Monitoring and Characterization of T-Lymphocyte Reconstitution after Allogeneic Stem Cell Transplantation

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To

my parents,

my wife Reem,

my twins Jana and Mutaz

and

for the coming twins.

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Abbreviations

Ag	Antigen
ALL	Acute lymphoblastic leukemia
AML	Acute lymphocytic leukemia
APC	Allophycocyanin
APCs	Antigen-presenting cells
CD	Cluster of differentiation
CML	Chronic myelogenous leukemia
CMV	Cytomegalovirus
CTL , Tc	Cytotoxic T lymphocytes
DMSO	Dimethylsulfoxid
ELISPOT	Enzyme-linked immunoSPOT
FACS	Fluorescence activated cell sorter
FCS	Fetal calf serum
FITC	Fluorescein- isothiocyanate
gp100	Melanoma-specific antigen
GvHD	Graft versus host disease
GvL	Graft versus leukemia
HLA	Human leukocyte antigen
HY	Male-specific histocompatibility antigen
IC	Intracellular
ICC	Intracellular cytokine
IFN-γ	Interferon gamma
IL	Interleukin
Iono.	Ionomycin
MDS	Myelodysplastic syndrome
mHAg	Minor histocompatibility antigen
MHC	Major histocompatibility complex
MLR	Mixed lymphocytes reaction
MM	Multiple myeloma
NHL	Non-Hodgkin's lymphomas
PBMCs	Peripheral blood mononuclear cells
PE	R-Phycoerythrin
PerCP	Peridin chlorophyll protein
PMA	Phorbol myristate acetate
pp65	Phosphoprotein antigen
Pt.	Patient
RT-PCR	Real time-polymerase chain reaction
SEB	Staphylococcal enterotoxin B
Th	Helper T lymphocytes
TM	Tetramer
Tx	Transplantation
WT1	Wilms' tumour suppressor gene

1. Introduction

1.1 Stem cell transplantation (SCT)

It was apparent from the early mouse studies that there was potential application of chemo-irradiation and marrow grafting for therapy of leukemia and other blood diseases. The notion of a transplantable stem cell from which all hematopoiesis could be generated led to widespread application of marrow transplantation for hematologic malignancies using intensive irradiation and intravenous (i.v.) infusion of marrow to protect the recipient from the inevitable lethal marrow aplasia.[1]

However, hemopoietic stem cell transplantation (SCT) refers to the use of marrow, peripheral or umbilical cord blood as the source of self-renewing progenitor cells capable of differentiating into blood cells of all lineages.[2,3] In general, the bone marrow transplantation was first attempted, albeit unsuccessfully, when human bone marrow cells were injected intravenously to treat a patient with aplastic anemia.[4]

The first studies of human SCT were pioneered by Thomas E. Donnall and colleagues in the late 1950s.[5,6] Although all the early clinical transplantation efforts failed, most probably due to poor human leukocyte antigen (HLA) matching, research continued and more successful transplantations were reported in the early 1970s.[7,8] For his pioneer work in this field, Thomas E. Donnall received the Nobel Prize in medicine in 1990. Today, SCT is a well-established treatment method for hematological malignancies (e.g. leukemia, lymphoma and myeloma), nonmalignant bone marrow disorders (e.g. aplastic anemia) and genetic diseases associated with abnormal hematopoiesis and function (e.g. thalassemia, sickle cell anemia and severe combined immunodeficiency).[8-11]

As SCT is considered the best treatment option for many hematological malignancies, the transplant numbers have increased five-fold during the last decade. Moreover, to monitor the fast increase in adopting the SCT as a treatment, data from 118,167 SCT (36% allogeneic, 64% autologous) collected within the EBMT activity survey from 1990 to 2001 were used to assess trends over time, transplant rates and coefficient of variation (CV) of transplant rates among European countries for acute myeloid leukemia (AML), acute lymphocytic leukemia (ALL), chronic myeloid leukemia (CML), myelodysplastic syndromes (MDS), lymphoproliferative disorders (LPS) and multiple myeloma (MM). Transplant rates increased

in all European countries and for all indications from 1990 to 2001, for example, from 1.7-fold (CML) to 24.8-fold (MM).[12]

1.1.1 Stem Cell Sources

The source of the stem cells used for transplantation depends on the type of tumor, the presence of bone marrow involvement, the patient's age and the availability of a suitable donor. Hemopoietic stem cell donors can be the patients themselves as in an autologous transplantation, a genetically identical twin in a syngeneic transplantation, or a related or non-related HLA matched donor for an allogeneic transplantation. Hemopoietic stem cells also may be collected from placental or umbilical cord blood. Cord blood banks may provide donors for a larger number of patients who require allografts but do not have access to "conventional" donors. It is expected that cord blood cell use will increase because of the low incidence of immunological complications experienced by the recipients.[2]

1.1.2 Autologous SCT

Autologous transplantations most frequently are used for myeloma, autoimmune diseases, germ cell tumors, the acute and chronic leukemias, the non-Hodgkin's lymphomas and Hodgkin's disease, as well as some solid tumors such as testicular, ovarian and breast malignancies.[2,3,12]

There has been a dramatic increase in the number of autologous peripheral blood stem cell transplants over the last decade, for example in 2000, it is estimated that 25,000 autologous transplantations were completed.[2] Moreover, the autologous peripheral blood cell has many advantages over the bone marrow autografts such as the faster recovery of cell counts, lesser transplant morbidity, shorter hospital stay and reduced cost.[3] So it was rational, due to all of these advantages, to increase the autologous peripheral blood stem cell transplants.

The advantages of an autologous SCT include lack of a need to find a suitable donor and lack of graft immunoreactions against the host, since the patient is the donor. The disadvantages of autologous SCT include the possibility of infusing the patient's own malignant cells as part of the transplantation and the absence of "graft-versus-tumor" effect.

1.1.3 Allogeneic SCT

Allogeneic transplantations most frequently are used for acute and chronic leukemias, myelodysplasia and nonmalignant diseases (e.g. aplastic anemia, immunodeficiencies,

inherited metabolic disorders).[4,12] Even recently, the highest proportion of allogeneic transplants was found in AML and MDS, the lowest for MM and LPS.[12] Worldwide, there were 78,022 registered allogeneic transplantations from 1970 to 2001, and at least in 2000, it estimated that 15,000 allogeneic transplantations were completed.[2] Moreover, the numbers of allogeneic SCT carried out in Europe for the hematological malignancies from 1990 to 2001 was 42,868 composing 36% of all the total 118,167 SCTs.[12]

The choice between the more risky allogeneic transplant and an autologous procedure depends on patient age, the underlying disease, donor availability and institutional preference. For patients whose diseases or medical conditions are not applicable for the autologous transplantation, a suitable donor must be located for allogeneic transplantation. The inevitable immunological mismatch of allogeneic transplants can be beneficial to some patients for the resulting graft-versus-leukemia (GvL) effect or can generate adverse sequelae due to the resulting graft-versus-host disease (GvHD) (Table 1).

Complications resulting from infections are the most common cause of morbidity and mortality immediately after allogeneic transplantation. GvHD and infection followed by recurrence or progression of primary disease are the leading causes of death after the peritransplantation period.[2] On the other hand, as most of the allogeneic SCTs use stem cells from a matched or identical HLA donor, the cure rates following allogeneic SCT with HLA-matched siblings exceed 85% for some otherwise lethal diseases.[1]

In this study new methods will be established to monitor the contradictory GvHD and the GvL effect following the allogeneic SCT.

Transplantation Type	Stem Cell Source	Donor Search	GvHD*	GvL Effect*	Malignant Cell Infusion*
Allogeneic	Matched donor	Yes	Yes	Yes	No
Syngeneic	Identical twin	No	No	No	No
Autologous	Patient	No	No	No	Yes
* Possible sequelae.					

Table 1: Stem cell sources and sequelae.

1.2 Cellular reconstitution after SCT

The establishment of the donor immune system in the recipient (i.e. reconstitution) takes months to years to complete and functional immunocompetent T cells are reconstituted.[4] It initially involves the expansion of a postthymic donor T cell repertoire showing many unusual phenotypic and functional features. Normalization of the immune system in the recipient requires the emergence of tolerized T cells processed from precursors through the recipient thymus. This event is delayed and may be incomplete in older recipients.[13] In the first few months following bone marrow or blood SCT, the immune repertoire is dominated by T cells expanding from transplanted T cells derived from the donor's peripheral blood T cell compartment. This consists predominantly of central and effector memory cells with a smaller population of naïve T cells and endstage effector cells. It is these postthymic cells that are largely responsible for the success or failure of the transplant through their impact on engraftment, GvHD, GVL, and reactivating viruses (Figure 1).[13]

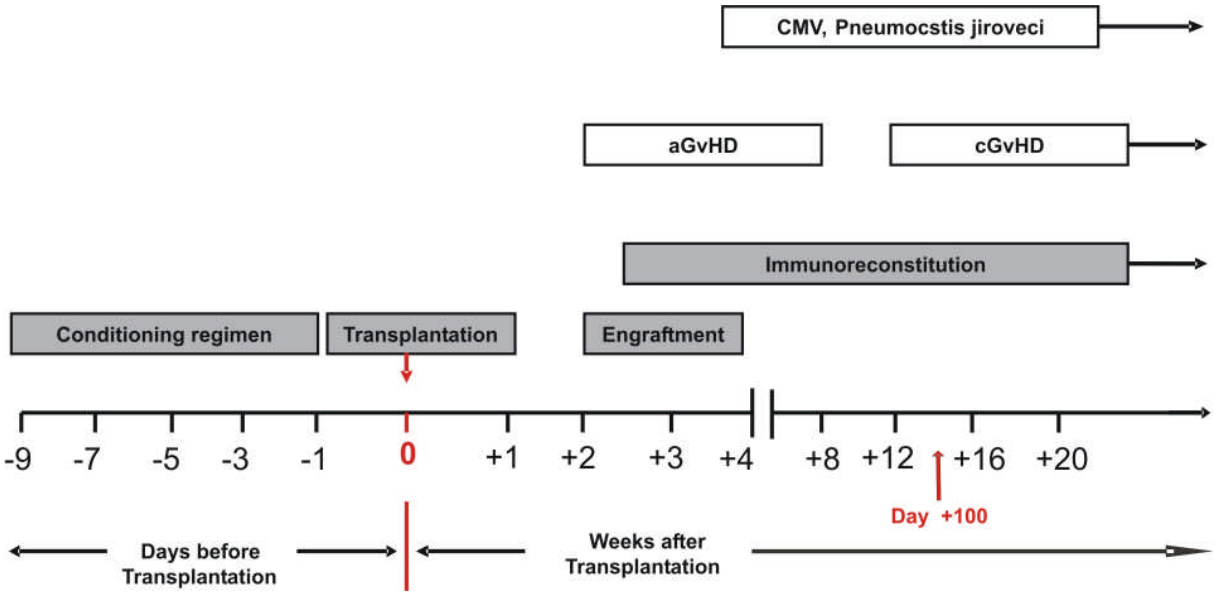

Figure 1: Course of events and risks associated with allogeneic transplantation. Gray boxes represent the 4 components of transplantation as outlined in the text; open boxes represent some risks at different stages.

Antigens (Ags) eliciting T cell responses in donor cells can be classified as (1) major or minor histocompatibility antigens (MHC or mHAgs) that can be either tissue restricted or widely distributed on many tissues, (2) normal (nonalleleic) protein sequences overexpressed

or aberrantly expressed in malignant cells, or (3) Ags representing a unique tumor specific peptide sequence. It should also be remembered that donor T cells contain a repertoire of memory cells responding to Ags of herpes group viruses (e.g. cytomegalovirus (CMV), Epstein Barr virus (EBV), herpes simplex virus (HSV)) resident in the recipient (Table 2).[13]

Non-tissue-restricted

Minor histocompatibility antigens (mHAgs)

Major histocompatibility (MHC) antigens directly and indirectly presented

Tissue-restricted/aberrantly expressed

mHAgs: HY, HA-1, HA-2, HB-1

Nonalleleic: proteinase 3, WT-1, telomerase

Malignant-cell-restricted

Products of chromosome translocation: t9:22, t15:17

Virus-specific

CMV: pp65, IE1

EBV : EBNA 1-3, LMP-1, LMP-2

Table 2: Classification of well-characterized antigens driving donor T-cell responses.
HY, male-specific minor histocompatibility antigen; HA, minor histocompatibility antigen; HB-1, B cell minor histocompatibility antigen-1; WT1, Wilms' tumor; t, translocation; pp,65 phosphoprotein 65 antigen; IE1, intermediate-early 1 antigen; EBNA, Epstein Barr nuclear antigen; LMP, latent membrane protein.

More detailed information on the reconstitution of Ag-specific responses has been derived from intracellular cytokine flow cytometry, cytokine secretion assays [enzyme-linked immunospot (ELISPOT), matrix affinity technology] and class I HLA-peptide tetramer labelling.[14] But little is done in the field of molecular biology to assess the reconstitution of Ag-specific T cells after SCT. Therefore, in this study, the reconstitution of CMV- and mHAg-specific T cells (e.g. anti-HY and anti-WT1 T cells) will be monitored in allogeneic transplanted patients following SCT.

1.3 Cytomegalovirus (CMV) infection after allogeneic SCT

Cytomegalovirus (CMV) is a frequent pathogen in humans and is usually associated with asymptomatic infection, followed by a state of viral persistence or latency. Human CMV

establishes persistent lifelong infections in most (50%-85%) individuals.[15,16] The virus primarily infects endothelial cells in a range of tissues and, after a lytic cycle, establishes an asymptomatic latent infection.[15] The principal site of virus latency in the peripheral circulation is likely the monocyte.[17]

CMV infection still remains a major cause of morbidity and mortality after SCT. In contrast to patients treated with high-dose chemotherapy and autologous SCT, patients after allogeneic SCT are at a much higher risk of active CMV infection because of the delayed recovery of T- and B-cell functions.[18] In the context of immunologic impairment due to conditioning for SCT, 60% to 70% of high-risk (CMV-seropositive) or CMV-seronegative patients who receive transplants from a seropositive donor; if no preventive measures are taken, will be under the risk of developing CMV disease during the first 100 days after conventional or nonmyeloablative SCT. And moreover, approximately ~20–30% will develop CMV disease during the first year, unless preemptive strategies were adopted..[17,18] In the early days of allogeneic SCT, CMV can be observed to reactivate 30 to 60 days after transplantation, and disease occurred in approximately one half of patients.[19] The time of onset of disease increased to approximately six months after allogeneic SCT, and mortality due to late-onset CMV caused the preponderance of deaths at a rate approaching 10% for allogeneic SCT recipients.[20] Effective antiviral prophylaxis and early intervention has led to decrease the active CMV infection and disease after day 100 after transplantation. Patients developing late-onset CMV disease are characterized by a delayed reconstitution of CMV-specific T-cell responses.[21,22]

As CMV infection after allogeneic SCT is frequently associated with life-threatening invasive lung and visceral disease,[23-25] monitoring the CMV load and the presence of the CMV-specific T cells are too important. Accurate monitoring of CMV-specific T-cell reconstitution is required for appropriate decision on treatment, such as anti-viral drugs, which have adverse effects. As many researchers took in consideration the reconstitution of CMV-specific T cells after allogeneic SCT,[26-28] it was found that the threshold level for protection from CMV reactivation was estimated (e.g. over 1×10^6 cells/l peripheral blood with the IFN-γ-ELISPOT assay).[26] Recurrence of CMV infection occurred only in the patients who failed to generate a cytotoxic T lymphocyte (CTL) response to the virus,[27] so the monitoring of CMV-specific CTLs may help in identifying the subset of patients at risk from recurrent infection or disease. In a pilot study in a limited number of patients at high risk for late-onset CMV disease, a single transfusion of a donor-derived *ex vivo* expanded

polyclonal CMV-specific T-cell line was found to be associated with clearance of the viral load from the blood and reconstitution of CMV-specific T-cell responses in some of the patients, indicating a potential strategy to prevent late CMV disease.[29]

There are many methods in use for detecting CMV in the body fluids such as rapid culture, antibody assays, antigenemia assays, and DNA detection methods.[30,31] The effect of pre-emptive CMV treatment based on early detection of CMV reactivation - by methods including antigenemia assays,[30,31] polymerase chain reaction (PCR)[30,32-34] or recently by the reverse transcription-PCR[31,35-38] - has been found to increase the time to onset of CMV disease, with the result that late disease is the current main CMV-related problem in SCT.[39-41]

In this study, the reconstitution of CMV-specific T-cells will be monitored to predict the patients' protection against the CMV. In addition, new functional assay will be established to assess the CMV-specific T cells in the patients' peripheral blood after allogeneic SCT.

1.4 Graft-versus-host disease (GvHD)

Billingham and Brent described how injection of newborn mice with viable spleen cells from adult donors of a different strain resulted in the development of what they termed runt disease,[42] but it was the Danish physician Morton Simonsen who introduced the name graft-versus-host disease (GvHD). Normally, GvHD divided into two types, a) acute GvHD (aGvHD) which occurs within the first 100 days after transplantation and b) chronic GvHD (cGvHD) is distinguished from aGvHD by clinical symptoms that can resemble an overlap of several connective tissue diseases (e.g. lupus erythematosus, mixed connective tissue disease, scleroderma, Sjogren syndrome, biliary cirrhosis, idiopathic pulmonary fibrosis). The classical definition of cGvHD is GvHD that persists or occurs *de novo* beyond 80-100 days post SCT (Figure 1).[43]

Despite adequate post-transplantation immunosuppressive therapy, GvHD remains a major cause of morbidity and mortality in the allogeneic SCT setting, even in patients who receive HLA-identical sibling grafts.[44] Up to 30% of the recipients of stem cells or bone marrow transplantation from HLA-identical related donors and most patients who receive cells from other sources (e.g. matched, unrelated, non-HLA-identical siblings; cord blood) will develop ≥ Grade 2 aGvHD despite immunosuppressive prophylaxis.[45,46]

GvHD can occur when transplanted donor-derived T cells recognize MHC or mHAgs proteins and their associated peptides expressed by antigen-presenting cells (APC). A widely

accepted paradigm for the pathophysiology of GvHD is based on the existence of 3 sequential phases (Figure 2).

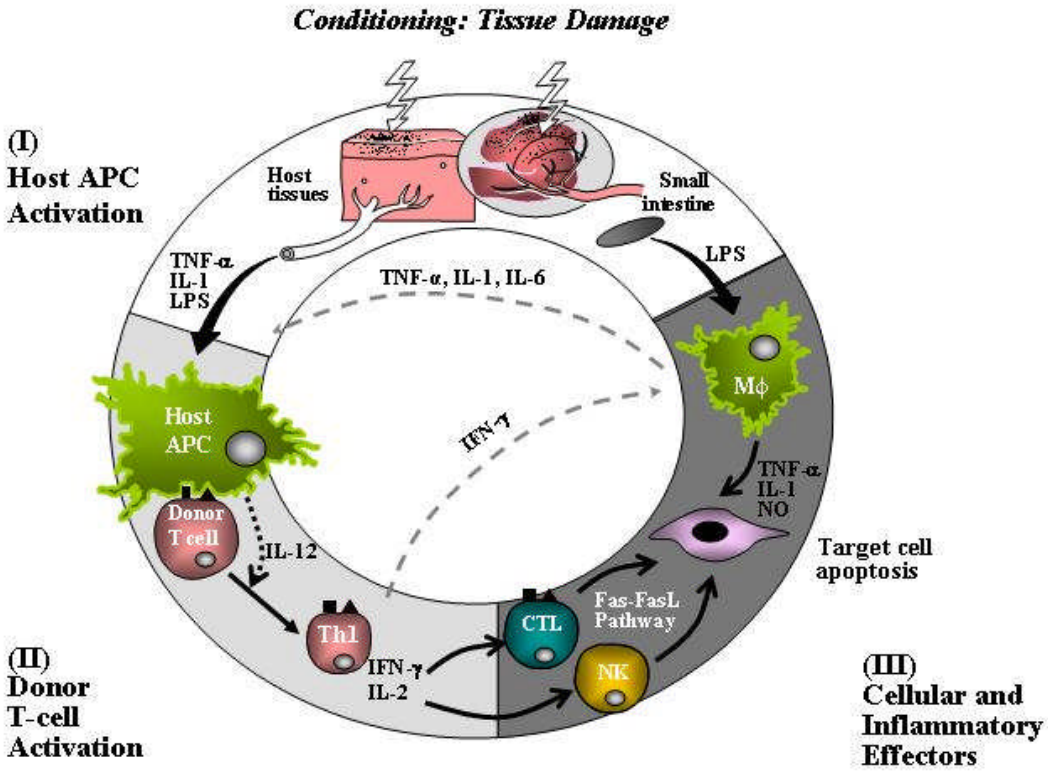

Figure 2: GVHD Pathophysiology

GvHD remains a potentially fatal complication of allogeneic SCT that involves the gut, liver and skin.[47,48] Many cellular mediators of GvHD are known such as dendritic cells (DCs), T cells, NK cells, macrophages, cytokines and surface markers on immune cells (i.e. MHC and mHAgs) in the recipient.[43] GvHD is initiated by the action of donor-derived T cells that have been suggested to polarize into type 1 T cells after being stimulated with interleukin (IL)-12 from APC. These type 1 T cells, comprising CD4+ T helper (Th1) and CD8+ T cytotoxic cells (Tc1), produce interferon (IFN)-γ , tumor necrosis factor (TNF)-α and Fas ligand that, in combination, severely injure multiple organs, leading to GvHD.[49-51] On the other hand, recently substantial attention has been focused on the potential for regulatory T (Treg) cells that may be capable of suppressing alloreactivity in the setting of murine and human allogeneic transplantation.[52-54]

To eliminate or reduce the lethal complications of GvHD in early time points after the onset of disease, great attention is paid to detect GvHD after human SCT. As the standard

methods used normally to diagnose GvHD, which are the histological methods, overlapped with viral infections, so other methods was established. For example, host-reactive lymphocytes with broad specificity have been observed in GvHD patients using the limiting dilution techniques, which are time consuming for detecting functional T cell analysis. In the meantime, many techniques were established to predict GvHD such as, T lymphocyte precursors frequency analysis,[55] some serum markers (e.g. the levels of TNF-α , IFN-γ , IL-10, soluble Fas, and IL-18),[49,51,56,57] polymorphism of IL-10[58] and transforming growth factor (TGF)-β1[59] genes, ELISPOT assays,[47,60] and T cell receptor (TCR)-Vβ clonotypic analysis.[60-62] In addition, multimers technology has been developed which allows flow cytometric detection of specific T cells independently of their activation state.[63] But most of these analysis methods have not been reported to be necessarily predicting GvHD. Thus, there are contradictory results among these methods and there still remain problems with attempts to use these parameters as reliable and sensitive markers of GvHD. Therefore, in this study, the monitoring of alloreactive T cells in the patients peripheral blood will be monitored by establishing two assays including the detection of intracellular cytokine (ICC) by flow cytometer and real time-polymerase chain reaction (RT-PCR) assay.

1.5 Graft-versus-leukemia (GvL) effect

There is compelling evidence, much of which is derived from the results of allogeneic SCT, that human leukemias can also be recognized and eliminated by T cells. The immunologically mediated graft-versus-leukemia (GvL) effect that was predicted by animal model studies of allogeneic SCT has been documented in clinical trials. Patients who receive an allogeneic transplant for advanced leukemia have a lower probability of leukemic relapse if they develop acute and/or chronic GvHD as a complication of the transplant.[64,65] Also, Kolb and Holler were able to prove that donor transfused lymphocytes exhibits a GvL effect and increases chimerism after bone marrow transplantation.[66] The risk of leukemic relapse is increased after syngeneic SCT or T-cell depleted allogeneic SCT, suggesting a critical role for donor T cells specific for allogeneic determinants in initiating or mediating the GvL effect.[67]

Target T-cell epitopes involved in the GvL reaction are either autologous tumor associated antigens (TAAs)[68] or allogeneic mHAgs[69] expressed by the tumor. In allogeneic SCT, T cells specific for mHAgs - which are peptides that differ between donor and recipient due to polymorphism in the genome - provide a potent GvL effect.[70] However, T cells specific for some mHAgs are also responsible for GvHD, and means of reliably segregating the

beneficial GvL effect from GvHD has not yet been well established (Figure 3).[71] For better understanding such segregation, recently it was found that T cells contribute to GvHD and GvL via membrane-bound or locally released TNF.[72] Moreover it was found that the cytolytic activity of T cells is primarily mediated through the Fas-Fas ligand and perforin-granzyme pathways.[73] In experiments with purely selected donor T cells, the FasL pathway was important for GvHD activity by both CD4(+) and CD8(+) T cells, whereas the perforin pathway was required for CD8-mediated GvL activity. These data demonstrate, in an allogeneic bone marrow transplanted murine model, that donor T cells mediate GvHD activity through the FasL pathway compared to GvL activity which use the perforin pathway. This suggests that donor T cells make differential use of cytolytic pathways and that the specific blockade of one cytotoxic pathway may be used to prevent GvHD without interfering with GvL activity.[74] On the other hand and surprisingly, it was found that T cells deficient for both Fas ligand and perforin can still exert GvL activity *in vivo* in mouse models.[75,76] This was resolved by Schmaltz *et al*. who found that the TNF-related apoptosis-inducing ligand (TRAIL) is mediating the GvL but not the GvHD. These data suggest that strategies to enhance TRAIL-mediated GvL activity could decrease relapse rates of malignancies after hematopoietic SCT without exacerbation of GvHD.[73]

In addition, there is evidence that effector mechanisms other than T cells may also contribute to GVL activity either directly or as a consequence of inflammation induced by allogeneic T cells. This was confirmed by the observation that natural killer (NK) cells can lyse leukemic cells *in vitro*. However, NK cells may be particularly effective for inducing GvL activity after T cell depleted haploidentical transplant where disparity between killer inhibitory receptors (KIRs) expressed by donor NK cells and HLA molecules on recipient leukemic cells favors NK activation.[77,78]

One approach for separating GvL from GvHD is to identify peptides that are recognized by T cells and presented by leukemic cells but not by tissues that are a target of GvHD. There are several broad categories of proteins that may give rise to Ags that could be targets of a selective GvL response. These include a) tumor-specific proteins resulting from chromosome translocations such as bcr/abl or PML/RAR, or from mutations such as p21 ras,[79-81] b) normal proteins that are overexpressed in leukemic cells such as WT-1 or proteinase 3,[82,83] and c) mHAgs that are selectively expressed in recipient hematopoietic cells including leukemic cells but not in nonhematopoietic cells.[84-86]

In this study, the reconstitution of WT1-specific T cells, as a GvL model, will be monitored in the peripheral blood of allogeneic transplanted patients. In addition, a sensitive and new RT-PCR assay will be established to monitor the effector cells of the GvL effect.

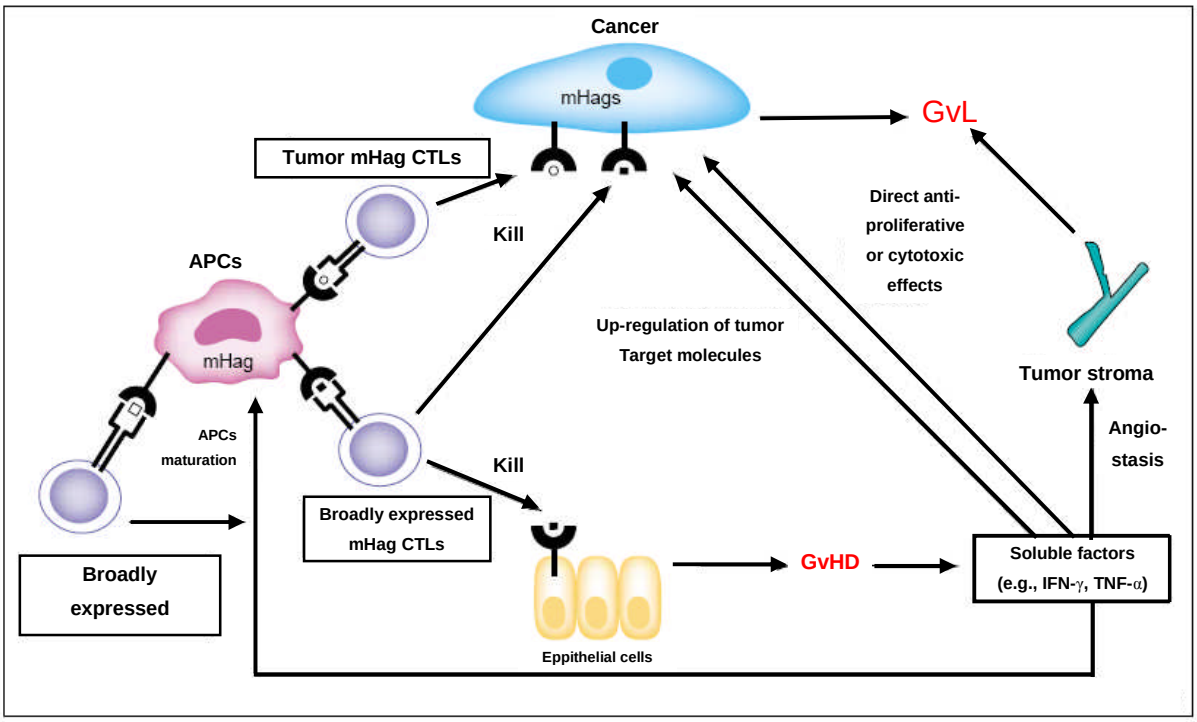

Figure 3: The supportive influence of GvHD on the GvL effect in the effector phase of mHAg CTLs.

1.6 Minor histocompatibility antigens (mHAgs)

The observations that inbred strains of mice were able to reject both tumors and skin grafts from MHC identical donors, led Snell *et al*. to propose that additional transplantation Ags must be encoded by genes other than the MHC.[87,88] Since graft rejection produced as a result of these genetic differences was significantly slower than in case of MHC disparities, they were described as minor histocompatibility antigens (mHAgs). Though these observations were later confirmed in humans by the rejection of skin grafts from HLA-identical siblings,[89] it was with the advent of clinical allogeneic STC that mHAgs came to the spotlight and provided an impetus for further investigation.[90-92] Currently many of these mHAgs are well identified.[90,91,93] Moreover, Hambach and Goulmy suggested a cancer immunotherapy by targeting these mHAgs.[94]

It was found that some mHAg-specific T cells can contribute to a selective GvL effect after allogeneic SCT.[95] The GvL effect has been associated with an expansion of mHAg-

specific T cells that also exhibit a suppressive effect on the growth of leukemic precursors *in vitro*.[86,96] Moreover, isolation,[97] generation[98,99] and expansion [100] of the mHAgs-specific T cells are visible recently. On the other hand and unfortunately, many mHAgs are ubiquitously expressed, thus T cells may also target normal tissues leading to GvHD.[101-103] In fact, mHAg-specific T cells are detectable in patients with active GvHD.[104] Furthermore it was found that pregnancy is able to prime mHAg-specific T-cell responses for both autosomal (e.g. HA-1) and anti-HY (male specific) mHAgs.[105,106] Therefore, the WT1- and HY-specific T cells, as mHAg models, will be monitored in this study especially their reconstitution following allogeneic SCT.

1.7 Immune monitoring approaches

1.7.1 Cytokines

In general, cytokines provide a direct measure of the effector function of T lymphocytes.[107] For example, T cells that produce interleukin (IL)-2, tumor necrosis factor (TNF)-α and interferon (IFN)-γ are considered to be of the Th_1 phenotype, compared with T cells that produce predominantly IL-4, IL-5, IL-10, and IL-13, which define the Th_2 phenotype.[50]

In the past decade, the analysis of cytokine production became increasingly important in unraveling the course of an immune response, in the evaluation of specific therapies, and in the search of the pathophysiologic mechanisms at the base of many diseases. Fore example, TNF-α has been implicated in the pathophysiology of GvHD at several steps in the process, including induction of apoptosis in target tissues through the TNF-α receptor; activation of macrophages, neutrophils, eosinophils, B cells, and T cells; stimulating production of additional inflammatory cytokines (IL-1, IL-6, IL-10, IL-12, and TNF-α itself); increased expression of HLA; and the facilitation of T-lymphocyte lysis.[72,108-110] In addition, IFN-γ is another pro-inflammatory cytokine that can be secreted in GvHD pathophysiology (Figure 2) and in response to CMV.[47,111] Therefore, in addition to IFN-γ, some cytokines will be traced in many of the assays adopted in this study. But it should be kept in mind that the cytokines may be similar in both GvHD and GvL because they will be secreted in response to Ag whether it was alloantigen (in the case of GvHD) or tumor Ag (in the GvL). So, to solve this problem, the secreted cytokines should be correlated to the applied Ag.

1.7.2 Mixed lymphocytes reaction (MLR)

When lymphocytes from genetically different individuals are mixed together in tissue culture blast transformation occurs, a reaction known as the mixed lymphocyte reaction (MLR). The MLR is a clinically relevant *in vitro* assay where lymphocytes from one individual (responders, R) are incubated with the lymphocytes of another individual (stimulator, S) which have been previously rendered incapable of blast transformation by gamma-irradiation.[112] It is presumed that the MLR is an *in vitro* analog of *in vivo* alloreactivity, and is widely used in transplantation immunology to measure recipient T-cell responses against donor tissues due to the mismatch of MHC antigens (especially class II).[113] The MLR ability to predict possible rejection of the donor organ in the transplant recipient can be used in the allo-settings in healthy individuals.[114-116] So, a modified MLR assay will be established and adopted to assess the alloreactive T cells in the allogeneic transplanted patients following SCT. This will establish an assay which, hopefully, will predict the GvHD.

1.7.3 Tetramer (TM) staining

Recently developed MHC multimer technologies allow visualization and isolation of Ag-specific T cells. Fore example, the introduction of peptide–MHC class I tetrameric complex technology initiated a profound revolution in the field of cellular immunology.[117] Class I HLA-peptide tetramers (TM) are soluble complexes of four synthetic HLA molecules associated with a specific peptide that fits the peptide-binding groove of the HLA molecule under study. TMs are conjugated with a fluorochrome to allow their visualization by flow cytometry.[118]

Peptide-MHC class I tetrameric complexes are proving invaluable as fluorescent reagents for enumeration, characterization, and isolation of peptide-specific T cells and have afforded many advantages over previous techniques, particularly the ability to directly quantify and phenotype Ag-specific T cells with minimal *in vitro* manipulation.[119] However, functional analysis and *in vivo* transfer of MHC multimer-stained cells is hampered by the persistence of TCR-MHC interactions and subsequently induced signaling events.[118,120] But interestingly, new types of MHC multimers were generated, which can be monomerized in the presence of a competitor, resulting in rapid loss of the staining reagent allowing “reversible” T-cell staining procedure.[121]

However, a cell is not characterized so much by what lies on its surface (e.g. TM), but by what resides inside, where most of its biology takes place and which is likely to reflect its

functional phenotype. Therefore the phenotypical TM staining in addition to other functional assays will be adopted to monitor the reconstituted T cells.

1.7.4 Intracellular cytokines (ICC) flow cytometry

The most popular method to assess cytokines is the enzyme-linked immunosorbent assay (ELISA), which is applied to measure cytokine secretion in supernatants, whereas flow cytometry is used to determine intracellular cytokine (ICC) production.[122] Moreover, it was found that the circulating lymphocyte subsets linked to the ICC profiles in normal humans.[123] ICC flow cytometry is based on direct detection of ICC expression with fluorochrome-conjugated anticytokine antibodies after short periods of activation with various stimuli (e.g. 5-6h). Stimulation can be performed with peripheral blood mononuclear cells (PBMCs), whole blood, lymph nodes, or other biologic fluids.[124] Staining of the ICC depend on the identification of cytokine-specific monoclonal antibodies which are compatible with a fixation-permeabilization procedure.[123]

It is well known that the ICC assays have some disadvantages such as the low sensitivity (10^{-4}) - compared to ELISPOT - and the cell-fixation which limits further functional assays to be performed.[107] Although there is no absolute quantitative measurement of the produced cytokine by the ICC flow cytometric method, but it can easily identifies the cytokine-producing cell type by phenotyping for many cell markers (e.g. cell lineage, activation and apoptosis). For the fast and phenotypical criteria of the ICC assays, it will be used in this study to monitor the reconstitution of T cells in allogeneic transplanted patients.

1.7.5 Enzyme-linked immunospot (ELISPOT)

The enzyme-linked immunospot (ELISPOT) assay is based on the principle of the ELISA detecting antigen-induced secretion of cytokines trapped by an immobilized antibody and visualized by an enzyme-coupled second antibody.[125] In a recent study, IFN-γ-ELISPOT assay showed to have good reproducibility for the determination of Ag-specific T cells in different laboratories.[126] The ELISPOT assay has the advantage of detecting only activated/memory T cells and the cytokine release can be detected at the single cell level, allowing direct determination of T cell frequencies.[127] Furthermore, this assay has been found to be more sensitive than ELISA (e.g. detection limit of 10-200 times lower) and ICC staining.[128,129] The high sensitivity and easy performance, allowing a direct enumeration of peptide-reactive T cells without prior *in vitro* expansion, makes the ELISPOT assay

eminently well suited to monitor and measure T-cell responses.[130] For these advantages, the ELISPOT assay will be adopted to monitor the reconstitution of T cells after allogeneic SCT.

1.7.6 Real time-polymerase chain reaction (RT-PCR)

The real time-polymerase chain reaction (RT-PCR) assay is based on the principle that amplification of cDNA by the polymerase chain reaction (PCR) follows a strict mathematical equation whereby with each cycle of amplification two copies are made from each individual. Thus, the amount of cDNA amplified after a given number of cycles will be directly proportional to the log2 of the starting amount of template. This quantitation is achieved with a gene-specific nucleotide probe complementary to a region of DNA nested between the PCR primers. This probe is labeled with a reporter fluorochrome and also with a quencher that can absorb fluorescence. The quencher can only quench the reporter fluorescence when the two dyes are close to each other. During amplification the probe is removed from the DNA strand and degraded by the 5'-3' exonuclease activity of Taq DNA polymerase and the fluorochrome is separated from the quencher yielding one unit of fluorescence for each cycle of amplification. By recording incremental fluorescence at each PCR cycle it is, therefore, possible to calculate the starting amount of cDNA template. [124,131,132] Also, the RT-PCR instruments allow “real time” detection of PCR products as they accumulate during PCR cycles. Thus, by RT-PCR it is possible to gather quantitative information about gene expression in any given specimen.

Many significant advantages to the use of RT-PCR for immune monitoring were described such as its flexibility, sensitivity and reliability.[124,127,133] Also, the RT-PCR can be considered the method of choice for the rapid and reproducible measurement of gene expression in small samples.[134] In addition to its sensitivity, RT-PCR also provides flexibility of analysis since cDNA is quite stable for future analysis.[127] Moreover, recently the RT-PCR assay was developed to assess the many cytokines in the murine as well in human.[133,135] For example, serial sampling of fine-needle aspirates of metastases from melanoma patients receiving IL-2-based vaccinations has been performed to assess changes in expression of IL-10, TGF-β, and IFN-γ mRNA levels by RT-PCR assay.[136]

This tool offers unique advantages and should be considered as part of a repertoire used to design a comprehensive immune monitoring strategy.[124] Moreover, it was suggested that RT-PCR represents a useful tool for the monitoring of patients undergoing immune manipulation (e.g. SCT). However, little information is available in the literature about the

utilization of RT-PCR for immune monitoring, as this methodology has been only recently applied to this field. Therefore, the RT-PCR assay will be adopted in this study to monitor the reconstitution of donor T cells in the allogeneic transplanted patient's body.

The present study strived to investigate, using various techniques, whether Ag-specific T cells could be monitored in allogeneic transplanted patients, and if so, how the used techniques correlate to each other. Four main questions were addressed:

1) Can the reconstitution of CMV-specific T cells after allogeneic SCT be monitored using phenotypical and functional techniques?
Approach: using TM, ICC, ELISPOT and RT-PCR techniques to monitor the reconstituted CMV-reactivity by the aid of CMV pp65-peptide and/or pp65-protein.

2) Can the GvL effect be monitored after allogeneic SCT using functional techniques especially the RT-PCR? This was address by monitoring the reconstitution of WT1-reactive T cells using ELISPOT and RT-PCR techniques using two HLA-A2-restricted peptides, namely: WH187 and Db126.

3) Can the mHAgs-reactive T cells be monitored after allogeneic SCT using functional techniques especially the RT-PCR? This was addressed by monitoring the HY-reactive T cells using ELISPOT and RT-PCR techniques using SMCY-derived HY peptide.

4) Can the alloreactive T cells in the allogeneic transplanted patients' peripheral blood be monitored by intracellular (IC) cytokine staining? This was investigated by first establishing and optimizing an alloreactive mixed lymphocyte reaction (MLR) model that can simulate the GvHD settings, then testing some allogeneic transplanted patients' samples for detecting the presence of alloreactive T cells.

2. Materials and Methods

2.1 Materials

2.1.1 Equipment

Instrument	Provider
Applied Biosystems 7900HS Fast Real-Time PCR System	Applied Biosystems, Darmstadt, Germany
Biofuge pico	Heraeus, Hanau, Germany
BIOREADER® 2000	BIO-SYS, Karben, Germany
Centrifuge	Hettich, Vlotho, Germany
FACSCalibur	Becton-Dickinson, San Jose, USA
Filtermate 196 Packard Harvester	Canberra Packard, Zurich, Switzerland
Improved Neubauer Hemocytometer	Paul Marienfeld, Lauda-Koenigshofen, Germany
Incubator	Heraeus, Hanau, Germany
Inverted microscope	Zeiss, Heidelberg, Germany
Laminar air flow cabinet	Heraeus, Hanau, Germany
Microplate Scintillation Counter Topcount	Canberra Packard, Zurich, Switzerland
PCR Thermal Cycler	Perkin Elmer, Überlingen, Germany
pH-Meter	Knick, Berlin, Germany
Pipettes	Eppendorf, Hamburg, Germany
Spectrophotometer	Eppendorf, Hamburg, Germany
Water bath	Fried Electronic, Haifa, Israel

2.1.2 Chemicals and disposables

Product	Provider
Alkaline phosphatase conjugate substrate kit	Bio-Rad Laboratories, Munich, Germany
CMV-pp65 peptide (NLVPMVATV)	Clinalfa, Laeufelfingen, Switzerland
CMV-pp65 tetramer	Beckman Coulter, San Diego, USA
CMV-pp65 recombinant protein	Milenia Biotec, Bad Nauheim, Germany

Cytofix/Cytoperm® kit	Becton Dickinson, Heidelberg, Germany
Dimethyl sulfoxide (DMSO)	Merck, Darmstadt, Germany
Distilled water	B. Braun Melsungen, Melsungen, Germany
DNase I	Roche, Basel, Switzerland
Endoglubin	Baxter, Unterschleissheim, Germany
FACS tubes	Becton Dickinson, Meylan Cedex, France
Fetal calf serum (FCS)	PAA Laboratories, Pasching, Austria
Ficoll-Paque™ Plus	Amersham Biosciences, Uppsala, Sweden
gp100 peptide (TLGPGPVTA)	Bachem, Bubendorf, Germany
Human AB serum	Cambrex Bio Science, Verviers, Belgium
HY-311-319 peptide (FIDSYICQV)	ProImmune, Oxford, U.K
Ionomycin	Sigma-Aldrich, Munich, Germany
Iscove's medium	Biochrom, Berlin, Germany
L-Glutamine	Invitrogen, New York, USA
[Methyl-^{3}H]-thymidine	Hartmann Analytics, Braunschweig, Germany
Nitrocellulose 96-well plates	Millipore, Schwalbach, Germany
Penicillin / Streptomycin	Invitrogen, New York, USA
Phorbol myristyl acetate (PMA)	Sigma-Aldrich, Munich, Germany
Phosphate buffered saline (PBS) w/o Mg^{2+}, Ca^{2+}	Gibco, Karlsruhe, Germany
PKH26 red fluorescence cell linker kit	Sigma-Aldrich, Munich, Germany
Reverse transcription system	Promega, Mannheim, Germany
RNeasy micro kit	Qiagen, Hilden, Germany
RPMI 1640 medium	Cambrex Bio Science, Verviers, Belgium
Sodium azide	Carl Roth, Karlsruhe Germany
Staphylococcal enterotoxin B (SEB)	Sigma-Aldrich, Munich, Germany
Streptavidin-alkaline phosphatase	Bio-Rad Laboratories, Munich, Germany
TaqMan well-plates	ABgene, Hamburg, Germany
Trypan-blue	Sigma-Aldrich, Munich, Germany
Tween	Merck, Darmstadt, Germany
U-bottom 96-wells culture plates	Becton Dickinson, Meylan Cedex, France
UniFilter-96 GF/C	PerkinElmer, Rodgau - Jügesheim, Germany
WT1-Db126-134 (R**M**FPNAPY**L**) peptide	Clinalfa, Laeufelfingen, Switzerland
WT1-WH187-195 (S**L**GEQQYS**V**) peptide	Clinalfa, Laeufelfingen, Switzerland

2.1.3 Monoclonal antibodies

Product	Clone	Fluorochrome	Provider
Anti-CD3	SK7	PerCP[1]	Becton Dickinson, Heidelberg, Germany
Anti-CD3	S4.1	Alxa Fluor 488	Caltag Laboratories, Hamburg, Germany
Anti-CD3	S4.1	PE-Cy5.5[2]	Caltag Laboratories, Hamburg, Germany
Anti-CD4	SK3	APC[3]	Becton Dickinson, Heidelberg, Germany
Anti-CD8	RPA-T8	APC	Becton Dickinson, Heidelberg, Germany
Anti-IFN-γ	B27	FITC[1]	Becton Dickinson, Heidelberg, Germany
Anti- IFN-γ	NIB42	-	Becton Dickinson, Heidelberg, Germany
Anti- IL-4	MP4-25D2	FITC[4]	Becton Dickinson, Heidelberg, Germany
Anti- Perforin	deltaG9	PE	Hoelzl Diagnostika, Cologne, Germany
Anti- TNF-α	MP9-20A4	PE	Caltag Laboratories, Hamburg, Germany
Biotinylated anti-IFN-γ	4S.B3	-	Becton Dickinson, Heidelberg, Germany

[1] Peridin chlorophyll protein
[2] R-Phycoerythrin
[3] Allophycocyanin
[4] Fluorescein-isothiocyanate

2.1.4 CD8 and IFN-γ primers

Primer	Sequence	Provider
CD8 (forward)	5'-CCCTGAGCAACTCCATCATGT	Applied Biosystems, Darmstadt, Germany
CD8 (reverse)	5'-GTGGGCTTCGCTGGCA	Applied Biosystems, Darmstadt, Germany
IFN-γ (forward)	5'-AGCTCTGCATCGTTTTGGGTT	Applied Biosystems, Darmstadt, Germany
IFN-γ (reverse)	5'-GTTCCATTATCCGCTACATCTGAA	Applied Biosystems, Darmstadt, Germany

2.1.5 CD8 and IFN-γ probes

Probe	Sequence	Provider
CD8	**FAM**-TCAGCCACTTCGTGCCGGTCTTC-**TAMRA**	Applied Biosystems, Darmstadt, Germany
IFN-γ	**FAM**-TCTTGGCTGTTACTGCCAGGACCCA-**TAMRA**	Applied Biosystems, Darmstadt, Germany

2.1.6 Kits

BD Cytofix/Cytoperm™ kit was used to perforate the cells to allow IC cytokines detection. In addition, total RNA was isolated from PBMCs by using RNeasy Micro kit.

2.1.7 Media

Both RPMI1640 and Iscove's media were used and were called "'complete" when containing the following:

Additives	Final Concentration
Pooled human AB serum	10%
Penicillin / Streptomycin	1%
L-Glutamine	2%

Also a freezing medium, composed of 90% fetal calf serum (FCS) and 10% DMSO, was used to freeze cells under liquid nitrogen.

2.1.8 Cell lines

The human HLA-A*0201-positive leukemia B-cell line C1R.A2 (expressing a transfected genomic clone of HLA.A2) was used as Ag presenting cells. It is unable to present endogenous Ags so it just expresses surface peptide-pulsed HLA-A2 molecules. These cells were cultured in complete-RPMI 1640 medium until peptide pulsing.

2.2 Patients

All the included patients underwent allogeneic SCT, and their donors were mandatory matched for the HLA-A,-B and -C and optional matched for the HLA-DR and -DP allele. All patients and their donors gave informed consent approved by local ethics committee (IRB).

2.2.1 Analysis of T-cell reactivity to CMV in patients

Eighteen patients (n=18) were tested for the reconstitution of CMV-specific T cells post transplantation. The CMV serological status was identified for all patients and donors. CMV pp65 protein was used as stimulus due to its dominant Ag recognition by CD4 T cells. In addition, one attractive candidate stimulating Ag the immunodominant HLA-A2 peptide epitope CMV pp65 (495-503) was used.[137,138] T-cell reactivity for the CMV Ags was tested

in all of the patients in the experimental-group (i.e. HLA-A2 positive) and control-group (i.e. HLA-A2 negative). PBMCs from ten patients were monitored at serial time point to follow the reconstitution of CMV pp65-specific T cells. After testing most of the patients' PBMCs by TM staining, ICC flow cytometry, ELISPOT assay and RT-PCR assay, the correlation between the different assays was calculated. The tested combinations of CMV serological status were as shown in table 3 :

	CMV Status	
	Patient	**Donor**
HLA-A2+	+	+
	-	+
	+	-
HLA-A2-	+	+
	-	+
	+	-

Table 3: Patient-Donor CMV serological combinations used to detect reconstituted CMV-specific CD8 T cells.

PBMCs were co-cultured in final concentration of 10µg/ml for the pp65 peptide,[139] and 20µl/ml for the CMV pp65 protein (i.e. no stock concentration was given by the provider). The incubation time was 3h (RT-PCR), 6h (ICC) and 24h (ELISPOT), respectively.

2.2.2 Analysis of a potential GvL effect (e.g. WT1) in patients

Patients with leukemia in which the WT1 Ag is potentially over-expressed on the leukemic blasts [140,141] were enrolled in this study. PBMCs from twelve patients with AML (n=9), ALL (n=2) and CML (n=1) leukemia were tested for the reconstitution of WT1-specific T cells after SCT. Two 9-mer WT1 peptides containing the major anchor motifs essential for binding to HLA-A2 molecules, were used to detect WT1-specific T cells. These two peptides (Db126: R**M**FPNAPY**L**, WH187: S**L**GEQQYS**V**, bold letters represent anchor motifs) are well known to exhibit high binding affinity[140,142]. All patients in the experimental-group were HLA-A2-positive compared to HLA-A2-negative in the control-group. Moreover, another negative controls were used namely the peptide-dissolving media and the HLA-A2-restricted gp100 peptide which is derived from the melanocyte lineage-specific protein PMEL 17 (256-264). Five patients were analyzed at different time points to

monitor the kinetic of WT1-specific T cells. PBMCs reactivity was tested by the ELISPOT and RT-PCR assays.

PBMCs were co-cultured with the peptides in the final concentrations of 10µg/ml, 5µg/ml and 10µg/ml of WT1-WH187, WT1-DB126 and gp100, respectively. The incubation time was 3h for RT-PCR assay and 24h for the ELISPOT assay.

2.2.3 Analysis of T-cell reactivity to mHAg (e.g. HY) in patients

Ten male patients (n=10) who received SCT from female donors were analyzed, to monitor the HY-specific T cells as the recipients reconstituted immune system is newly exposed to Y chromosome proteins after transplantation. T-cell reactivity for the HLA-A2-restricted SMYK-derived HY peptide was tested in all of the patients in the experimental-group (i.e. HLA-A2 positive) and control-group (i.e. HLA-A2 negative).[97,100] In addition, two experimental negative controls were tested in parallel, namely the peptide-dissolving media and the HLA-A2-restricted gp100 peptide. In general, T-cell reactivity against the HY peptide was tested by ELISPOT and RT-PCR assays. In seven patients T-cell reactivity was analyzed at different serial time to follow the reconstitution of HY-specific T cells.

PBMCs were co-cultured with the peptides in the final concentrations of 10µg/ml and 10µg/ml of the HY and gp100, respectively. The incubation time was 3h for the RT-PCR assay compared to 24h for the ELISPOT assay.

2.2.4 Analysis of patients for GvHD

PBMCs from nine patients (n=9), who were diagnosed to have GvHD were collected and used in the MLR settings. The MLR read out was IC cytokines (e.g. IFN-γ and IL-4). The 1:2 ratio, of responder (R): stimulator (S) cells, was used in the MLR assays. This ratio was adopted after performing preliminary experiments (see below).

2.3 Methods

2.3.1 Cells processing

2.3.1.1 Isolation of PBMCs

After informed consent and the approval of local Ethical Committee, heparinized peripheral blood was withdrawn from patients. Peripheral blood mononuclear cells (PBMCs) were isolated by density gradient centrifugation over Ficoll-Hypaque Plus® according to the manufacturer recommendations with few modifications. In brief, blood was diluted with PBS at a 1:1 ratio, then overlayed over Ficoll solution. After centrifugation at 380g for 20min at room temperature, the interphase was transferred into a new tube. After washing the cells once with PBS, the erythrocytes were removed by treatment with lysis buffer, another washing step in PBS was performed.

2.3.1.2 Freezing and storage

After counting on hemocytometer, PBMCs were resuspended in freezing medium and aliquoted in cryovials. The cryovials were transferred immediately to slow-freeze containers (i.e. “Mr. Frosty”) which were placed in a -80°C freezer for 4h to 24h. Then the cryovials were transferred in liquid nitrogen.

2.3.1.3 Thawing and resting

PBMCs were thawed in a 37°C water bath, and then transferred to at least ten folds of complete RPMI medium. Cells were centrifuged at 300g for 10min at 4ºC, resuspended in complete RPMI medium and rested overnight at 37°C in polypropylene tubes. Finally, the recovered PBMCs were counted and viability was determined by hemocytometer and trypan blue vital staining respectively. In general, 60-80% of the rested cells were viable.

2.3.2 MLR

An experimental system was established to simulate the sensitization process in patients with GvHD patient’s body. In this system HLA-mismatched healthy PBMCs were used to simulate the alloreactions in GVHD patients.[114] The sensitization step is called as “primary

MLR", and the real alloreaction is called "secondary MLR" in this thesis. Normally the primary MLR was performed for seven days and the secondary MLR for 16h.

The PBMCs were plated in U-bottomed 96-well culture plates and incubated at 37°C in 5% CO_2. For primary MLR, responder cells (R) were mixed with irradiated (75Gy) stimulator cells (iS) in R:S ratio of 1:2. After seven days, the sensitized responder cells (sR) were harvested and a secondary MLR was conducted using the same sR:iS ratio (i.e. 1:2). The secondary MLR cells were harvested after 3h or 6h, and the IFN-γ mRNA or IC cytokines were determined by RT-PCR or flow cytometry, respectively (see below). As positive control, PBMCs were stimulated with 2µg/ml phorbol 12-myristate 13-acetate (PMA) and 100µM ionomycin (Iono.).

Classical MLR, using ^{3}H-thymidine incorporation, was performed to check the capability of sR to proliferate in response to second round of stimulation with iS[115]. Briefly, responder PBMC ($0.1x10^6$ cells/well), together with irradiated (75Gy) stimulator PBMC ($0.2x10^6$ cells/well) were incubated for 7 days in 96-well round-bottom plates in a final culture medium volume of 200µl/well. On day 7 of culture, 0.037 MBq/ml [methyl-^{3}H]-thymidine was added to the wells and the plates were incubated for 16h at 37° and 5% CO_2 in a humidified chamber. DNA was harvested on a filter using the Packard Harvester and dried. Subsequently, scintillation fluid was added and the filters were sealed. The incorporated [^{3}H]-thymidine was measured using a liquid scintillation counter. Counts per minute (cpm.) were used as readout for proliferation.

2.3.3 Flow cytometry

2.3.3.1 Cell Surface immunophenotyping

The cell surface markers analyzed in this thesis are shown in table 4. All of cell surface staining steps were conducted for 15min at room temperature in the dark.

Marker	Cell Type
CD3	Pan T cells
CD4	Th cells
CD8	Tc cells

Table 4 : T-cells subpopulations and identifying markers.

Cells were washed with FACS wash buffer. The indicated monoclonal antibodies were added onto the cell pellet, and incubated for 15min at room temperature. A final washing step was performed with FACS wash buffer.

2.3.3.2 ICC staining

Intracellular cytokine production was measured in responding cells after inhibiting their secretion. PBMCs were incubated for 6h after adding the peptide and/or protein then ICC was performed. In the MLR settings, the incubation time was expanded to 16h before ICC measurement. As positive control, a combination of 2µg/ml PMA and 100µM ionomycin was used to activate T cells for cytokines production. The secretion of cytokines was stopped by adding either GolgiPlug (i.e. Brefeldin A). These protein transport inhibitors accumulate cytokines in the intracellular compartments (e.g. Golgi apparatus and endoplasmic reticulum) allowing the measurement of intracellular cytokines by FACS. For tracking the accumulated cytokines, the BD Cytofix/Cytoperm Plus™ kit was used as recommended by the manufacturer. In brief, activated PBMCs were harvested and washed with FACS buffer to block Abs non-specific binding in the following steps. After staining for cell surface markers, cells were fixed and permeabilized by thoroughly resuspending in 250µl of BD Cytofix/Cytoperm solution for 20min at 4°C. After washing in 1× BD Perm/Wash solution, fixed/permeabilized cells were resuspended in 50µl of BD Perm/Wash solution containing anti-cytokine antibody (e.g. IFN-γ or TNF-α or IL-4 or IL-10 or Perforin). Cells were incubated for 30min at 4°C in the dark followed by a washing step with 1× BD Perm/Wash solution, which was also used as staining buffer prior to flow cytometric analysis.

2.3.3.3 TM staining

HLA-A2-restricted CMVpp65-TM loaded with soluble peptide sequence was used to assess the frequency of reconstituted CMVpp65-specific Tc cells in blood. The staining protocol was adopted from the CMVpp65-TM manufacturer, with some modifications. In brief, $1x10^6$ PBMCs were washed and resuspended in 50µl FACS wash buffer. Then PE-labeled CMVpp65-TM in addition to monoclonal anti-CD3 and anti-CD8 antibodies were added. After an incubation step for 30min at room temperature in the dark, cells were washed with 2ml FACS wash buffer and analyzed by FACS. At least $0.5x10^6$ viable leukocytes were acquired.

2.3.3.4 PKH_{26} staining

The stimulator cells in the secondary MLR settings were labelled with PKH_{26}, a red fluorescent dye that stably integrates into the cell membrane. This step is necessary to distinguish between stimulator and responder cells [143]. Before staining, stimulator PBMCs were washed twice with serum-free medium. The cells were then resuspended in 200µl loading buffer (an aqueous, osmolarity-regulating solution containing no Ca^{2+} or other physiological salts). 200µl of freshly prepared PKH_{26} was added to reach a final concentration of 2.5µM. After an incubation period for 30min at room temperature the staining reaction was stopped by incubation with 2ml of human serum for 2min. This step is recommended to bind most of the residual lipophilic PKH_{26} dye to serum proteins. Following centrifugation, for 5min at 300g, cells were washed twice with 25ml of complete RPMI-1640 medium, and cell recovery was determined by cell counting.

2.3.3.5 Flow cytometric analysis

For the flow cytometry analysis, $0.3x10^6$ PBMCs were acquired to monitor the ICC production compared to $0.5x10^6$ for the TM or all viable lymphocytes in the MLR settings. Flow cytometric analysis was performed using FACS Calibur apparatus and Cellquest Pro software.

2.3.4 RT-PCR

2.3.4.1 Cell isolation

Cells were harvested by centrifugation at 300g for 10min at 4ºC in eppendorf tubes. Cells were either stored at -80ºC or were used immediately for RNA extraction.

2.3.4.2 RNA isolation

Cell lysis and total RNA isolation was performed as previously described.[144] Total RNA was isolated from test samples using the Qiagen RNeasy micro kit with minor modifications. Briefly, after disrupting cells by RLT buffer and vortexing, the homogenized cell lysate was mixed with 1 volume of 70% ethanol. After applying onto RNeasy MinElute Spin Columns and centrifuging for 15s at 8,000g, the flow-through was discarded and the columns were washed by adding RW1 buffer and centrifuged for 15s at 8,000g. This step was repeated

again with replacing the RW1 buffer by RPE buffer, and then the silica-gel membranes were dried by adding 500µl of 80% ethanol followed by longer centrifugation step for 3min at 8,000g. After discarding the flow-through, the columns were centrifuged again in microcentrifuge for 5min at maximum speed followed by discarding the flow-through. Finally, total RNA was eluted by pipetting 14-25µl RNase-free water onto the silica-gel membrane and centrifuging for 1min at maximum speed.

The concentration of eluted total RNA was determined by a UV spectrophotometer. Purified total RNA was either stored at –20°C in RNase-free water or was used immediately to prepare cDNA.

2.3.4.3 cDNA synthesis

Reverse transcription of mRNA into complementary DNA (cDNA) was carried out using the Promega Reverse Transcription System. For reverse transcription of IFN-γ and CD8 mRNA, 0.2-0.5µg of total mRNA was mixed with MgCl2, 10X reverse transcription buffer, dNTP mixture, recombinant RNasin® ribonuclease inhibitor, AMV reverse transcriptase, oligo (dT) 15 primer and nuclease-free water. The mixture was incubated for 60min at 42ºC, then 5min at 95ºC and finally for 5min at 4ºC. The synthesized cDNA was stored at -20°C until usage or immediately used for RT-PCR.

2.3.4.4 RT-PCR (TaqMan®) procedure

Measurement of IFN-γ mRNA gene expression was performed using the Applied Biosystems ABI Prism 7900 Sequence Detection system as described previously.[144] The feasibility of this approach for the analysis of Ag-specific T-cell responses has been shown previously.[145]

PCR primers for IFN-γ, CD8, and Taqman Probes were designed to span exon-exon junctions to prevent transcription of genomic DNA. After generation of cDNA by reverse transcription of mRNA, the number of cDNA copies was calculated using the molecular weight of each gene-specific amplicon. To generate standard curves, serial dilutions of the amplified gene at known concentrations were tested. CDNA specimens, cDNA standards, and water, as negative control, were mixed in total volumes of 20µl with Taqman master mix, 400nM primers and 200nM probes.

Thermal cycling was as follows: 2min at 50°C, 10min at 95°C, 40-cycles of 15s at 95°C and finally 1min at 60°C. Standard curves extrapolation of both IFN-γ and CD8 was

performed using the copy number unit. Sample data were normalized by dividing the IFN-γ transcripts copy number by the CD8 transcripts copy number.

2.3.4.5 Results analysis

A 2-fold difference in gene expression was found to be within the discrimination ability of the assay. All RT-PCR assays were performed in duplicates and reported as the mean ± standard deviation.

2.3.5 ELISPOT

The Letsch *et al.*[125] ELISPOT assay was adopted with some modifications.[130] In brief, ELISPOT plates were coated with 15μg/ml mouse-anti-human IFN-γ-mAb for overnight at 4ºC. Then blocking was performed with complete Iscove's medium for 2h at 37ºC. Overnight rested PBMCs were cultured in final concentration of $0.05\text{-}0.4\times10^6$ cells per well into the coated ELISPOT plates in triplicates when there were enough cells. Stimulation was performed as follows:

(*i*) 10μg/ml for CMVpp65, WT1-WH187, HY and gp100.
(*ii*) 5μg/ml for WT1-Db126.
(*iii*) 20μl/ml for CMVpp65 protein (stock concentration was not provided by the provider).
(*iv*) 0.5-1μg/ml PMA and 0.25-0.5nM ionomycin as positive control.

After 24h of incubation at 37°C in 5% CO_2, cells were removed by washing six times with solution of PBS and 0.05% Tween-20. Then, the plates were incubated with 0.626μg/ml anti-human-IFN-γ biotinylated mAb for 24h at 4°C. After washing the plate six times with PBS, streptavidin-alkaline phosphatase diluted 1/1,600 was added for 2h at room temperature. Following another six PBS washes, plates were incubated with BCIP/NBT substrate for 30min. Color development was stopped by washing five times with distilled water. Plates were dried overnight at room temperature and colored spots were counted using an automated ELISPOT reader.

2.3 Data evaluation and statistics

2.4.1 Flow cytometry

Acquired cells were compared to negative controls, and then plotted in dot plots in comparison to negative control.

2.4.2 Classical MLR

Results were presented as c.p.m. means ± S.D. Significant cell proliferation ($p<0.05$) in the R+S settings was determined by students *t* test in comparison to the R cells alone as negative control.

2.4.3 ELISPOT

Results were expressed as the mean ± standard deviation of counted IFN-γ spots number after incubation for 24h. A T-cell response was considered positive if the number of spots with peptide exceeds the number of spots by 10 and the difference between the single values containing peptide and control is statistically significant at a level of $p \leq 0.05$ using the *t* test.[124]

2.4.4 RT-PCR

To determine specific response to stimulation, mRNA for IFN-γ from stimulated PBMCs versus non-stimulated (background) was detected by RT-PCR. The IFN-γ mRNA copy number was first corrected for CD8 mRNA. A cutoff value (stimulation index) of 2.0 for the IFN-γ mRNA ratio obtained from stimulated with relevant to non-stimulated PBMCs was considered to be evidence of epitope specificity. In other words, to minimize the possibility of falsely considering PBMCs immunoreactivity, a 2-fold increase in stimulated:unstimulated IFN-γ transcript ratio was assumed as evidence of epitope-specific reactivity.

2.4.5 Linear regression and correlations

The linear regression and correlation analysis of the read outs were calculated using the Pearson correlation analysis with two-tailed P values. Statistical significance was achieved when $P < 0.05$ using Prism 4 software.

3. Results

3.1 Reconstitution of donor CMV-reactive T cells

There are many techniques described in the literature that are capable of monitoring CMV-specific T cells, such as TM staining,[111] ELISPOT assay,[22] MLRs,[146] cytotoxic assays[111,146] and ICC flow cytometry.[111,138,146] But little is done to monitor the reconstitution of CMV-specific T cells after allogeneic SCT especially on a molecular bases.[145] For testing patients' PBMCs for the presence of CMV-specific CTLs, certain patient's criteria should be considered such as the CMV serotyping, of patient and donor, as well as the HLA-A2 allele genotyping. A nomenclature system concerning the CMV serotyping and HLA-A2 allele genotyping was established (Table 5).

	CMV-Serotyping		HLA-A2 allele
	Patient	Donor	
CMV++A2+	+	+	+
CMV-+A2+	-	+	+
CMV+-A2+	+	-	+
CMV++A2-	+	+	-
CMV-+A2-	-	+	-
CMV+-A2-	+	-	-

Table 5 : Nomenclature of CMV serotyping and HLA-A2 typing for the tested patients who underwent SCT and were monitored for the presence of CMV-reactive CTLs. (+): represents the seropositivity and/or HLA-A2 allele holding. (-): represents the seronegativity and/or HLA-A2 allele non-holding.

The eighteen patients that were enrolled in this study were subdivided into two groups. The so called testing-group with HLA-A2 positive allele was compared to the control-group which included the HLA-A2 negative patients. Many combinations of patient-donor serotyping were tested to cover all the expected reconstituted CMV-reactive CTLs. Many patients were followed-up in longitudinal manner covering many time points. The patients' PBMCs which were collected in monthly time-intervals are shown in table 6. These patients' time points covered the range of forty days up to four years six months after Tx.

			Time after Tx		
Pt.	**CMV Serotyping**	**Immunosuppression**	**Y**	**M**	**D**
1a	**CMV++A2-**	**-**	**-**	**4**	**26**
1b		**-**	**-**	**7**	**10**
2a	**CMV++A2+**	**-**	**2**	**6**	**20**
2b		**-**	**2**	**7**	**11**
2c		**-**	**2**	**8**	**8**
3a	**CMV++A2+**	**-**	**1**	**7**	**4**
3b		**-**	**1**	**8**	**16**
5a	**CMV-+A2+**	**-**	**-**	**8**	**2**
5b		**-**	**-**	**9**	**13**
5d		**-**	**-**	**10**	**11**
6a	**CMV-+A2-**	**-**	**-**	**8**	**15**
7a	**CMV++A2-**	**-**	**3**	**6**	**3**
10a	**CMV++A2+**	**-**	**2**	**8**	**14**
11a	**CMV++A2+**	**+**	**1**	**5**	**27**
11b		**+**	**1**	**6**	**17**
11c		**+**	**1**	**7**	**24**
12a	**CMV++A2+**	**+**	**-**	**6**	**23**
12b		**+**	**-**	**7**	**14**
12c		**+**	**-**	**7**	**28**
12d		**++**	**-**	**8**	**12**
14c	**CMV-+A2+**	**-**	**1**	**2**	**9**
15a	**CMV-+A2+**	**+**	**4**	**4**	**18**
15b		**+**	**4**	**5**	**25**
16a	**CMV++A2+**	**++**	**-**	**1**	**10**
22a	**CMV-+A2+**	**-**	**2**	**11**	**6**
23a	**CMV+ -A2-**	**+**	**-**	**-**	**40**
23b		**+**	**-**	**-**	**55**
24a	**CMV+-A2+**	**-**	**-**	**1**	**19**
25a	**CMV-+A2-**	**+**	**-**	**7**	**12**
26a	**CMV++A2-**	**-**	**1**	**11**	**20**
27a	**CMV++A2+**	**++**	**-**	**1**	**19**

Table 6 : Patient's criteria tested for CMV-reactivity. Y: years, M: months, D: days.

3.1.1 TM detection assay was able to detect CMV-reactive T cells

MHC class I specific TMs are a well known tool to monitor Ag-specific T cells. After PBMCs TM staining and FACS acquiring, the flow cytometry gating strategy was took in consideration gating viable leukocytes (Fig. 4-a), which further gated in pan T lymphocytes gate (Fig. 4-b), and finally the target TM (or cytokines in the other sections) positive cells were plotted vs. Tc cells (Fig. 4-c). For monitoring CMV pp65-specific T cells, tetrameric staining was performed in the testing-group (i.e. HLA-A2 positive) as well as the control-group (i.e. HLA-A2 negative). Even in the CMV++ pair, some of the patients' PBMCs did

not bind the pp65 peptide-TM complex reflecting the absence of CMV pp65 peptide reactive CTLs (Fig. 5-a). Other patients showed abundant CMV pp65 peptide-specific CTLs in the peripheral blood (Fig. 5-b). In the control-group, no Tc could bind the pp65 peptide TM, reflecting the specificity of the used TM to recognize the HLA-A2 allele.

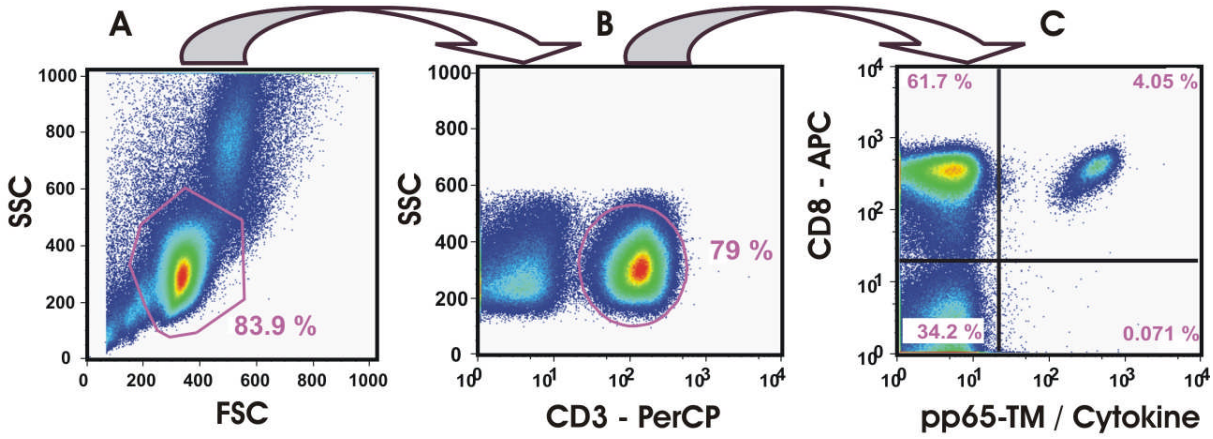

Figure 4 : Schematic diagram showing the gating strategy of target cells, starting with forward scatter (FSC) vs. side scatter (SSC) (A), then CD3 vs. SSC (B), and finally plotting the CD8 vs. the target TM or cytokines (C).

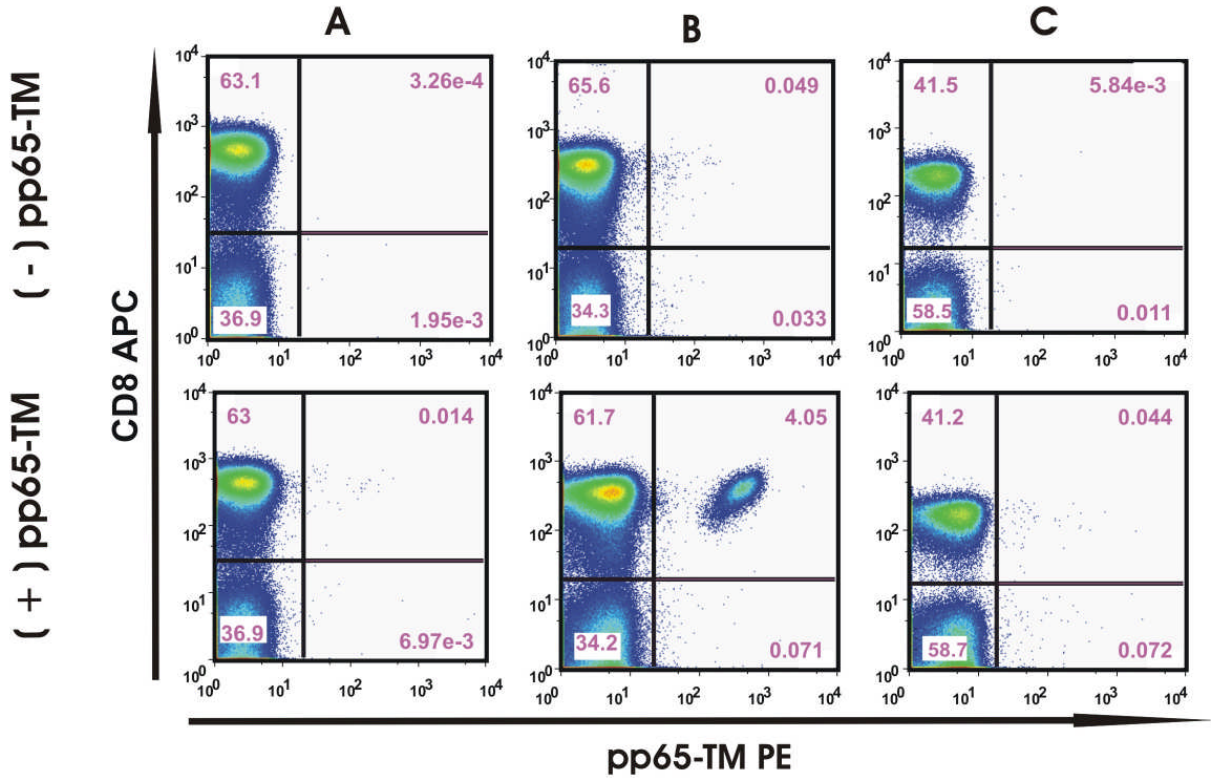

Figure 5 : Pp65-TM staining of CMV++ patients having HLA-A2 positive (A & B) or HLA-A2 negative (C) showed variable forms of detection.

In summary, eight patients out of eleven (≈ 73%) in the testing-group showed detectable CMV pp65 TM-reactive CTLs (Fig. 6-a), in comparison to three patient who did not (≈ 27%). On the other hand, no one of the six control-group patients (0%) had CMV pp65 TM-reactive CTLs (Fig. 6-b). These results confirm the specificity of the used TM as monitoring method.

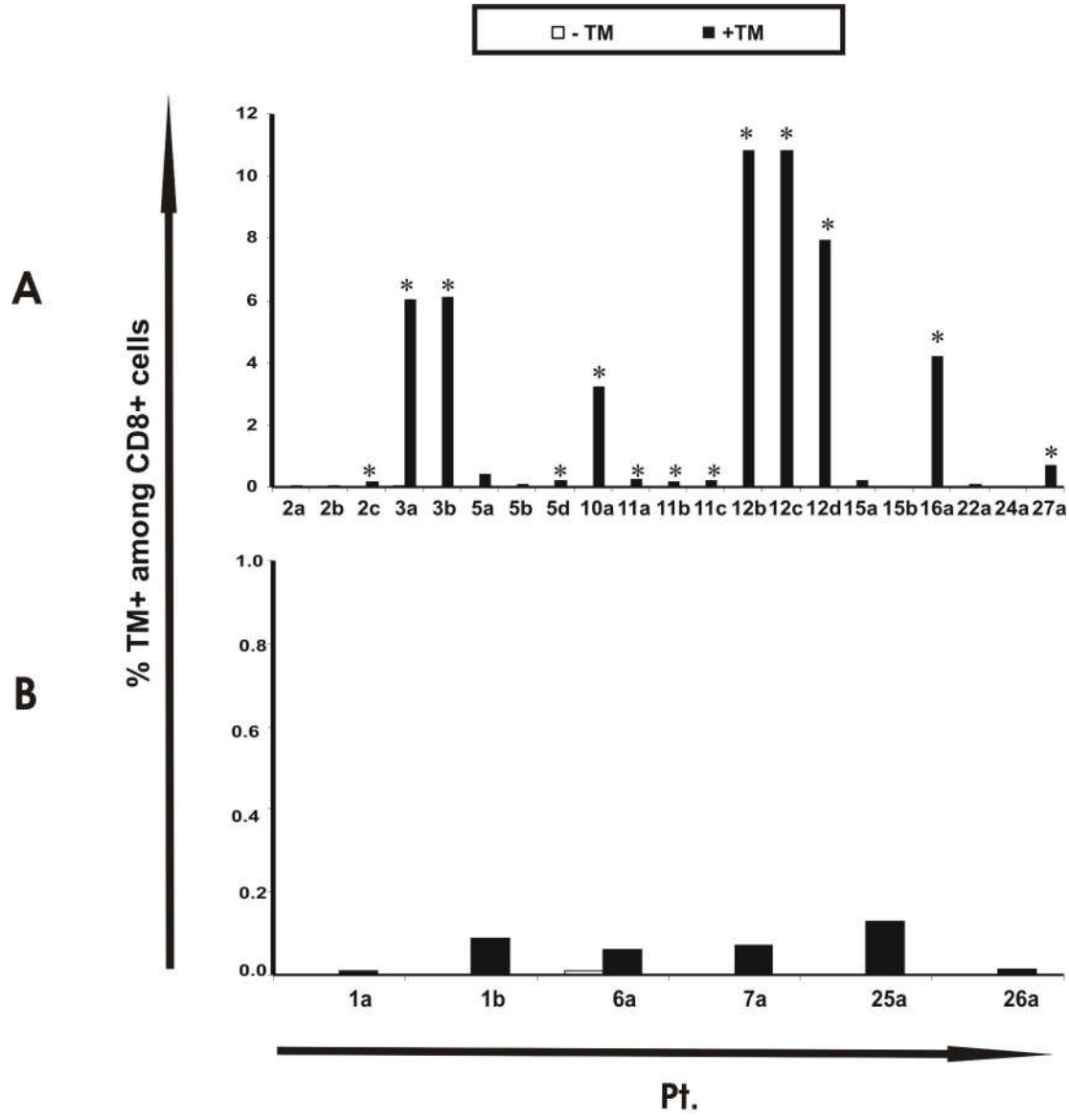

Figure 6 : TM staining of CMV-specific CD8 T cells in both HLA-A2 positive (A) and HLA-A2 negative (B) patients' PBMCs. (*): significant increase of TM+ cells compared to negative control ($p<0.05$).

There is increasing evidence that also some functionally defective CMV-specific T cells can be detected by TM staining, thus it is essential to perform additional assays (e.g. ICC and/or ELISPOT assays) to test the functional abilities of detected T cells.[147]

3.1.2 IC IFN-γ assay can detect CMV-reactive CTLs

IC IFN-γ was detectable by flow cytometry after 6h reactivation period (Fig. 7). Pt.15a, who has a HLA-A2 allele, did not have detectable CMV-specific CTLs even after four years after Tx (Fig. 7-a). This patient did not show any reactive CTLs for neither CMV pp65 peptide nor protein. On the other hand, Pt.2a showed a strong reactivity for CMV pp65 protein but not the peptide. This reflects the presence of CTLs that recognized other epitopes, of the pp65 protein, rather than the used peptide (Fig. 7-b). Some patients like Pt.3a showed strong CMV reactivity for both tested CMV Ags (Fig. 7-c). This reflects the presence of many CMV-specific CTLs in the patients' peripheral blood. In this patient even some CMV-reactive Th could be seen in the lower right quadrant, which secreted IFN-γ in MHC class II manner. In the patients' control-group, having no HLA-A2 allele, IFN-γ production seemed to vary. For example in Pt.26a no detectable IFN-γ production in either pp65 peptide or pp65 protein panels (Fig. 7-d), even though this patient underwent SCT two years ago. On the other hand other patients such as Pt.1a, who underwent SCT five months, showed a significant IFN-γ production after stimulation with pp65 protein but not with the pp65 peptide (Fig. 7-e). So this patient has CMV-specific CTLs recognizing many of the pp65 protein epitopes, but not the tested pp65 peptide which can not be presented by this patient's antigen presenting cells due to its HLA-A2 specificity.

A longitudinal follow-up was in scope as PBMCs from many patients were collected on monthly base. For the longitudinal follow-up, some patients did not show any IFN-γ production after both viral Ags stimulations (Fig. 8-a). This reactivity was not detectable even after four years and four months (Pt.15a) or five months (Pt.15b) of Tx. Other patients developed CMV pp65 protein-reactivity and produced IFN-γ in response to only pp65 protein, but not the HLA-2-restricted peptide. For example, Pt.11a PBMCs showed pp65 reactivity for the protein, but not the peptide, after one year and half (Fig. 8-b). In addition to the presence of pp65 protein reactive CTLs in peripheral blood, some patients lately developed CMV reactivity for the pp65 peptide. Eight months after Tx, Pt.5 found to has CTLs that were able to produce IFN-γ in response to pp65 protein, but not pp-65-peptide (Fig. 8-c) after Tx. But this patient developed CMV reactivity for the pp65 peptide after nine months of Tx. Other patients' PBMCs were able to produce IFN-γ in response to both pp65 peptide and pp65 protein. Even PBMCs from the Pt.12b were able to start their CMV reactivity as early as the seventh month after Tx (Fig. 8-d). So, using the pp-65 protein is better than using the pp65 HLA-restricted peptides to monitor the CMV reactivity.

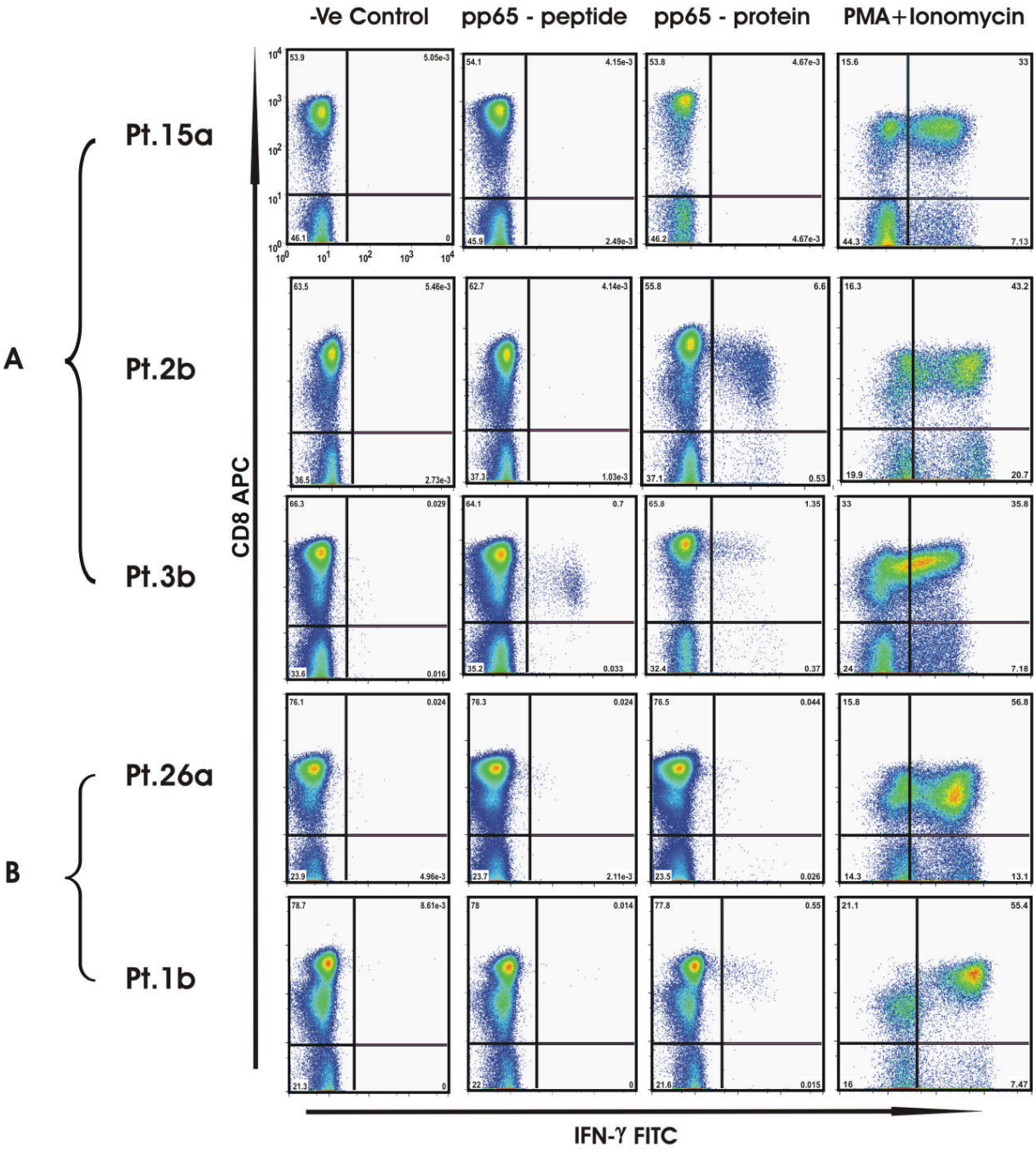

Figure 7 : IC IFN-γ production after stimulation with pp65 peptide, pp65 protein and PMA and ionomycin in comparison to negative control. Both patient types, HLA-A2 positive (A) and HLA-A2 negative (B) were tested.

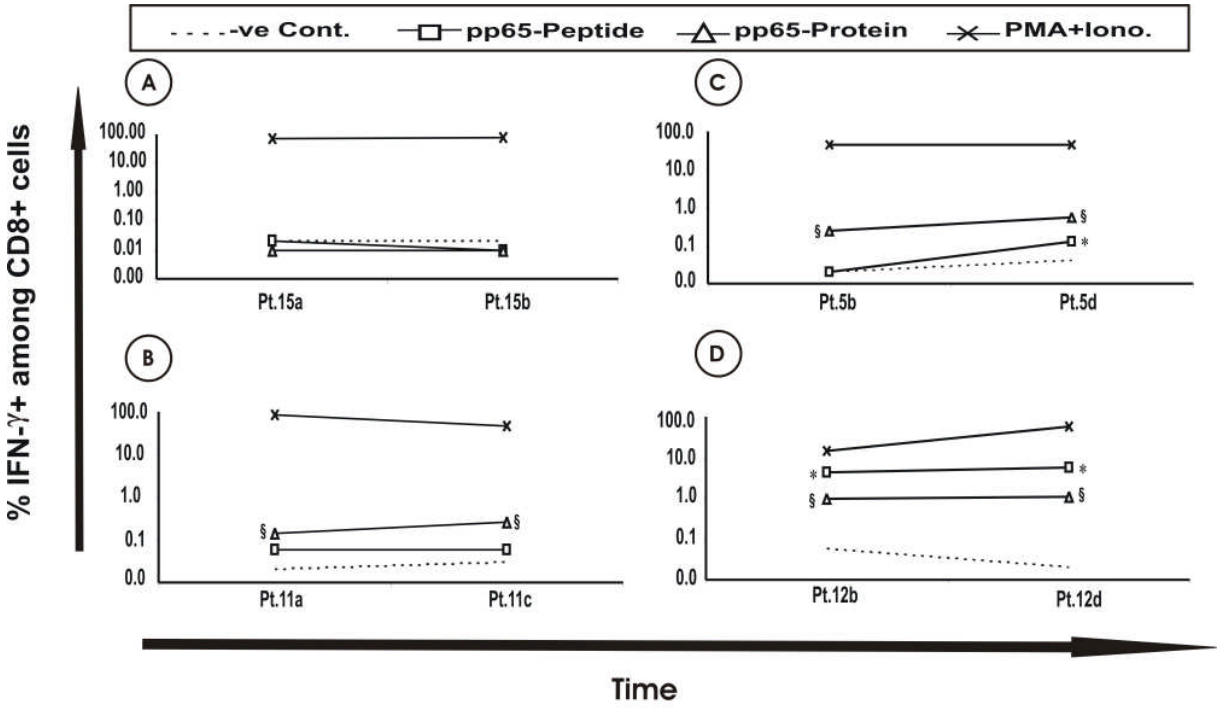

Figure 8 : Time kinetics follow-up for some patients after activation with pp65 peptide and pp65 protein. CMV reactivity was not detectable in response to both (A) or one (B) CMV-Ags, but on the other hand some samples shown reactivity for both Ags (C & D). Significance was considered if $P<0.05$ when pp65 peptide (*) or pp65 protein (§) induced IFN-γ production was compared to negative control.

Other cytokines, such as TNF-α, IL-2, IL-4, IL-6, IL-10, granzymes and perforin, can be used to monitor Ag-specific T cell functions. For example the CTLs cytolytic function is important to lyse viral infected or leukemic cells. For such monitoring, perforin and/or granzyme B production can be tested after CMV Ags reactivation. Accumulation of IC perforin after activation with the two CMV Ags was monitored by flow cytometry. Perforin was detected only in PBMCs from a patient who was stimulated with CMV pp65 protein (Fig. 9). On the other hand, no activation was detected using the CMV pp65 peptide. In these experiments perforin was detectable in significant quantities in both testing-group (Fig. 9-a) and control-group (Fig. 9-b). This may reflect the broad expression of pp65 protein epitopes which can be presented by the PBMCs antigen presenting cells. In Pt.2c and Pt.5d both IFN-γ and perforin were detectable which is reflecting the presence of CTLs with pro-inflammatory and cytolytic functions, respectively.

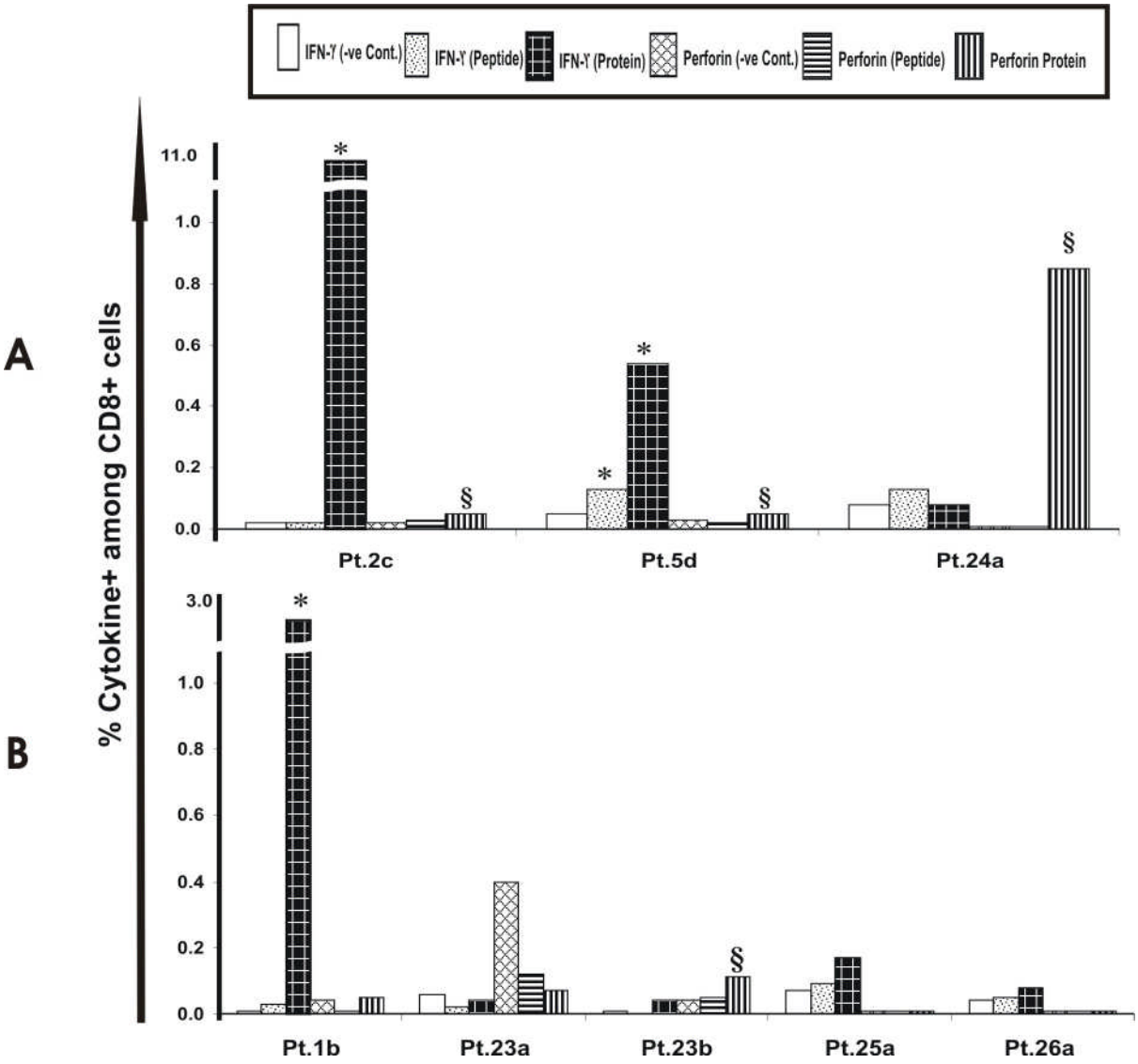

Figure 9 : CTLs capacity to produce IFN-γ as well as perforin after viral Ags exposure. HLA-A2 positive (A) and HLA-A2 negative (B) patients' PBMCs were stimulated. Significance was considered if $P<0.05$ when pp65 peptide (*) or pp65 protein (§) induced IFN-γ production was compared to negative control.

Another approach was used to enhance the Ags presenting capacity of the patients' PBMCs, using the C1R cell line.[148] The C1R cell line is a human plasma leukemia cell line that does not express endogenous HLA-A or B antigens. HLA-A2 positive C1R-A2 cells are C1R cells that express a transfected genomic clone of HLA-A2. C1R-A2 cell line was used as antigen presenting cells that can present only the HLA-A2-restricted peptides. Although this cell line can not present endogenous peptides, HLA-A2 peptides that are added to the culture media can only presented to the patients' PBMCs. The use of C1R-A2 cell line enhanced the

production of IC IFN-γ in the patients' PBMCs (Fig. 10-a). The IC IFN-γ production in patients' PBMCs alone (upper panel) was increased by 95% when PBMCs were mixed with C1R-A2 cells (lower panel). This was compared with PBMCs from a healthy donor who was HLA-A2 positive and CMV seropositive (Fig. 10-b). The healthy donor's PBMCs IC IFN-γ production was increased by 38% when mixed with C1R-A2 cells (lower panel) in comparison to PBMCs alone (upper panel).

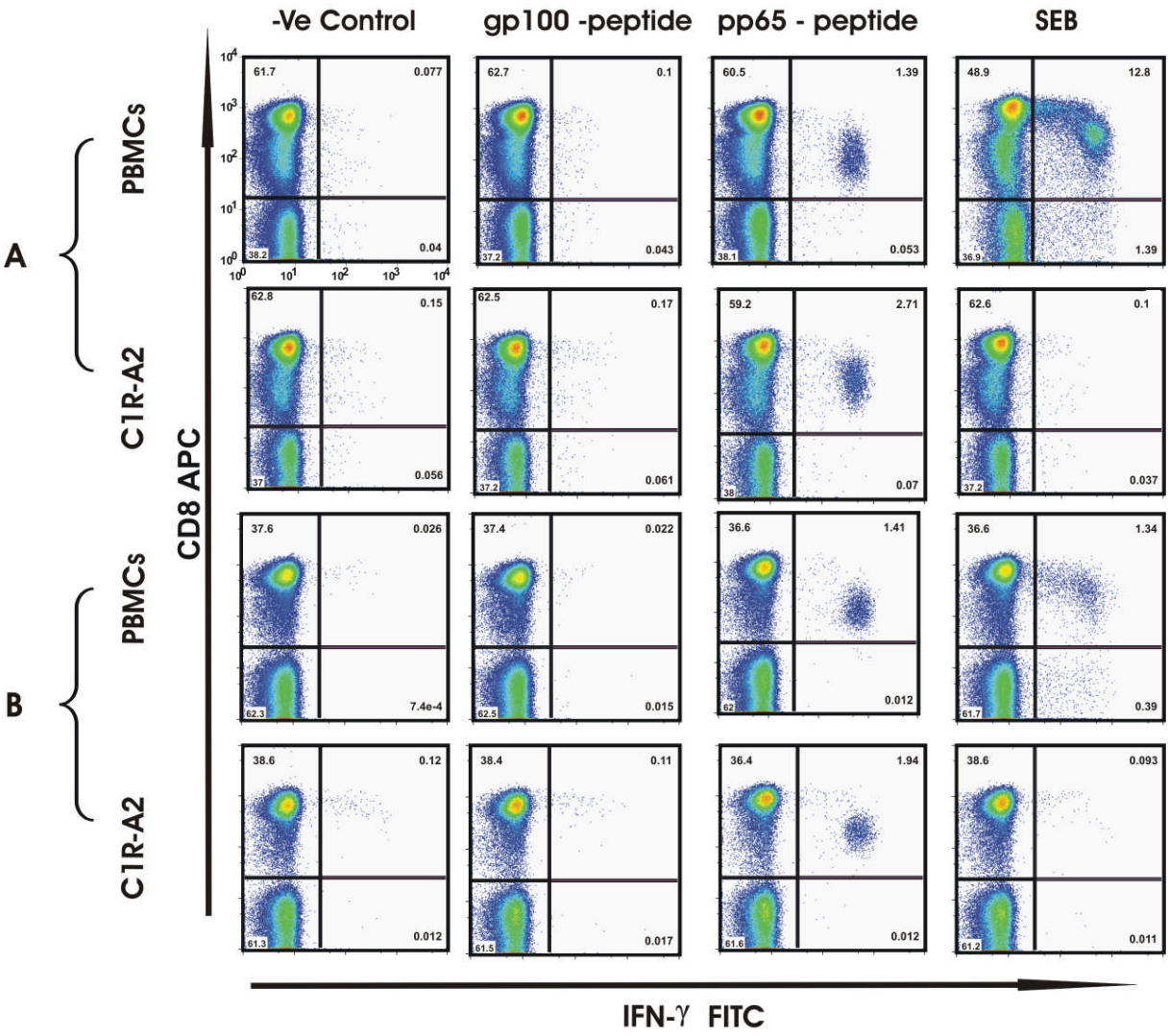

Figure 10 : Comparison of Ag presenting capacity for PBMCs alone versus combination of PBMCs and C1R-A2. The production of IC IFN-γ by patients' (A) or healthy donors' (B) PBMCs alone was stimulated with CMV pp65 peptide (upper panel) and compared to CMV pp65 peptide pulsed C1R-A2 cells (lower panel).

In the total eighteen patients who were monitored for their CMV-reactive CTLs, eight of the twelve HLA-A2 positive PBMCs (≈ 67%) produced IFN-γ in response to one or both

CMV-pp65 Ags (Fig. 11-a). These results confirm the reconstitution of CMV reactive CTLs in patients' peripheral blood after the transplantation. Also, these CTLs are specific for both pp65 HLA-A2-restricted peptide as well as the other epitopes compromising the pp65 epitopes. On the other hand, two of the HLA-A2 negative patients' PBMCs (33 %) only produced IFN-γ when stimulated with the pp65 protein but not with the pp65 peptide (Fig. 11-b).

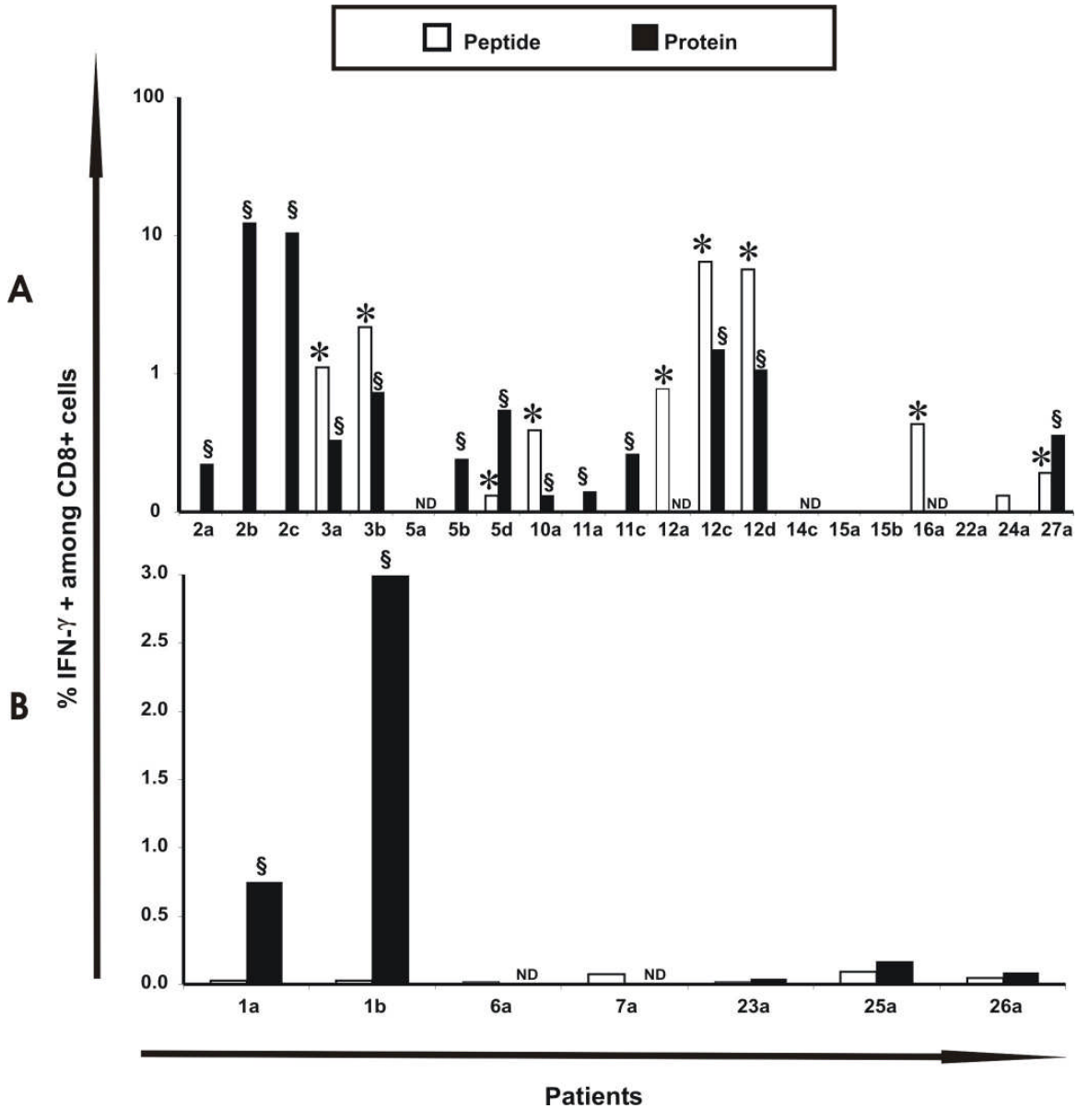

Figure 11 : CTLs capacity to produce IFN-γ after viral Ags exposure. HLA-A2 positive (A) and HLA-A2 negative (B) patients' PBMCs produced IFN-γ after stimulation with pp65 peptide and/or pp65 protein. Significance was considered if $P<0.05$ when pp65 peptide (*) or pp65 protein (§) induced IFN-γ production was compared to negative control. ND: not determined.

3.1.3 RT-PCR detection assay can monitor CMV-reactive T cells

Most of the assessments of cellular immunity to the human CMV in patients who undergo allogeneic SCT rely on the CMV-specific MHC class I TMs, ELISPOT assay and ICC flow cytometry,[17] and little is done to use molecular approaches in monitoring the reconstitution process. In literature many molecular approaches are described to detect the CMV viral load before[149,150] and after[151-153] SCT or solid-organ transplantation. For example, Gallez-Hawkins *et al.*[154] used the PCR to measure the CMV DNA, in the plasma of allogeneic transplanted patients, as a marker for the CMV infection. Also they used two methods to identify the reconstituted CD8+ T cells namely: IC IFN-γ flow cytometry and CMV-specific TM. In addition, Hempel *et al.* [155] found that the immune response measured in the peripheral blood of mice by RT-PCR or ELISPOT assays showed a significant correlation with the response measured in the spleen ($P = 0.001$). So the hypothesis that RT-PCR assay has some correlations with human peripheral blood monitoring methods was suggested and tested in this doctoral thesis.

IFN-γ mRNA transcripts were detectable by RT-PCR assay in various manners as can be seen in Fig. 12-a. Some patients who have HLA-A2 allele did not have any detectable CMV-specific CTLs. For example Pt.15a PBMCs did not show any detectable CMV-specific CTLs, for neither CMV pp65 peptide nor pp65 protein, even after four years of Tx. Other patients such as Pt.2a showed a strong reactivity for CMV pp65 protein but not the pp65 peptide. This reflected the presence of CTLs in the patients' peripheral blood that recognized other pp65 epitopes, rather than the used HLA-restricted peptide. Some patients like Pt.3a, showed high expression of the IFN-γ mRNA in response to both tested CMV Ags. In general this indicates the presence of many CMV-specific CTLs in the patients' peripheral blood and the reconstitution of strong CMV reactivity. In this patient even some CMV-reactive Th could be seen in the IC flow cytometry which secreted IFN-γ in MHC class II manner (Fig. 7-c, lower right quadrant). So the detected IFN-γ mRNA by RT-PCR assay was the combined reactivity of both CD8+ and CD8- T cells in the case of pp65 protein. Even in the patients' control-group, IFN-γ mRNA transcripts were clearly monitored. For example in Pt.26a and Pt.1a, who underwent SCT before two years or five months, respectively, CMV-specific CTLs directed to the pp65 protein were detectable (Fig. 12-b). On the other hand, both of these patients did not have any IFN-γ mRNA transcripts due to the pp65 peptide stimulation, confirming the specificity of the HLA-A2 peptide.

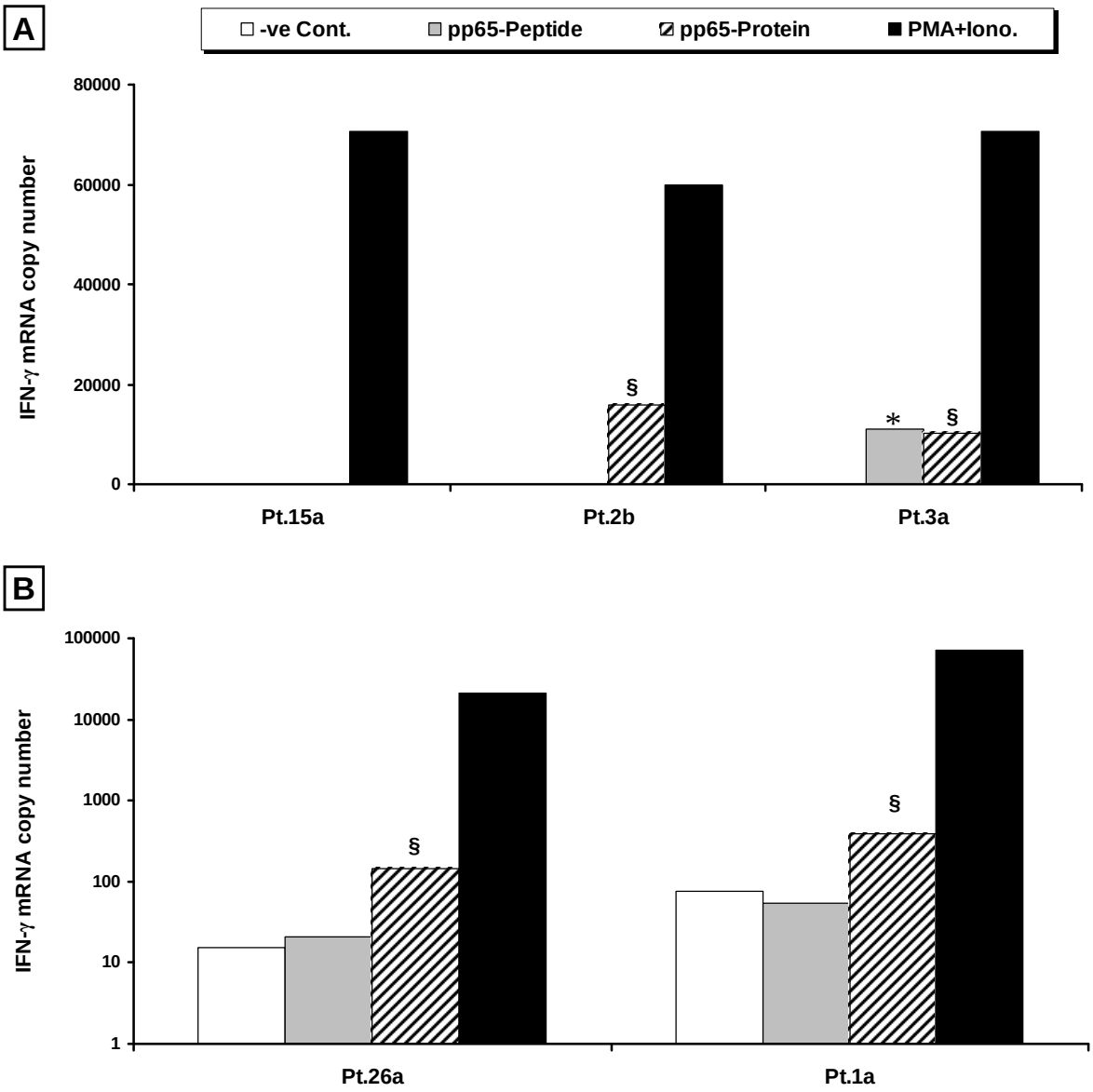

Figure 12 : Patients' CTLs, having the HLA-A2 allele (A) and others who did not (B), were tested for IFN-γ mRNA expression after viral Ags exposure. Significance was considered if $P<0.05$ when pp65 peptide (*) or pp65 protein (§) induced IFN-γ production was compared to negative control.

In the longitudinal follow-up, some patients did not show any IFN-γ mRNA expression after both viral Ags stimulation (Fig. 13-a). Although the donor was CMV-seropositive, CMV reactivity was not detectable even after more than four years (Pt.15a-b) after transplantation. Other patients developed CMV pp65 protein-reactivity and expressed IFN-γ mRNA in response to only pp65 protein, but not the HLA-2-restricted peptide. For example, figure 13-b show PBMCs with reactivity for pp65 protein (Pt.2a-c) but not pp65 peptide (Pt.2a-b) after 30 and 31 months of Tx. But this patient expressed IFN-γ mRNA in response to the pp65 peptide

at the 32 months after Tx. In addition to the presence of pp65 protein reactive CTLs in peripheral blood, some patients lately developed CMV reactivity for the pp65 peptide. Eight months after Tx, Pt.5 shown to have CTLs that expressed IFN-γ mRNA in response to pp65 protein, but not pp-65-peptide (Fig. 13-c). But this patient developed CMV reactivity for the pp65 peptide at the ninth month after Tx. Finally, some patients PBMCs such as Pt.12b were able to express IFN-γ mRNA in response to both pp65 peptide and pp65 protein (Fig. 13-d). Even this patient started his CMV reactivity as early as the seventh month after Tx. As a summary, the use of pp-65 protein is better than the pp65 HLA-restricted peptides to monitor the CMV-reactivity by the RT-PCR assay.

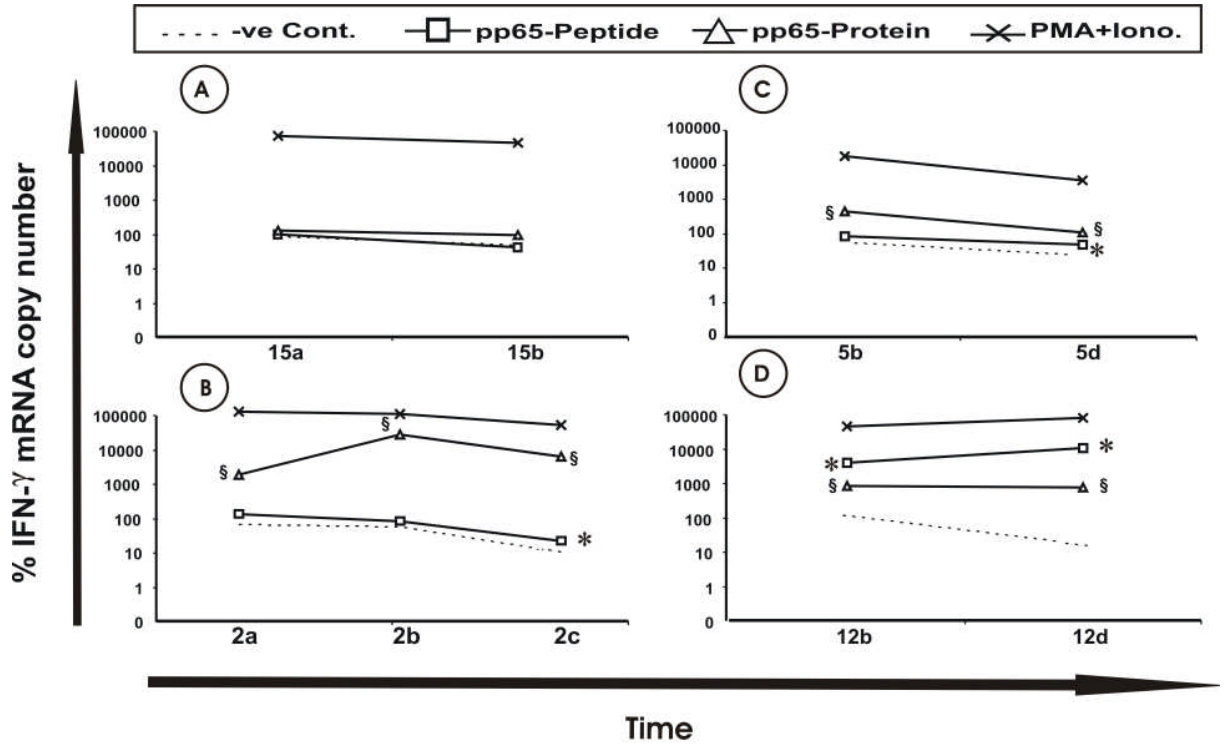

Figure 13 : CMV-reactivity monitoring time kinetics follow-up in some patients using the RT-PCR assay. CMV-reactivity was not detectable for both Ags (A), or found in addition to all pp65 protein time points (B) in some of pp65 peptide treatment (C). Also in some patients, CMV-reactivity was found in all time points after both pp65 protein and pp65 peptide (D). Significance was considered if $P<0.05$ when pp65 peptide (*) or pp65 protein (§) induced IFN-γ production was compared to negative control.

Blood samples from thirty patients were monitored for the presence of CMV-reactive CTLs. In the HLA-A2 positive patients' samples, fifteen out of twenty two (68%) expressed

IFN-γ mRNA in response to pp65 protein, compared to sixteen out nineteen (84%) in response to pp65 protein (Fig. 14-a). These results confirm the detection capability of RT-PCR assay for the reconstituted CMV-reactive CTLs in the patients' peripheral blood. Also, these CTLs were more specific for the epitopes compromising the pp65 protein more than the pp65 HLA-A2-restricted peptide. On the other hand, in the HLA-A2 negative patients' samples, no one of the eight samples (0%) expressed IFN-γ mRNA in response to pp65 peptide compared to six out of six (100%) in response to pp65–protein (Fig. 14-b).

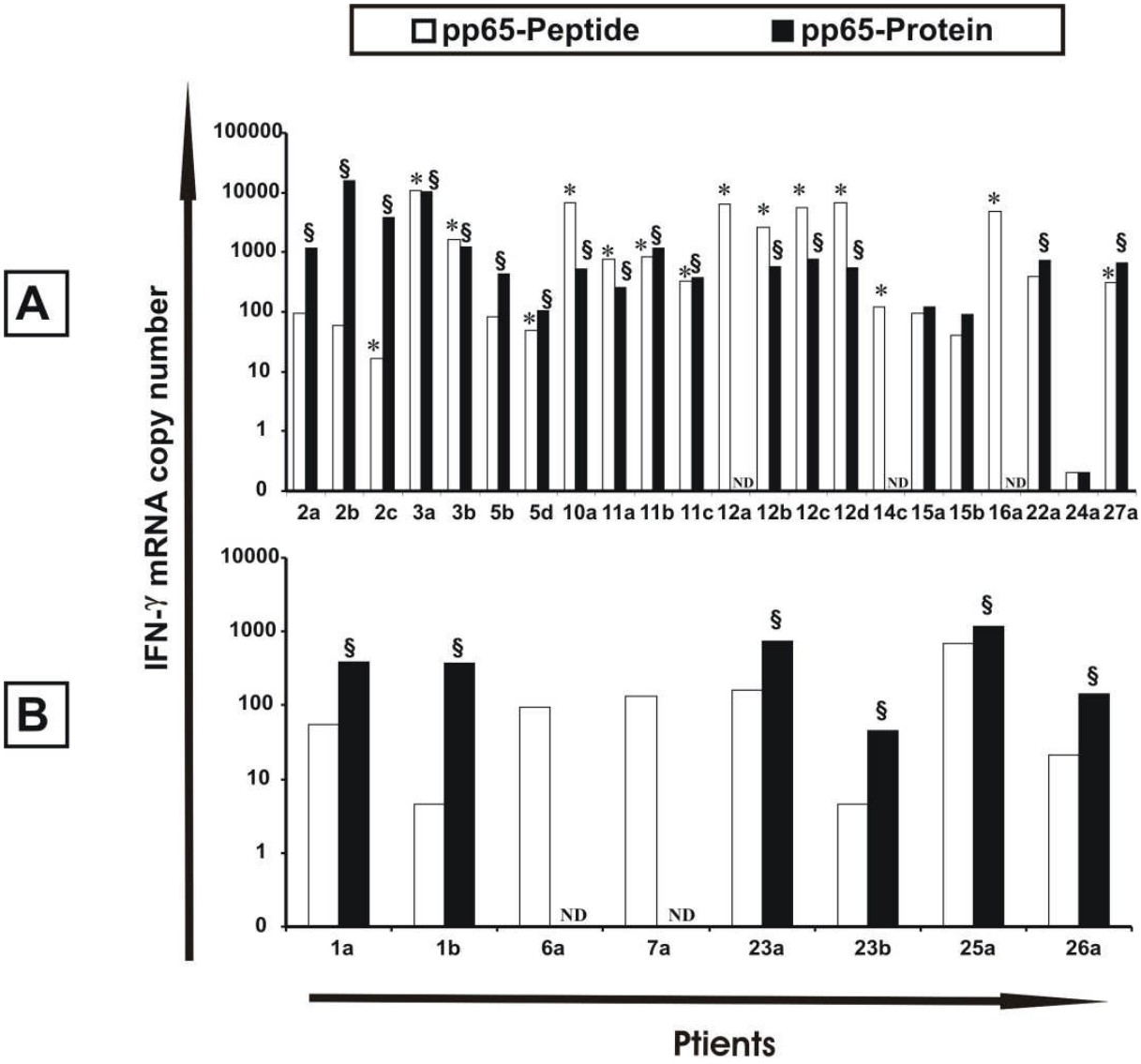

Figure 14 : IFN-γ mRNA regulation after viral Ags exposure. HLA-A2 positive (A) and HLA-A2 negative (B) PBMCs were tested for CMV-reactivity. Significance was considered if $P<0.05$ when pp65 peptide (*) or pp65 protein (§) induced IFN-γ production was compared to negative control. ND: not determined.

3.1.4 ELISPOT assay detected CMV-reactive CTLs

As the ELISPOT assay is well known to have the highest sensitivity for detecting reactive T lymphocytes,[125] this technique was adopted for monitoring of CMV-reactive T lymphocytes. The PBMCs from the same two patients-groups, testing and control, were tested for their capacity to secrete IFN-γ after stimulation with both pp65 Ags. After 24h of stimulation period, IFN-γ secretion was measured by ELISPOT assay and the results presented as "IFN-γ spots number".

In the HLA-A2 positive tested PBMCs (Fig. 15-a), few patients' PBMCs were not able to secrete any IFN-γ regardless of the used CMV stimulating Ag. For example, Pt.15 PBMCs did not response to both pp65 Ags even after four years and six months following Tx. Other patients such as Pt.2b showed IFN-γ secretion in response to pp65 protein but not the pp65 peptide. This reflected the presence of other epitopes that can be presented even though they were not HLA-A2 restricted. The pp65 protein reactivity was in less degree than other patients (e.g. Pt.5b) reflecting that the used pp65 peptide has a variability in stimulating CMV-specific CTLs. PBMCs from other patients (e.g. Pt.11c) were able to secrete IFN-γ in response to both pp65 Ags. But such secretion was noticed to be more abundant in response to the pp65 protein than the pp65 peptide, which may reflect the presence of many CMV-specific clones in the patients' peripheral blood that can recognize the CMV pp65 protein.

On the other hand, the HLA-A2 negative patients' control-group (Fig. 15-b), no IFN-γ was secreted significantly after the pp65 peptide pulsing, confirming the HLA-A2-restriction for the used pp65 peptide. But this did not mean that these patients do not have reconstituted CMV-specific CTLs. These patients proved to have CMV-reactive T cells directed against other epitopes of the pp65 protein. But on the contrary this was noticed by the ability of their PBMCs to secrete IFN-γ when stimulated with the pp65 protein for 24h.

In the longitudinal follow-up, some HLA-A2 positive patients did not show any IFN-γ mRNA expression after both viral Ags stimulation. This reactivity was not detectable even after four years and four months (Pt.15a) or five months (Pt.15b) of Tx (Fig. 16-a). Other HLA-A2 positive patients' PBMCs (e.g. Pt.2b-d) secreted IFN-γ only in response to the pp65 peptide, but not the protein (Fig. 16-b). In others like Pt.5, PBMCs were able to secrete IFN-γ in response to pp65 protein, but not pp65 peptide, nine months after Tx (Fig. 16-c). Other HLA-A2 positive patients' PBMCs were able to secrete IFN-γ in response to both pp65 peptide and pp65 protein (Fig. 16-d). On the other hand, some of the HLA-A2 negative control-group patients' PBMCs were able to secrete detectable and significant quantities of

IFN-γ after stimulation with pp65 protein, but not pp65 peptide (Fig. 16-e). As a summary, the use of pp-65 protein is better than the pp65 HLA-restricted peptides to monitor the CMV-reactivity.

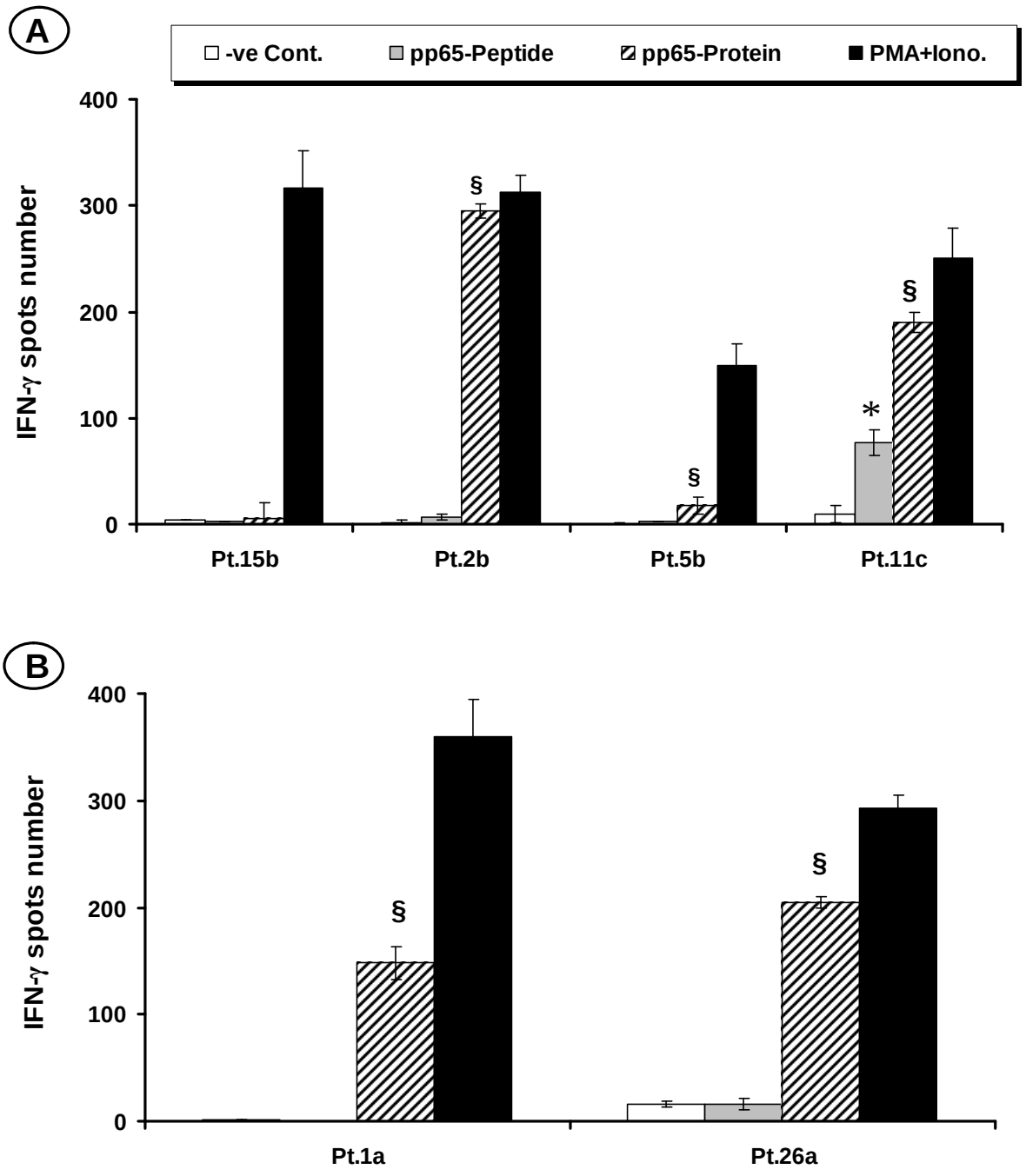

Figure 15 : ELISPOT assay detection of secreted IFN-γ from CTLs after viral Ags exposure. PBMCs from HLA-A2 positive (A) and HLA-A2 negative (B) patients can secrete IFN-γ after CMV Ags stimulation for 24h. Significance was considered if $P<0.05$ when pp65 peptide (*) or pp65 protein (§) induced IFN-γ production was compared to negative control.

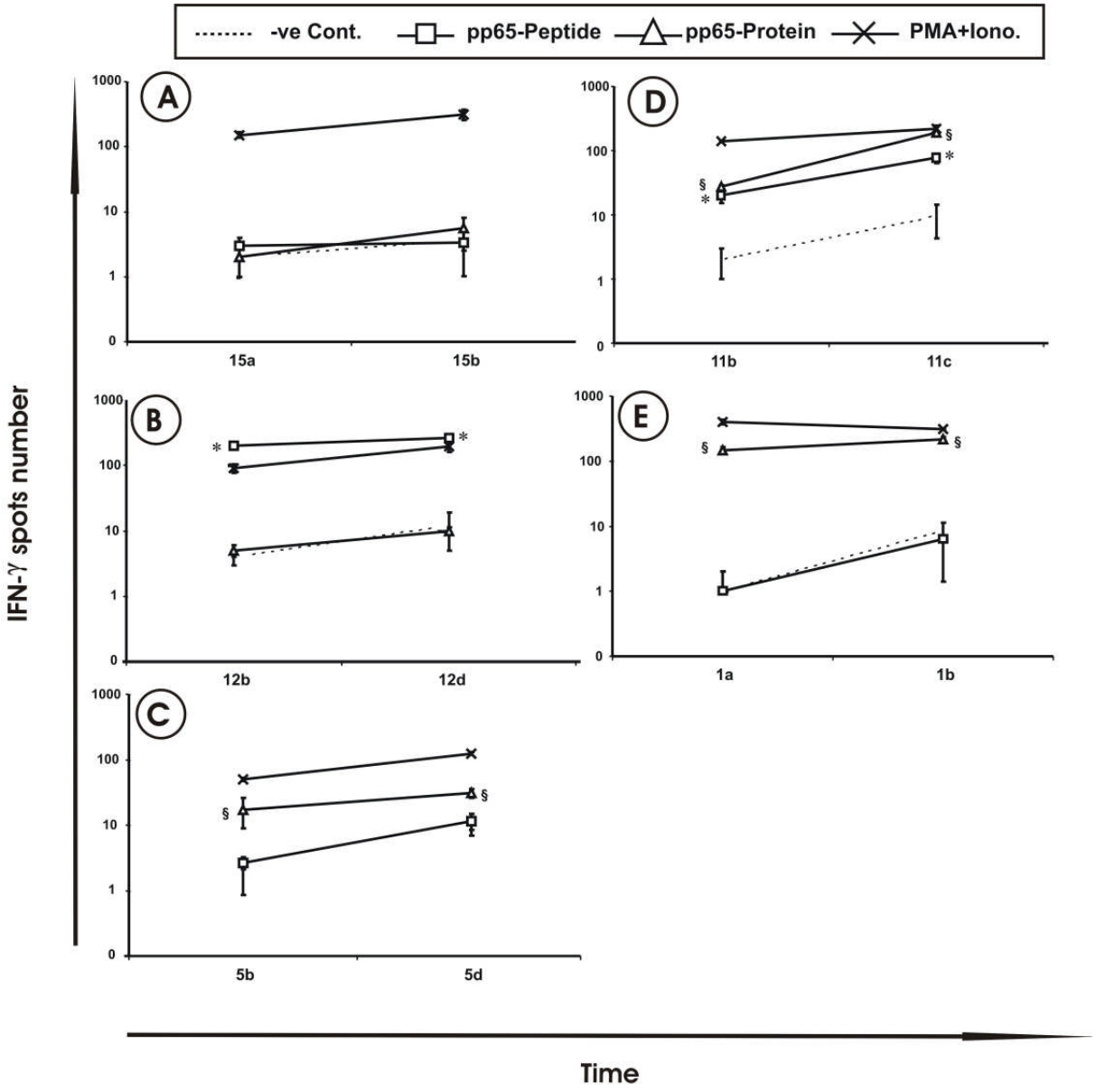

Figure 16 : ELISPOT assay time kinetics follow-up for some patients having the HLA-A2 positive (A, B, C & D) and HLA-A2 negative (E) alleles after activation with two CMV Ags. Significance was considered if $P<0.05$ when pp65 peptide (*) or pp65 protein (§) induced IFN-γ production was compared to negative control.

Ten HLA-A2 positive patients were tested in many time points for the presence of CMV-reactive T lymphocytes in their peripheral blood. Pp65 peptide reactive CTLs in the patients were detectable in 50% (5/10) compared to 50% (5/10) who were reactive to pp65 protein (Fig. 17-a). Also, as a sum 70% (7/10) were reactive for at least one of the two CMV Ags. These results were compared to two patients who were HLA-A2 negative (Fig. 17-b), in which all of them showed that IFN-γ was detectable in significant quantities when the PBMCs

were stimulated with pp65 protein (100%). On the other hand, none of those patients PBMCs were able to secrete IFN-γ in response to stimulation with pp65 peptide (0%).

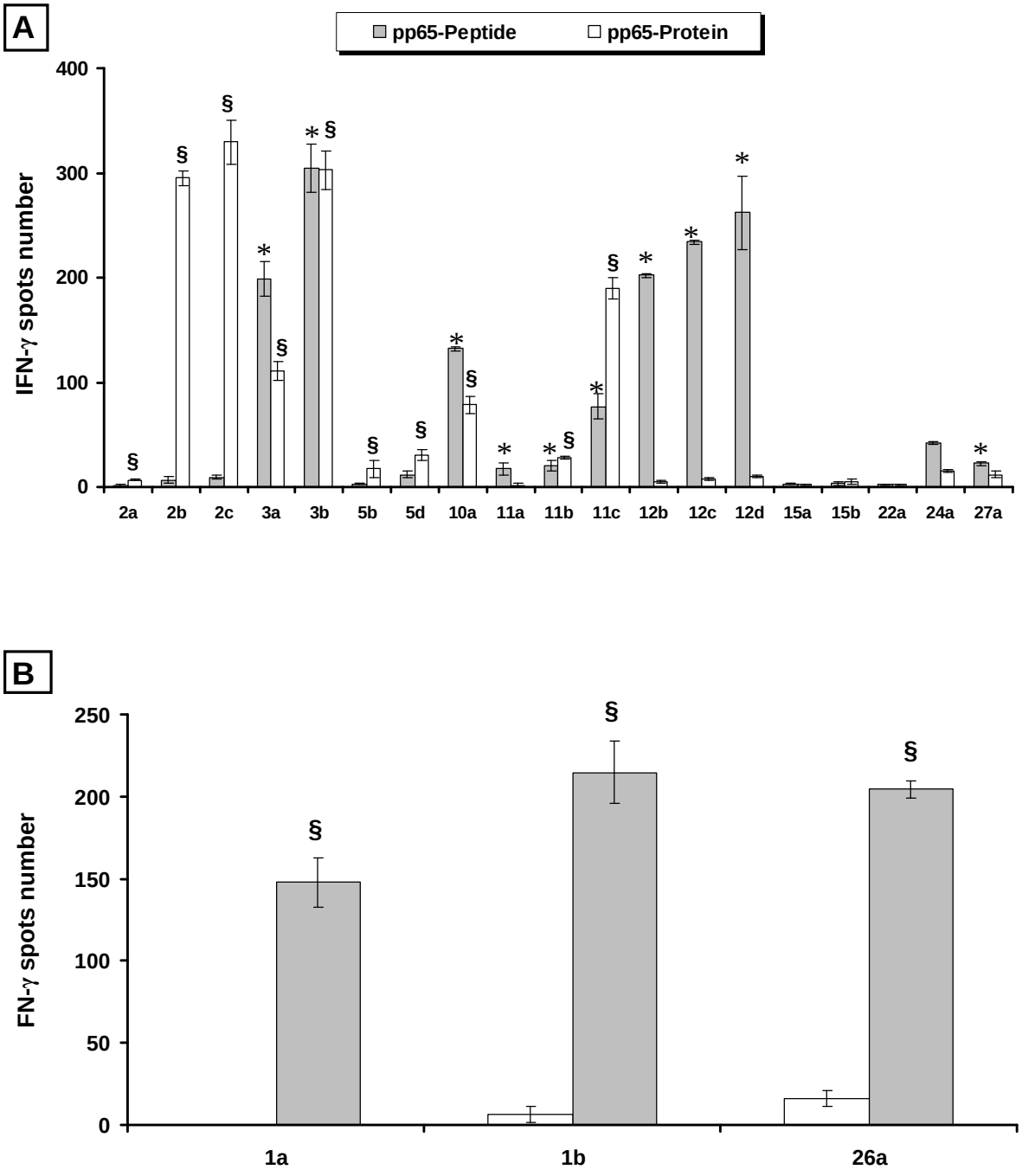

Figure 17 : Detection of IFN-γ secretion by ELISPOT assay after viral Ags exposure. Both types of patients' PBMCs, HLA-A2 positive (A) and HLA-A2 negative (B), were stimulated with CMV Ags. Significance was considered if $P<0.05$ when pp65 peptide (*) or pp65 protein (§) induced IFN-γ production was compared to negative control.

As a summary, CMV-specific T cells were detectable by all of the tested assays, but the RT-PCR shown an inferior sensitivity in detecting very low frequencies of CMV-specific T cells compared to the other tested assays. In addition, after proving the HLA-A2 restriction of pp65 peptide, this restriction was overcome by the use of pp65 protein. Although the adopted ELISPOT assay shown good detection limit, but unfortunately it was not very sensitive to detect the very low frequencies of CMV-specific T cells. Table 7 summarizes all of the results concerning the reconstitution of CMV-reactive T cells in patients' PBMCs.

		pp65-Peptide				pp65-Protein		
Pt.	**CMV Serotype**	**TM**	**ICC**	**ELISPOT**	**RT-PCR**	**ICC**	**ELISPOT**	**RT-PCR**
1a	**CMV++A2-**	-	-	-	-	+	+	+
1b		-	-	-	-	+	+	+
2a	**CMV++A2+**	-	-	-	-	+	+	+
2b		-	-	-	-	+	+	+
2c		+	-	-	+	+	+	+
3a	**CMV++A2+**	+	+	+	+	+	+	+
3b		+	+	+	+	+	+	+
5a	**CMV-+A2+**	-	-	**ND**	**ND**	**ND**	**ND**	**ND**
5b		-	-	-	-	+	+	+
5d		+	+	-	+	+	+	+
6a	**CMV-+A2-**	-	-	**ND**	-	**ND**	**ND**	**ND**
7a	**CMV++A2-**	-	-	**ND**	-	**ND**	**ND**	**ND**
10a	**CMV++A2+**	+	+	+	+	+	+	+
11a	**CMV++A2+**	+	-	+	+	+	-	+
11b		+	-	+	+	+	+	+
11c		+	-	+	+	+	+	+
12a	**CMV++A2+**	**ND**	+	**ND**	+	**ND**	**ND**	**ND**
12b		+	+	+	+	+	-	+
12c		+	+	+	+	+	-	+
12d		+	+	+	+	+	-	+
14c	**CMV-+A2+**	**ND**	-	**ND**	+	**ND**	**ND**	**ND**
15a	**CMV-+A2+**	-	-	-	-	-	-	-
15b		-	-	-	-	-	-	-
16a	**CMV++A2+**	+	+	**ND**	+	**ND**	**ND**	**ND**
22a	**CMV-+A2+**	-	-	-	-	-	-	+
23a	**CMV+ -A2-**	**ND**	-	**ND**	-	-	**ND**	+
23b		**ND**	-	**ND**	-	-	**ND**	+
24a	**CMV+-A2+**	-	-	-	-	-	-	-
25a	**CMV-+A2-**	-	-	**ND**	-	+	**ND**	+
26a	**CMV++A2-**	-	-	**ND**	-	-	**ND**	+
27a	**CMV++A2+**	+	+	+	+	+	-	+

Table 7 : Detection of CMV-specific T cells in the peripheral blood of allogeneic transplanted patients using TM, ICC, ELISPOT and RT-PCR and assays. Significant (+) and not significant (-) detection of IFN-γ is shown. ND: not determined.

3.1.5 Comparison of CMV-monitoring methods in transplanted patients

3.1.5.1 Sensitivity comparisons between monitoring assays

As many techniques, namely: TM staining, ICC flow cytometry, RT-PCR and ELISPOT assays, were tested to monitor the CMV-specific CTLs reconstitution, sensitivity of each technique should be compared to the others. After considering each patient's time point as individual sample, the sensitivity was presented as: the percentage of significantly positive values among all of the tested samples. As showed in table 8, specificity for both HLA-A2 positive and HLA-A2 negative patients' samples was calculated for all of the tested four techniques used to monitor the CMV-reactive T lymphocytes.

When the pp65 peptide was used, among the HLA-A2 positive testing-group samples, ICC technique showed the lowest sensitivity (39%) compared to TM staining (62%), RT-PCR (68%) and ELISPOT assays (53%). This was not the case when the pp65 protein was used, since the ICC flow cytometry sensitivity was (79%) compared to RT-PCR (84%) and ELISPOT assays (53%). On the other hand, in the HLA-A2 negative patients as control-group, pp65 peptide-specific T cells were not detectable with all of the techniques. This reflected the high specificity of the used pp65 peptide for the HLA-A2 allele. But again, ICC flow cytometry showed to have the lowest sensitivity (50%) compared to both RT-PCR and ELISPOT assay which have 100% for both, when the pp65 protein was used. In summary, the use of pp65 protein increased the sensitivity of both ICC and RT-PCR techniques. But this is not applicable to the TM staining, because of the allele restrictions for the multimers technologies. In the ELISPOT assay, no big difference was noticed when the peptide or protein was used.

	A2+		A2-	
	pp65 peptide	pp65 protein	pp65 peptide	pp65 protein
TM	**62% (13/21)**	-	**0% (0/6)**	-
IC IFN-γ	**39% (9/23)**	**79% (15/19)**	**0% (0/8)**	**50% (3/6)**
RT-PCR	**68% (15/22)**	**84% (16/19)**	**0% (0/8)**	**100% (6/6)**
ELISPOT	**53% (10/19)**	**53% (10/19)**	**0% (0/3)**	**100% (3/3)**

Table 8 : Comparison of sensitivity between CMV monitoring techniques.

3.1.5.2 Impact of serostatus on reconstitution of CMV-reactive CTLs

As CMV serostatus of the donor and the enumeration of functional CMV-specific CTLs in the graft may identify recipients at risk for developing CMV disease, many different combinations of serotyped patient/donor pairs were monitored. By calculating the percentage of significant positive read outs among the total sample number, each CMV-serotype pattern of the patient/donor was presented in table 9. The strongest reconstitution was found in CMV-seropositive patients given allografts from CMV-seropositive donors. Even this recovery of CMV-specific CTL can be rapid reaching up to 21% of all CD8+ T cells as found by Cwynarski *et al.*[152] In the second rank, delayed and moderate reconstitution of CMV-specific CD8+ T cells was observed in CMV-seronegative patients receiving a transplant from a CMV-seropositive donors. Finally, the last rank with the lowest reconstitution capacity was in CMV-seropositive patients who receive SCT from CMV-seronegative donors. This is rational since the patient immune system replaced by the donor's one after allogeneic SCT, and as any CMV-specific T cells were at the donor stem cells inoculum will be transferred to the patient's body. The other factor, which is the seropositivity of the patients, indicates that the patient had CMV particles which may be at the latent phase. So when the CMV break through the latent phase, this will give the proper Ags so the memory CMV-specific T cells allowing them to expand.

		A2+			A2-		
		CMV++	**CMV-+**	**CMV+-**	**CMV++**	**CMV-+**	**CMV+-**
TM	**peptide**	86% (12/14)	17% (1/6)	0% (0/1)	0% (0/4)	0% (0/2)	N.D
	protein	-	-	-	-	-	-
IC IFN-γ	**peptide**	60% (9/15)	14% (1/7)	0% (0/1)	0% (0/4)	0% (0/2)	0% (0/2)
	protein	100% (13/13)	40% (2/5)	0% (0/1)	67% (2/3)	100% (1/1)	0% (0/2)
RT-PCR	**peptide**	87% (13/15)	33% (2/6)	0% (0/1)	0% (0/4)	0% (0/2)	0% (0/2)
	protein	100% (13/13)	60% (3/5)	0% (0/1)	100% (3/3)	100% (1/1)	100% (2/2)
ELISPOT	**peptide**	77% (10/13)	0% (0/5)	0% (0/1)	0% (0/3)	N.D	N.D
	protein	62% (8/13)	40% (2/5)	0% (0/1)	100% (3/3)	N.D	N.D

Table 9 : Monitoring of different serotypes of CMV patient/donor.

3.1.5.3 Correlation of CMV monitoring assays

The use of HLA-restricted TMs is a phenotypic method that can not give any functional data about the detected cells. On the contrary, the RT-PCR assay can give precise information about the function but not the cell phenotypes. RT-PCR assays as new methods in which Ag-specific T cells can be detected should be compared to the well established methods such as the HLA-restricted tetramers which are popular means of detecting Ag-specific T cells. All patients PBMCs samples were tested for the CMV-reactivity by CMV pp65-TM and RT-PCR assay. For the correlation analysis, the RT-PCR values were presented as "Fold Change" in comparison to the negative control which considered as one fold. The Pearson correlation analysis showed significant positive correlation between TM and RT-PCR techniques with P value reaching 0.0001 (Fig. 18). Also the linear regression correlation was combined with R^2 of 0.8752. This significant correlation is promising for the implementation of RT-PCR assay as a molecular approach to monitor the reconstitution of CMV-specific T cells.

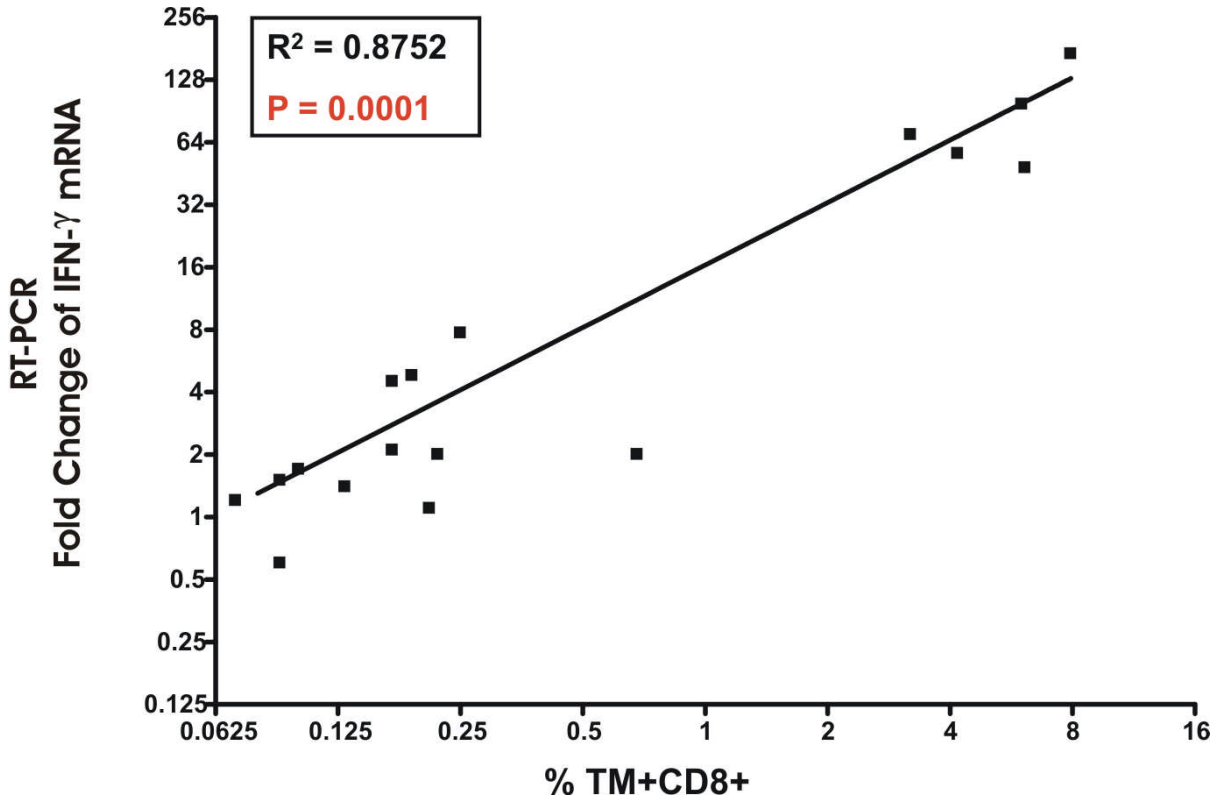

Figure 18 : Linear regression analysis of quantitative data to compare TM staining and RT-PCR assay.

On the other hand, as IC IFN-γ can be detected by flow cytometry after 6h, the up-regulations of IFN-γ mRNA can be detected by the RT-PCR assay within 3h after Ag pulsing. As both methods were used to detect CMV-specific T cells, correlation analysis was performed. For this type of correlation, the RT-PCR read outs were presented as "Fold Change" as in the previous performed TM:RT-PCR correlation analysis. By applying the Pearson correlation analysis, a significant positive correlation coefficient between the two methods was observed. As showed in figure 19 the P value was reaching less than 0.0001, indicating high significant correlation between the ICC and RT-PCR techniques as method to detect reconstituted T lymphocytes after allogeneic SCT. This correlation was confirmed by the linear regression which had R^2 of 0.753. This significant correlation between the ICC and RT-PCR is promising for the use of RT-PCR assay for monitoring of CMV-specific T-cells reconstitution.

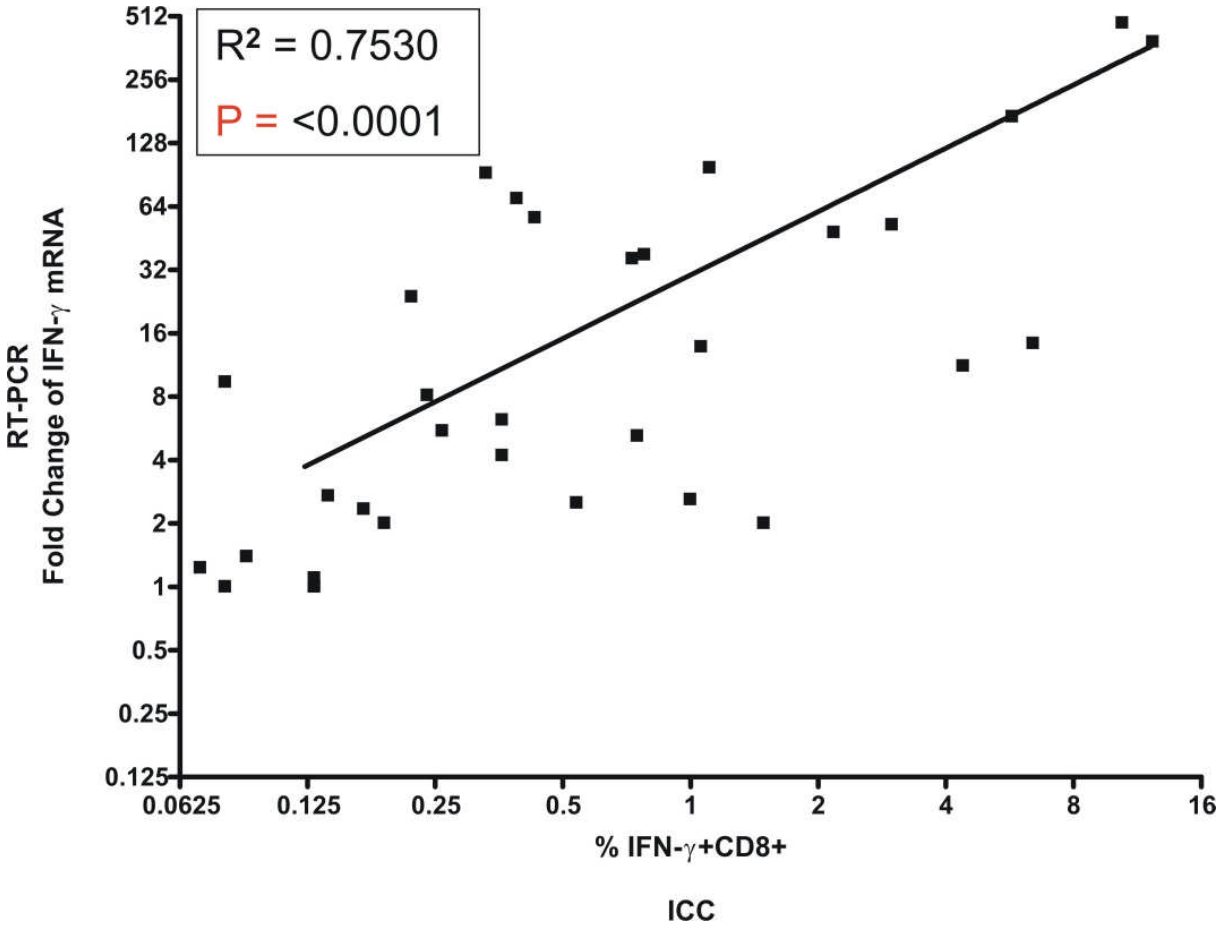

Figure 19 : Linear regression analysis of quantitative data to compare ICC flow cytometry and RT-PCR assay.

Presently, ELISPOT assay is widely used in monitoring antigen-specific responses in the context of infectious diseases, cancer, and autoimmunity. Also it described to have the highest sensitivity to detect Ag-specific T cells. But also the RT-PCR assay has high sensitivity which not so much tested in the field of detecting Ag-specific T cells. The long time consumption as limitation of ELISPOT assay can be overcame by the RT-PCR which only needs 3h. As most of the PBMCs samples were tested with both techniques, correlation study was necessary to adopt the RT-PCR assay as a mean of monitoring method for cells reconstitution after allogeneic SCT. By the Pearson correlation analysis, a significant positive correlation coefficient between the two methods was observed. As showed in figure 20 the P value was reaching less than 0.0001, indicating high significant correlation between the ELISPOT and RT-PCR assays as method to detect T lymphocytes. Also, the linear regression correlation showed a R^2 of 0.4061. This significant correlation is promising for the use of RT-PCR assay in monitoring the reconstitution of Ag-specific T cells.

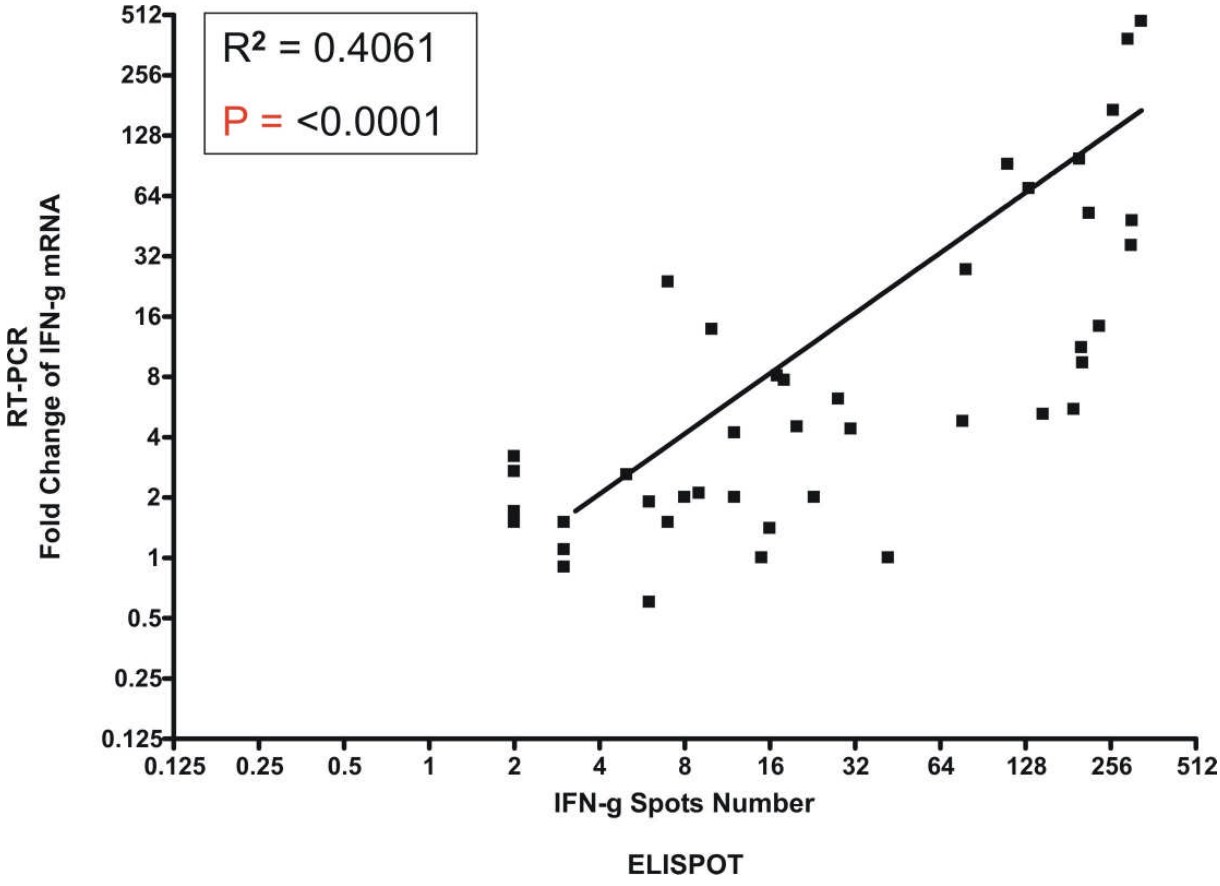

Figure 20 : Linear regression analysis of quantitative data to compare ELISPOT and RT-PCR assays.

3.2 Monitoring of WT1-specific T cells

WT1 protein is over-expressed in some leukemia and various types of solid tumors, and it is considered to be an attractive target antigen for immunotherapy against these malignancies. Normally WT1-reactivity is measures by TM staining,[156] cytoplasmic granzyme B[157] and IFN-γ staining assay,[156,157] ELISPOT assay,[157] RT-PCR assay,[145] and cytotoxicity assays (^{51}Cr release assays).[158] To monitor WT1-specitfic T cells in relevance to GVL effect, the IFN-γ production and IFN-γ mRNA gene expression were measured by ELISPOT[157] and RT-PCR assays[145], respectively. Twelve patients who underwent allogeneic SCT were selected to perform the WT1-reactivity testing. These patients were selected to have hematopoietic malignancies, before allogeneic SCT, which upregulates the WT1 protein on their malignant cells. The patients were followed-up in time kinetics to detect the WT1 reactivity. The criteria of patients who expected to have WT1-reactive T cells are in table 10.

				Time after Tx		
Pt.	**Diagnosis**	**HLA-A2**	**Immunosuppression**	**Y**	**M**	**D**
1b	**AML**	**-**	**-**	**-**	**7**	**10**
2c	**CML**	**+**	**-**	**2**	**8**	**8**
3b	**AML**	**+**	**-**	**1**	**8**	**15**
4a	**AML**	**+**	**-**	**-**	**5**	**1**
4b		**+**	**-**	**-**	**5**	**23**
4c		**+**	**-**	**-**	**6**	**18**
4d		**+**	**-**	**-**	**6**	**27**
4e		**+**	**-**	**-**	**7**	**10**
5b	**ALL**	**+**	**-**	**-**	**9**	**13**
5c		**+**	**-**	**-**	**10**	**2**
8a	**AML**	**+**	**-**	**-**	**5**	**15**
8b		**+**	**-**	**-**	**6**	**22**
8d		**+**	**-**	**-**	**8**	**23**
11a	**AML**	**+**	**+**	**1**	**5**	**27**
11b		**+**	**+**	**1**	**6**	**17**
11c		**+**	**+**	**1**	**7**	**24**
14a	**ALL**	**+**	**-**	**1**	**-**	**20**
14b		**+**	**-**	**1**	**3**	**22**
18	**AML**	**-**	**++**	**-**	**5**	**15**
19	**AML**	**-**	**-**	**5**	**1**	**25**
24	**AML**	**+**	**-**	**-**	**1**	**19**
26	**AML**	**-**	**-**	**1**	**11**	**20**

Table 10 : Selected patients' criteria for testing the presence of WT1-reactive T cells. Y: years, M: months, D: days, AML: acute lymphocytic leukemia, CML: chronic myelogenous leukemia, ALL: acute lymphoblastic leukemia.

3.2.1 RT-PCR assay can detect WT1-reactive T cells

By using the RT-PCR assay, WT1 CTLs can be monitored to trace the GvL effect in a quantitative manner. This method was described by Rezvani *et al*. to detect functional leukemia-associated antigen-specific memory CD8+T cells existing in CML patients.[145] In the tested allogeneic SCT transplanted patients, GvL effect was detectable by the RT-PCR assay in some patients (Fig. 21), reflecting the presence of functional CTLs in the patient's peripheral blood.

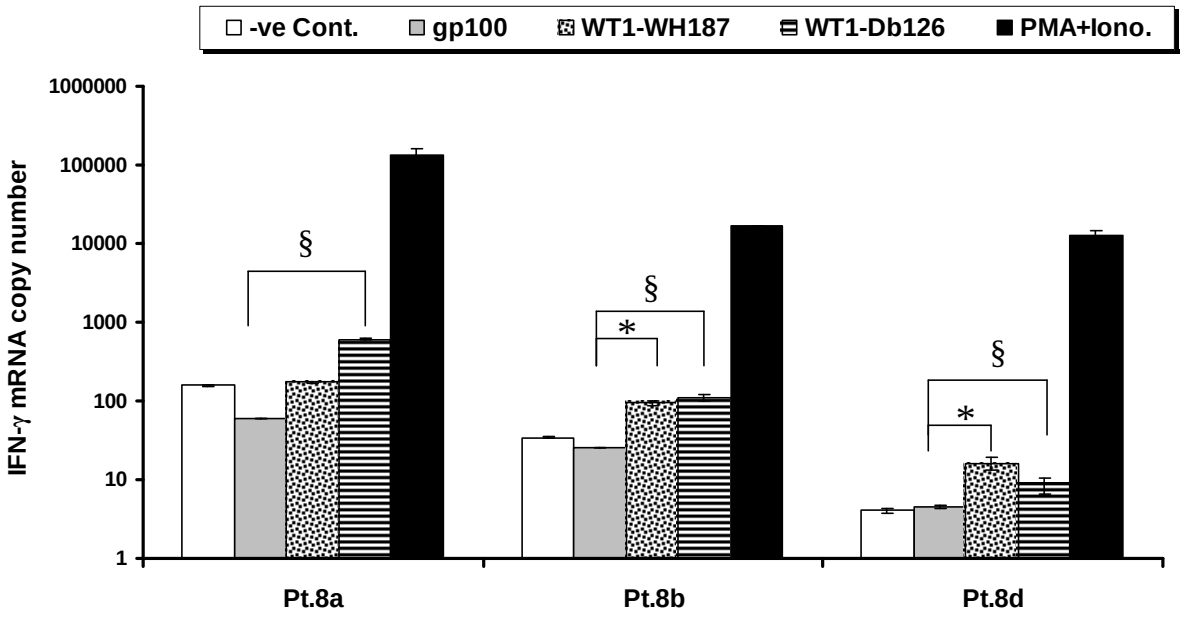

Figure 21 : WT1-specific CTLs found in AML patient. Significance was considered if P<0.05 when WH187 (*) or (**§**) Db126 peptides induced INF-γ was compared to gp100 peptide.

There are many immunodominant WT1 peptides that can be used as target antigens, but Makita *et al.* used four HLA-A24 restricted WT1 peptides, namely: WT1-228, -235, -356 and -417, to generate WT1-specific CTLs.[158] Others used HLA-A2-restricted WT1 peptides such as WT1-187 and -126.[140] On the other hand even HLA class II WT1-restricted peptides can be used to monitor WT1-specific Th cells.[159] However, the two HLA-A2-restricted WT1 peptides tested here, showed a RT-PCR dual reactivity for both WH187 and Db126 peptides in some patients (Fig. 22). This reactivity was conserved in the DB126 peptide, but not the WH187, for the same patient with other time points even it was one month later. So, both of the WH1-specific CTLs can be detected using the RT-PCR assay.

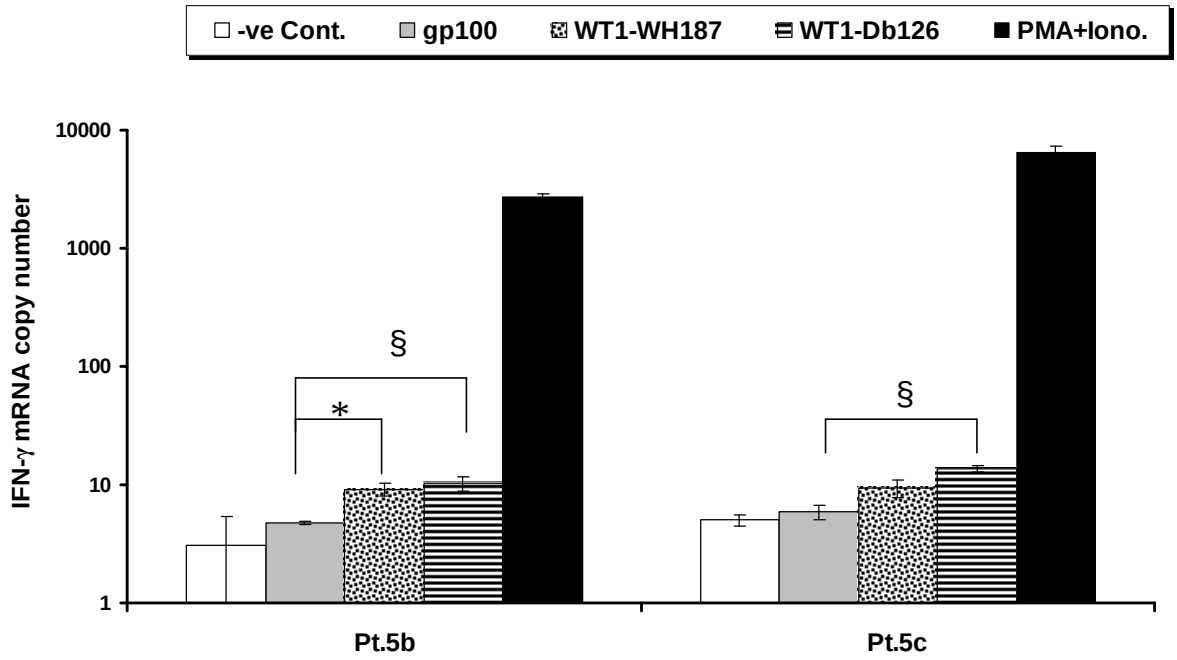

Figure 22 : Expression of IFN-γ mRNA after reactivation with two WT1 peptides. Significance was considered if P<0.05 when WH187-peptide (*) or (§) Db126-peptide induced INF-γ was compared to gp100 peptide.

For performing a longitudinal WT1-reactivity follow-up, PBMCs in many time points were tested in a time kinetics manner. Most of the patients donate PBMCs in different time points at least in a month time intervals, then their PBMCs were isolated and frozen until testing. In the RT-PCR assay, IFN-γ mRNA expression of WT1-reactive CTLs was detectable within 3h after peptides stimulation. Once patients' PBMCs shown reactivity for one or both WT1 peptides, another time point was checked by RT-PCR and ELISPOT assays to continue a longitudinal follow-up. The IFN-γ mRNA up-regulation in response to WT1 peptides was compared to the gp100 control peptide to determine the significance (P<0.05). Two types of comparison were performed to compare the WT1-Db126 (*) or WT1-WH187 (§) peptides to the gp100 peptide at the same day. In one patient who was suffering from AML, PBMCs shown a significant up-regulation of IFN-γ mRNA after stimulation with both WT1 peptides (Fig. 23-a). These significant RT-PCR positive results reflect the presence of WT1-specific CTLs in patients' peripheral blood as early as five months after Tx. On the other hand, a second patient who was suffering from ALL, PBMCs shown a significant up-regulation of IFN-γ mRNA after WT1 peptide pulsing compared to gp100 (Fig. 23-b). This patient's WT1 reactivity was documented after nine months of Tx, but unfortunately no earlier time points could be tested to determine the beginning of this WT1 reactivity.

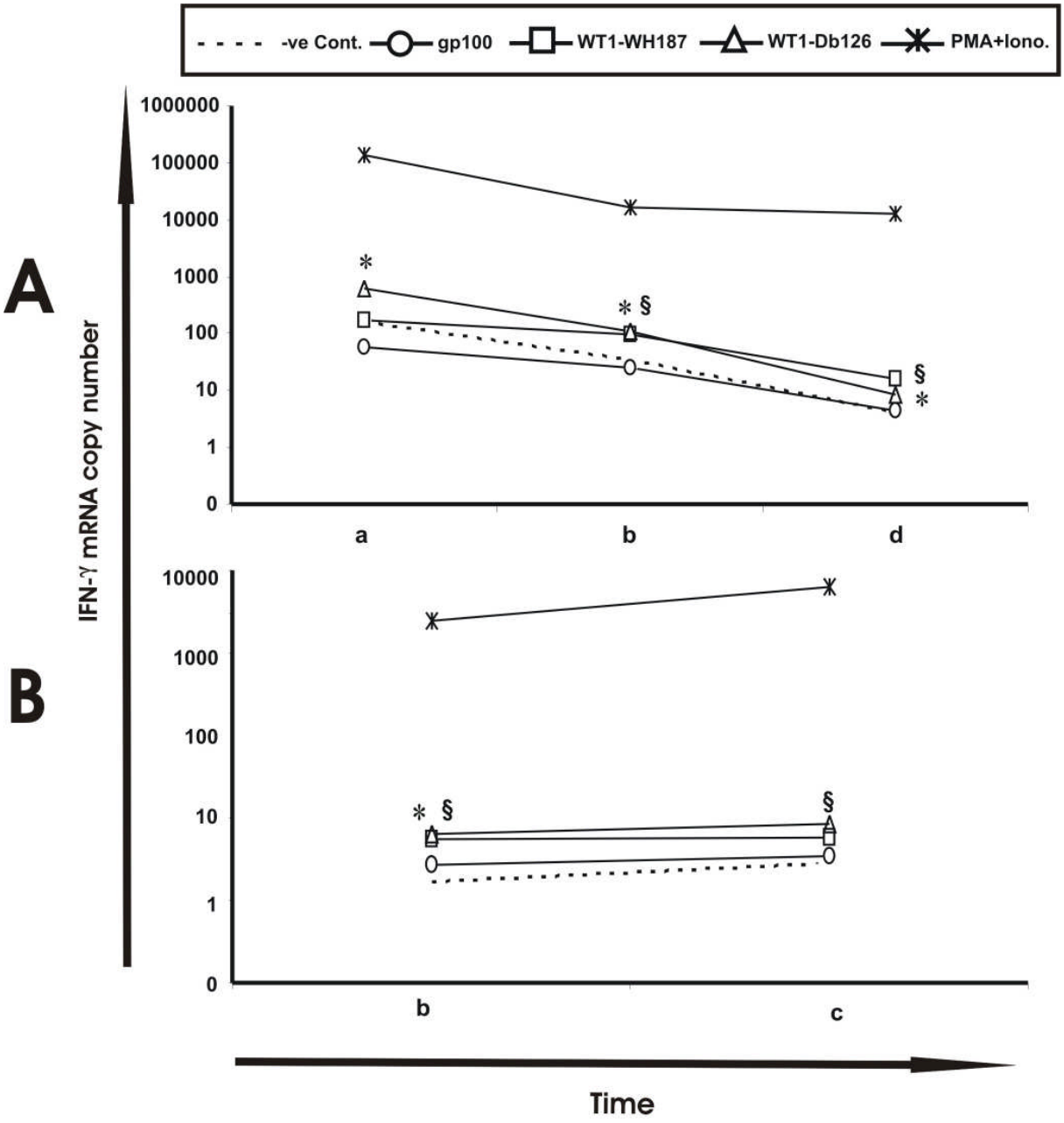

Figure 23 : RT-PCR time kinetic follow-up of two patients namely: Pt.8 (A) and Pt.5 (B). WT1-reactive CTLs are detectable significantly when WH187 peptide (*) or Db126 peptide (§) induced INF-γ in comparison to gp100 peptide ($P<0.05$).

Not all of the tested eight HLA-A2 positive patients shown to have WT1-reactive CTLs (Fig. 24). This has been expected and fits to the observation of Rezvani et *al.*[145] who used even pure CD8 selected cells from CML patients after allogeneic SCT. Nevertheless, the WT1 restricted CTLs were detectable in two patients out of eight.

For quality control considerations, HLA-A2 negative patients were selected to confirm the superior performance of the tested RT-PCR method as well as the HLA-A2-restriction of the

used WH187 and Db126 WT1 peptides. After pulsing the HLA-A2 negative patients' PBMCs with both peptides, IFN-γ mRNA measurements were not significant in all samples in comparison to the gp100 control peptide (Fig. 25).

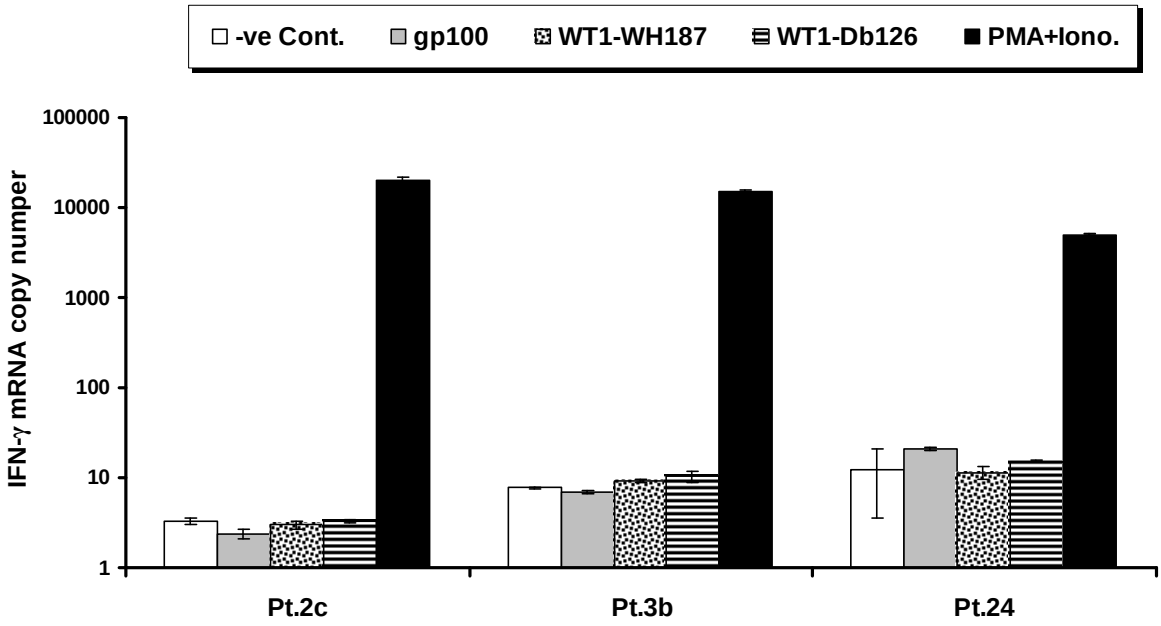

Figure 24 : IFN-γ gene expression analysis of three HLA-A2 positive patients after WT1 peptides pulsing by the RT-PCR assay.

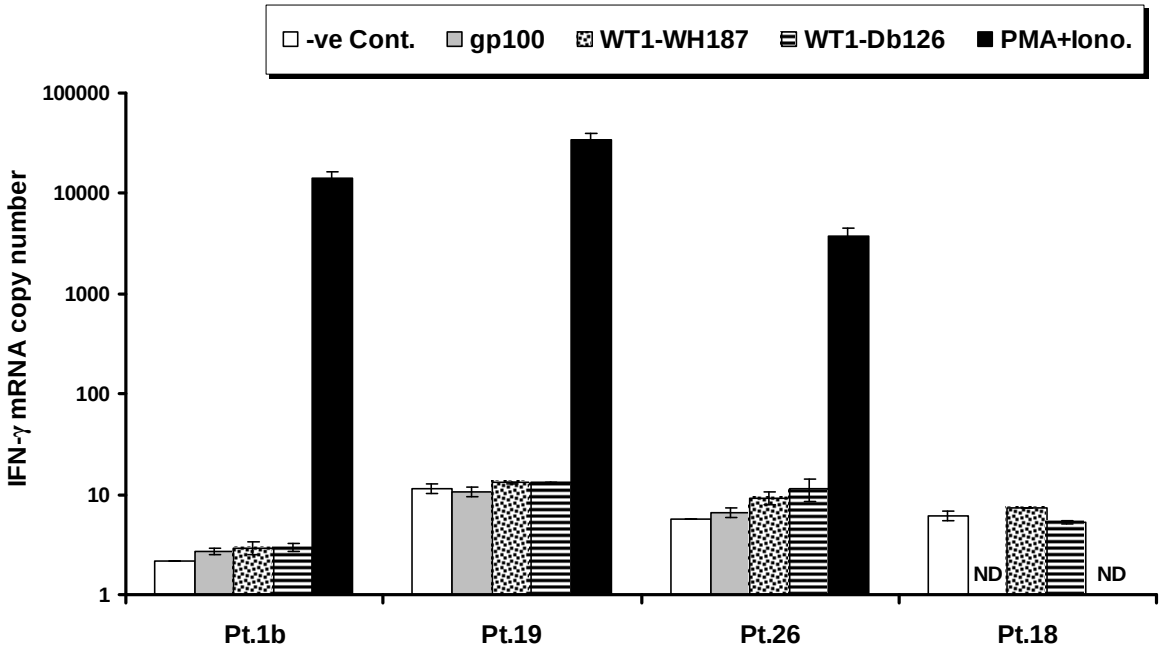

Figure 25: IFN-γ gene expression analysis of four HLA-A2 negative patients after WT1 peptides pulsing by the RT-PCR assay.

3.2.2 ELISPOT assay can not assess WT1-reactive CTLs

As described by Scheibenbogen *et al.*,[157] WT1 specific CTLs can be detected in the AML patient by ELISPOT assay. So, the same assay was used to detect the presence of WT1-specific CTLs in the peripheral blood of the tested allogeneic STC. The patients' PBMCs, who were described in table 9, were used to monitor WT1-spesific CTLs by ELISPOT assay. This was performed in parallel to the RT-PCR experiments to check the correlation between the two methods. Taking in consideration the ELISPOT assay long time of cytokines-secretion period (e.g. 24h), PBMCs IFN-γ production was tested in response to co-culturing with WT1 peptides. Rezvani *et al.*[145] used WT1 peptide concentrations ranging from 0.1µg/ml to 10µg/ml, but here two WT1 peptide concentrations were used namely: 5µg/ml and 10µg/ml. The production of IFN-γ after 24h in response to one or both WT1 peptides was compared to the gp100 control peptide as well as medium alone. Unfortunately, the ELISPOT assay was not sensitive enough to detect the IFN-γ in many patients in comparison to the RT-PCR assay (Fig. 26). This dose also applies to the sample patient (i.e. Pt.8) who was positive in the RT-PCR assay (Fig. 21).

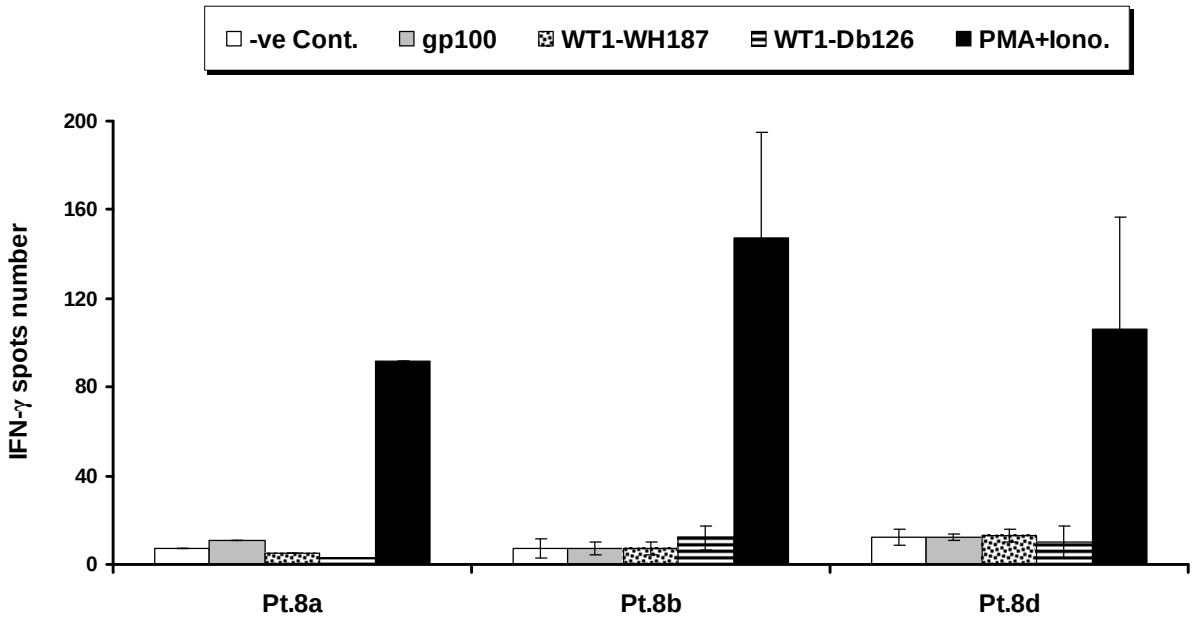

Figure 26 : WT1-specific CTLs detection by ELISPOT assay in three patients.

Unlike in RT-PCR (Fig. 22), IFN-γ in Pt.5 was not detectable by the ELISPOT assay in his PBMCs sample (Fig. 27). Both of the WT1 peptides did not induce detectable amounts of IFN-γ secretion even after 24h of activation. This is comparable with what has been achieved

by Scheibenbogen *et al.*[157], since they could detect very low frequency of WT1-reactive CTLs in AML patients. They found that thirteen out of fifteen AML patients did not have WT1-reactive T cells (87 %).

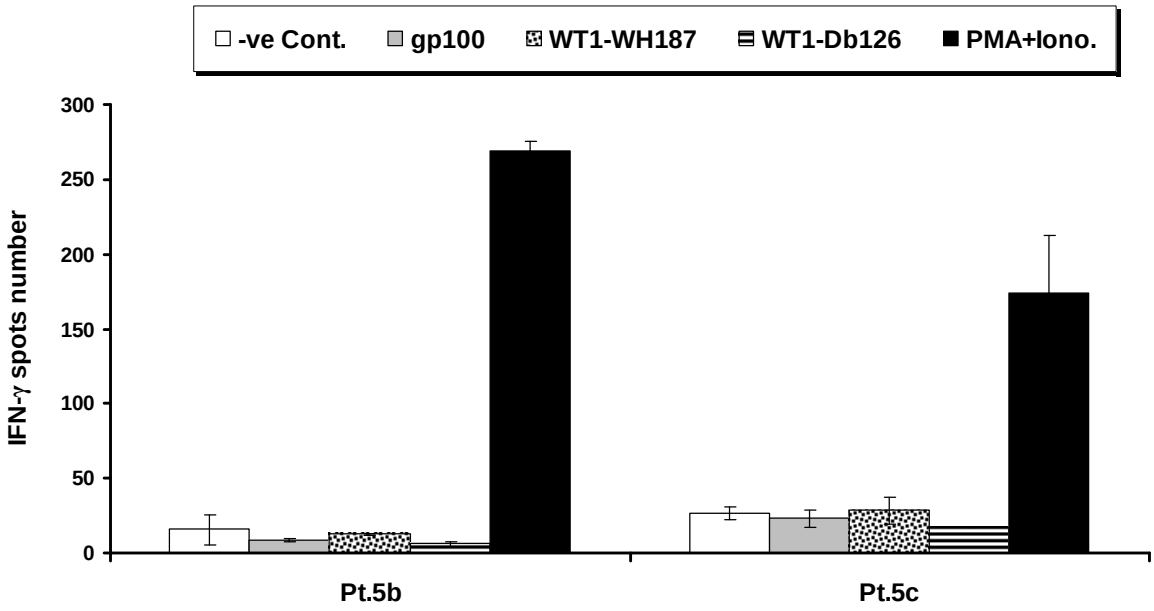

Figure 27 : Detection of secreted IFN-γ by ELISPOT assay after activation with WT1 peptides.

Using ELISPOT and ICC assays to monitor WT1- and proteinase 3-specific T cells in AML patients, reveled that small number of the patients have WT1-specific CTLs (e.g. 13%) in their peripheral blood.[157] This suggested very low CTLs frequencies but sensitive ELISPOT assay. In the same adopted ELISPOT assay, no significant IFN-γ production in many HLA-A2 positive patients was found after a long secretion period (Fig. 28). In addition, this was confirmed by the RT-PCR results obtained from the same patients (Fig. 24). These ELISPOT results, in addition to the RT-PCR, reflected the very low frequencies of WT1-specific CTLs in the peripheral blood of allogeneic SCT patients.

In addition to the use of gp100 as the experimental-control peptide, another patient-control was necessary therefore HLA-A2 negative patients' PBMCs were tested. These PBMCs were not able to secrete detectable IFN-γ in response to both WT1 peptides (Fig. 29). The IFN-γ basal production in the two experimental-controls, media and gp100, were equivalent to the two WT1 peptide-treated HLA-A2 cells. In addition, these results were confirmed by the RT-PCR experiments for the same patients (Fig. 28). This result for the

HLA-A2 negative patients' PBMCs confirms the specificity of both peptides to the HLA-A2 alleles, and that the gp100 peptide is appropriate as an experimental-control.

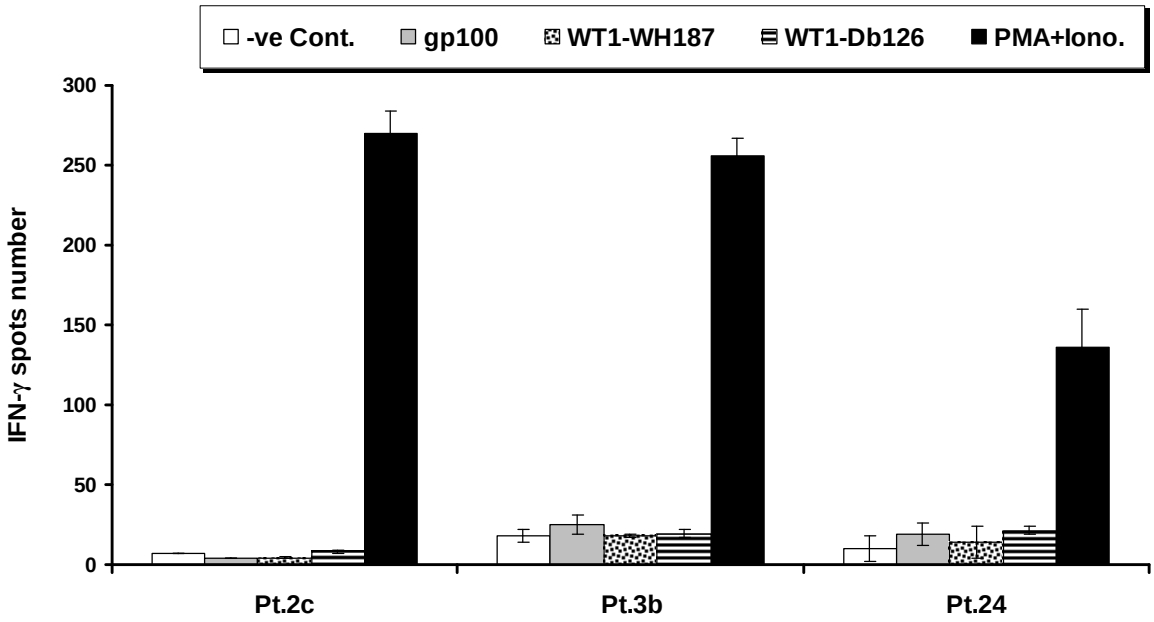

Figure 28 : HLA-A2 positive patients IFN-γ production after activation with WT1 peptides.

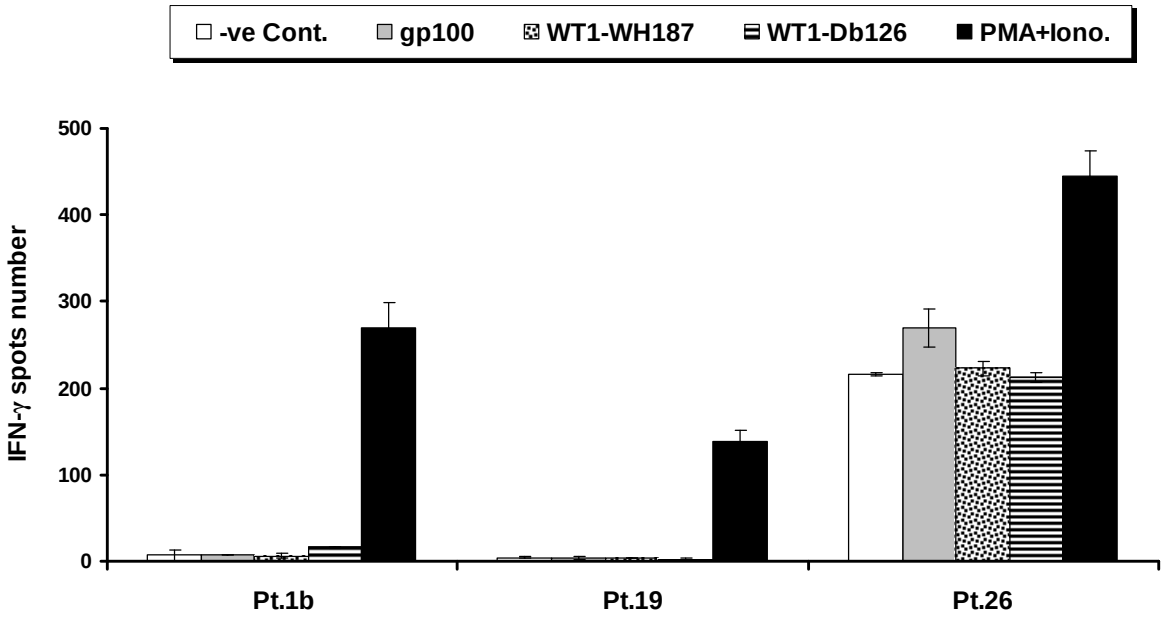

Figure 29 : HLA-A2 negative patients IFN-γ production after activation with WT1 peptides.

As a summary, WT1-specific CTLs were detectable in 25% (two patients) of all tested HLA-A2 positive patients (eight patients) who underwent allogeneic SCT. These two patients have detectable INF-γ mRNA levels, which could be traced in the RT-PCR assay. In contrary, IFN-γ could not be detected using the ELISPOT assay as a monitoring tool, reflecting an inferior sensitivity in detecting very low frequencies of WT1-specific T cells. In addition, both assays were comparable by not detecting any reactivity for the HLA-A2 negative patients. Table 11 summarizes all of the results concerning the reconstitution of WT1-reactive T cells in patients' PBMCs.

			WT1-WH187		WT1-Db126	
Pt.	**Diagnosis**	**HLA-A2**	**ELISPOT**	**RT-PCR**	**ELISPOT**	**RT-PCR**
1b	**AML**	-	-	-	-	-
2c	**CML**	+	-	-	-	-
3b	**AML**	+	-	-	-	-
4a	**AML**	+	-	-	ND	ND
4b		+	-	-	ND	ND
4c		+	-	-	ND	ND
4d		+	-	-	ND	ND
4e		+	-	-	ND	ND
5b	**ALL**	+	-	+	-	+
5c		+	-	-	-	+
8a	**AML**	+	-	-	-	+
8b		+	-	+	-	+
8d		+	-	+	-	+
11a	**AML**	+	-	-	-	-
11b		+	-	-	-	-
11c		+	-	-	-	-
14a	**ALL**	+	-	-	-	-
14b		+	-	-	-	-
18	**AML**	-	-	-	-	-
19	**AML**	-	-	-	-	-
24	**AML**	+	-	-	-	-
26	**AML**	-	-	-	-	-

Table 11 : Detection of WT1-specific CTLs in the peripheral blood of allogeneic transplanted patients using RT-PCR and ELISPOT assays. Significant (+) and not significant (-) detection of IFN-γ is shown. AML: acute lymphocytic leukemia, CML: chronic myelogenous leukemia, ALL: acute lymphoblastic leukemia, +: HLA-A2 positive, -: HLA-A2 negative and ND: not determined.

3.3 Monitoring of mHAg-reactive T cells

As a reconstitution model of mHAgs-specific cells, HY antigen-specific T cell reconstitution was monitored in allogeneic-transplanted patients. To monitor HY-specific T-cells, IFN-γ mRNA gene expression levels and the protein production were measured by RT-PCR [145] and ELISPOT assays,[125] respectively. All of the patients who enrolled in this study were male recipients who received allogeneic SCT from female donors. PBMCs from ten patients were tested for their HY reactivity, seven of them were HLA-A2 positive and the rest were HLA-A2 negative as control-group. The criteria of patients who were expected to have HY-reactive T cells are showed in table 12.

				Time after Tx		
Pt.	**Diagnosis**	**HLA-A2**	**Immunosuppression**	**Y**	**M**	**D**
4a	**AML**	**+**	**-**	**-**	**5**	**1**
4b		**+**	**-**	**-**	**5**	**23**
4c		**+**	**-**	**-**	**6**	**18**
4d		**+**	**-**	**-**	**6**	**27**
4e		**+**	**-**	**-**	**7**	**10**
8a	**AML**	**+**	**+**	**-**	**5**	**15**
8b		**+**	**+**	**-**	**6**	**22**
8c		**+**	**-**	**-**	**7**	**19**
8d		**+**	**-**	**-**	**8**	**23**
9	**NHL**	**-**	**+**	**3**	**6**	**7**
13a	**NHL**	**+**	**+**	**1**	**-**	**22**
13c		**+**	**+**	**1**	**1**	**7**
13d		**+**	**+**	**1**	**2**	**15**
13e		**+**	**+**	**1**	**2**	**29**
13f		**+**	**+**	**1**	**3**	**13**
13g		**+**	**+**	**1**	**4**	**9**
14a	**ALL**	**+**	**-**	**1**	**-**	**20**
14b		**+**	**-**	**1**	**3**	**22**
17a	**NHL**	**+**	**-**	**-**	**9**	**8**
17b		**+**	**-**	**-**	**10**	**25**
18	**AML**	**-**	**++**	**-**	**5**	**15**
19	**AML**	**-**	**-**	**5**	**1**	**25**
20a	**NHL**	**+**	**-**	**2**	**4**	**5**
20b		**+**	**-**	**2**	**6**	**7**
21a	**NHL**	**+**	**+**	**1**	**7**	**24**
21b		**+**	**+**	**1**	**8**	**8**
21c		**+**	**++**	**1**	**8**	**29**
21d		**+**	**+**	**1**	**9**	**12**

Table 12 : Criteria of selected patients to detect HY-reactive T cells. Y: years, M: months, D: days, AML: acute lymphocytic leukemia, CML: chronic myelogenous leukemia, ALL: acute lymphoblastic leukemia.

3.3.1 RT-PCR assay can detect HY-reactive T cells

As described by Rufer *et al.*,[97] SMCY-derived HY peptide was discovered to be an immunodominant mHAg after bone marrow transplantation. They isolated some HY-specific T cell clones during acute GvHD after allogeneic bone marrow transplantation. On the other hand, methods describing the detection of HY-reactive T cells vary between CTL assays,[97] ^{3}H-thymidine incorporation assay,[160] cytotoxicity (^{51}Cr release) assay,[100,160] TM and IC-IFN-γ flow cytometry,[105] and ELISPOT assay (in mice).[161] No study describes the detection of the HY-reactivity using RT-PCT technology so far.

Among the seven HLA-A2 positive allogeneic SCT transplanted patients, HY-reactive CTLs were detectable in one patient by the RT-PCR assay (Fig. 30), reflecting the presence of functional CTL cells in this patients' peripheral blood.

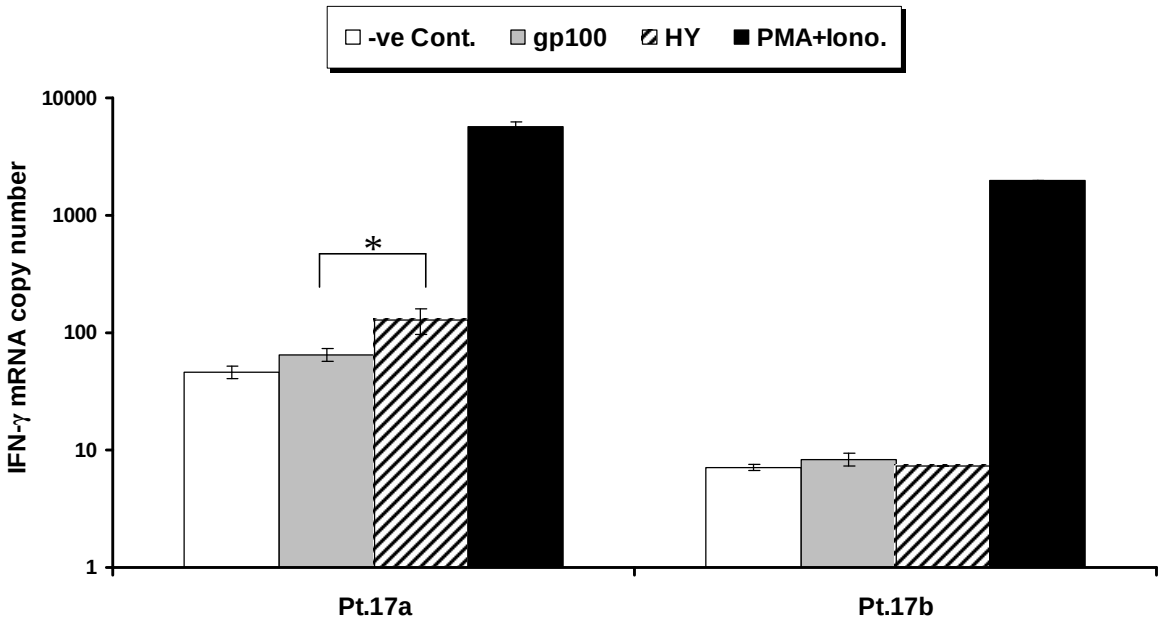

Figure 30 : HY-specific CTLs found in male patient. (*): Significance was considered if $P<0.05$ when HY peptide induced INF-γ mRNA up-regulation compared to gp100 peptide.

The detection limit of Ag-specific cells is based on two factors, first the frequency of the cells, and second the sensitivity of the detection method. Therefore, the highly sensitive RT-PCR assay was used to monitor the presence of HY-specific T cells in patients' peripheral blood. Although the RT-PCR assay detected significant increase in IFN-γ mRNA in one patient, many others did not show any HY-reactivity even if they were HLA-A2 positive patients (Fig. 31). This may be due to the low frequency of HY-specific CTLs in the peripheral blood of the tested patients, or due to the presence of HY-specific T cells recognizing other HY epitopes rather than the tested peptide.

Besides the two experimental-controls, namely media alone and gp100 peptide, an additional control was used. This control was the HLA-A2 negative patients' PBMCs which should not at least theoretically recognize the HY peptide which is HLA-A2-restricted. The RT-PCR testing system showed that the used HY peptide was MHC class I (i.e. HLA-A2) restricted. This was confirmed by the non-significant IFN-γ mRNA detection in the HLA-A2 negative patients (Fig. 32).

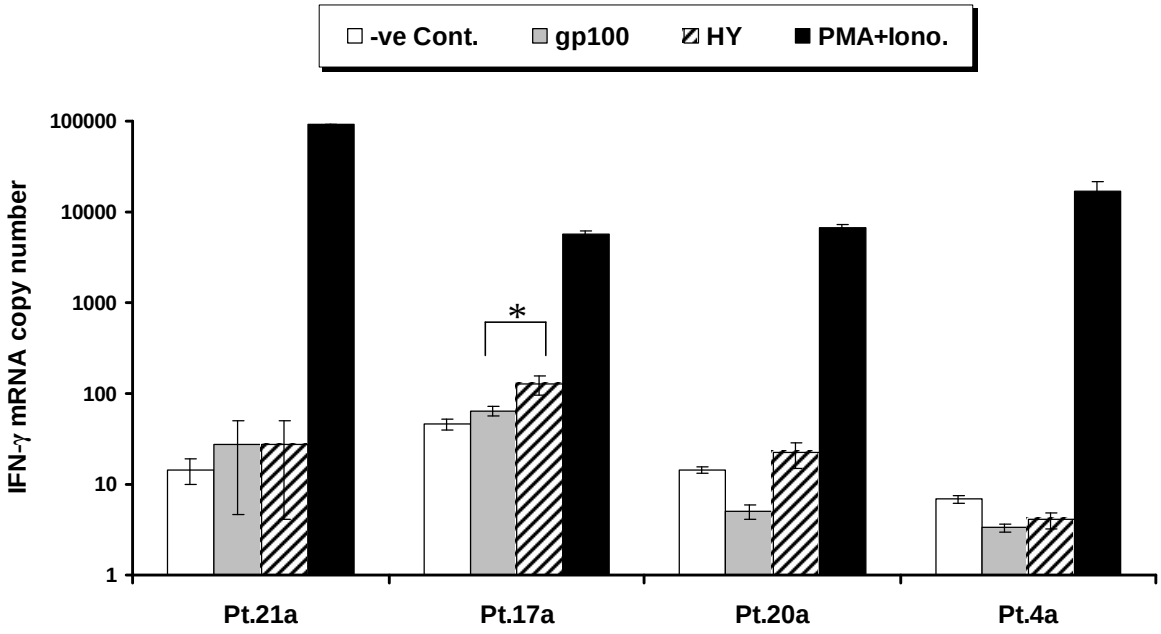

Figure 31 : RT-PCR monitoring of some HLA-A2 positive patients after HY peptide pulsing. (*): Significant INF-γ mRNA up-regulation when HY compared to gp100 ($P<0.05$).

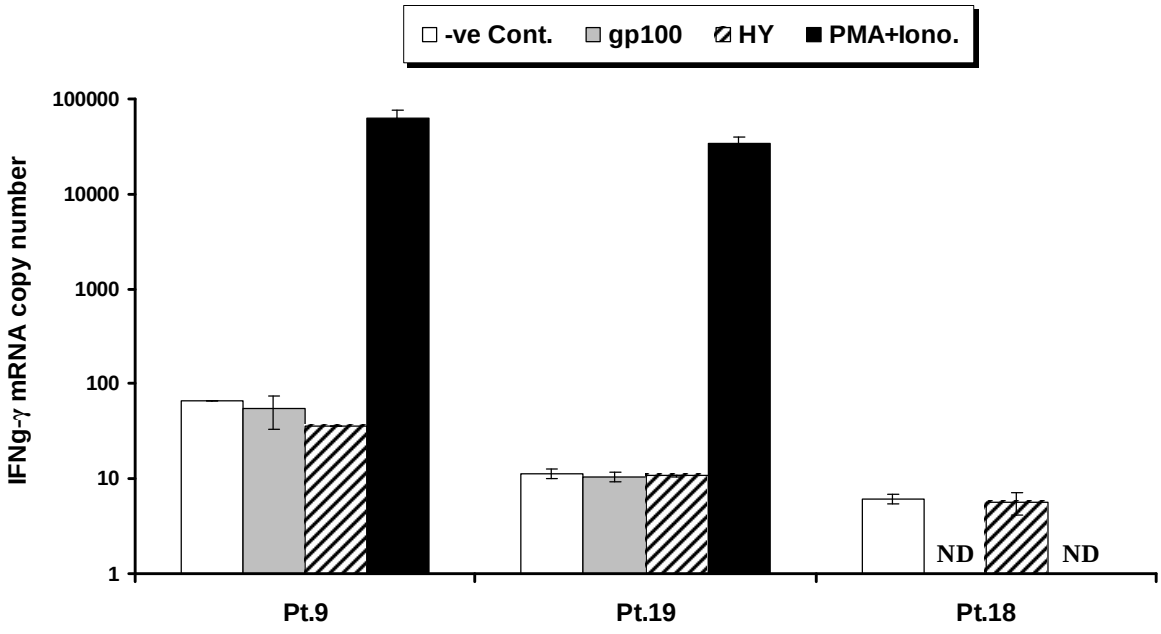

Figure 32 : RT-PCR monitoring of some HLA-A2 negative patients after HY peptide pulsing. ND: not determined.

To elaborate the generation of HY-specific CTLs, a longitudinal follow-up for the patients was took place. This was done by collecting PBMCs at many time points to cover a time range where HY-reactive T cells expected to be in patients' peripheral blood. Nine patients PBMCs did not show any IFN-γ mRNA expression after HY-peptide exposure (Fig. 33).

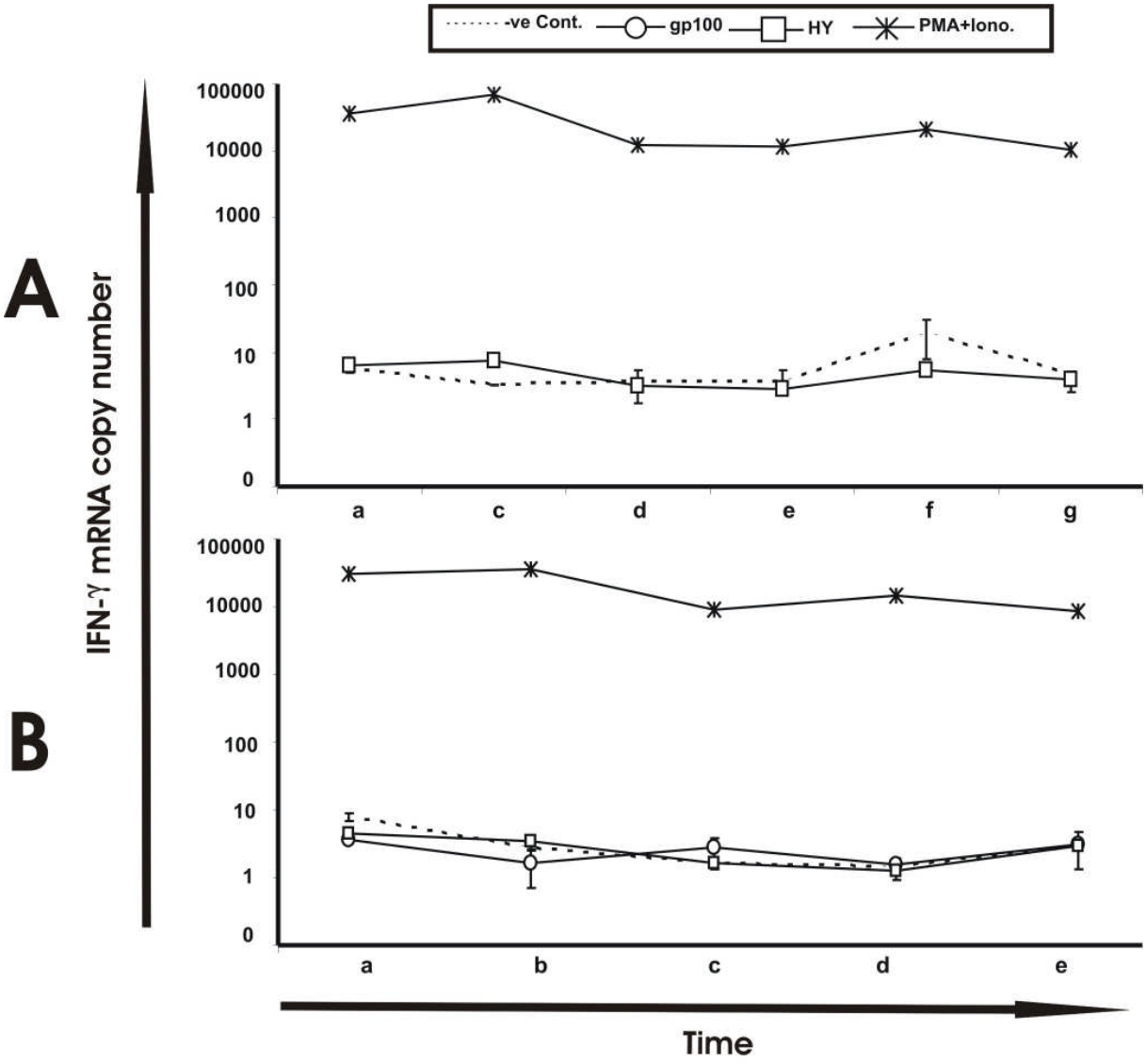

Figure 33 : IFN-γ mRNA expression time kinetics follow-up for two patients' PBMCs namely: Pt.13 (A) and Pt.4 (B), after activation with HY peptide.

3.3.2 ELISPOT assay can not detect HY-reactive CTLs

Many ELISPOT assays are well established to detect the presence of human Ag-specific CTLs in the peripheral blood of tested allogeneic STC. A study was done by James *et al.*[161] used ELISPOT assay to detect mice HY peptides-reactive T cells after skin allografting. The same patients in table 11 were used to monitor HY-specific CTLs. This has been performed

by taking the advantage of secreting IFN-γ for long time (e.g. 24h) after reactivation with the peptides of interest. IFN-γ spots produced in response to HY-peptide were compared to the gp100 control peptide as well as the medium alone, which was considered as the background.

Even in the same patient who was positive in the RT-PCR assay and significant IFN-γ mRNA was detectable (Fig. 30), the ELISPOT assay did not detect any IFN-γ production even after 23h of activation (Fig.34). This may reflect the higher sensitivity of RT-PCR assay compared to the ELISPOT assay.

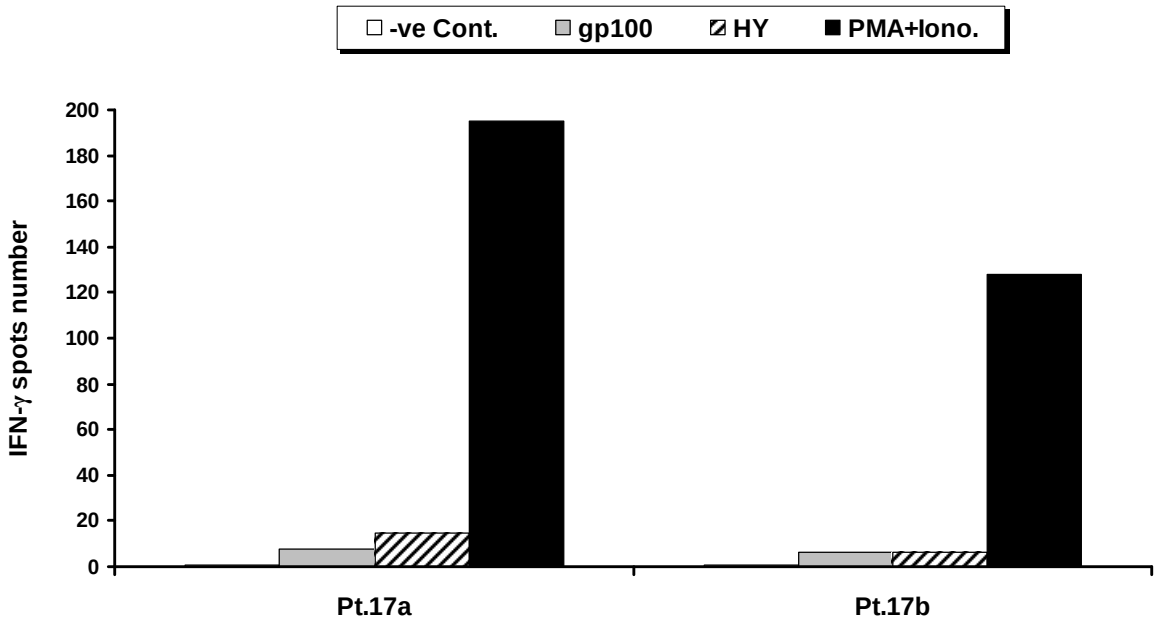

Figure 34 : ELISPOT assay for the patient who has positive results at the mRNA level.

As Rufer *et al.* [97] showed that the SMCY-derived HY peptide is an immunodominant mHAg after bone marrow transplantation. They indicated that this HY peptide recognized in the context of HLA-A2. As HLA-A2-restricted peptide, most of the selected patients were selected to possess the HLA-A2 allele. In the HLA-A2 positive patients, the ELISPOT assay did not detect significant IFN-γ production in many patients (Fig. 35), which was confirmed by the RT-PCR assay (Fig. 31). The negative results of both ELISPOT and RT-PCR assays reflecting the very low frequencies of HY-specific CTLs in the peripheral blood of male patients after allogeneic SCT from female donors.

As patients' control-group, PBMCs from HLA-A2 negative patients were enrolled in this study to compare the efficiency of both methods to detect any HY-specific CTLs. No significant number of IFN-γ spots was detected in the HLA-A2 negative patient's PBMCs after reactivation with HY-peptide (Fig. 36). The very low IFN-γ spot number due to HY-peptide was comparable to media alone and/or gp100 control peptide. This confirms the

restriction of HY-peptide to the HLA-A2 allele. In addition, these results were confirmed by the RT-PCR experiments for the same patients (Fig. 32).

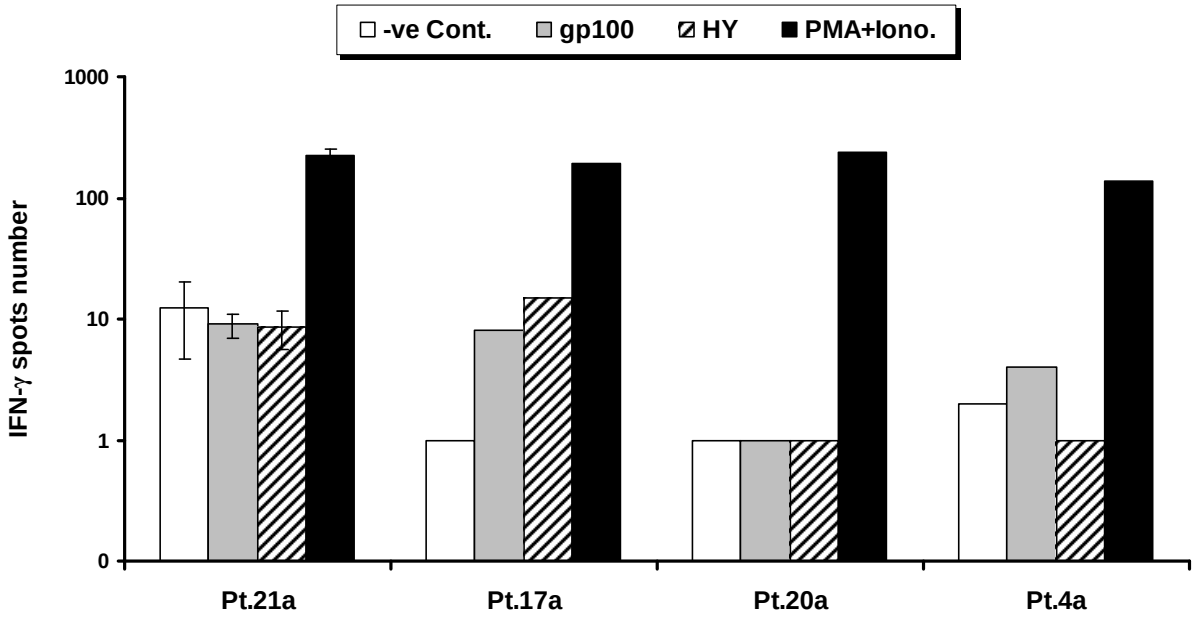

Figure 35 : ELISPOT assay monitoring for some HLA-A2 positive patients after HY-peptide pulsing.

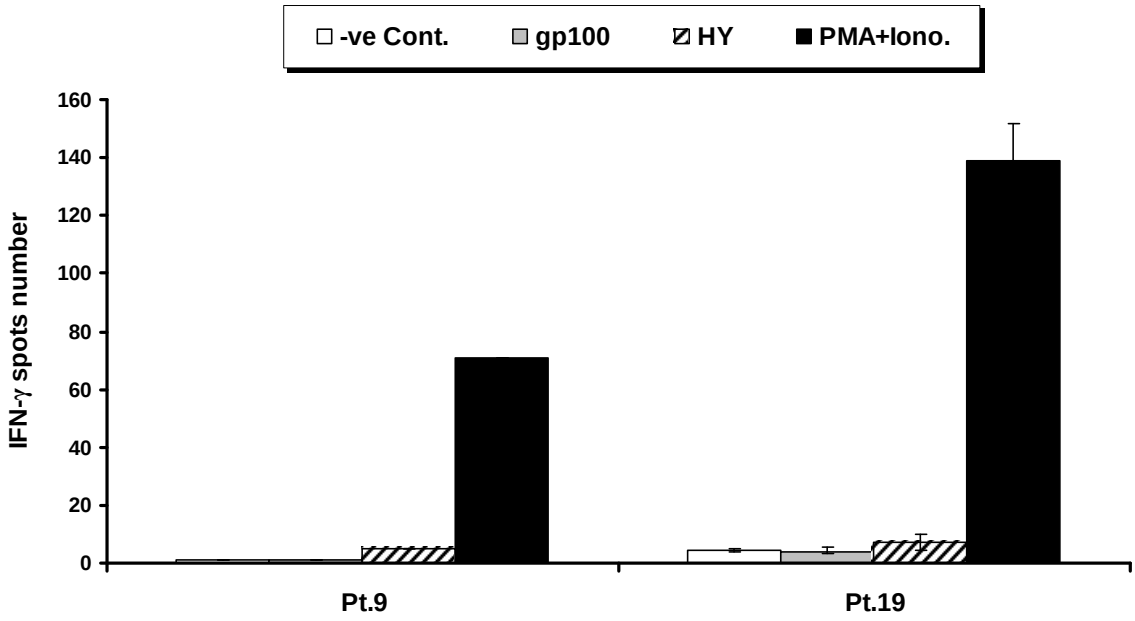

Figure 36: ELISPOT assay monitoring for some HLA-A2 negative patients after HY-peptide pulsing.

Time kinetics follow-up of seven patients were performed to trace HY-specific CTLs in peripheral blood, if any. Many patients' PBMCs were monitored in monthly basis to monitor any formation of HY-reactive T cells. Unfortunately, these patients' PBMCs did not show and IFN-γ production after HY-peptide exposure (Fig. 37). Most of the tested samples kept a low number of IFN-γ spots after HY-peptide pulsing. It was in the same range of the experimental-controls, which were the media alone and/or gp100 peptide (i.e. background). This may be interpreted by the low frequencies of HY-specific CTLs in the tested patients' PBMCs, which even did not increase after two and half years (e.g. Pt.20b).

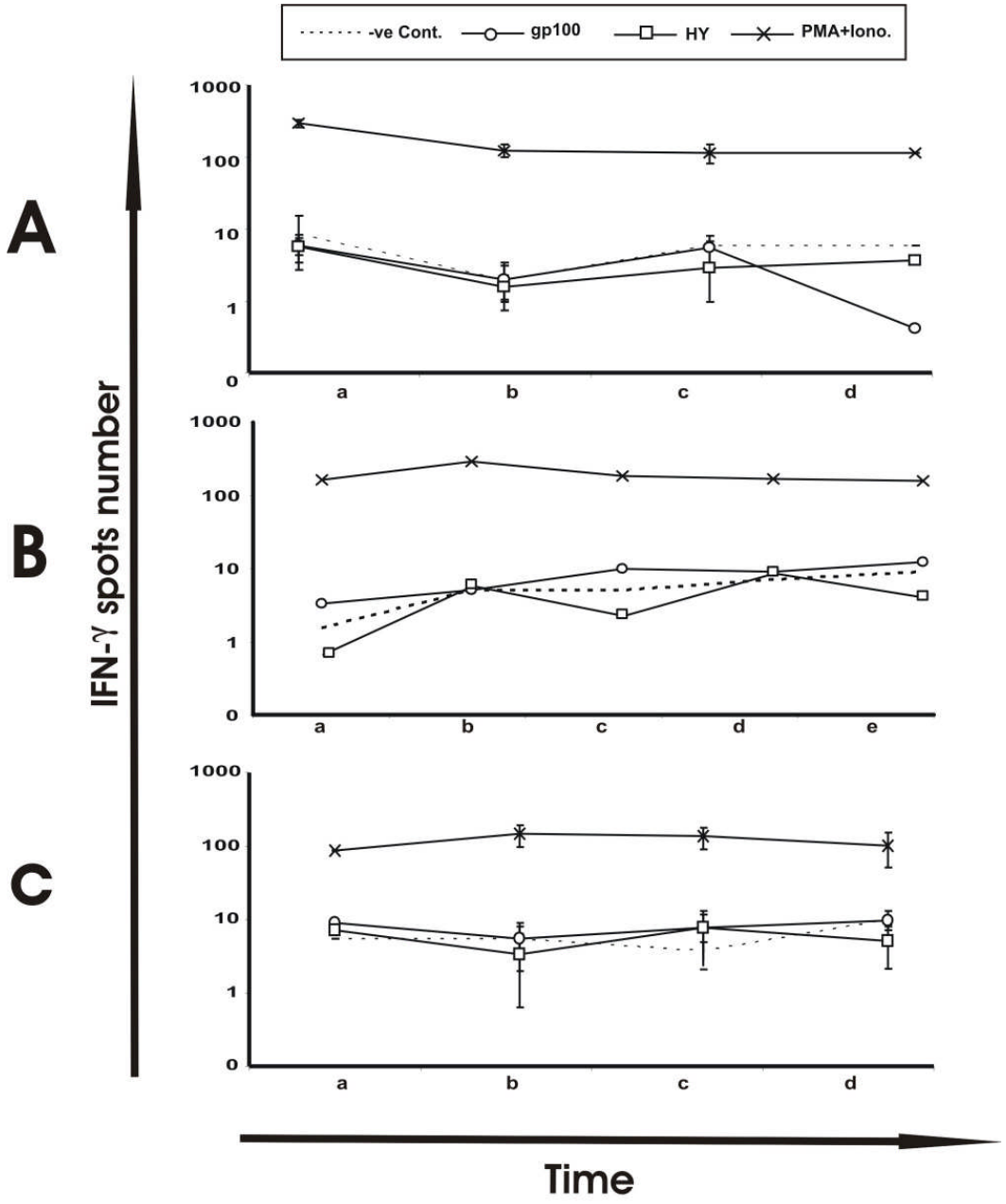

Figure 37 : Time kinetics follow-up of three patients' PBMCs namely: 21 (A), 4 (B) and 8 (C), for HY-reactive CTLs after reactivation with immunogenic HY-peptide.

As a summary, HY-specific CTLs were detectable in 14% (one patient) of all male tested HLA-A2 positive patients (seven patients), who received stem cells from female donors. In this patient sample, IFN-γ mRNA was detectable by the RT-PCR assay but not the ELISPOT assay. This shows the higher sensitivity of the RT-PCR assay to monitor HY-specific T cells following an adequate stimulus. In addition, both assays were comparable by not detecting any reactivity for the HLA-A2 negative patients. This confirms the HLA-A2-restriction of the HY-peptide. Table 13 summarizes all the results concerning the monitoring of HY-reactive CTLs.

			Time after Tx				
Pt.	**Diagnosis**	**HLA-A2**	**Y**	**M**	**D**	**ELISPOT**	**RT-PCR**
4a	**AML**	**+**	-	5	1	**-**	**-**
4b		**+**	-	5	23	**-**	**-**
4c		**+**	-	6	18	**-**	**-**
4d		**+**	-	6	27	**-**	**-**
4e		**+**	-	7	10	**-**	**-**
8a	**AML**	**+**	-	5	15	**-**	**-**
8b		**+**	-	6	22	**-**	**-**
8c		**+**	-	7	19	**-**	**-**
8d		**+**	-	8	23	**-**	**-**
9	**NHL**	**-**	3	6	7	**-**	**-**
13a	**NHL**	**+**	1	-	22	**-**	**-**
13c		**+**	1	1	7	**-**	**-**
13d		**+**	1	2	15	**-**	**-**
13e		**+**	1	2	29	**-**	**-**
13f		**+**	1	3	13	**-**	**-**
13g		**+**	1	4	9	**-**	**-**
14a	**ALL**	**+**	1	-	20	**ND**	**-**
14b		**+**	1	3	22	**-**	**-**
17a	**NHL**	**+**	-	9	8	**-**	**+**
17b		**+**	-	10	25	**-**	**-**
18	**AML**	**-**	-	5	15	**ND**	**-**
19	**AML**	**-**	5	1	25	**-**	**-**
20a	**NHL**	**+**	2	4	5	**-**	**-**
20b		**+**	2	6	7	**-**	**-**
21a	**NHL**	**+**	1	7	24	**-**	**-**
21b		**+**	1	8	8	**-**	-
21c		**+**	1	8	29	**-**	-
21d		**+**	1	9	12	**-**	**-**

Table 13 : Detection of HY-specific CTLs, in the peripheral blood of allogeneic SCT patients, using RT-PCR and ELISPOT assays. Significant (+) and not significant (-) detection of IFN-γ is shown. Y: years, M: months, D: days, AML: acute lymphocytic leukemia, NHL: Non-Hodgkin's lymphomas, ALL: acute lymphoblastic leukemia. ND: not determined.

3.4 Monitoring of GvHD

3.4.1 Detection of IFN-γ by IC flow cytometry assay

Although alloantigen specific T cells partially resemble virus-specific T cells, they do differ from the latter in several aspects. First, not only do they recognize alloantigeneic peptides in a self-MHC restricted way, through the so-called indirect pathway, but they can also recognize the antigen in a non-MHC restricted way (the so-called direct pathway). Second, not only naive CD45RA+ but also memory CD45RO+ T cells may respond to a newly introduced alloantigen. For simulating the GvHD's alloantigen specific T cells at the patients' body, MLR settings were optimized for PBMCs from two mismatched healthy donors to generate alloantigeneic sensitized T cells.[115] These alloantigeneic cells were stimulated by the alloantigen again and then the some cytokines were detected intracellularly.

3.4.1.1 Establishment of GvHD model

Nikolaeva *et al.*, showed that both CD4+ and CD8+ T cells had an early and vigorous response to allogeneic stimulation in mismatched healthy individuals' MLRs.[114] Their MLR protocol was adopted with some modifications such as the PKH_{26} use instead of CFSE.[114] PBMCs from healthy individuals were co-cultured with allogeneic irradiated stimulator cells.

The flow cytometry gating strategy was took in consideration gating-out the PKH_{26} labeled cells (Fig. 38-a). Then viable leukocytes (Fig. 38-b) were further gated in pan T lymphocytes gate (Fig. 38-c), and finally the target cytokines were plotted vs. CD4 (Fig. 38-d).

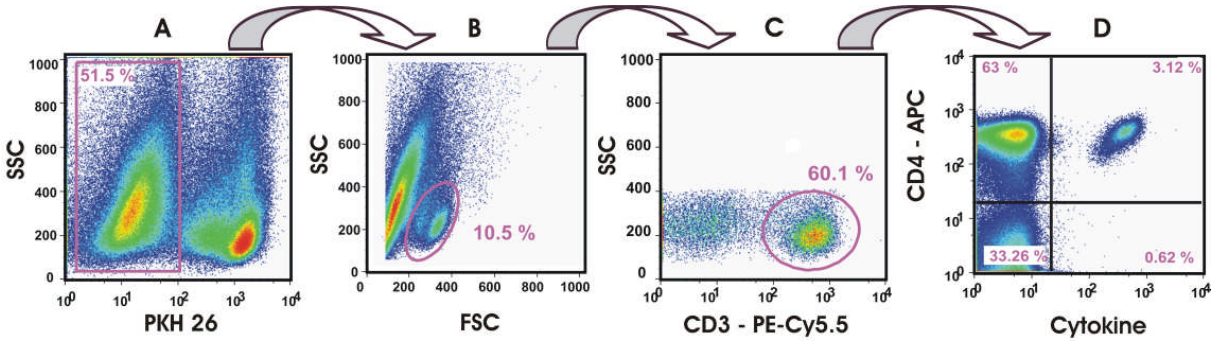

Figure 38: Schematic diagram showing the gating strategy of responder cells. Starting with gating the cells of interest at the PKH_{26} vs. SSC gate (A), followed by FSC vs. SSC gating (B), then CD3 vs. SSC (C) and finally plotting the CD4 vs. the cytokines (D).

The alloantigeneic sensitized responder cells were not able to produce any IFN-γ by themselves if no adequate stimulus was applied (Fig. 39-a). Even the IC IFN-γ of alloantigeneic sensitized responder cells were not detectable by flow cytometer when the irradiated stimulator cells did not counterstained by PKH_{26} dye (Fig. 39-b). Also IFN-γ production was not detectable when alloantigeneic responder cells were co-cultured with PKH_{26}-labled but non-irradiated stimulator cells (Fig. 39-c). This reflected the importance of stimulator PBMCs to be irradiated (e.g. 75Gy) so it can not secrete any mediators at the media that can inhibit the responder cells' function. Finally, the IFN-γ was detected significantly when both irradiated and PKH_{26}-labled stimulator cells were used (Fig. 39-d). This indicated that the stimulator cells should not only be irradiated but also gated out from the flow cytometer analysis.

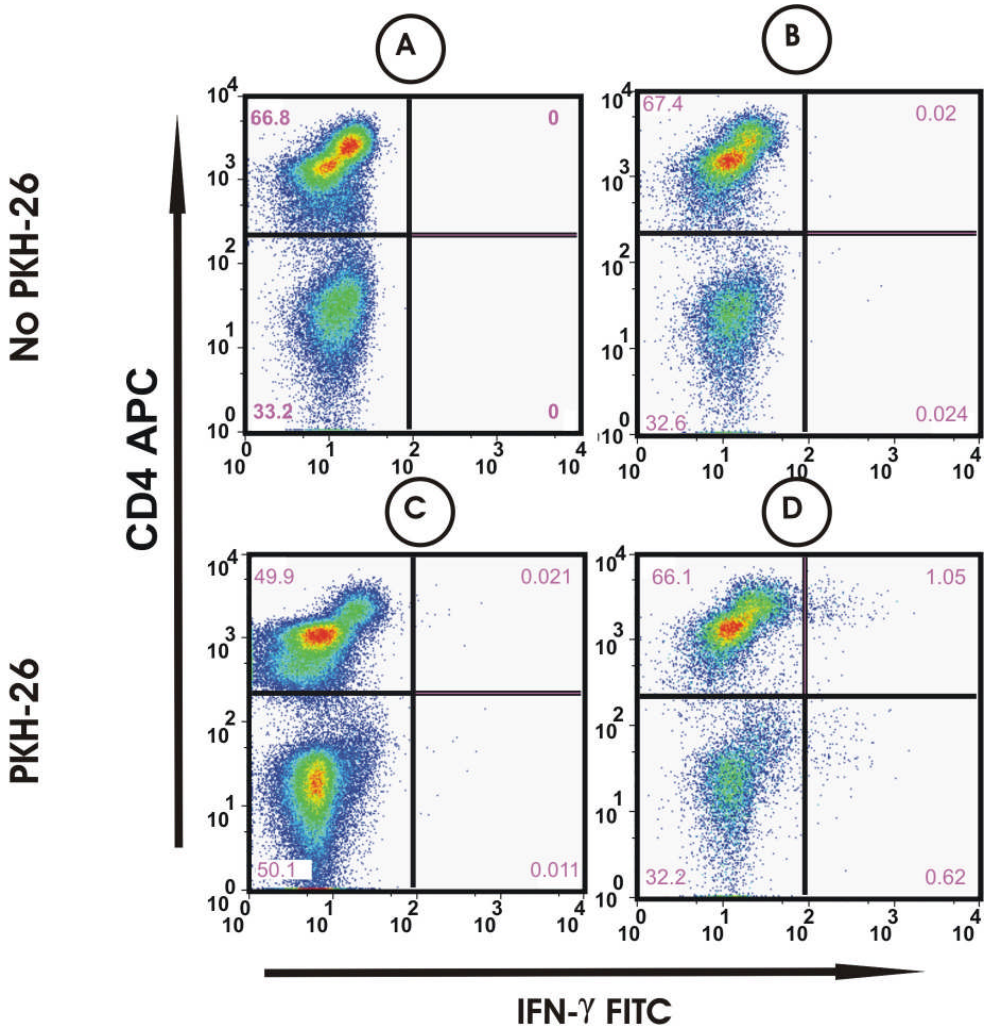

Figure 39 : IC IFN-γ detection in the second MLR by flow cytometry. Sensitized responder cells (sR) were incubated with irradiated (iS) or non-irradiated (S) stimulator cells in the following combinations: sR (A), sR + iS (B & D), sR + S (C).

Simulating the patient's PBMCs isolation, freezing and thawing protocols were important to confirm the functionality of stored alloantigeneic T cells. After performing first and second MLRs some aliquots of sensitized responder cells after first MLR were frozen, thawed and rested before initiating second MLR. These frozen, thawed and rested cells showed significant IC IFN-γ as the fresh cells who were not submitted to freezing thawing processes (Fig. 40). This reflected the ability of frozen alloantigeneic sensitized cells to secrete IFN-γ after thawing and resting before undergoing alloantigeneic stimulation process.

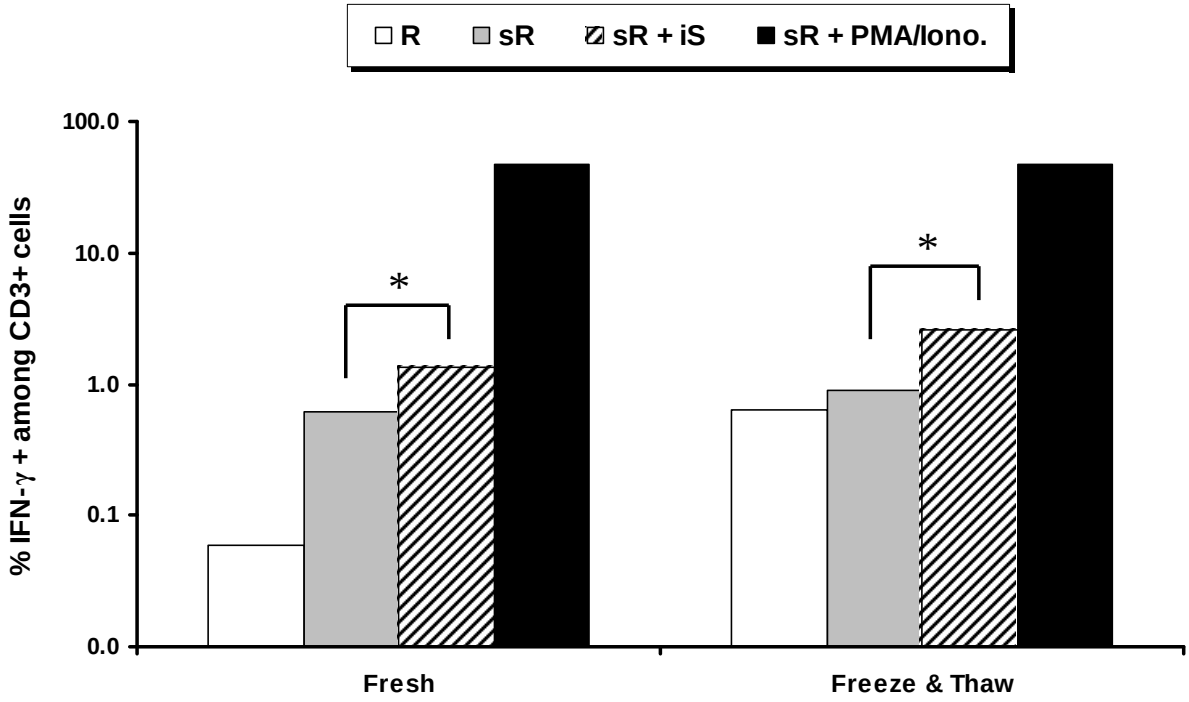

Figure 40 : Effect of freezing and thawing processes of sensitized cells on IC IFN-γ production. (*): Significant INF-γ production compared to sR (n=3) ($P<0.05$).

Other cytokines such as perforin, TNF-α, IL-4 and IL-10 were tested in the established MLR settings. In CD4+ (Th) and CD4- (Tc) T cell populations, IC TNF-α was not detected in significant amounts after 16h of second MLR (Fig. 41-a). Other cytokines such as perforin were not detectable in the CD4+ T cell population because this cytokine is well known to be CTL-restricted (Fig. 41-b). But on the other hand, perforin was produced in higher amounts in the CD4- fraction, but this was not significant. The well known Th_2 cytokine IL-4 was not detected significantly after stimulation of alloantigeneic sensitized responders cells even with PMA/ionomycin (Fig. 41-c). Finally, due to high background, IL-10 showed the same non-significant detectable amounts after stimulation in the second MLR (Fig. 41-d).

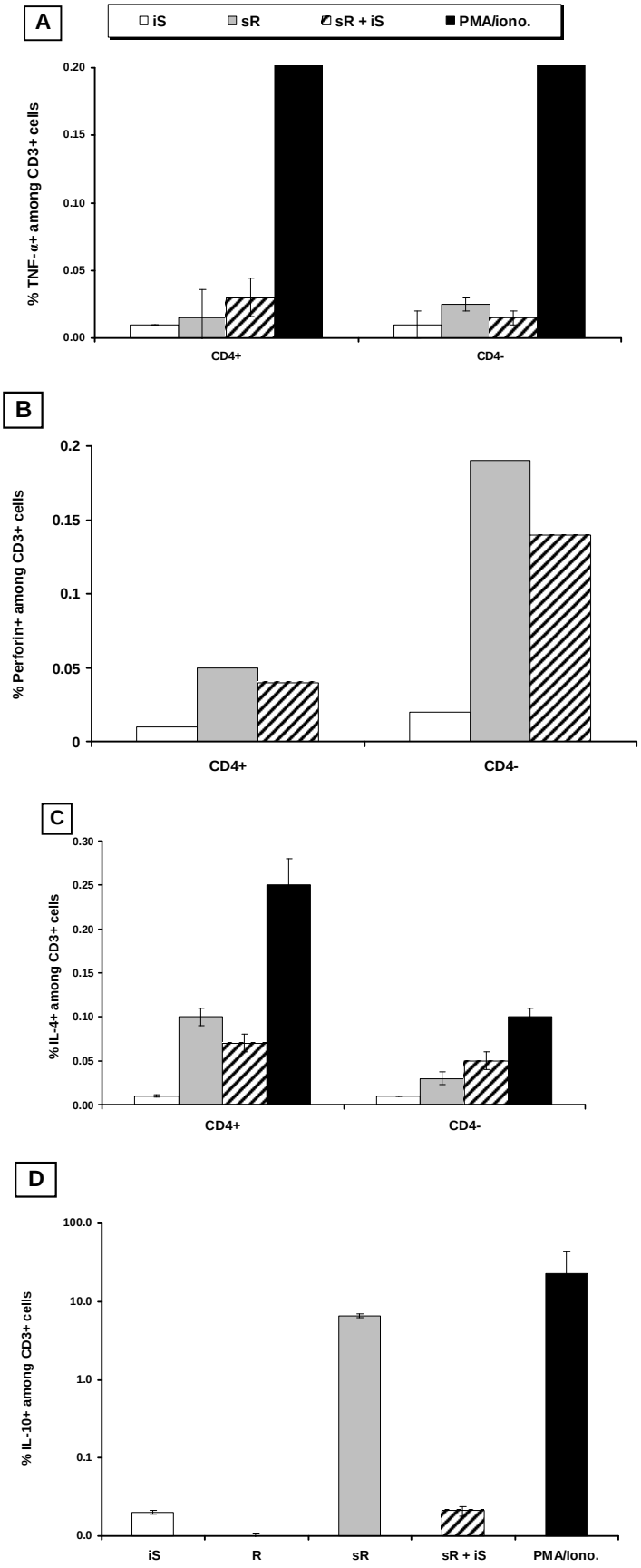

Figure 41 : IC TNF-α (A), perforin (B), IL-4 (C) and IL-10 (D) testing after second MLR (n=3 for all except Perforin n=1) .

The MLR is a clinically relevant *in vitro* assay where lymphocytes from one individual (responder) are incubated with the lymphocytes of another individual (stimulator) which have been previously rendered incapable of blast transformation by gamma-irradiation.[112] Using this concept classical MLR was used to monitor the PBMCs cell-division after allogeneic stimulus was given. In the first MLR settings alloantigeneic responder cells showed proliferation at the fifth day of co-culturing with irradiated stimulator cells (Fig. 42). The detected proliferation process indicated the occurrence of blast transformation at the responder cells. At the second MLR, alloantigeneic sensitized responder cells were able to proliferate in response to the same stimulator cells used in the first MLR. This proliferation was also detected significantly in the second MLR after 24h of co-culturing as well as 48h. But the capability of the alloantigeneic responder cells to proliferate declined after 48h of starting the second MLR, but it was sill significant compared to the non-sensitized responder cells.

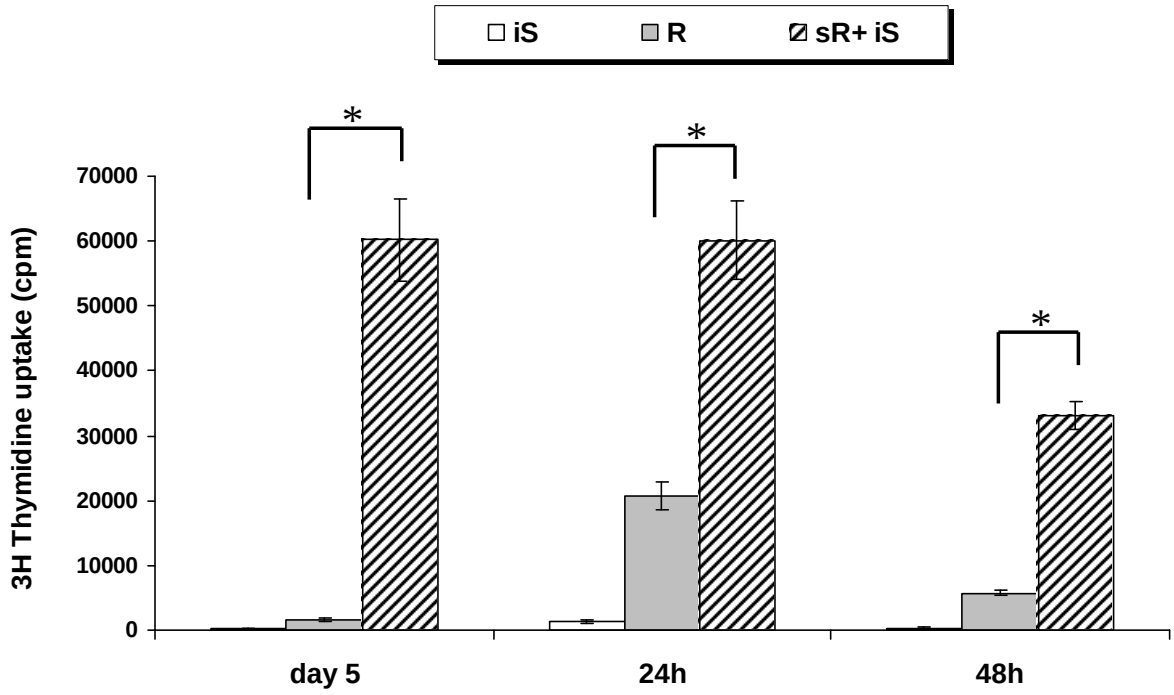

Figure 42 : Classical MLR for the fifth day at the first MLR and after 24h and 48h at the second MLR. iS: irradiated stimulators, R responders and sR: sensitized responders. (*): Significant cell proliferation compared to R ($P<0.05$).

3.4.1.2 IC flow cytometry can not detect alloreactive T cell in GvHD patients

After establishing the MLR settings to detect alloantigeneic sensitised T cells, PBMCs samples from patients who had undergone allogeneic SCT were chosen to be tested for any reactive T cells. The PBMC samples of the patients after SCT (AT) were tested for any reactivity against the patients' PBMCs before transplantation (BT), and as a negative control these cells were also tested against the donor (D) cells. But treatment with PMA/ionomycin was included as positive control to check the viability and capability of producing IFN-γ. The criteria for the selected patients are shown in table 14.

				GvHD		
Pt.	**Diagnosis**	**Immunosuppression**	**Days after Tx**	**Type**	**Stage**	**Tissue**
40	**AML**	**++**	**+32**	**a**	**III**	**skin, gut**
41	**MDS**	**++**	**+42**	**a**	**II**	**skin**
42	**T-NHL**	**+**	**+48**	**-**	**-**	**-**
43	**AML**	**++**	**+38**	**a**	**III-IV**	**skin, gut, liver**
44	**MDS**	**+**	**+36**	**-**	**-**	**-**
45	**MM**	**-**	**+41**	**-**	**-**	**-**
46a	**AML**	**++**	**+34**	**a**	**I**	**skin**
46b		**++**	**+79**	**a**	**III**	**skin, gut**
46c		**+**	**+102**	**c**	**extensive**	**skin, liver**
46d		**+**	**+365**	**c**	**extensive**	**skin, gut, liver**
47a	**ALL**	**+**	**+141**	**c**	**extensive**	**skin**
47b		**++**	**+192**	**c**	**limited**	**skin**
47c		**+**	**+365**	**c**	**extensive**	**skin**
48	**Myeloma**	**+**	**+46**	**a**	**II**	**skin, gut**

Table 14 : Criteria of selected patients who tested for GvHD. AML: acute lymphocytic leukemia, MDS: myelodysplastic syndrome, NHL: non-Hodgkin's lymphomas, MM: multiple myeloma, ALL: acute lymphoblastic leukemia, a: acute, c: chronic.

For detecting anti-patient allogeneic primed T cells, IC IFN-γ was measured after 16h of MLR setting in the presence of GolgiStop as a secretion inhibitor. In this part, the responder cells (AT) or irradiated stimulators (BT or D) were labeled with PKH_{26} then gated in or out, respectively. Either IC IFN-γ or IL-4 was detected only in responder cells but not in stimulator cells as background. With few exceptions, all patients PBMCs were able to produce IFN-γ in response to PMA/Iono. confirming the viability and functionality of all the responder PBMCs after thawing (Fig. 43). By tracking the CD4- responder cells, which are

mainly the CTLs, significant amounts of IC IFN-γ were not detectable in all of the patient cells when the responder cells (AT) co-cultured with either BT (i.e. allogeneic) or D (autologous) as stimulator cells (Fig. 43-a). Moreover, no significant amounts of IC IFN-γ was detected when the CD4+ responder cells - which contain all of the Th cells - co-cultured with the same mentioned stimulator cells (BT and D) (Fig. 43-b).

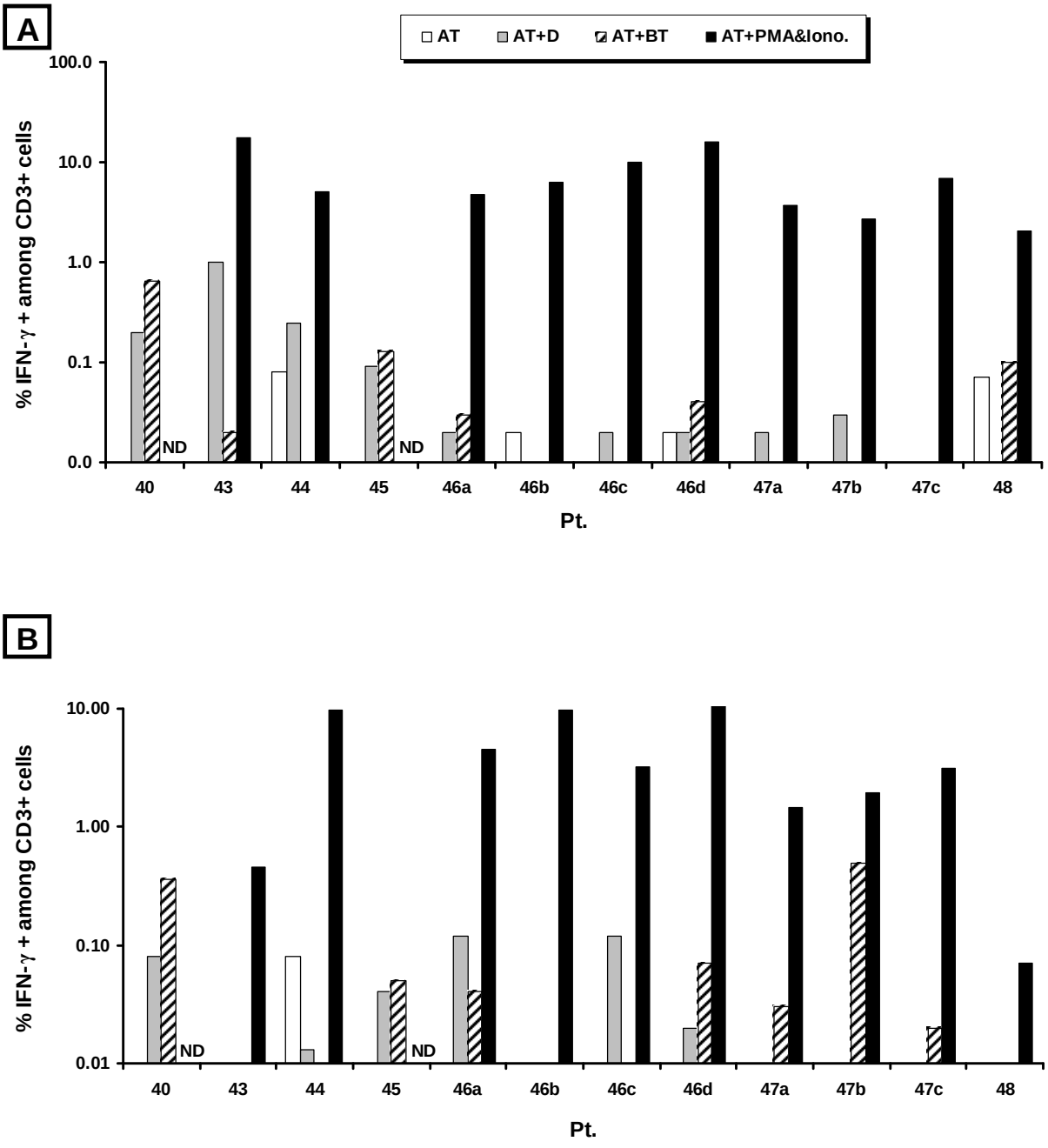

Figure 43 : Induced IC IFN-γ production after 16h of MLR. Both CD4- (A) and CD4+ (B) T cells were tested for IFN-γ production. ND: not determined.

As all of the monitored patients in figure 43 did not show any alloreactivity against donor cells, this was better confirmed by using donor cells and BT alone as background (Fig. 44). Finally, alloreactive T cells' IC IFN-γ was detectable significantly only in two patients' samples out of nine reflecting a sensitivity of 22%.

Other cytokines such as IL-4 were tested in parallel to IFN-γ in two patients' PBMCs samples. The patient cells after Tx (i.e. AT) did not show any IL-4 production after co-culturing with the patient cells before transplantation (i.e. BT) (Fig. 45). This reflected the absence of the Th_2 pathway in the examined patients' samples. But two facts should be considered when interpreting these results, firstly, IL-4 is not easily detected by the flow cytometry and SEB or PMA/ionomycin is not a good positive control to induce IL-4 production.

For longitudinal follow-up, PBMCs from two patients were sufficient to monitor IC IFN-γ production. The time points picked for Pt.46 were 34, 79, 102 and 365 days after transplantation, which covers the aGvHD as well as the cGvHD periods (Fig. 46-a). On the other hand, Pt.47 time points were 141, 192 and 365days after transplantation which covers only the cGvHD period (Fig. 46-b). No significant IFN-γ production in the MLRs of both patients was detected after co-culturing of the AT cells with BT cells in the presence of brefeldin A for 16h.

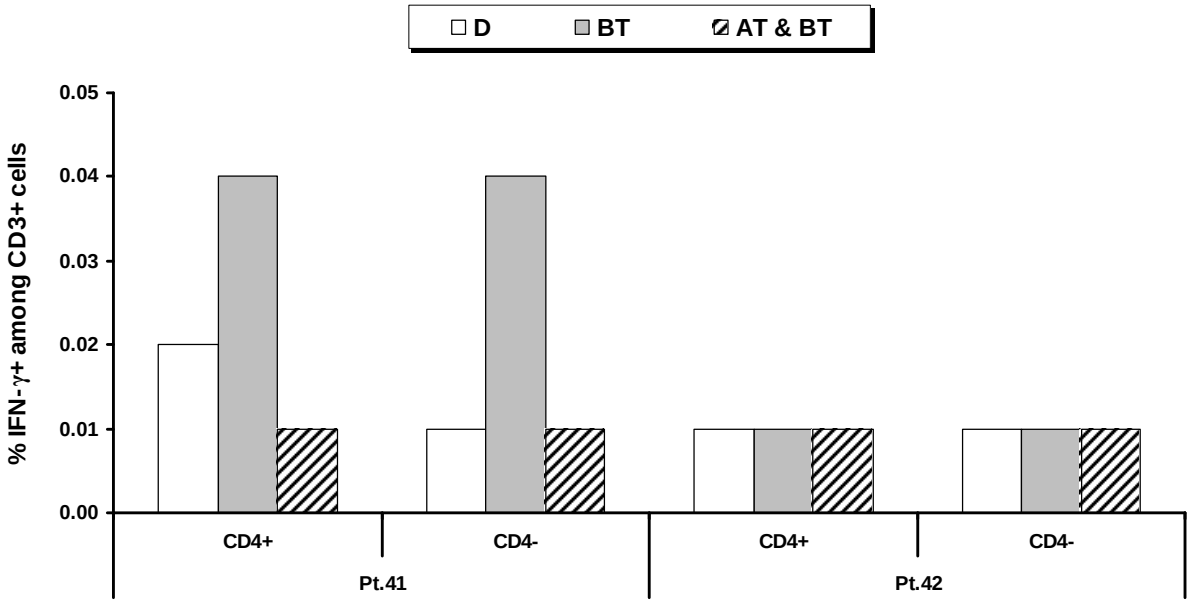

Figure 44 : Induced IC IFN-γ production after 16h of MLR in two patients.

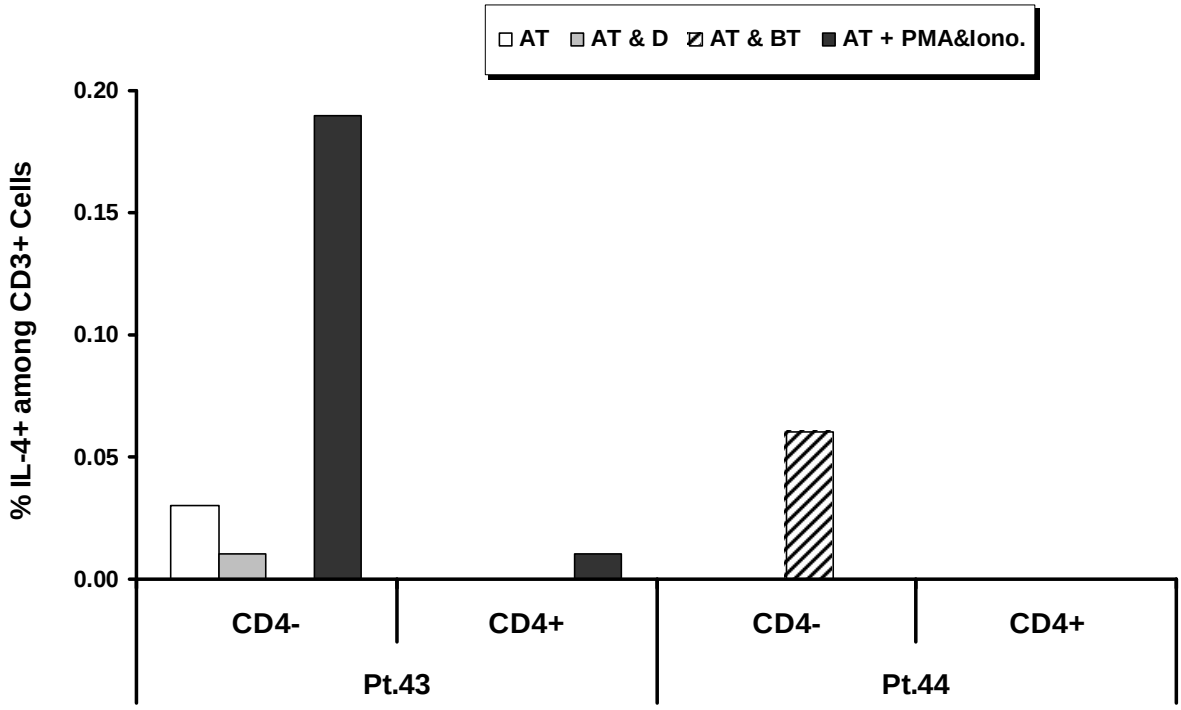

Figure 45 : Induced IC IL-4 production after 16h of patients MLR.

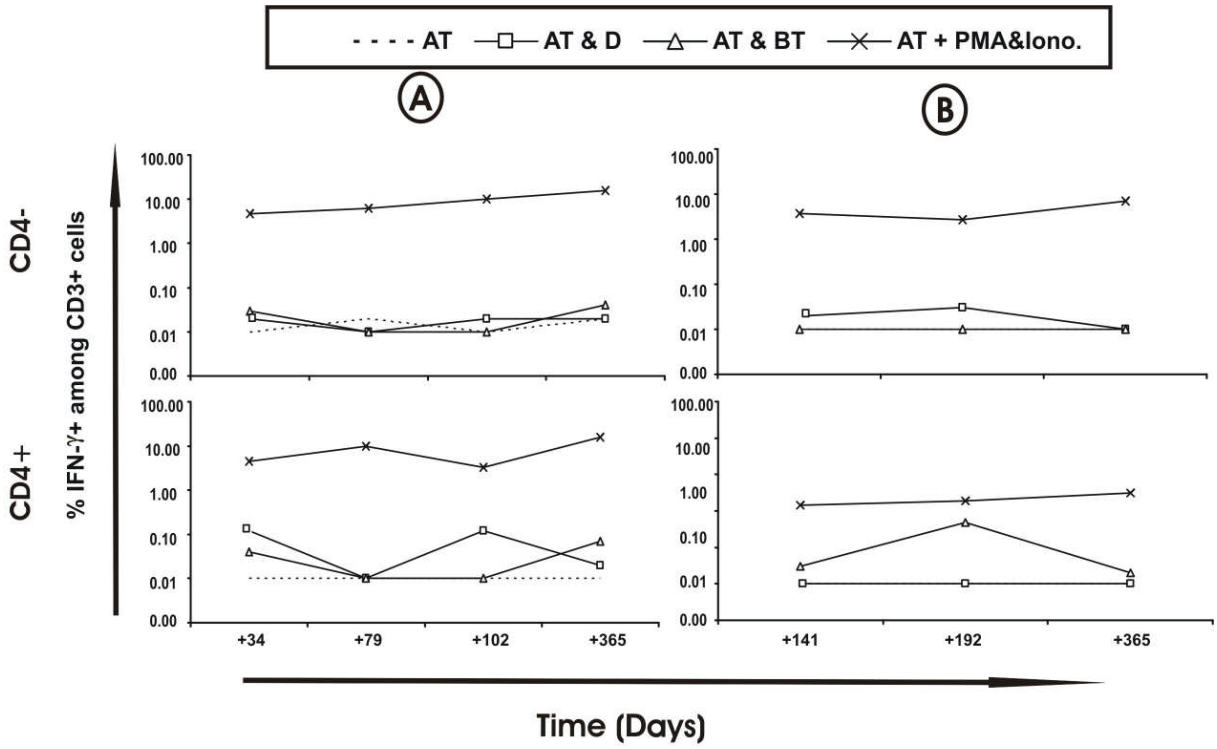

Figure 46 : Longitudinal follow-up of IC IFN-γ by flow cytometer in two patient' PBMCs. Pt.46 (A) and Pt.47 (B) samples were tested for reactivity after incubation with BT cells.

3.4.2 RT-PCR can detect IFN-γ mRNA in the healthy MLR settings

To know if the RT-PCR assay can detect sensitized T cells in the patients suffering from GvHD, the established MLR settings were applied and IFN-γ mRNA expression was monitored by the RT-PCR assay. After 3h of co-culturing of alloantigeneic sensitized responder cells with irradiated stimulator cells, IFN-γ mRNA was up-regulated significantly in comparison to the sensitized responder cells alone (Fig. 47). As this was reproducible in three healthy MLR settings, so the RT-PCR assay was confirmed to be eligible for monitoring GvHD patient samples.

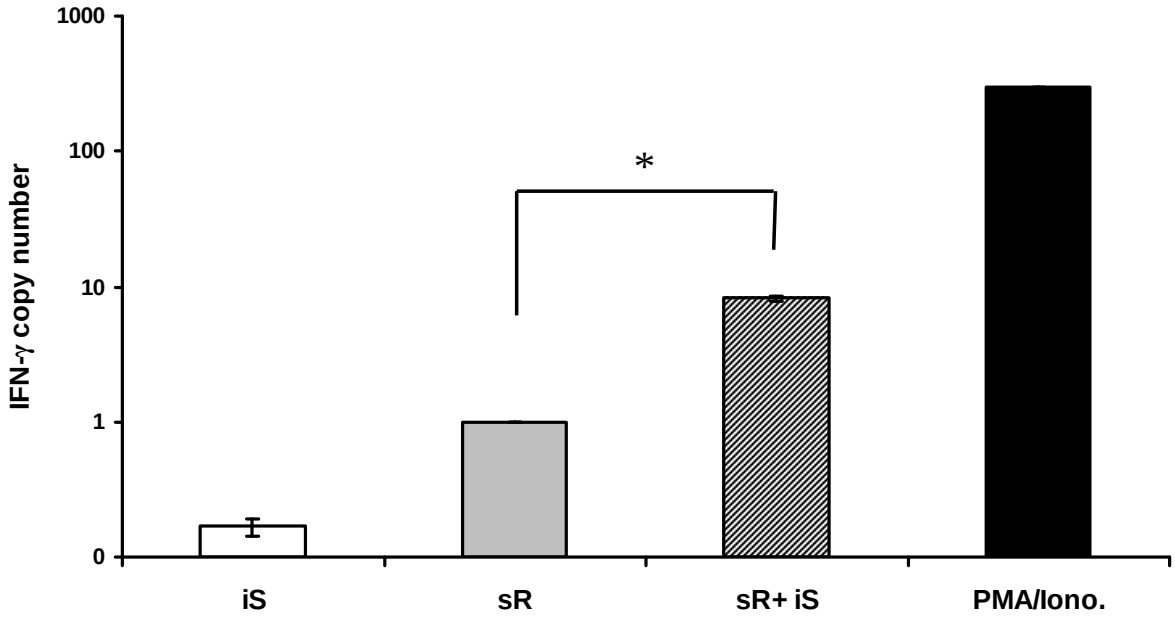

Figure 47 : Detection of IFN-γ mRNA by the RT-PCR assay after 3h of second MLR. (*): Significant IFN-γ mRNA up-regulation compared to sR ($P<0.05$).

3.4.3 RT-PCR can assess alloreactive T cells in GvHD patients

Twelve patient samples were tested by the RT-PCR for detecting alloreactive T cells. Significant up-regulation of the IFN-γ gene expression was detected in four samples reflecting the presence of cell activation. The up-regulated IFN-γ mRNA was assessed after co-culturing the patients' PBMCs after transplantation with PBMCs before transplantation (i.e. allogeneic). This was not the case in comparison to donors' PBMCs (i.e. autologous) (Fig. 48). Due to the clinically identified aGvHD (e.g. Pt.41) and cGvHD (Pt.46) in the patients at the time of PBMCs donation, this confirms that the IFN-γ was produced from alloreactive T cells. Although the alloreactive T cells in Pt.46 were not able to produce detectable IFN-γ

mRNA at early time point (i.e. +34 days) after transplantation, but it started that lately (i.e. 102 days) and kept the IFN-γ production even after one year of transplantation. This reflects that the alloreactive T cells were more primed in the patient body. Although Pt.44 PBMCs up-regulated IFN-γ mRNA without diagnosed GvHD, this patient had complicated medical state which should be considered.

Finally, it is seems clearly that the RT-PCR assay is more sensitive and promising than the ICC flow cytometry for detecting the primed alloreactive T cells in the allogeneic transplanted patients who suffering GvHD. But this assay can be further implemented in predicting the alloreactivity in the patients after allogeneic SCT.

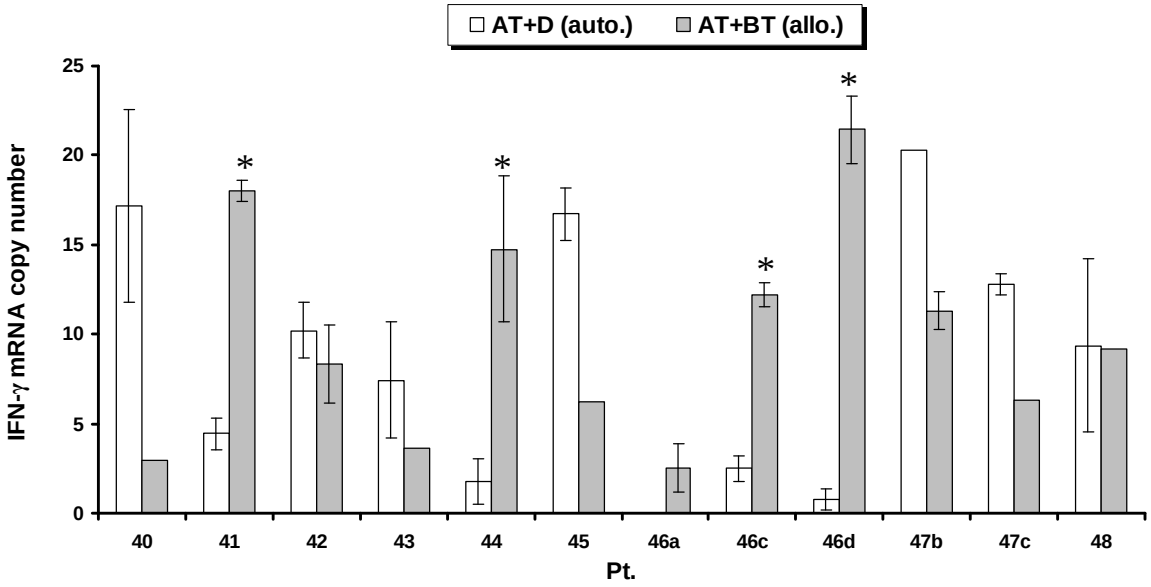

Figure 48 : Detection of IFN-γ mRNA by the RT-PCR assay after 3h. (*): Significant IFN-γ mRNA up-regulation compared to AT+D ($P<0.05$).

3.5 Impact of clinical parameters on T cell functionality

It should be kept in mind that the tested allogeneic transplanted patients may have some complications after their SCT (e.g. GvHD) and/or might be subjected to immunosuppressive drug treatments (e.g. steroids, cyclosporine A). Therefore the immunocompromised cells may have functional impairment. Also, the GvHD may induce many inflammatory pathways which may results in general pan T-cell activation. So the clinical data of the patients including the GvHD and/ or the immunosuppression state should be considered in the interpretation of obtained results. All of the tested patients' immunosuppression states were

classified in three groups namely; negative (-), weak (+) and strong (++), according to the dose of immunosuppressive drugs they were taking within two weeks prior to blood donation. In parallel to the patients' immunosuppression states, the GvHD type, stage and the affected tissue were documented.

In the CMV settings, many patients have detectable and functional CMV-reactive T cells even if they were under immunosuppressive treatments and/or had GvHD (table 15). For example, Pt.12d shown detectable CMV-reactive T cells by almost all of the monitoring assays even though he was under strong immunosuppressive drug treatments and limited cGvHD. On the other hand, no CMV reactivity was detected in Pt.15a under immunosuppressive drug treatments and extensive cGvHD. So, although some of the patients who were tested for CMV reactivity were suffering GvHD and/ or under immunosuppressive treatment at the time of testing, significant functional CMV-reactive T cells were detectable after activation with pp65 peptide and/or pp65 protein as CMV Ags. In summary, the data suggest that the patients' GvHD and/or immunosuppressive states were not limiting factors for detecting the CMV-reactive T cells after allogeneic SCT.

In the WT1 settings, all of the patients' samples showing detectable WT1-reactive T cells, were not under immunosuppressive treatments (table 16). Also, except for the Pt.5c who was suffering from limited cGvHD, all of them were not suffering from any GvHD at the time of blood donation. Among all of the tested samples, only two patients were under immunosuppressive treatments one of them was HLA-A2+ (Pt.11) and the other was HLA-A2- (Pt.18). Similar to the WT1 results, the HY-reactive T cells were detectable in Pt.17a who was neither under immunosuppressive treatments nor having GvHD at the time of testing (table 17). The rest of patients in the HY monitoring part did not show any detectable HY-reactive T cells whatever the states of GvHD and/or immunosuppressive medicaments were. This observations may indicate that weaker T cells responses may be suppressed in patients with more sever immunosuppression or immunodeviation due to GvHD.

The same clinical data were followed in the patients who were tested for detecting the alloreactive T cells. As expected, most of the tested patients had GvHD and were under immunosuppressive drug treatments to resolve their GvHD symptoms. Although all of the patients were taking immunosuppressant medications, the functional alloreactive T cells were detectable by the RT-PCR assay (table 18). For example, in Pt.46b who was suffering stage III aGvHD, significant up-regulation of IFN-γ mRNA was detectable by the RT-PCR even the

patient was under strong immunosuppressive drug treatments. Finally, the immunosuppressive state was not a limiting factor for detecting the alloreactive T cells.

		GvHD			pp65-Peptide Results				pp65-Protein Results		
Pt.	Immuno-suppression	Type	Stage	Tissue	TM	ICC	ELISPOT	RT-PCR	ICC	ELISPOT	RT-PCR
1a	-	-	-	-	-	-	-	-	+	+	+
1b	-	-	-	-	-	-	-	-	+	+	+
2a	-	-	-	-	-	-	-	-	+	+	+
2b	-	-	-	-	-	-	-	-	+	+	+
2c	-	-	-	-	+	-	-	+	+	+	+
3a	-	-	-	-	+	+	+	+	+	+	+
3b	-	-	-	-	+	+	+	+	+	+	+
5a	-	-	-	-	-	-	ND	ND	ND	ND	ND
5b	-	-	-	-	-	-	-	-	+	+	+
5d	-	c	limited	skin	+	+	-	+	+	+	+
6a	-	-	-	-	-	-	ND	-	ND	ND	ND
7a	-	-	-	-	-	-	ND	-	ND	ND	ND
10a	-	c	limited	skin	+	+	+	+	+	+	+
11a	+	c	extensive	lung	+	-	+	+	+	-	+
11b	+	c	extensive	lung	+	-	+	+	+	+	+
11c	+	c	extensive	lung	+	-	+	+	+	+	+
12a	+	-	-	-	ND	+	ND	+	ND	ND	ND
12b	+	-	-	-	+	+	+	+	+	-	+
12c	+	-	-	-	+	+	+	+	+	-	+
12d	++	c	limited	skin	+	+	+	+	+	-	+
14c	-	-	-	-	ND	-	ND	+	ND	ND	ND
15a	+	c	extensive	lung	-	-	-	-	-	-	-
15b	+	c	extensive	lung	-	-	-	-	-	-	-
16a	++	a	I	skin	+	+	ND	+	ND	ND	ND
22a	-	-	-	-	-	-	-	-	-	-	+
23a	+	-	-	-	ND	-	ND	-	-	ND	+
23b	+	-	-	-	ND	-	ND	-	-	ND	+
24a	-	a	-	gut	-	-	-	-	-	-	-
25a	+	-	-	-	-	-	ND	-	+	ND	+
26a	-	-	-	-	-	-	ND	-	-	ND	+
27a	++	a	II	liver	+	+	+	+	+	-	+

Table 15 : Clinical data for the tested patients at the time of CMV-reactivity testing. (+): positive, (++): strong positive, (-): negative and (ND): not determined.

		GvHD			WT1-WH187		WT1-Db126	
Pt.	**Immunosuppression**	**Type**	**Stage**	**Tissue**	**ELISPOT**	**RT-PCR**	**ELISPOT**	**RT-PCR**
1b	-	-	-	-	-	-	-	-
2c	-	-	-	-	-	-	-	-
3b	-	-	-	-	-	-	-	-
4a	-	-	-	-	-	-	ND	ND
4b	-	-	-	-	-	-	ND	ND
4c	-	-	-	-	-	-	ND	ND
4d	-	-	-	-	-	-	ND	ND
4e	-	-	-	-	-	-	ND	ND
5b	-	-	-	-	-	+	-	+
5c	-	c	limited	skin	-	-	-	+
8a	-	-	-	-	-	-	-	+
8b	-	-	-	-	-	+	-	+
8d	-	-	-	-	-	+	-	+
11a	+	c	extensive	lung	-	-	-	-
11b	+	c	extensive	lung	-	-	-	-
11c	+	c	extensive	lung	-	-	-	-
14a	-	-	-	-	-	-	-	-
14b	-	-	-	-	-	-	-	-
18	++	c	extensive	liver	-	-	-	-
19	-	-	-	-	-	-	-	-
24	-	a	I	gut	-	-	-	-
26	-	-	-	-	-	-	-	-

Table 16 : Clinical data for the tested patients at the time of WT1-reactivity testing. (+): positive, (++): strong positive, (-): negative and (ND): not determined.

		GvHD			Results	
Pt.	**Immunosuppression**	**Type**	**Stage**	**Tissue**	**ELISPOT**	**RT-PCR**
4a	-	-	-	-	-	-
4b	-	-	-	-	-	-
4c	-	-	-	-	-	-
4d	-	-	-	-	-	-
4e	-	-	-	-	-	-
8a	+	-	-	-	-	-
8b	+	-	-	-	-	-
8c	-	-	-	-	-	-
8d	-	-	-	-	-	-
9	+	-	-	-	-	-
13a	+	**c**	**limited**	**skin**	-	-
13c	+	**c**	**limited**	**skin**	-	-
13d	+	**c**	**limited**	**skin**	-	-
13e	+	**c**	**limited**	**skin**	-	-
13f	+	**c**	**limited**	**skin**	-	-
13g	+	**c**	**limited**	**skin**	-	-
14a	-	-	-	-	**ND**	-
14b	-	-	-	-	-	-
17a	-	-	-	-	-	+
17b	-	-	-	-	-	-
18	++	**c**	**extensive**	**liver**	**ND**	-
19	-	-	-	-	-	-
20a	-	-	-	-	-	-
20b	-	-	-	-	-	-
21a	+	-	-	-	-	-
21b	+	-	-	-	-	-
21c	++	**c**	**limited**	**skin**	-	-
21d	+	**c**	**limited**	**skin**	-	-

Table 17 : Clinical data for the tested patients at the time of HY-reactivity testing. (+): positive, (++): strong positive, (-): negative and (ND): not determined.

		GvHD			Results	
Pt.	**Immunosuppression**	**Type**	**Stage**	**Tissue**	**ICC**	**RT-PCR**
40	++	**a**	**III**	**skin, gut**	-	-
41	++	**a**	**II**	**skin**	-	+
42	+	-	-	-	-	-
43	++	**a**	**III-IV**	**skin, gut, liver**	-	-
44	+	-	-	-	-	+
45	-	-	-	-	-	-
46a	++	**a**	**I**	**skin**	-	-
46b	++	**a**	**III**	**skin, gut**	-	+
46c	+	**c**	**extensive**	**skin, liver**	-	+
46d	+	**c**	**extensive**	**skin, gut, liver**	-	-
47a	+	**c**	**extensive**	**skin**	-	-
47b	++	**c**	**limited**	**skin**	-	-
47c	+	**c**	**extensive**	**skin**	-	-
48	+	**a**	**II**	**skin, gut**	-	-

Table 18 : Clinical data for the tested patients at the time of alloreactivity testing. (+): positive, (++): strong positive, (-): negative and (ND): not determined.

4. Discussion

4.1 Monitoring of CMV-reactive T cells

After SCT engraftment, active CMV infection occurs in approximately 60–70% of CMV-seropositive patients or CMV-seronegative patients who receive transplants from a seropositive donor - even if no preventive measures are taken - the risk of developing CMV disease is 20–30%.[18] In some patients given allografts, recovery of CMV-specific cytotoxic T lymphocytes (CTLs) was rapid and reached up to 21% of all CD8+ T cells.[152] However, there are many techniques described to monitor CMV-specific T cells, such as cytokine secretion assay (CSA),[111] tetramer (TM) staining,[111,162] enzyme-linked immunospot (ELISPOT) assay,[22] mixed lymphocytes reaction (MLR),[146] cytotoxic assays,[111,146] and intracellular cytokine (ICC) flow cytometry.[111,138,146] But little is reported to monitor the reconstitution of CMV-specific T cells after allogeneic SCT on molecular basis.[145]

To assess the reconstitution of CMV-specific T cells in patients after SCT, representative patient-donor pairs were selected to be monitored. These patient-donor pairs covered all of the CMV-serostatus which expected to induce variable CMV-specific T cell reconstitution types.[163] Overall 31 blood samples from allogeneic transplanted patients covering 18 patients with two HLA-A2 alleles were tested in this study. Well known methods such as TM staining , ICC flow cytometry and ELISPOT assay were used in addition to a new established RT-PCR assay to monitor the reconstitution of CMV-reactive T cells in the allogeneic transplanted patients' peripheral blood samples.

As there are many TM complexes for detecting CMV-specific T cells,[164,165] the HLA-A2-restricted TM was selected to monitor the CMV-specific CTLs which can recognize the immunodominant $pp56_{495\text{-}503}$ peptide epitope. The TM staining showed nicely separated viable CTLs that can recognize the HLA-A2-restricted peptide. Moreover, the TM technology could detect eight HLA-A2 positive patients, among eleven, who had CMV-specific CTLs at least in one of the tested time points. This indicates that TM staining can detect the CMV-specific T cells in allogeneic transplanted patients' peripheral blood efficiently. Taking in consideration that the TM staining detected these T cells as early as 40 days following transplantation, this reflects the ability of this technique to detect early reconstitution of CMV-specific CTLs. But unfortunately no earlier time points were available to monitor the initiation of reconstitution for these CMV-specific CTLs. Interestingly, there is increasing

evidence that also functionally defect CMV-specific CTLs can be detected by TM staining, thus it is essential to perform additional functional assays (e.g. ICC, ELISPOT and RT-PCR assays) to confirm functionality of the TM detected T cells.[147]

The detection of specific IFN-γ-producing T cells by flow cytometry usually requires high T cell frequencies (minimum 0.02% specific T cells in the CD3+CD8+ T-cell subpopulation, corresponding to approximately 40 specific T cells per one million PBMC).[125] In this study, the ICC staining detected eight HLA-A2 positive patients, among twelve, who have CMV-specific T cells at least in one of the tested time points. This indicates that ICC staining can detect the CMV-specific T cells in allogeneic transplanted patients' peripheral blood efficiently. Taking the advantage of phenotyping CMV-reactive T cells by ICC, it was clear that these cells were CD8+ T cells reflecting the CTLs phenotype. But in some patients CD8- T cells (e.g. helper T lymphocyte (Th)) showed a capability to secrete IFN-γ when stimulated with the recombinant pp65 protein. Taking all together, the detected CMV-specific T cells were found to be a combination of a majority of CTLs and a minority of Ths. This may be due to the presence of CD4+ Th cells in 10- to 100-fold less than CD8+ Tc cells.[107] Although CTLs were detectable by activation with pp65 peptide and/or recombinant protein, Ths were only detectable when the pp65 protein was used. These results which are in concordance with others,[138] reflect the specificity of the used peptide to the HLA-A2 allele, and the presence of other CMV-specific T cells in the patients' peripheral blood that can not be detected by the TM staining such as the Ths. Furthermore, the use of pp65 protein was found to be more efficient than pp65 peptide as re-stimulating Ag for the CMV-specific T cells. In addition to the IFN-γ assessment, the cytotoxic functional perforin was assessed in the reconstituted CMV-specific T cells. Significant IC perforin was detectable in some patients' samples, indicating that the detected CMV-specific T cells were really functional CTLs that have cytotoxic abilities in addition to the type 1 cytokines pathway (e.g. IFN-γ).

In the longitudinal follow-up, many forms of CMV responses were seen using the pp65 peptide and/or protein as stimulus. In some experiments, the initiation of reconstitution for CMV-specific T cells was detectable using pp65 protein but not the peptide which was detected later. These results indicate that the CMV-reactivity can be monitored by IC flow cytometry using both the pp65 peptide and protein, but the later can detect the CMV-specific T cells earlier. This may be interpreted as that the pp65 protein contains many antigenic epitopes that can induce the generation of many CMV-specific T-cell clones, which are not only HLA-A2-specific.[138] In order to enhance the CMV Ags presentation, the C1R-A2 cell

lines were loaded with pp65 peptide then co-cultured with the PBMCs that contain the CMV-specific T cells. This model showed enhancement of detection for IC IFN-γ by flow cytometer, which not have been published before. This was in agreement with Arlen *et al*. concerning the enhancing the ELISPOT assays by using the same concept.(148)

In general, in IC flow cytometry it is possible to routinely identify positive populations on the order of 0.1% or even slightly less. But populations significantly lower, on the order of 0.01%, will not be distinguishable from background, because they reach the average level of spontaneous cytokine-secreting cells in peripheral blood.(124) On the other hand, ELISPOT assay is the "*ex vivo*" assay with the lowest limit of detection of IFN-γ-producing T cells, allowing the reliable detection of as few as 1-10 specific T-cells per one million PBMC.(125,166,166) To further define the CMV-specific T cells a sensitive ELISPOT assay was adopted.(17) In this study, ELISPOT assay detected, at least in one of the tested time points, fifteen HLA-A2 positive patients' samples among nineteen covering seven out of ten patients who have CMV-specific T cells. This indicates that the ELISPOT assay can monitor the reconstitution of CMV-specific T cells in allogeneic transplanted patients' peripheral blood efficiently. Again, pp65 peptide and protein induced different forms of IFN-γ secretion. With more focusing, IFN-γ-spots were generated in response to either pp56 peptide or protein, but some times they were for both CMV Ags. In view of these results, the reconstituted cells were found to compose from different CMV-specific T cell clones that can recognize different epitopes from the pp65 protein. These cells expected to be a mix of different memory T cells that can respond fast when counteracting with their Ag for the second time.(167) Although the ELISPOT assay can monitor the reconstituted CMV-specific T cells in high sensitivity, it has many disadvantages such as the time consuming protocols (i.e. 4 days) and the incapability of phenotyping the CMV-specific effector cells.(167) Moreover the main disadvantage is that, even with the aid of specialized imaging equipment, there is a certain degree of subjectivity in the interpretation of results as a threshold for the size, intensity, and gradient of the spots are user-defined.(127)

As the sensitivities of ICC and ELISPOT assays are dependent on the background from T cells spontaneously producing cytokines, various procedures help to reduce background staining.(125) To get rid of background effect in the ELISPOT assay, Hempel found that the immune response measured in the peripheral blood of mice by RT-PCR and ELISPOT showed a significant correlation to the response measured in the spleen (P=0.001).(155) In addition to the new introduction of RT-PCR assays as monitoring assays for Ag-specific T

cells and due to their high sensitivity and reproducibility,[127] this approach was adopted. Thus, the following hypothesis was proposed: can the RT-PCR assay detect patients' peripheral blood CMV-specific T cells with correlation to the other adopted monitoring assays? At least in one of the tested time points, the RT-PCR assay detected nineteen HLA-A2 positive patient samples among twenty one, covering ten out of twelve patients, who have CMV-specific T cells. Simply these data indicate that RT-PCR assay can efficiently monitor the reconstitution of CMV-specific T cells in the peripheral blood of allogeneic transplanted patients following SCT. With few exceptions, significant up-regulation of IFN-γ mRNA expression was noticed in the entire patient samples treated with pp65 protein. On the other hand, the up-regulation of IFN-γ gene expression was induced in some samples when re-stimulated with pp65 peptide. Even it was noticed that the pp65 peptide always induced cell-activation in parallel to pp65 protein, but not the other way around. These results indicate that the RT-PCR assay can monitor the CMV-specific T cells even in lower frequencies than detected by TM, ICC and ELISPOT.

In the longitudinal time follow-up study, the RT-PCR assay was able to monitor the already found or newly initiated CMV-reactivity. In addition, the RT-PCR assay was able to detect the reconstitution of CMV-specific T cells as early as forty days after SCT. Taking in consideration the results of the two CMV Ags, more detection sensitivity was shown by the use of pp65 protein rather than the peptide. In view of these results, the different reconstituted CMV-specific T cell clones composing all of the different pp65 protein epitopes were detectable by this very sensitive RT-PCR assay. In addition to the ability of RT-PCR assay to monitor the reconstituted CMV-specific T cells in high sensitivity, also it has many other advantages such as the short stimulation time (i.e. 3 hours). But unfortunately, the RT-PCR detected CMV-specific effector cells can not be isolated or phenotyped.[127]

Interestingly, the CMV-reactive T cells were detectable by ICC flow cytometry, ELISPOT, and RT-PCR assays but not TM staining in the control group patients who do not have the HLA-A2 allele to present the HLA-A2-restrected pp65 peptide. This was performed by the use of pp65 protein as CMV stimulus that can be processed by the patients' antigen presenting cells (APCs). After the pp65 protein processing in the exogenous pathway, all of the pp65 protein immunodominant epitopes presented to all of the CMV-specific T cells found in the PBMCs mixture. This gave the ability to detect the non-HLA-A2-restricted CMV-reactive T cells. Unfortunately this is not applicable by the TM staining since the

multimers technology depends on the pre-selection of the HLA-restricted peptides to be loaded into the TM-peptide complexes.[118,120]

The sensitivity of the adopted detection assays is very important to monitor the Ag-specific T cells in the peripheral blood of the allogeneic transplanted patients. As four techniques were adopted to monitor the reconstitution of CMV-specific T cells, the sensitivity for each technique was determined. The previous described high sensitivity for the TM staining[137] was reproduced in this study when the pp65 peptide was used as an Ag. Moreover, the same high sensitivity was found in the RT-PCR assay, in contrary to the ICC staining which showed the lowest sensitivity compared to TM staining and RT-PCR assay. This confirms that established functional RT-PCR assay has the same high sensitivity of the phenotypic TM staining. On the other hand, all of the techniques did not detect any pp65 peptide reactivity in the HLA-A2 negative patients' samples, confirming the specificity of the techniques in addition to the HLA-A2-restriction for the used peptide. Assessment of the CMV reactivity using the pp65 protein increased the sensitivities in ICC flow cytometry and RT-PCR assay, reflecting better monitoring ability using the pp65 protein rather than the HLA-A2-restricted peptide. Interestingly, the non-HLA-A2-restricted CMV-specific T cells could be monitored in the HLA-A2 negative patients by ICC staining, ELISPOT assay, and RT-PCR assay when the pp65 protein was used as stimulating Ag. Taking all together, the new established RT-PCR assay can monitor the reconstitution of CMV-specific T cells with high specificity and without HLA-restriction if the antigenic protein was used.

Normally when the RT-PCR assays newly introduced to test new target gene, comparisons should be performed in parallel to the already existing methods, and then the correlation should be determined.[168,169] Although the established RT-PCR assay has high sensitivity similar to that found in the other methods, another testing strategy was adopted by checking the correlation of the established RT-PCR assay with the other methods. In general, the RT-PCR assay was correlated significantly with the TM staining ($P=0.005$), which was not yet been published elsewhere. Even a higher correlation was found when the RT-PCR assay results were correlated to the ELISPOT and ICC ($P=0.0001$). Moreover, the Pearson correlation coefficient (R^2) was high when the RT-PCR assay was compared to ICC staining, reflecting good linearity between the two techniques. This was not the case when the RT-PCR assay was compared to the ELISPOT assay and TM staining. In view of these results, the higher correlation coefficient can be explained by the higher frequencies of CMV-specific T cells in the allogeneic transplanted patients following SCT.

In healthy CMV-seropositive individuals, up to 40% of all T cells in the peripheral blood can be specific for CMV,[170] underlining the importance of a strong CMV-specific cellular immunity in containing persistent CMV infection. As the CMV-serostatus of the donors influence the outcome of allogeneic SCT in some patient categories,[163] the results from the four monitoring techniques were correlated to the CMV-serostatus of the tested patients and their donors. In concordance with Cwynarski *et al.* who found that the CMV-reactive T cells can reach up to 21% of all CD8+ T cells,[152] the recovery of CMV-specific CTLs was rapid when both the patient and the donor were seropositive for CMV before SCT. In addition to these results, the tested pp65 protein enhanced the detection limit up to 100% in most of the tested patients. For the first time, with few exceptions, this could be achieved in both HLA-A2 positive and negative patients by ICC flow cytometry, ELISPOT and RT-PCR assays, but could not be applied to the TM staining. On the other hand, early reconstitution was not observed if either the donor or recipient was seronegative for CMV, especially when the patient was CMV-seronegative and his donor was CMV-seropositive. In the CMV-seropositive patients received transplant from CMV-seronegative donors displayed, delayed reconstitution of CMV-specific T cells were seen which have been documented elsewhere.[152] These results illustrate a novel concept concerning the use of the established RT-PCR assay as a monitoring tool for the reconstitution of CMV-specific T cells in patients at risk due their own or their donors CMV-serostatus.

As a summary these results concerning the monitoring of allogeneic transplanted patients, confirm the ability of monitoring CMV-reactive T cells by the classical methods (e.g. TM staining, ICC staining, and ELISPOT assay) as well as the new established RT-PCR assay. Moreover, in addition to the fast assessment, the RT-PCR showed a high sensitivity which correlates to the other known methods. Although functionally defective CMV-specific T cells can be detected by TM staining,[147] the RT-PCR assay has the advantage of detecting the functionality of the reconstituted CMV-specific T cells with a similar sensitivity to the TM staining. By demonstrating the high correlations between the RT-PCR and the classical methods, the detection of CMV-reactive T cells in low frequencies by RT-PCR assay is no more under doubt. Finally, the use of whole antigenic and immunodominant CMV pp65 protein gave a novel approach to monitor CMV-specific T cells in regardless of the patients HLA typing.

4.2 Monitoring of WT1-reactive T cells

Enrolling representative patient samples is also important for covering all of the possibilities of detecting WT1-reactive T cells in peripheral blood. Therefore, assessment of twenty two leukemic samples covering twelve patients, were eligible to be used. In addition, most of the well known blood malignancies that over-express the WT1 antigen were selected to be monitored including AML, ALL and CML.[140,142,171] Moreover, the patient samples covered a range of time between five months and five years and two months following allogeneic SCT. In this model all of the expected HY-reactive T cells should be in scope, so the other determinant for monitoring was the sensitivity of the testing techniques.

Most of the tools used to detect WT1-reactive T cells are TM staining,[171,172] immunohistochemistry,[172] ELISPOT assay,[157] ICC flow cytometry,[157,171] cytotoxicity assays,[158,173] and recently a molecular approach was established.[145] As Scheibenbogen *et al.* could detect WT1-specific T cells by high sensitive ELISPOT assay[157] this assay was adopted. Although the adopted ELISPOT assay reported to have high sensitivity, unfortunately it did not detect any WT1-reactive T cells in patient samples. To overcome the failure of ELISPOT assay to detect the WT1-specific T cells, a sensitive RT-PCR assay was in scope. The adopted assay was established by Rezvani *et al.* with high sensitivity to detect frequencies of 1 responding T cell / 100,000 T cells.[145] Some of the RT-PCR assay results were significant positive for IFN-γ mRNA gene expression indicating the presence of WT1-reactive T cells in the patients peripheral blood following allogeneic SCT. Taken together the detection of WT1-reactive T cells by the RT-PCR, but not the ELISPOT, confirms the higher sensitivity of the RT-PCR.

By using the highly sensitive ELISPOT assay, Scheibenbogen *et al.* did not detect any WH187-reactive T cells in all of the fifteen AML patients they tested, compared to two patient samples that secreted IFN-γ upon stimulation with WT1-Db126 peptide.[157] According to the present study, interestingly the non-detectable WT1-reactive T cells in the ELISPOT assay was in concordance with Scheibenbogen *et al.* who barely could detect these CTLs. In an effort to eradicate doubt concerning the ability of the ELISPOT assay to detect the WT1-reactive T cells, a significant up-regulation of IFN-γ mRNA upon stimulation with both peptides was detected using the RT-PCR assay. In line with these results, obvious evidence that the two HLA-A2-restricted WT1 peptides differ in their reactivity was observed, since the WH187 peptide reactivity was detected by RT-PCR in 17% of the eighteen patient samples compared to 28% in response to the Db126 peptide. According to

the present study WT1-Db126 peptide showed a higher immunogenicity than WT1-WH187. Consequently, the RT-PCR is good to be used as a monitoring tool for detecting the generation of GvL-specific T cells.

Differences in detecting IFN-γ mRNA after stimulation with the two WT1 HLA-A2-restricted peptides, may be interpreted due to differences in the immunogenicity of both peptides. In murine model, coincidentally Db126 peptide demonstrated the same order of binding affinity as that of viral Ags, which is the strongest Ags for CTLs induction. Moreover, the cytotoxic activity of Db126-specific CTLs showed to have half the maximal lysis in the range of nanomolar of the peptide.(173) Thus, only the Db126 peptide with the highest binding affinity for HLA-A2 molecules could elicit CTL responses. The same consideration should be in mind when viewing the vaccination trials against some leukemias which adopted the Db126 peptide as a vaccine in the HLA-A2 positive patients.(156,171) Even in a case study, vaccination with Db126 peptide induced complete remission in a patient with recurrent AML by in the absence of toxicity.(171) In view of these results, Db126 peptide should be considered to be the most promising target peptide to induce CTL responses against WT1 in HLA-A2 positive patients with malignant neoplasms.

In contrast to ELISPOT assay, which did not detect any WT1-reactive T cells, RT-PCR was able to detect significant increase of IFN-γ mRNA in response to WT1 peptides. Doubts were raised about the detection limit of ELISPOT assay concerning the following question: dose the ELISPOT assay has enough detection limit to detect WT1-specific T cells at patients peripheral blood samples? In general, the “limit of detection” of a method is the capacity of the method to detect small amounts of a substance with some assurance, and it is known that the limit of detection by ELISPOT was reported to be 10–200 times lower than ELISA performed on culture supernatants.(124) On the other hand, the detection of specific IFN-γ-producing T cells by ELISPOT assay is better than flow cytometry which is usually requires higher T cell frequencies (approximately 40 specific T cells per one million PBMC).[125] However, the ELISPOT assay sensitivity is depending on the spontaneous secretion of cytokines by T cells as a background. Although not so many researchers used the ELISPOT assay to monitor WT1-reactive T cells,(157) approximately the same results were obtained in comparison to this work. This may be due to the low frequencies of WT1-specific CTLs in the peripheral blood of the tested patients, or due to the presence of WT1-specific T cells recognizing other WT1 epitopes rather than the tested peptides.

In general, RT-PCR assays are usually used to diagnose the leukemias and to assess the WT1 expression in tumor's blasts,[156,158,174] but not monitoring the reconstituted WT1-reactive T cells. Recently the detection of WT1-reactive T cells by RT-PCR was established in purely selected CD8 positive T cells, but not in PBMCs.[145] Despite that the ELISPOT assay did not detect WT1-reactive T cells among all of the patient PBMC samples, RT-PCR demonstrated significant up-regulation of IFN-γ mRNA in response to WT1 peptides. In terms of adopting RT-PCR protocol with a high sensitivity and capability of detecting Ag-specific T cells at frequencies in the order of 1/100,000 CD8+ T cells,[145] so the RT-PCR results should be more reliable than the ELISPOT. In line with this high sensitivity, the significant up-regulation of IFN-γ mRNA after WT1 peptide pulsing confirming the presence of WT1-reactive T cells at the selected patients' peripheral blood. These results illustrate a novel concept of monitoring WT1-reactive T cells by RT-PCR assay even if the frequency is below the detection limit of the most powerful technique to detect Ag-specific T cells, which is the ELISPOT assay.

Although the presence of WT1-reactive T cells in the peripheral blood of allogeneic transplanted patients is important, the reconstitution time after transplantation is important too. In this study, the minimal time required to detect reconstituted WT1-reactive T cells was five months and fifteen days, but in another patient it was nine moths and thirteen days following transplantation. Unfortunately, no earlier blood samples were available for both patients to detect any earlier reconstitution of the GvL effector T cells. For both patients, the detection of WT1-reactive T cells by RT-PCR assay was in concordance with what have been found by others.[145] Interestingly and for the first time WT1-reactive T cells were detected by molecular approach in AML and ALL patients. These results can be further expanded to screen other groups of patients to monitor the reconstitution of WT1-reactive T cell during the first five months following allogeneic SCT.

These results indicate that the GvL effect can be monitored by RT-PCR but not ELISPOT assay. This is due to the higher sensitivity of the RT-PCR assay which can detect very low frequencies of Ag-specific T cells.[145] Also the present study clearly indicates a tight junction between the selected WT1 peptides and the up-regulation of IFN-γ mRNA as a final readout for the established RT-PCR assay. Concomitantly, WT1-Db126 peptide seems to have more immunogenicity compared to the WT1-WH187 peptide. Moreover in this model, the GvL effector T cells that can recognize the tumor cells having the WT1 protein can be monitored as early as five months following SCT, but earlier time follow-up is suggested.

4.3 Monitoring of HY-reactive T cells

Allogeneic stem cell donors can be incompatible at different levels. Even in the HLA-identical pairs it will be still incompatible for numerous minor histocompatibility antigens (mHAg). Nevertheless, some incompatibilities are found to be associated with an increased risk of graft-versus-host disease (GvHD), which could be related to the way the immune system recognizes these antigens. In HLA-identical bone marrow transplantation, GvHD may be induced by disparities in mHAgs between the donor and the recipient, with the antigen being present in the recipient and not in the donor. CTLs specific for mHags of the recipients can be isolated from the blood of recipients with GvHD.[97]

To enroll representative patient's samples to detect HY-reactive T cells in peripheral blood, all of the selected patients were male recipients who received SCT from female donors. In addition to the selected patients who were suffering AML, ALL and NHL before SCT, samples covered a range of time between five months up to five years and two months after allogeneic SCT. Assessments of twenty eight patient samples from ten patients were eligible to be used. In this model all of the expected HY-reactive T cells should be in scope, so the other determinant for monitoring will be the sensitivity of the testing assays. Although twenty five HLA-A2 positive patient samples were monitored, only one sample was positive for the HY-reactivity. This confirms the eligibility of the monitoring assays to detect HY-reactive T cells in the patient's peripheral blood.

There are many methods describing the detection of HY-reactive T cells such as CTL assays,[97] ^{3}H-thymidine incorporation assay,[160] cytotoxicity (51Cr release) assay,[160,100] TM staining[175] and IC-IFN-γ flow cytometry,[105] but no study described the detection of the human HY-reactive T cells using molecular technologies so far. Interestingly, James *et al.* and others could detect HY-specific T cells in mice using high sensitive ELISPOT assay.[161,176] Although high sensitive ELISPOT assay was adopted as monitoring tool, unfortunately no detection of HY-specific T cells was achieved in all the patients' samples. On the other hand, as the HY-specific T cells were documented to be detectable in mice by using the RT-PCR assay, the same concept was adopted to monitor the reconstitution of HY-specific T cells in allogeneic transplanted patients.[177] In contrast to the ELISPOT negative results, the adopted RT-PCR assay which showed previously a higher detection sensitivity, was able to detect the activated T cells after stimulation with the HY peptide. Concomitantly, the RT-PCR is good to be used as a monitoring tool for detecting the generation of mHAg-specific T cells.

In the GvHD models, coincidentally H2-D^k epitope[175] and FIDSYICQV SMCY-derived [100,105] HY peptides demonstrated the strongest mHAgs for CTLs induction in mice and human, respectively. In this model the RT-PCR assay positive results were specific to the HY peptide stimulation when compared to the low background IFN-γ mRNA expressed in response to the gp100 HLA-A2-restricted peptide. In general, this significant up-regulation of IFN-γ mRNA after stimulation with the HY HLA-A2-restricted peptide may be interpreted by the immunogenicity of HY peptide. By demonstrating detectable HY-reactive T cells in one patient among seven, these patient's PBMCs were able to produce IFN-γ after activation with the HY peptide which may reflect that the HY antigen promotes cell-mediated immunity rather than antibody production and humoral immunity.[50] In view of these results, HY peptide should be considered in monitoring of reconstitution of mHAgs-specific T cells.

In contrast to ELISPOT assay, which did not detect any HY-reactive T cells, RT-PCR was able to detect significant increase of IFN-γ mRNA in response to HY peptide. Again, doubt rises about the detection limit of ELISPOT assay and the following question appeared: can the ELISPOT assay detect HY-reactive T cells in patients' peripheral blood? To answer this question we should keep in mind that the ELISPOT detection limit was reported to be lower than ELISA and ICC, and the sensitivity of ELISPOT assay is depending on the background which is the spontaneous secretion of cytokines by T cells. Although no researchers used the ELISPOT assay to monitor human HY-reactive T cells, these ELISPOT results are believed to be realistic as this assay showed high sensitivity in detecting CMV-reactive T cells. The non-detectable HY-specific T cells by the ELISPOT assay may be interpreted by the low frequency of these CTLs in the peripheral blood of the tested patients, or due to the presence of HY-specific T cells recognizing other HY epitopes rather than the tested peptide.

Recently RT-PCR assays were established to detect ex vivo generated and expanded HY-reactive T cells in murine models.[175,177] Due to fact that no literature was found concerning the monitoring of reconstituted HY-reactive T cells in allogeneic transplanted patients' peripheral blood, so the RT-PCR assay was adopted. Despite that the ELISPOT assay did not detect any HY-reactive T cells among all of the tested patients' PBMC samples, RT-PCR demonstrated significant up-regulation of IFN-γ mRNA in response to the HY peptide in one patient. Moreover, this patient developed eosinophilia at the time of testing which is considered as an early marker of GvHD.[178,179] In terms of adopting the RT-PCR assay which has high sensitivity and capability to detect Ag-specific T cells at low frequencies, the RT-PCR assay results showed more reliability than the ELISPOT assay. In line with this high

sensitivity, the significant up-regulation of IFN-γ mRNA after HY peptide pulsing confirming the presence of HY-reactive T cells at the selected patients' peripheral blood. These results illustrate a novel concept of monitoring HY-reactive T cells by RT-PCR even if the frequency is below the detection limit of the ELISPOT assay.

Not only the presence of HY-reactive T cells in the peripheral blood of allogeneic transplanted patients was in the focus of this study, but also the reconstitution time required for these T cells to be detectable was important too. In viewing the RT-PCR results, the HY-reactive T cells were detectable only in one patient's blood sample with a reconstitution time of nine moths and eight days following transplantation. Concerning the absence of the HY-reactivity two months later in the same patient, this may be interpreted by the administration of immunosuppressive drugs to overcome the GvHD symptoms. Unfortunately, no earlier blood samples were available for this patient to detect any earlier reconstitution of the HY-specific T cells. For this patient, the detection of HY-reactive T cells was in accordance with what has been found by others.[97] Interestingly, for the first time HY-reactive T cells were detected by molecular approach (e.g. RT-PCR) in allogeneic transplanted patients which have not yet been published. These results can be further expanded to screen other groups of patients to monitor the reconstitution of HY-reactive T cell during the first nine months following allogeneic SCT.

Taking all together, these results indicate that the HY-reactivity can be monitored by RT-PCR assay but not the ELISPOT assay. This is because of the higher sensitivity of the established RT-PCR assay compared to the ELISOT assay. Also the present study clearly indicates a tight junction between the selected HY peptide and the up-regulation of IFN-γ mRNA as a final readout for the established RT-PCR assay. Concomitantly, the RT-PCR assay confirmed that the FIDSYICQV SMCY-derived HY peptide is immunodominant mHAg after allogeneic SCT. Moreover in this mHAg model, the HY effector T cells which can recognizes the autosomal HY protein on the male patient's tissues could be monitored as early as nine months following transplantation, but earlier time follow-up is suggested for future work. However, the present data provide evidence that the emergence and activation of HY-specific T cell in allogeneic transplanted patients can be monitored to predict any developing GvHD in parallel to eosinophilia. In conclusion, these findings provide evidence that HY-specific CTLs may be induced in male patients given a stem cell graft from a female donor, which may lead to the development of either GvL reactions or GvHD or both.

4.4 Monitoring of alloreactive T cells

Great attention is paid to the detection of GvHD and after human SCT. For example, host-reactive lymphocytes with broad specificity have been observed in GvHD patients using the limiting dilution techniques, which are time consuming for detecting functional T cell analysis. In the meantime, many techniques were established to predict GvHD such as, first: the *in vitro* skin explant assay,[180] second: T lymphocyte precursors frequency analysis,[55] third: some serum markers, such as the levels of TNF-α , IFN-γ , IL-10, soluble Fas, and IL-18,[49,51,51,56,57] fourth: polymorphism of IL-10,[58] transforming growth factor (TGF)-β1[59] and other genes[181], fifth: ELISPOT assays,[47,60] and fifth: T cell's TCR-Vβ clonotypic analysis.[60-62] In addition, TM technology has been developed which allows flow cytometric detection of specific T cells independently of their activation state.[63] But many of these analysis methods have not been reported to be necessarily predicting GvHD, but it may detect many inflammation states. Thus, there are contradictory results among these methods and there still remain problems with attempts to use these parameters as reliable and sensitive markers of GvHD.

In general, systematic analyses of alloreactive lymphocytes in large cohorts of patients have only been performed in bone marrow grafts before transplantation,[55] whereas only very small cohorts of patients have been analyzed for the presence of circulating alloreactive lymphocytes after transplantation.[182] Thus, a valid *in vitro* immune monitoring of GvHD patients seems essential for further improvements in allogeneic stem cell transplantation. In this study, a mixed lymphocyte reaction (MLR) model was established to simulate the generation of alloreactive T cells in the GvHD patients' peripheral blood. Successfully, this model produced alloreactive T cells that can produce IFN-γ when counteracting with their stimulator cells which bearing their specific allo-antigens (allo-Ags). Many optimizing steps were done successfully, including freezing-thawing protocols, gating-out stimulator cells from the FACS analysis, and testing the ability of secreting many cytokines.

The MLR-generated allospecific T cells were able to produce significant IFN-γ after co-culturing with their specific allo-Ag. This IC IFN-γ production was in concordance with others.[114,183] To assess this IC IFN-γ production by flow cytometer, irradiation and counterstaining of the allo-Ag-holding stimulator cells were found to be essential, reflecting weak IFN-γ production in the alloreaction which can be detected significantly after gating-out the irradiated and counterstained allo-Ags-holding cells from the flow cytometric analysis. To simulate the effect of freezing-thawing on the allogeneic transplanted patient samples, MLR-

generated alloreactive T cells were frozen and thawed before testing. Despite this freezing-thawing step, the alloreactive T cells capability to produce IFN-γ was kept in the significant levels. Taking all together, this model was optimized to monitor the IFN-γ production in the situation of alloreactions. In addition to the monitoring of IFN-γ production in the generated alloreaction settings, TNF-α, IL-4, IL-10 and perforin production was tested in the same MLR settings. Unfortunately none of them were detectable significantly by IC staining and flow cytometric analysis, reflecting the generation of type 1 cytokines pathway in the established alloreactive MLR settings, but not the type 2 cytokines pathway.

Correlation of immunological and clinical data is essential to improve the understanding of the immunology of GvHD. As an optimized MLR testing system was established, monitoring of alloreactive T cell in allogeneic transplanted patients was eligible. So, nine allogeneic transplanted GvHD- and non-GvHD-suffering patients were monitored for the presence of alloreactive T cells in their peripheral blood. Unfortunately, none of the fourteen patient samples showed significant IC IFN-γ production after exposure to the patient cells before SCT. However it should be kept in mind that, in the patient experiments, IFN-γ signal was not easily distinguishable in the flow cytometeric analysis therefore the use of IC flow cytometry as monitoring tool should be more optimized before considering as eligible method to monitor GvHD patients.

On the other hand, the IL-4 production was not significant in all of the patients' tested CD4+ (i.e. Th) and CD4- (i.e. Tc) T cell subpopulations. Interestingly, these results can be interpreted as the alloreactive T cells in the allogeneic transplanted patients use the type 1 cytokines pathway more than the type 2, and this is with concordance with what have been found by Kasakura.[50] But these results should be carefully considered since IL-4 can not be detected easily by flow cytometers and as IL-4 signal normally appears as a shoulder to the CD4+ T cells more than a separated cell population.

As there are no molecular approaches to monitor the alloreactivity in the GvHD patients, the RT-PCR assay was established and adopted. Assessment of IFN-γ gene expression was monitored in the optimized healthy mismatched-MLR alloreaction settings. The up-regulation of IFN-γ mRNA expression after the second exposure to allo-Ag reflects the ability of RT-PCR assay to monitor the alloreactive T cells. Interestingly, significant up-regulation of IFN-γ mRNA expression in some patient samples after transplantation (AT) was seen after co-culturing with the patient cells before transplantation (BT). This indicates the ability of detecting alloreactive T cells in the some patients suffering GvHD. These results indicates,

for the first time, that the RT-PCR is promising tool for monitoring the alloreactive T cells in the peripheral blood of allogeneic transplanted patients especially predicting GvHD.

4.5 Impact of the clinical parameters on the detected T-cell properties

Unlike the majority of the tested patients for CMV-, WT1- and HY-reactive T cells, few patients were under immunosuppressive drug treatments and/or suffering GvHD. The immunosuppressive state of the patients should be considered since it may cause the impairment of T-cell functionality. This is possible especially when a high dose of corticosteroids (e.g. Prednisolone) was applied, which can suppress all of the immunocompetent reactive T cells in the patient.[4,42] As seen in the CMV and GvHD monitoring parts, the immunosuppression state was not a limiting factor for detecting the functional T cells. But it may clarify why some patients' PBMCs did not show any IFN-γ production after a proper Ag stimulation. The negative results may be really due to the absence of the Ag-reactive T cells in the tested samples, but also it could be due to the impairment of the T cells in the donated patients' peripheral blood.

Another factor which may affect the interpretation of the CMV-, WT1- and HY-reactivity results is the presence of GvHD. Although some of the tested patients had this disease at the time of blood donation, positive and negative results could be obtained. As the GvHD can occur in many of the allogeneic transplanted patients, these results should be carefully interpreted for any other Ag-specific T cells rather than the alloreactive T cells. But this does not mean that the result is falsely positive since the used Ags were very specific and can not be detected by pan-reactive T cells.

4.6 Limitations of the used monitoring assays

Each monitoring assay has advantages and limitations which should be considered. For example, the TM staining gives only phenotypical but not functional data about the tested cells.[167] On the other hand, the ELISPOT assay can give the functional but not phenotypical data about the Ag-reactive T cells.[167] Also, the ICC and ELISPOT assays do not allow the Ag-specific T cells isolation, since the cell should be perforated or discarded to give the functional data.[107] Although the RT-PCR assay gave the best results and showed the superior sensitivity, unfortunately no phenotypical data can be obtained from this assay.[127] So, the IFN-γ mRNA up-regulation after applying certain Ag can not be correlated to specific T-cell sub-population. Concomitantly and as a compromise, the RT-PCR assay should be performed

in parallel with phenotypical assays (e.g. TM staining and ICC detection) that can identify the Ag-reactive T cell sub-population.

4.7 Further suggested work

Although the monitoring assays in this work were promising, the results need confirmation by further testing. For example, more patients should be tested for the reconstitution of WT1-, HY- and allo-specific T cells. This will increase the confidence about the already found results. Also, testing patients in earlier time points after SCT will give better view concerning the reconstitution of specific T cells after allogeneic SCT. In addition, other cytokines such as the TNF-α, IL-4, perforin and IL-2 are strongly suggested to be traced in the patient settings.[109] This will give better view concerning the cytokine pathways used by the immune cells in the settings of GvHD (e.g. Th1, Th2 and Tc cells functional).[50] Finally, patients under strong immunosuppressive drug treatments should be excluded from any further experiments for monitoring the WT1- and HY-reactive T cells. Another solution can be the blood donation before the administration of the immunosuppressive medicaments. By this approach, the negative results will be directly correlated to the cells' Ag-reactivity and not to the impairment of tested cells due to the administration of immunosuppressive medicaments (e.g. cyclosporine, corticosteroids, and methotrexate).[2]

5. Summary

After administration of the hematopoietic stem cells inoculum into the eligible patients in the allogeneic setting, many hematopoietic cells reconstitute in the following months to years after transplantation. The reconstituted cells can be pathogen-specific (e.g. cytomegalovirus (CMV)-reactive T cells), leukemia-specific (i.e. graft versus leukemia (GvL) effect) or recipient-specific (e.g. graft-versus-host disease (GvHD)). Thus, valid *in vitro* immune monitoring strategies should broaden the understanding of CMV immunity, GvL and GvHD, which seems essential for further improvements in allogeneic stem cell transplantation (SCT). Furthermore, approaches of this type should help to dissect allo-specific from tumor-specific immune responses and will help to clarify, how both mechanisms are interconnected and how they can be best put in action for therapeutic purposes.

The CMV-specific T cells reconstitution was monitored efficiently, in allogeneic transplanted patients, by classical approaches such as tetramer (TM) staining, intracellular cytokine (ICC) flow cytometry and enzyme-linked immunospot (ELISPOT) assay, in addition to the newly introduced real time-polymerase chain reaction (RT-PCR) assay. In general, the RT-PCR assay was highly sensitive in comparison to the other methods, and was also correlated to all of them. Interestingly, the use of the pp65 protein as CMV antigen enhanced the monitoring capabilities in comparison to the pp65 HLA-A2-restricted peptide, and allowed testing of HLA-A2 negative patients.

As an example for assessment of the GvL effect in allogeneic transplanted patients, the Wilms' tumor suppressor gene (WT1)-specific T cells were monitored. Unfortunately the WT1-specific T cells were not detectable by a sensitive ELISPOT assay, but on the other hand and for the first time the established RT-PCR assay was able to track two types of WT1-reactive T cell clones in the same samples. In addition and as not described before, the RT-PCR assay was able to monitor the reconstitution of WT1-specific T cells in patients who were suffering AML and ALL before transplantation.

As the minor histocompatibility antigens (mHAgs) play a dual role in the GvL and GvHD, the reconstitution of male-specific mHags (HY)-specific T cells were monitored. The adopted

sensitive ELISPOT assay did not detect any HY-specific T cells in all of the tested patients' samples. On the contrary, the established RT-PCR assay successfully detected the HY-specific T cells in one patient who later developed eosinophilia as a GvHD complication. As for the first time these cells were detectable by RT-PCR assay in the allogeneic transplanted patients' peripheral blood, this give a novel concept of predicting GvHD due to the generation of mHAg-specific T cells in the patients body.

Due to the still insufficient knowledge on mechanisms involved in GvHD, it was considered a major aim to tailor the allo-reactive T cells in the patients' peripheral blood in order to avoid fatal courses of GvHD. An allogeneic mixed lymphocyte reaction (MLR) model was established and optimized to simulate the presence of the allo-reactive T cells in the GvHD patients' blood. After applying the established MLR model on some patients' samples, weak but significant allo-reactive T cells could be monitored in few samples by using the RT-PCR assay but not ICC flow cytometry. For better detection of the allo-reactive T cells in the patients' peripheral blood, the use of RT-PCR assay is strongly advised for monitoring GvHD.

Finally more patients and other cytokines are suggested to be further monitored to give better view. Overall, the results of this work strongly support that monitoring methods can be established to be used in clinical practice in the near future.

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Erklärung

Hiermit erkläre ich ehrenwörtlich, dass ich die vorliegende Arbeit selbst angefertigt habe und keine anderen als die von mir angegebenen Quellen und Hilfsmittel benutzt habe. Die aus fremden Quellen übernommenen ldeen sind als solche kenntlich gemacht. Die vorliegende Arbeit wurde noch keiner Prüfungsbehörde vorgelegt.

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