GI and liver aGVHD, and 11 patients (15.2%) had combined skin, GI, and liver aGVHD. The overall clinical grading of aGVHD was as follows: grade I, n = 8 (11.1%); grade II, n = 29 (40%); grade III, n = 17 (23.6%); and grade IV, n = 18 (25%).

Sixty-three of 72 patients (87.5%) were given 2 mg/kg/day of MP as first-line therapy. The median dose of steroid at beginning of ECP was 2 mg/kg/day. In detail, IS therapies before ECP were CsA, n = 9; CsA plus steroid (2 mg/kg), n = 42; tacrolimus plus steroid (2 mg/kg), n = 12; and CsA or tacrolimus plus MMF plus steroid, n = 9. ECP was used as first-line therapy in 8 of 72 patients, as second line therapy in 43 of 72 patients (among them, 1 haploidentical HSCT was treated only with CsA), as third-line in 15 of 72 patients, and in 6 of 72 patients as fourth-line therapy.

Median duration of treatment was 4 months (range 1.1-10.2) for a median number of 18 procedures (range 8-90). Forty-one out of 72 patients (57%) stopped ECP early because of

CR to ECP (20 patients), NR (8 patients), relapse of primary disease (6 patients), clinical contraindications (such as sepsis in 6 patients), and anaphylaxis (1 patient).

Clinical Response to ECP

At the end of ECP treatment, 52 of 72 (72%) patients had a CR, 8 of 72 (11%) had a PR, and 12 of 72 (17%) were NR. Among the 52 patients showing a CR, 7 patients had aGVHD grade I, 22 patients had grade II, 12 had grade III, and 11 had grade IV. The CR rate for patients with aGVHD grades I and II and grades III and IV were 78% and 66%, respectively (p *ns*), whereas the PR rate for patients with aGVHD grades I and II and grades II to IV were 5% and 17%, respectively (p ns).

No significant difference in CR rate was observed according to indication for ECP (SR, 67%; SD, 81%; IC groups, 70%).

At ECP discontinuation, complete resolution of aGVHD manifestations on skin, gut, and liver was observed in 78%, 76%, and 84% of patients, respectively. Maximal response to ECP was observed after 8 weeks of treatment (16 procedures). At the end of ECP treatment, IS therapy withdrawal was possible in 12 patients (17%) and its reduction was possible in 44 patients (61%). In the 63 patients treated with 2 mg/kg/day MP before ECP, the steroid dose was reduced by 80% after 1 month of treatment, 84% after 2 months, and 88% after 3 months.

The median Lansky/Karnofsky performance score improved from 70% before ECP to 100% after treatment's completion. No significant difference was detected between responders and NR to ECP according to the major clinical risk factors affecting the probability to develop aGvHD.

cGVHD

Twenty-three of 72 patients (32%) developed clinic manifestations of cGVHD after ECP's discontinuation. In detail, 19 patients (26%) had progressive cGVHD (11 NR and 8 PR to ECP) and 4 patients (5%) had quiescent cGVHD onset after a median of 6 months (range, 5 days to 16 months) from the end of ECP. Overall grading of cGVHD, based on the National Institutes of Health Consensus,[25] was mild for 6 patients, moderate for 10, and severe for 7. Among the patients with progressive cGVHD, ECP was used with other IS therapies in 4 of 19 patients, obtaining CR in only 1 of them. Overall, 10 of 19 patients were alive at the last follow-up: 9 of 10 had no cGVHD and discontinued IS therapy, whereas only 1 patient presenting with cGVHD was still in treatment.

All the patients with quiescent cGVHD were alive at the last follow-up: 2 patients were free from cGVHD and without IS therapy and the other 2 patients had cGVHD and were still on treatment with IS therapy plus ECP.

Survival

Overall TRM of the patients' cohort at day +180 was 4% (SE, 1%). TRM significantly differed between responders (3%, SE, 2%) and NR (20%, SE 13%) to ECP ($p < .0001$). Overall TRM at last follow-up was 11% (SE, 4%), with a maintained significant difference between responders and NR (3%, SE 2% and 58%, SE 20%, respectively) ($p < .0001$) (Figure 1).

The 5-year OS was 71% (SE 5%) and significantly differed between responders and NR (78%; SE, 5% versus 30%; SE, 14%, respectively; $P = .0004$) (Figure 2).

The 5-year PFS of primary disease of the entire cohort was 72% (SE 5%), with a significant difference ($P = .0007$) between responders (79%; SE 5%) and NR (30%; SE, 14%) (Figure 3).

The 5-year TTP of underlying disease was 81% (SE 5%), with no difference between the 2 groups (responders: 82%; SE 5% versus NR: 78%; SE, 14%; $P = .65$)
(Figure 4).

The overall 5-year CI of relapse of the underlying disease was 20% (SE 5%); there was no difference between responders and NR to ECP (Figure 5).

At last follow-up (median time from HSCT of 5 years; range, .18-17.6), 51 patients were alive (71%); 48 of them (94%) were GVHD free and without any IS therapy. Twenty-one patients (29%) died: 14 because of underlying disease's relapse and 7 for other reasons (NRM).

Causes of NRM were sepsis (1 patient with aGVHD, day +65 after HSCT); CMV pneumonia (1 case, with cGvHD), acute hepatitis from HCV infection (1 case with cGvHD), encephalopathy (1 case with cGvHD), and multiorgan failure (2 cases with cGvHD); 1 patient died because of CMV pneumonia at day +135 from HSCT, without any evidence of cGVHD.

Complications

Side effects observed during ECP treatment were generally mild and more frequent in low-weight children. They consisted in mild hypotension associated with abdominal pain (10 patients, 16 episodes out of 1382 apheresis sessions), which resulted the procedure's suspension, transient reduction in haemoglobin, platelet, and/or WBC count in 26, 20, and 25 patients, respectively, heart failure for fluid overload after the procedure (in a condition of pre-existing cardiac impairment), which promptly responded to adequate therapy, and a

case of anaphylaxis (cough, vomiting, abdominal pain, hypotension, and palpebral edema) a few minutes after the end of 8-MOP irradiated bag infusion (after 10 ECP procedures, and with a prompt response to antihistamine and steroid therapy). One patient with grade IV aGVHD on high-dose steroid therapy (5 mg/kg/day) experienced acute GI bleeding after the second course of ECP: GI endoscopy showed multiple ulcers in the stomach.

5. Discussion

cGvHD is a well-established indication for ECP, [26, 27] whereas no randomized trials have been performed exploring its role in treatment of aGvHD. Given the paucity of studies on the topic, we aimed to retrospectively evaluate the efficacy of ECP for the treatment of aGVHD at our Institution.

The majority of knowledge about feasibility and efficacy of ECP in treatment of aGVHD derives from studies in adults[14]. So far, data on 207 paediatric patients treated with ECP for aGVHD have been reported, showing an overall CR rate ranging from 32% to 73% and a survival rate ranging

from 44% to 85% [18,20,28-34].

Our data compare favourably with a multicentre retrospective study of the Italian Association for Paediatric Haematology/ Oncology (AIEOP), since we observed a higher percentage of response (72% versus 54%).[18]

We hypothesized that an earlier beginning of ECP treatment (22 days after aGvHD diagnosis versus 30 days in AEIOP study) may have improved the response rate, but further studies are needed to address this topic.

In our cohort, the probability of response to ECP was not influenced by major clinical risk factors for aGVHD development, the grade of aGvHD (grade I and II, 78%; versus grade III and IV, 66%; P = .70) and indication for ECP (SR, 67%; SD, 81%; IC, 70%; P = .91).

Our results showed better response rate than those reported in literature for advanced stages of disease. Nevertheless, higher CR rates were observed in grade II GVHD, suggesting that an early start of ECP sessions may be beneficial.

ECP proved to be effective in all involved organs and allowed a steroid dose reduction of 80%, 84%, and 88% after 1, 2, and 3 months, respectively from beginning of treatment.

ECP was performed as single agent, front-line therapy for aGvHD in 8 patients with fungal infection and viral reactivation, with complete response in 7 of them. This is a previously never reported finding.

Notably, IS therapy was either discontinued or reduced in 78% of responding patients, and this is an important indicator of success of the procedure given the obvious impact of immunosuppression on the risk of infectious complications and relapse of underlying disease.[1,2,18,20,28,35]

In our Centre's experience, ECP was well tolerated, with few and mild adverse effects, the most frequent being mild hypotension, abdominal pain, and headache. The majority of side effects were observed in the earliest period in which ECP was performed at our Institution, and this observation support the idea that there has been a learning process for the management of technical issues and side effects. In our experience, ECP was feasible even in 15 very young children with low body weight (<15 kg). Currently used ECP techniques include the "off-line" and the "in-line" devices.[19] In our centre, both techniques were used in different time periods with no difference in the observed response rate. The number of WBC collected and MNC reinfused did not affect clinical outcome. Notably, all patients underwent the procedure with the bilumen central venous line already in place (Hickman-Broviac Bard Access Systems, Salt Lake City, UT, USA), with no need for a larger central venous line (for instance, Quinton) placement, which is praxis in the majority of centres.

The reported CI of cGVHD in paediatric population ranges from 6% to 65% according to the source of stem cells (HLA-identical sibling cord blood versus matched unrelated donor peripheral

blood),[37,38] whereas in the AIEOP experience, the CI of cGVHD was reported to be 27%.[39] In our small series, the incidence of cGVHD was 32%, the majority of cases being of progressive cGVHD (26%) and a few quiescent cGVHD (5%). Hence it is hard to determine if patients previously treated with ECP for aGVHD could benefit from retreatment.

In our cohort, ECP treatment seemed to positively impact on TRM, OS and PFS, whereas it did not impact on TTP and CI of underlying disease relapse. Such observations need to be confirmed in a larger, prospectively selected cohort, but encourage us in exploiting this promising approach for aGVHD and prompt to plan randomized trials, given the urgent need for a standardized approach to ECP in paediatric patients.

References

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Table 1: Clinical characteristics of patients treated with ECP.

Characteristic	No. Patients	Group by Reason for ECP			
		SR	SD	IC-A	IC-B
	72	21	21	9	21
Sex (M/F)	44/28	12/9	14/7	7/2	11/10
Disease					
ALL	37	8	13	6	10
AML	16	9	3	0	4
MDS/AML secondary	6	1	2	1	2
CML	4	1	2	0	1
NHL	4	2	1	0	1
Others	5	0	0	2	3
Disease status at HSCT					
CR1/CR2/CR3/other	26/30/3/13	12/6/1/2	6/11/0/4	2/2/2/3	6/11/0/4
Source of HSC					
URD	54	17	11	7	19
BM; PBSC; CB	40; 8; 6	13; 1; 3	8; 2; 1	6; 1; 0	13; 4; 2
HLA identical sibling	15	3	9	1	2
BM; CB	14; 1	3; 0	8; 1	1; 0	2; 0
HLA-identical familiar donor	1	1	0	0	0
Haplo, TCRαβCD19 depleted	2	0	1	1	0
Donor					
Age, median (range), yr	28 (1-54)	28 (16-49)	27 (2-54)	27 (1-40)	27 (10-44)
Match/mismatched*	41/31	12/9	13/8	4/5	12/9
Sex mismatched	25/72	6/21	7/21	3/9	9/21
Female donor/male recipient	10/72	2/21	3/21	2/9	3/21
Conditioning regimen					
Myeloablative: yes/no	70/2	21/0	21/0	8/1	20/1
TBI: yes/no	44/28	13/8	14/7	5/4	13/8
aGVHD: overall clinical grade at start of ECP					
Grade I; II; III; IV	8; 29; 17; 18	0; 4; 6; 11	2; 9; 6; 4	3; 5; 1; 0	3; 11; 4; 3
aGVHD: organ involvement and grade at start of ECP					
Skin	64	19	20	6	19
Grade I; II; III; IV	10; 21; 20; 13	2; 3; 8; 6	3; 8; 5; 4	2; 4; 0; 0	3; 6; 7; 3
Gut	55	18	17	6	14
Grade I; II; III; IV	27; 18; 2; 8	4; 5; 1; 8	9; 8; 0; 0	5; 0; 1; 0	9; 5; 0; 0
Liver	12	7	2	1	2
Grade I; II; III; IV	5; 4; 3; 0	1; 3; 3; 0	2; 0; 0; 0	1; 0; 0; 0	1; 1; 0; 0
Therapies before ECP					
CsA (no steroid)	9	0	0	9	0
Steroids (+ others)	63	21	21	0	21
Age at ECP, median (range), yr	8.3 (.9-20.3)	7.9 (1.5-17.9)	8.3 (.9-20.3)	8.3 (1.8-17.1)	7.9 (1.6-18.3)
Body weight at ECP, median (range), kg	25 (7-98)	25 (9.6-85)	25 (7-98)	24 (13-38)	25 (10-52)
Interval HSCT to aGVHD, median (range), d	16 (6-64)	15 (6-32)	16 (8; 41)	17 (14-64)	16 (12-50)
Interval aGVHD to ECP, median (range), d	22 (4-81)	24 (4-63)	22 (14-81)	18 (5-29)	22 (5-56)

M indicates male; F, female; ALL, acute lymphoblastic leukemia; AML, acute myeloid leukemia; MDS, myelodysplastic syndrome; CML, chronic myeloid leukemia; NHL, non-Hodgkin lymphoma; HSC, hematopoietic stem cell; URD, unrelated donor; BM, bone marrow; PBSC, peripheral blood stem cell; CB, cord blood; Haplo, haploidentical parental donor; TBI, total body irradiation.

IC-A group is those with infectious complications and no steroid before ECP; the IC-B group is those with infectious complications and steroid before ECP.

* HLA match considered 6/6.

Table 2: Outcome of patients treated with ECP according to overall grading of aGvHD and organ involvement.

Outcomes of Patients Treated with ECP according to Overall Grading of aGVHD and Organ Involvement

	No. of Patients	At Day +28			At Day +56			Stop ECP		
		CR	PR	NR	CR	PR	NR	CR	PR	NR
Overall grade										
Grade I	8	6	0	2	7	0	1	7	0	1
Grade II	29	6	8	15	19	5	5	22	2	5
Grade III	17	5	8	4	9	4	4	12	2	3
Grade IV	18	4	12	2	11	5	2	11	4	3
Total (%)	72	21 (29%)	28 (39%)	23 (32%)	46 (64%)	14 (19%)	12 (17%)	52 (72%)	8 (11%)	12 (17%)
Organ involvement										
Skin										
Grade I	10	5	0	5	7	0	3	9	0	1
Grade II	21	8	8	5	16	4	1	17	2	2
Grade III	20	10	10	0	14	6	0	16	4	0
Grade IV	13	3	9	1	8	3	2	8	2	3
Total (%)	64 (100%)	26 (41%)	21 (42%)	11 (17%)	45 (70%)	13 (20%)	6 (10%)	50 (78%)	8 (13%)	6 (9%)
Gut										
Grade I	27	15	0	12	22	0	5	23	0	4
Grade II	18	10	4	4	12	2	4	14	1	3
Grade III	2	1	0	1	1	0	1	1	0	1
Grade IV	8	3	4	1	4	3	1	4	4	0
Total (%)	55 (100%)	29 (53%)	8 (14%)	18 (33%)	39 (71%)	5 (9%)	11 (20%)	42 (76%)	5 (9%)	8 (15%)
Liver										
Grade I	5	4	0	1	5	0	0	5	0	0
Grade II	4	2	1	1	3	0	1	3	0	1
Grade III	3	0	3	0	2	1	0	2	1	0
Grade IV	0	0	0	0	0	0	0	0	0	0
Total (%)	12	6 (50%)	4 (33%)	2 (17%)	10 (84%)	1 (8%)	1 (8%)	10 (84%)	1 (8%)	1 (8%)

Figure Legends

Figure 1: Cumulative incidence of transplant-related mortality (TRM) of the entire cohort (A) and of responders to ECP compared to non-responders (B).

Figure 2: Overall Survival (OS) of the entire cohort (A) and of responders to ECP compared to non-responders (B).

Figure 3: Progression Free Survival (PFS), cumulative (A) and of responders to ECP compared to non-responders (B).

Figure 4: Time to progression (TTP) of the entire cohort (A) and of responders to ECP compared to non-responders (B).

Figure 5: Cumulative incidence of relapse, global (A) and stratified according to response to ECP (B).

Figure 1

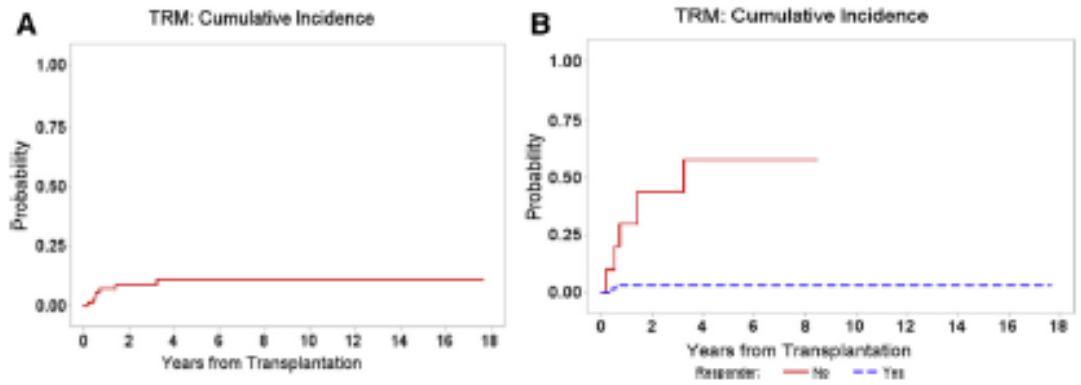

Figure 2

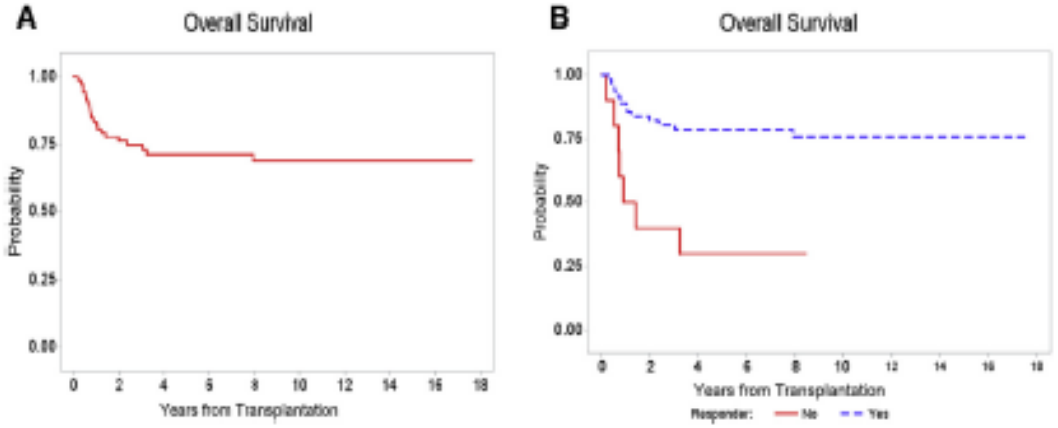

Figure 3

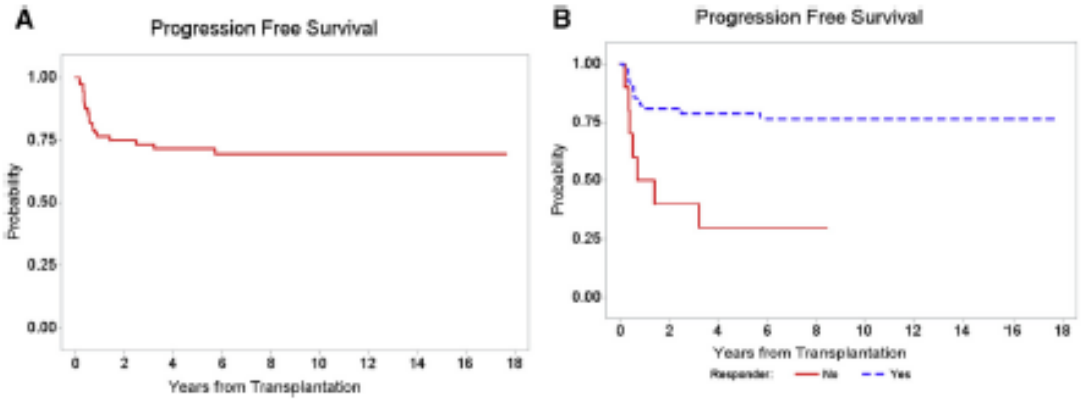

Figure 4

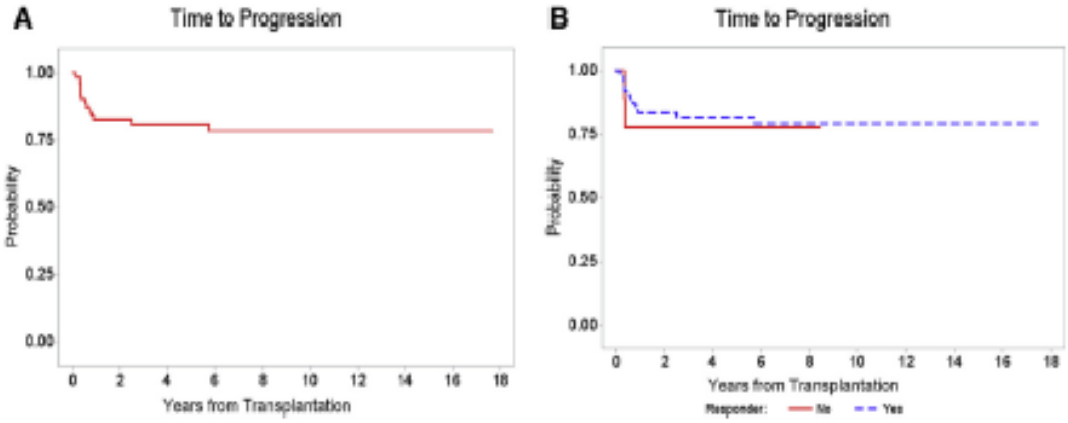

Figure 5