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TITOLO DELLA TESI DI DOTTORATO

IFN-γ induced by PHA stimulation as new marker for GvHD prediction in patients undergoing allogeneic hematopoietic stem cell transplantation (alloHSCT)

S.S.D. MED 04

Coordinatore: Prof. Christian Chiamulera

Firma _____

Tutor: Dott.ssa Vladia Monsurrò

Firma _____

Dottorando: Dott. Enrico Morello

Firma _____

Summary

Introduction

Graft versus host disease (GvHD) is caused by donor T-cells expanding in response to allo- and autoantigens and leads to tissue damage in the graft recipient (Ferrara 2009). Acute GvHD is responsible for 15-40% of mortality and it is a major cause of morbidity after allogeneic alloHSCT (Ball 2008). Chronic GvHD (cGvHD), occurring in up to 50% of patients who survive three months after alloHSCT, is the most serious and common long term complication of alloHSCT causing death from organ failure or infection and impairing quality of life of surviving patients (Filipovich 2005). Among opportunistic infections during the immune system reconstitution after HSCT, CMV infection has been clearly associated to the pathogenesis of GvHD (Ferrara 2009, Schlomchik 2007, Young 2008). Enhanced transcription of interferon gamma (INF-γ) has been reported in people with GvDH (Poloni 2010) whereas the concentration of other cytokines were not significantly increased (Yang 2005). Although there are different immune-suppressive schedules, it is difficult to achieve the desirable degree of immune-suppression: sufficient to prevent GvDH but not too severe to completely inhibit the antiviral immune response and to impair the anti-neoplastic surveillance (Graft versus Tumor effect – GvT) (Soiffer 2008, Welniak 2006, Li 2009).

GvHD is a clinical diagnosis supported by histological confirmation if requested, but, at present, there are no tests that could predict the onset of GvHD and clinicians and researchers are not able to understand how to separate GvHD from GvL (Ferrara 2009, Schultz 2006, Li 2009). Several studies are ongoing in order to control GvHD without impairing GvL effect, but the immunological connection between these two phenomena is too strong and the outcome is still poor (Li 2009, Lu 2009).

Immune reconstitution after alloHSCT mimic the immune-system ontogenesis and its activation is enhanced at the time of immune-suppressive drugs tapering (Soiffer 2008, Hess 2010): the reduction in the intensity of immune-suppression could determine the activation of T cells with consequent production of several cytokines such as IL-2, IL-17 or INF-γ (Ferrara 2009). On this basis the monitoring of INF-γ after stimulation with a mitogen (PHA-phytohemoagglutinin) in the patients undergone to alloHSCT could help the management and the prediction of GvHD.

Pathogenesis and biomarkers of GvHD

In order to reduce the risk of GvHD the suitable donor should be possibly HLA (Human

Leukocyte Antigens) identical (Ferrara 2009, Schlomchik 2007). High resolution PCR techniques could recognize differences at the HLA loci in order to identify the best donor ("A, B, C, DRB1 matched"), although a partially matched donor should be considered if a fully matched donor is not suitable (cord blood transplantation, haploidentical transplantation, mismatched unrelated donor) (Sociè 2009, Ferrara 2009, Welniak 2006).

Class I proteins are expressed on nucleated cells and class II proteins are mainly expressed by hematopoietic antigen presenting cells (APC), however their expression on some other cells could be induced by inflammatory stimuli. GvHD occurs even if the donor is an identical fully matched donor (about 40% of Matched Related Donor transplantation developed acute GvHD) (Ferrara 2009). This immunoreactivity is determined by the non-HLA genetic differences (Ferrara 2009):

- minor histocompatibility antigens (HA-3, HY expressed on all tissues and HA-1 and HA-2 expressed mainly on hematopoietic cells are recognized by donor T cells either in GvHD direction, nor in GvL direction)
- several cytokine genes polimorphisms (TNF-α, INF-γ, IL-10) are associated with an increased risk of GvHD in several studies (but non all) (Dickinson 2005).
- Polimorphisms of innate immunity proteins (nucleotide oligomerization domain 2 and Keratin 18 receptors), have also been associated with GvHD (Ferrara 2009).

The acute GvHD pathogenesis has been classically described as a three steps multifactorial mechanism (Ferrara 2009, Schlomchik 2007, Wolniak 2006):

- activation of APC: the underlying disease, the conditioning regimen or the infections during the peri-transplantation period induced an inflammatory response to a tissue damage. The damaged tissue produces cytokines, chemokines and exposition of MHC antigens on host APC. The damage of the gastrointestinal tract is crucial in the pathogenesis of acute GvHD because it allows for the translocation of bacteria determining systemic inflammatory stimuli such LPS or other pathogens molecules that could enhance APC activation. In addition APCs recognizes pathogens through conserved patterns named PAMPs (pathogen-associated molecular patterns) (Penack 2010). Toll like receptors (TLR) are the best defined receptors for these patterns. TLRs could also recognize viral nucleic acids and might enhance APC response: this mechanism could explain the strong association between onset of GvHD and viral infections, mainly due to herpesviruses (CMV) (Jasperson 2009, Ferrara 2009).
- donor T cell activation: donor T cells proliferate and differentiate in response to APC

mainly through costimulatory molecules (CD28/B7 and CD40/CD40L) (Kwon 2010). In mouse models CD4+ T lymphocyes induce GvHD to MHC class II antigens and CD8+ to MHC class I, however in humans this separation is not clear: if the donor is HLA identical, CD4+ and CD8+ could react both to minor histocompatibility complex antigens. Several subset of T cells interact with CD4+ and CD8+ T cells (Ferrara 2009):

- Regulatory T cells (Treg) suppress the proliferation of T cells in response to APC and could prevent GvHD in animal models (Kohrt 2010). Treg secrete antiinflammatory cytokines (IL-10 and TGF-β) and inhibit directly APCs.
- NKT 1.1+ cells of donor and host origin could suppress GvHD through IL-4 in the mouse model and total lymphoid irradiation enhances NKT function in humans, preventing acute GvHD without impairing GvL effect (Kohrt 2010).

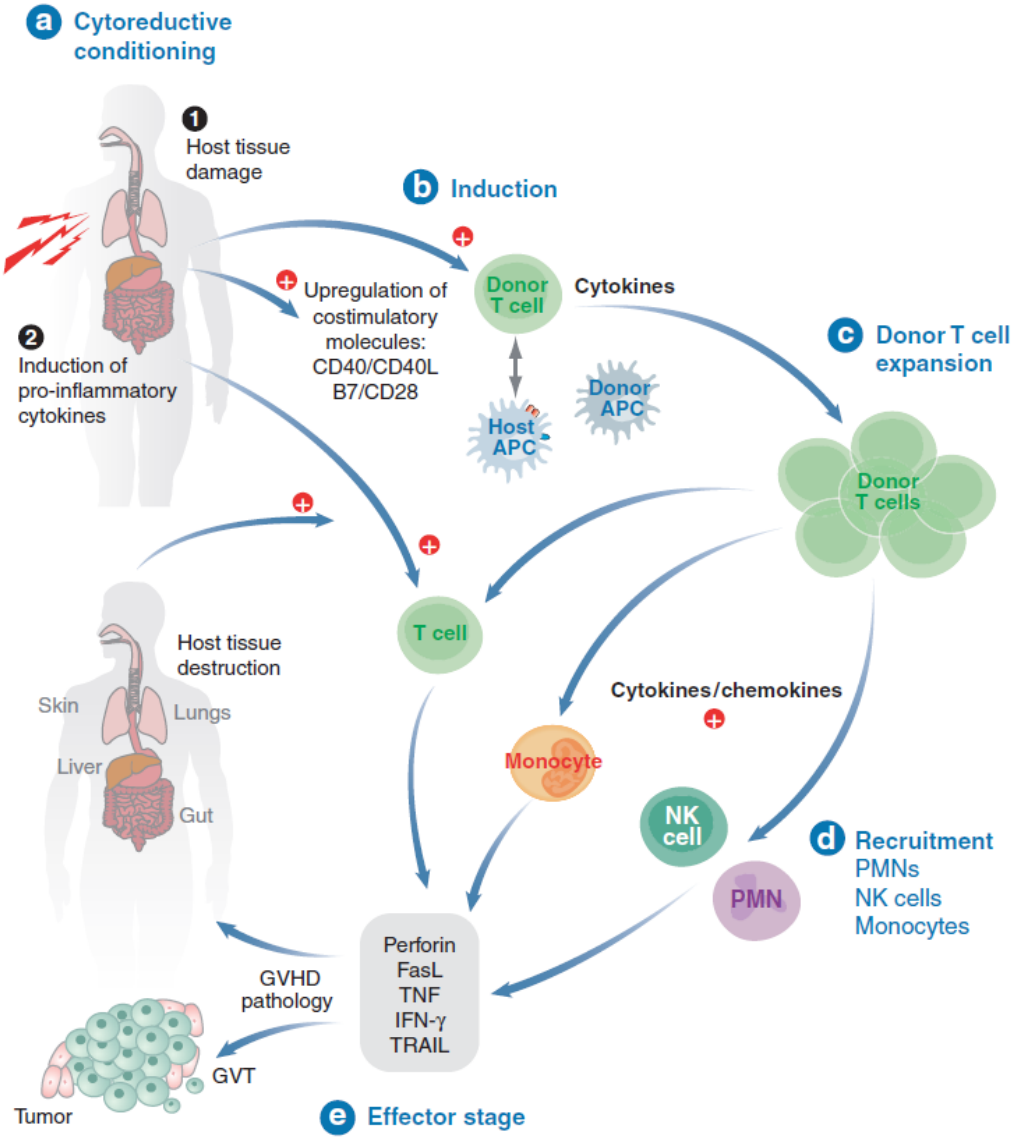
Cytokine storm in acute GvHD is a central issue. Th1 cytokines (INF-γ, IL-2, TNF-α) are involved in activation and pro-inflammatory differentiation of T-cells (Ferrara 2009).

- IL-2 promotes T-cell activation through CD25 (IL-2 receptor), but emerging data suggest a feed-back role due to the activation of Treg cells. Thus an IL-2 directed anti-GvHD therapy could impair tolerance after allogeneic alloHSCT (Ferrara 2009, Welniak 2006).
- The role of INF-γ is controversial (Holler 2002, Yang 2005, Lu 2009): it might enhance GvHD by increasing chemokine receptors, MHC proteins and adhesion molecules on the surface of involved cells, increasing sensitivity to inflammatory stimuli in Macrophages and Monocytes with the direct induction of damage of GI tract and amplifying the skin inflammatory reaction. Conversely it might reduce GvHD hastening apoptosis of activated donor T cells. Elispot assay demonstrate an INF-γ increase in humans with acute GvHD grade 2 to 4 but not in infections or mild cGvHD (Hirayama 2006). The dichotomous role of INF-γ in the activation of inflammatory response and in the Treg cell activation was described also in allograft rejection (Feng 2011).
- IL-10 may regulate acute GvHD suppressing immune-response (as reported in the previous paragraph) whereas TGF-β suppress acute GvHD and exacerbate cGvHD (Ferrara 2009).

The role of different cytokines depends on the timing and the duration of its secretion after allogeneic alloHSCT (Skert 2009, Ferrara 2009).

- The cellular and inflammatory effector phase is a complex cascade mediated by Cytotoxic T cells, NK cells and inflammatory mediators (INF-γ, TNF-a, IL-1, TRAIL and NO) that can induce tissue destruction (Ferrara 2009).
- Effector cells and chemokines: CTLs use preferentially Fas/FasL pathway to induce liver damage and Perforin/Granzyme pathway to induce GI and skin damage (Ferrara 2009). Chemokines and macrophage inflammatory protein-1a (MIP-1a) are over-expressed in murine models and enhances effector cell homing to the involved tissue. CCL3 is also involved in GvHD in mice, but not in GvL; thus, blockage of CCL3 trough Evasin 1 could prevent GvHD in transplanted mice (Castor 2010). Immunohistochemistry on GvHD biopsies revealed over-expression of CCR5 and INF-γ in humans (Palmer 2010).
- LPS or other microbial products induces host and donor cells to produce cytokine secretion through the TLR pathway. TNF-a is crucial in the tissue injury (mainly in the GI tract) activating APCs and enhancing alloantigens presentation, recruiting effector cells and damaging the tissue by itself (Ferrara 2009).

Figure 1 GvHD pathogenesis (Welniak 2006)



Chronic GvHD pathogenesis has been described as an autoimmune disease driven by allo-antigens, but the origin and the mechanism of action of the involved alloreactive cells remains not fully understood (Horwitz 2006, Martin 2008, Filipovich 2008, Tyndall 2008). From the animal models 4 hypothesis regarding chronic GvHD pathogenesis have been described (Martin 2008):

- the thymic damage caused by acute GvHD resulted in an impaired deletion of alloreactive T cells (Krenger 2008)
- the role of TGF-β is crucial but controversial (high levels are reported in humans but gene expression in CD4+ and CD8+ is associated with a reduced risk of cGvHD);

furthermore increased activity of TGF-β in the first phase after transplantation is associated with a reduced risk of acute GvHD and consecutively a reduced risk of cGvHD.

- The role of B cells and antibody mediated mechanisms: autoantibodies formation has been reported in animal models and in clinical data. B cells could play a role in the pathogenesis of chronic GvHD acting as APC and inducing alloresponse of CD4+ T cells and antibodies production (Tyndall 2008).
- deficiency in number and function of Treg cells are reported during cGvHD in mice and humans (Matsuoka 2010). In mouse models Treg cells could prevent cGvHD (Kohrt 2010). Circulating Treg cells in humans are not clearly associated with chronic GvHD onset, although a low number of Treg cells (relative to the number of CD8+) was demonstrated in the gut mucosa of patients affected by GvHD, but efforts in order to try to expand Treg cells for adoptive therapy are ongoing (Pidala 2010).

Finally, emerging data about the role of Th17 in chronic GvHD should be integrated with these hypotheses:

Th17 in mouse are sufficient but not necessary to induce GvHD (Iclozan 2010); in humans are reported to increase in number in acute GvHD and chronic active GvHD; IL-17+/INF-γ+ cells infiltrate GvHD lesions expressing IL23R (Dander 2009).

Biomarkers of GvHD

The National Institute of Health define classification and applications of biomarkers associated to cGvHD (Schultz 2006). According to those guidelines, the following characteristics should be considered in order to evaluate the reliability of a biomarker:

1. predicting response to therapy
2. measuring disease activity and distinguishing irreversible damage from continued disease activity
3. predicting the risk of developing chronic GvHD
4. diagnosing chronic GvHD
5. predicting the prognosis of chronic GvHD
6. evaluating the balance between GvHD and GvL effects
7. serving as a surrogate end point for therapeutic response.

The methods applied to the monitoring of the aspecific activity of the immune-system after alloHSCT of GvHD could be based on genomic or proteomic methods (Hansen

2008).

The J.L.M. Ferrara group (Paczesny 2009) defines through a proteomic approach a pattern of 4 soluble proteins (IL-2r-a, TNF-R1, IL-8, HGF) that could discriminate optimally between patients with symptoms of acute GvHD and could predict survival independently to clinical severity of GvHD. Similar results were generated as well in the setting of skin GvHD (Paczesny2010).

Weissinger et al. implemented a CE-MS (Capillary electrophoresis Mass Spectrometry) proteomic analysis able to identify 31 polypetides allowing a correct diagnosis of GvHD (sensitivity 100% and specificity 98%)(Weissinger 2007). These data were confirmed by a blinded analysis on 599 blood samples (sensitivity 83% and specificity 75.6%). This finding are already translated into a randomised controlled trial with the goal of prophylactically treat patients with a proteomic pattern associated with an increased risk of acute GvHD (Weissinger 2010).

McGuirk et al. studied a proteomic pattern associated with chronic GvHD characterised by one group of proteins over-expressed (haptoglobin, alpha-1-antitrypsin, apolipoprotein A-IV, serum paraoxonase and Zn-alpha-glycoprotein) and one under-expressed (clusterin precursor, alpha-2-macroglobulin, serum amyloid protein precursor, sex hormone-binding globulin, serotransferrin and complement C4 in serum of patients affected by cGvHD); interestingly serum haptoglobin (HP) levels were higher in the sera(?) of patients with cGvHD ($p<0.01$) and 43.8% of these patients demonstrate a particular haptoglobin polimorphismn (HP 2-2 phenotype).

Skert et al. prospectively analyzed 30 patients after transplantation and found that 18 of them developed cGVHD: they discovered that types of lymphocytes and cytokines are changing after transplantation however they demonstrated that the presence of CD152+ Tcells and NK cells are negative predictors of cGvHD, whereas, among cytokines, higher levels of TNF-a predict onset of cGvHD (Skert 2009).

Scambi et al. reported a series of patients with sclerodermic chronic GvHD with serum detectable pneumococcal antibody crossreacting with double stranded DNA. Furthermore higher levels of Factor H were proved in serum Sclerodermic cGVHD patients and Systemic Sclerosis patients. (Scambi 2010)

In order to monitor the immune-response in GvHD Laurin et al. implemented an Elispot based method detecting INF-γ in response to minor histocompatibility antigens. Trought this test he was able to demonstrate a strong association between donor vs minor histocompatibiliy antigens response and GvL or GvHD at the third month after transplant.

(Laurin 2010)

Immuknow ® is a registered immunological assay that determines the CD4 functional status in immunosuppressed patients by quantitatively measuring the intracellular ATP levels in PHA-stimulated CD4+ T-helper cells in whole blood. However, the application of this assay to the study of alloHSCT patients do not reveal any association with the positivity of this test and the presence of GvHD. (Gesundheit 2010)

More similar to what we are proposing in the present study, Zhou et al. implemented a test for immune-monitoring of allo-response using a real-time polymerase chain reaction method based on IL-2 and INF-γ mRNA quantification upon stimulation of whole blood with allogeneic T cell-depleted peripheral blood mononuclear cells; (Zhou 2005). However this method was never tested on alloHSCT patients but only on solid organ transplanted patients.

On the other hand several studies are published about immune-monitoring of specific response to antigens, for example against CMV, using flow cytometric analysis (Gratama 2010, Eid 2009, Avetisyan 2007) or elispot methods (Ganepola 2007).

Type of study and aims

This study is a prospective observational study approved by the ethical local committees (Bolzano Central Hospital, Hematology Department and BMT Unit and Azienda Ospedaliera Spedali Civili di Brescia, BMT Unit). The participants were patients transplanted at the 2 institutions between 2007 and 2010 and with a 365 days of follow-up .
Inclusions criteria for this study were:

- allogeneic hematopoietic stem cell transplantation from a matched or partially matched donor
- life expectancy of more than 6 months

Exclusion criteria for this study were:

- Cord blood transplantation, this decision was due to the complexity of the immune reconstitution after this particular type of transplant and the few cases expected in the 3 years period.

The aim was to test reliability of IFNgamma-Y production of PBMCs after PHA-PC stimulation for predicting cGvHD according to NCI criteria (Schultz 2006).
TWe use the positive control of the QuantiFERON-CMV® was used.
Since the test was originally designed for immune surveillance against CMV, all the obtained samples were associated with samples for viral detection (antigenemia or DNA-emia) and specific anti-CMV-antigens immune response.

Enrolled patients: 36 patients undergoing allogeneic alloHSCT at 2 hematological departments from 2 different hospitals (Bolzano, Brescia – Italy).

Outcomes:
Primary outcome: to test reliability of IFN-Ygamma production of PBMCs after PHA (PHA-induced INF-γ)-PC stimulation for predicting cGvHD using the positive control of the QuantiFERON-CMV® .for predicting cGVHD and its outcome.

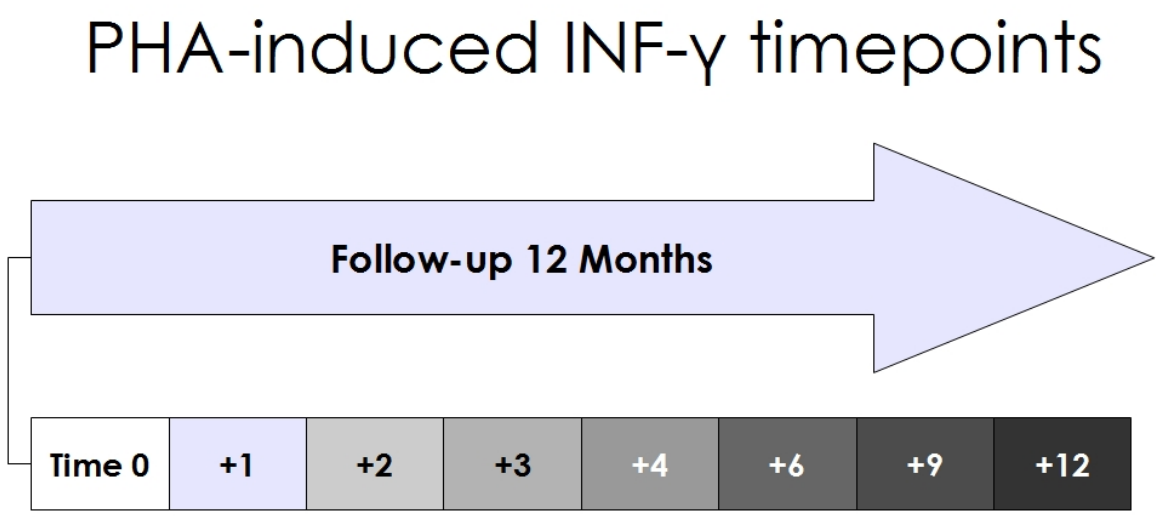
Secondary outcomes:

- to correlate time variation of PHA induced INF-γ with Overall Survival and Disease Free Survival
- to correlate time variation of PHA with Transplant Related Mortality (TRM)

Methods

Whole blood samples were collected at the 2 institutions according to the following schedule (figure 2).

Figure 2, sampling schedule after alloHSCT. FBC = Full Blood Count



Each 2-3 weeks	Weekly	At each visit
QuantiFERON-CMV ®	CMV-DNA	GVHD staging
Lymphocyte Subpopulations	pp65	FBC
Disease markers (WT-1)		Toxicity (CTC-WHO)

QuantiFERON-CMV ®

The determination of INF-γ-levels in patient samples will be done by using the recently developed in vitro ELISA test for measuring cell mediated immune responses to human cytomegalovirus (CMV) antigens (Cellestis). The method is based on the fact that individuals infected with CMV usually have CD8+ circulating lymphocytes that specifically recognize the viral antigens and therefore produce INF-γ. Samples will be processed as described by manufacturer. Briefly, whole blood samples are collected into three special tubes:

1) a Mitogen tube: positive control, for defining the individual's capability to produce INF-γ

2) the Nil control tube: negative control, to exclude the INF-γ-production due to other stimulation;

3) CMV antigen tube: CMV peptide cocktail simulating viral proteins, designed to target

CD8+ T cells, including HLA Class 1 Haplotypes A1, A2, A3, A11, A23, A24, A26, B7, B8, B27, B35, B40, B41, B44, B51, B52, B57, B58 and B60.
Specimen collection and incubation. For each subject 1 ml of blood must be collected by venepuncture directly into each QuantiFERON-CMV collection tube. Tubes must be transferred to a 37°C incubator within 16 hrs from collection and incubated for 16 to 24 hours. Subsequently collecting tubes are centrifuged at 3000 x g for 15 min for plasma harvesting. Human INF-γ level determination (ELISA). For each ELISA session a standard curve with 8 different concentrations has to be generated to be tested with the patient's samples. permitting the results' calculation. Results are obtained measuring the optical density (OD). Interpretation. A test is considered positive for an INF-γ response in the CMV-tube that is significantly above the Nil-tube value. Quantification will occur by correlating the OD with the INF-γ concentrations of the standard-curve blot. Indeterminate results must be correlated to a low response to Mitogen and the possibility of an inability of the patient's lymphocytes to generate INF-γ must be considered.

Preliminary data in a cohort of allogeneic transplanted patients (Morello 2008) demonstrate that the PHA stimulated INF-γ production is associated with GvHD and the monitoring of PHA stimulated INF-γ after allo-SCT seems to predict the onset of GvHD. The originality of this study is to assess the reliability of the positive control of the QuantiFERON®-CMV kit as new marker for GvHD early diagnosis. This easy test could help in the management of immunosuppressive treatment after allogeneic alloHSCT in order to balance the risk of GvHD with the risk of relapse. Subclinical chronic GvHD, characterized by an enhanced PHA-induced INF-γ production even at the time of immunosuppressive tapering, could reduce the risk of relapse, without impairing quality of life.
For the diagnosis of GvHD three cut-offs will be tested for sensitivity and specificity:
#1) 0,5 IU/mL as defined by manufacturer
#2) 9 IU/mL as experimentally defined by the median of the observations in our preliminary data set.
#3) 5 IU/mL as defined by the mean (rounded to the nearest entire value) of the observation in the first 4 months (time of immune-suppression tapering).
The patient will be monitored in order to define if PHA-PC became positive at the 3 cutoffs (from a value <0,5 IU/mL in the first 4 months) after transplant and thereafter if this value dropped after immune suppressive therapy.

Box 1 - Preliminary data (Morello 2008) – ASH 2008

Among 92 samples, 70 were positive for the PHA stimulated IFN-γ production according to the cut-off #1; 61% (43/70) were associated with GvHD whereas 27% (6/22) with lower PHA stimulated IFN-γ production were associated with GvHD. This difference was proved to be statistically significant (p=0.005). Using the cut-off #2, 46 samples out of 92 were positive for the PHA stimulated IFN-γ production; 71% (33/46) were associated with GvHD, whereas 34% (16/46) with lower PHA stimulated IFN-γ production were associated with GvHD. The difference was proved to be significant (p=0.000).
Among 10 patients monitored prospectively after transplant, 7 patients became positive for the PHA stimulated IFN-γ production and 6 developed subsequently chronic GvHD. The median time of the GvHD onset was 100 days from the first sample proved positive above the cut-off #1 and 33 days from the first sample proved positive above the cutoff#2. Four patients received steroid treatment for extensive chronic GvHD and their PHA stimulated IFN-γ production dropped after treatment.

CMV-DNAemia

Real time PCR for CMV quantification in peripheral blood:

nucleic acid extraction was performed by an automated bioMerieux NucliSens easyMAG system. A real time quantitative PCR was the method of choice for rapid detection and quantification of CMV in clinical specimens. The use of primers and probes make the method vulnerable to false negative results, caused by sequence diversity in the templates. In order to avoid inaccuracy and to evaluate the impact of CMV sequence variants in its detection and quantification in our patient population, we evaluate the clinical sensitivity of commercially available methods designed on different viral genome conserved regions by using control strains for reference. The tests was set-up on ABI Prism 7300 real time PCR system.

CMV genotyping. The amplified fragments were confirmed by sequencing on ABI 3130 Genetic Analyzer upon occasion in order to characterize the detected virus populations.

Quantification of pp-65-antigenemia

The quantification of pp-65-antigenemia is a routine method and is performed with the immunofluorescent in-house method on cytospin preparations of peripheral polymorphonuclear leukocytes using selected monoclonal antibodies to pp65-CMV (Clonab CMV, Biotest). Quantification is achieved by counting antigenemia-positive nuclei/ 400.000 PML/patient. The method was previously described in detail by Gerna et al., 1992.

Consideration about different methods in CMV isolation

Human cytomegalovirus (CMV) is the most important viral pathogen in transplant recipients. 40-70% of the population is persistently infected and without preventive measures have a 45-86% risk of CMV reactivation, and a 20-30% risk of CMV disease. Transmission occurs in 20-40% in case of a CMV seropositive donor (Ljungman 2008). Prophylaxis with antivirals and preemptive therapy prior to the appearance of clinical symptoms, reduce the risk of CMV disease but is still a major risk factor for the survival of alloHSCT recipients. A correlation between CMV antigenemia and CMV disease has been shown, therefore therapies were based on virological monitoring using the pp65-antigenemia assay (Ljungman 2008).

However, standardized and automated assays for quantification of CMV DNAemia better reflect virus replication and may provide cut-off DNA levels for accurate timing of the therapy to keep the balance between effective treatment and toxic side effects (Gimeno 2010). The technology of choice is a quantitative real-time PCR approach (Ljungman 2008). As the patients are at risk for CMV infection until adequate T-cell immunity is restored, additional screening of the virus-specific cellular immune response could be valuable to identify patients who cannot reconstitute a protective T-cell immunity (Ljungman 2008). Pp65-antigenemia cut-off values are widely accepted for guiding pre-emptive therapy. Some data are available for DNAemia cut-offs, but they have to be defined according to the molecular methods applied (Gimeno 2010). A randomised trial in allogeneic transplanted patients seems to demonstrate that the use of an adequate cutoff reduces the number of patients requiring antiviral treatment, but the threshold for pre-emptive therapy was referred to an home-made method and is not reproducible with commercial kits (Gerna 2008).

Lymphocyte subpopulations

Flow cytometric count of lymphocyte subset population will be performed according internal laboratory standards.

Clinical data

CMV disease, CMV infection, disease and management will be defined according to EBMT guidelines (Ljungman 2008).

Acute GvHD will be classified according to revised EBMT Criteria (Ball 2008) and reported

in table 1 and Chronic GvHD will be classified according to the NIH consensus (Filipovich 2005) as reported in table 3. The distinction between acute and chronic was classically based on a temporary criteria (<100 days and > 100 days after transplantation) and was referred to complications occurring after fully myeloablative bone marrow transplantation, but the introduction of new type of transplantation (reduced intensity), cell therapy (donor lymphocyte infusions), source of hematopoietic stem cell (peripheral blood and cord blood) has changed this clinical-pathological entity and overlap between acute and chronic. The classification of chronic GvHD implies a scoring system based of the involvement of several organs and its severity (Filipovich 2005).

Whenever possible a biopsy of the involved organ was performed in order to confirm the diagnosis of GvHD.

Table 1. Organ staging of acute GvHD and overall grading based on modified Keystone criteria

Stage	***Skin***	***Liver***	***Gut***
0	No rash due to GvHD	Bilirubin <2 mg/dL	*None*
I	Maculopapular rash <25% of body surface area without	Bilirubin 2-3 mg/dL	*Nausea and emesis; diarrhea <1000 mL*
II	Maculopapular rash or erythema with puritis or other symptoms . < 50% of body surface area or localized desquamation	Bilirubin 3-6 mg/dL	*Nausea and emesis; diarrhea <1500 mL*
III	Generalized erythroderma; symptomatic macular, papular or vesicular eruption with bullous formation or desquamation covering ≥ 50% of body surface area	Bilirubin 6-15 mg/dL	*Nausea and emesis; diarrhea ≥1500 mL*
IV	Generalized exfoliative dermatitis or bullous eruption	Bilirubin >15 mg/dL	*Nausea and emesis; diarrhea ≥1500 mL, ileus or abdominal pain*

Overall grading				
Grade	**Skin**	**Liver**	**Gut**	**PS**
0	0	0	0	0
1	1 - 2	0	0	0
2	1 - 3	1	1	1
3	2 - 3	2 - 3	2 - 4	2
4	2 - 4	2 - 4		2-4

Categories of Acute and Chronic GvHD

Category	**Time of Symptoms after alloHSCT or DLI**	**Presence of Acute GvHD features**	**Presence of Chronic GvHD features**
Acute GvHD			
Classical aGVHD	<100d	YES	NO
Persistent, recurrent or late onset aGVHD	>100d	YES	NO
Chronic GvHD			
Classic cGVHD	no time limits	NO	YES
Overlap syndrome	no time limits	YES	YES

Table 3, taken from Filipovich 2005 (next 2 pages)

Table 1. *Signs and Symptoms of Chronic GVHD*

Organ or Site	Diagnostic (Sufficient to Establish the Diagnosis of Chronic GVHD)	Distinctive (Seen in Chronic GVHD, but Insufficient Alone to Establish a Diagnosis of Chronic GVHD)	Other Features*	Common (Seen with Both Acute and Chronic GVHD)
Skin	Poikiloderma Lichen planus-like features Sclerotic features Morphea-like features Lichen sclerosus-like features	Depigmentation	Sweat impairment Ichthyosis Keratosis pilaris Hypopigmentation Hyperpigmentation	Erythema Maculopapular rash Pruritus
Nails		Dystrophy Longitudinal ridging, splitting, or brittle features Onycholysis Pterygium unguis Nail loss (usually symmetric; affects most nails)†		
Scalp and body hair		New onset of scarring or nonscarring scalp alopecia (after recovery from chemoradiotherapy) Scaling, papulosquamous lesions	Thinning scalp hair, typically patchy, coarse, or dull (not explained by endocrine or other causes) Premature gray hair	
Mouth	Lichen-type features Hyperkeratotic plaques Restriction of mouth opening from sclerosis	Xerostomia Mucocele Mucosal atrophy Pseudomembranes† Ulcers†		Gingivitis Mucositis Erythema Pain
Eyes		New onset dry, gritty, or painful eyes‡ Cicatricial conjunctivitis Keratoconjunctivitis sicca‡ Confluent areas of punctate keratopathy	Photophobia Periorbital hyperpigmentation Blepharitis (erythema of the eyelids with edema)	
Genitalia	Lichen planus-like features Vaginal scarring or stenosis	Erosions† Fissures† Ulcers†		
GI tract	Esophageal web Strictures or stenosis in the upper to mid third of the esophagus†		Exocrine pancreatic insufficiency	Anorexia Nausea Vomiting Diarrhea Weight loss Failure to thrive (infants and children)
Liver				Total bilirubin, alkaline phosphatase >2 × upper limit of normal† ALT or AST >2 × upper limit of normal†
Lung	Bronchiolitis obliterans diagnosed with lung biopsy	Bronchiolitis obliterans diagnosed with PFTs and radiology‡		BOOP
Muscles, fascia, joints	Fasciitis Joint stiffness or contractures secondary to sclerosis	Myositis or polymyositis‡	Edema Muscle cramps Arthralgia or arthritis	

Table I. *Continued*

Organ or Site	Diagnostic (Sufficient to Establish the Diagnosis of Chronic GVHD)	Distinctive (Seen in Chronic GVHD, but Insufficient Alone to Establish a Diagnosis of Chronic GVHD)	Other Features*	Common (Seen with Both Acute and Chronic GVHD)
Hematopoietic and immune			Thrombocytopenia Eosinophilia Lymphopenia Hypo- or hypergammaglobulinemia Autoantibodies (AIHA and ITP)	
Other			Pericardial or pleural effusions Ascites Peripheral neuropathy Nephrotic syndrome Myasthenia gravis Cardiac conduction abnormality or cardiomyopathy	

GVHD indicates graft-versus-host disease; ALT, alanine aminotransferase; AST, aspartate aminotransferase; BOOP, bronchiolitis obliterans-organizing pneumonia; PFTs, pulmonary function tests; AIHA, autoimmune hemolytic anemia; ITP, idiopathic thrombocytopenic purpura.

*Can be acknowledged as part of the chronic GVHD symptomatology if the diagnosis is confirmed.

†In all cases, infection, drug effects, malignancy, or other causes must be excluded.

‡Diagnosis of chronic GVHD requires biopsy or radiology confirmation (or Schirmer test for eyes).

A prognostic score based on performance status and typical organ or site involvement, was developed in order to uniform diagnosis, grading and treatment of chronic GvHD (Filipovich 2005).

Mild chronic GvHD:

- only 1 or 2 organ involved (except the lung) with no clinical significant impairment

Moderate chronic GvHD:

- at least 1 organ or site with clinically significant but no major disability (maximum score of 2 in any affected organ or site)
- 3 or more organs or sites with no clinically significant functional impairment (maximum score of 1 in all affected organs or sites). A lung score of 1 will also be considered moderate chronic GvHD.

Severe chronic GvHD:

- major disability caused by chronic GvHD (score of 3 in any organ or site). A lung score of 2 or greater will also be considered severe chronic GvHD.

Toxicity will be defined according to CTC-WHO Criteria.

Statistical analysis

Positive predictive value, sensitivity and specificity of the test will be calculated according with clinical-pathological diagnosis of chronic GvHD. ROC curves were designed for the association between values of PHA-induced INF-γ and presence of GvHD at the time of the sampling, 30 days or 100 days after the sampling.

Univariate analysis was performed with Fisher exact test for categoric variables, T-student and ANOVA for continuous variables. Log rank test was used for univariate analysis for time dependent variables. Multinomial logistic regression model was implemented for multivariate analysis in order to define an association between time independent variables and Cox regression model for time dependent variables.

In the multivariate analysis the following variables were used in a 2 step model:

- Pre-transplant variables
  - Sex
  - Age
  - Type of conditioning
  - Type of donor
  - Serostatus for CMV in recipients and donors
  - type of Anti-thymocyte globulin used or not
  - Source of stem cell
  - Immune-prophylaxis
  - Disease
  - Disease status at transplantation
  - pre-transplant PHA-induced INF-γ
- transplant variables
  - Moderate or severe infections
  - acute GvHD
  - Relapse
  - PHA-induced INF-γ (normalized for each month after transplant)
  - Immune-suppression (normalized as following for each month after transplant)
    - 1 tapering
    - 2 prophylaxis
    - 3 treatment

- 4 treatment for refractory GvHD

Results

Descriptive analysis

Thirty-six patients were enrolled in the study and demographic characteristics are presented in table 1. M/F ratio was 0,89 and median age was 50 (18-69).

Hematological diagnosis was acute leukemia and myelodisplastic syndrome in 25 patients, lymphoma in 7, myeloma in 2, severe aplastic anemia in one and chronic myeloid leukemia in one case; disease was in complete remission in the majority of the cases (64%). Six patients were in chemosensitive persistence of disease and 7 patients were transplanted with a refractory disease. The patients were conditioned mostly with non myeloablative regimens (64%), two myeloma patients were conditioned only with TBI200 rads and 11 patients with a fully myeloablative regimen. The donor was matched related in 11 cases, matched unrelated in 22 cases and mismatched in 3 cases. The GvHD prophylaxis was in the majority of cases the standard methotrexate/cyclosporinA immune-suppressive combination. Anti-thymocyte globulin (ATG Fresenius® or Thymoglobulin®) was added in case of matched unrelated or mismatched donors. Peripheral blood was the preferred source in 2/3 of this cohort (Table4).

Table 4 – demographic data of included patients

Patient ID	Sex	Conditioning	D IgG-CMV	Donor	TCD	Source	Diagnosis	Status at Tx	IS
BZ1	F	RIC	Positive	MUD	None	PB	AML and MDS	CR	CsA/MTX
BZ2	M	Micro	Positive	MUD	None	PB	Other	CR	CsA/MTX
BZ3	F	MA	Posivive	MUD	ATG	PB	ALL	Ref	CsA/MMF
BZ4	F	RIC	Negative	MUD	None	PB	Lymphoma	CR	CsA/MMF
BZ6	M	RIC	Positive	MUD	Thymo	PB	Lymphoma	CD	CsA/MMF
BZ7	M	RIC	Negative	MUD	ATG	BM	AML and MDS	CR	CsA/MMF
BZ8	F	RIC	Negative	MRD	None	PB	Lymphoma	CR	CsA/MTX
BZ9	F	RIC	Positive	MRD	None	PB	AML and MDS	Ref	CsA/MTX
BZ10	F	RIC	Negative	MUD	ATG	PB	AML and MDS	CR	CsA/MTX
BZ11	M	MA	Positive	MUD	ATG	BM	AML and MDS	CR	CsA/MTX
BZ12	F	RIC	Positive	MUD	ATG	PB	Lymphoma	CR	CsA/MTX
BZ13	M	RIC	Positive	MUD	ATG	PB	Lymphoma	CD	CsA/MMF
BZ14	M	MA	Positive	MMD	ATG	PB	ALL	CR	CsA/MTX
BZ15	M	RIC	Negative	MRD	None	BM	AML and MDS	CR	CsA/MTX

BZ16	F	RIC	Negative	MUD	ATG	BM	AML and MDS	CR	CsA/MTX
BZ17	F	RIC	Positive	MUD	ATG	PB	AML and MDS	Ref	CsA/MTX
BZ18	M	RIC	Negative	MRD	None	PB	AML and MDS	Ref	CsA/MTX
BZ19	M	RIC	Positive	MUD	ATG	BM	AML and MDS	CD	CsA/MTX
BZ20	F	RIC	Positive	MUD	Thymo	PB	Lymphoma	CR	CsA/MMF
BZ21	F	MA	Positive	MUD	Thymo	PB	AML and MDS	CR	CsA/MTX
BZ22	M	RIC	Negative	MUD	ATG	BM	AML and MDS	CD	CsA/MTX
BZ23	F	RIC	Positive	MRD	None	PB	AML and MDS	CR	CsA/MTX
BZ24	F	Micro	Positive	MRD	None	PB	Other	CD	CsA/MTX
BZ25	F	RIC	Negative	MUD	Thymo	BM	AML and MDS	CR	CsA/MTX
BS1	M	RIC	Negative	MRD	Thymo	BM	Lymphoma	CD	CsA/MTX
BS2	M	RIC	Positive	MRD	Thymo	BM	Other	CR	CsA/MTX
BS3	M	RIC	Positive	MUD	None	PB	ALL	CR	CsA/MTX
BS4	F	MA	Positive	MUD	Thymo	BM	ALL	CD	CsA/MTX
BS5	M	RIC	Positive	MMD	Thymo	PB	AML and MDS	CR	CsA/MTX
BS6	M	MA	Negative	MUD	Thymo	PB	ALL	CR	CsA/MTX
BS7	F	RIC	Positive	MMD	None	BM	AML and MDS	CR	CsA/MTX
BS8	F	MA	Positive	MRD	None	PB	ALL	CR	CsA/MTX
BS9	F	MA	Positive	MUD	Thymo	PB	ALL	CR	CsA/MTX
BS10	F	MA	Positive	MRD	None	PB	ALL	Ref	CsA/MTX
BS11	M	MA	Negative	MUD	Thymo	BM	AML and MDS	CR	CsA/MTX
BS12	M	MA	Positive	MRD	None	PB	Other	Ref	CsA/MTX

IS: Prophylaxis of GvHD MA: myeloablative RIC: reduced-intensity conditioning Micro: TBI200 cGy
MRD: Matched related Donor MUD: Matched unrelated Donor MMRD: Mismatched Related Donor
ATG Anti thymocyte globulin Fresenius ® Thymo: Thymoglobulin ®
PB: peripheral blood BM: bone marrow
AML and MDS: acute myeloid leukemia and myelodisplastic syndrome ALL: acute lymphoblastic leukemiaOther: Multiple Myeloma, Severe Aplastic Anemia, Chronic Myeloid Leukemia
CR: complete remission CD Chemosensitive Disease Ref Refractory Disease
CsA: Cyclosporin A MTX: methotrexate MMF: mycophenolate mofetil

Clinical data

Engraftment and disease control

All the patients engrafted at 30 days from transplantation, but 2 experienced a secondary graft failure, in one case recovered through donor lymphocyte infusions and in the second case the patient converted to a recipient hematopoiesis.

Relapse occurred in 3 patients at a median time of 98 days from transplant. All these patients died from leukemia progression shortly after relapse.

Twenty seven patients were in continuous complete remission at the end of follow-up.

Toxicity

Severe or moderate infections (CTC grade 3 or more) were reported in 16 patients during the follow-up, 1 patient died due to EBV infection and 1 due to gram negative septic shock. One patient died from myocardial infarction. Cumulative transplant related mortality at 365 days was 11% including one patient dead for acute GvHD.

CMV infection

CMV infection requiring treatment was diagnosed in 14 patients (39%), none developed CMV disease. Median time to anti-CMV treatment was 54 days from tranplantation.

Acute GvHD

Fifteen patients developed acute GvHD at a median time of 32 days from alloHSCT (clinical grade was 1 in one case, 2 in three cases, 3 in 10 and 1 grade 4). Acute GvHD was cause of death of a patient.

Chronic GvHD

Fifteen patients developed chronic GvHD at a median time of 166 days from alloHSCT. Mild cGvHD was diagnosed in 1 patient, moderate cGVHD in 7 patients and severe cGVHD in 7 patients. None died due to chronic cGVHD.

Histological data

Biopsy of the involved organ was performed in 14 cases of symptoms suggestive of a GvHD. In 12 cases the diagnosis was confirmed. The 2 unconfirmed cases resolved without immune-suppressive treatment: both express low levels of PHA-induced INF-γ at the time of histological sampling.

QuantiFERON-CMV®

After obtaining informed consent, and with the ethical committee approval, 178 whole blood samples from thirty six patients monitored through time were collected. According to the manufacturer specifications the test was positive in 95 cases, negative in 54 and indeterminate in 29. The indeterminate results were reported mostly in the first 2 months after transplantation and repeated negative tests were associated with moderate or severe infections (p=0,033) or TRM (p=0,02).

PHA-induced INF-γ production after alloHSCT

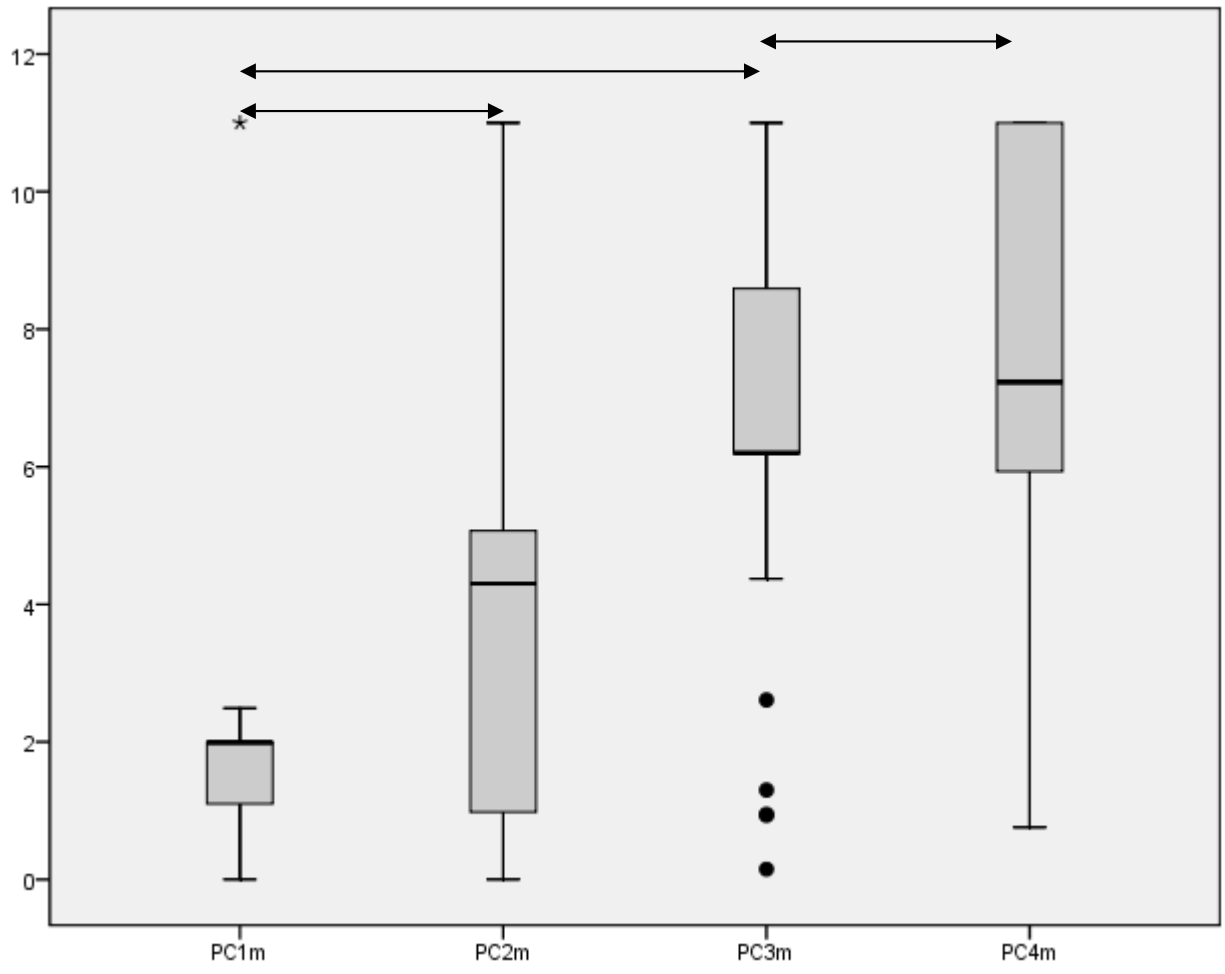
The values of PHA-induced INF-γ were normalized and grouped as following:

- First month after transplantation
- second month after transplantation
- third month after transplantation
- fourth month after transplantation
- fifth month after transplantation
- sixth-eighth months after transplantation
- ninth-twelfth months after transplantation

Missing data were analyzed for the multivariate analysis as the mean of the different categories.

Increase of PHA-induced INF-γ was observed (Figure 3) after alloHSCT and differences between all the possible combinations of the time points analysed was proved significant with the Shapiro-Wilk test (all p=0.000 except p=0.001 between third and fourth month).

Figure 3 Increase in PHA induced INF-γ (PC) at 1 month, 2 months, 3 months and 4 months after alloHSCT. Values expressed in UI/mL.



Descriptive and unviariate analysis

The increase of PHA-induced INF-γ after transplantation was detected in 24 patients after alloHSCT, 12 of them developed chronic GvHD in comparison to 4 patients out of 8 who do not experience increase in PHA-induced INF-γ (Odds Ratio 2,00, CI 0,473-8,462). A durable increase in PHA-induced INF-γ was associated with good outcome (less TRM, better rate of CCR, less CMV infections) however the differences were not proved significant.

PHA-induced INF-γ as diagnostic tool for chronic GvHD

Sensitivity and specificity of the test calculated with the ROC curve at the time of sampling obtaining an AUC of 0,61 with an high sensitivity for the cut-off provided by the manufacturer (97% at a value of less than 0.5 UI/mL).

Multivariate analysis

Acute GvHD

The cox regression model for time dependent variables identifies the following independent risk factors associated with acute GvHD:

- moderate or severe infections p=0,001

Chronic GvHD

In the Cox regression model for time dependent variables, the 2 step model fitted with our cohort of data and proved as independent risk factors for cGvHD the following variables

First step:

- female sex of recipient p=0.017
- age of recipient p=0.018
- source of stem cells p=0.031
- GvHD prophylaxis p=0.020
- infections p=0.026

Second step:

- increasing levels of PHA-induced INF-γ at first month p=0.021
- increasing levels of PHA-induced INF-γ at second month p=0.015
- increasing levels of PHA-induced INF-γ at third month p=0.027

GvHD

The same model was implemented for the onset of any grade or type of GvHD and the following variables were independently associated with GvHD:

First step:

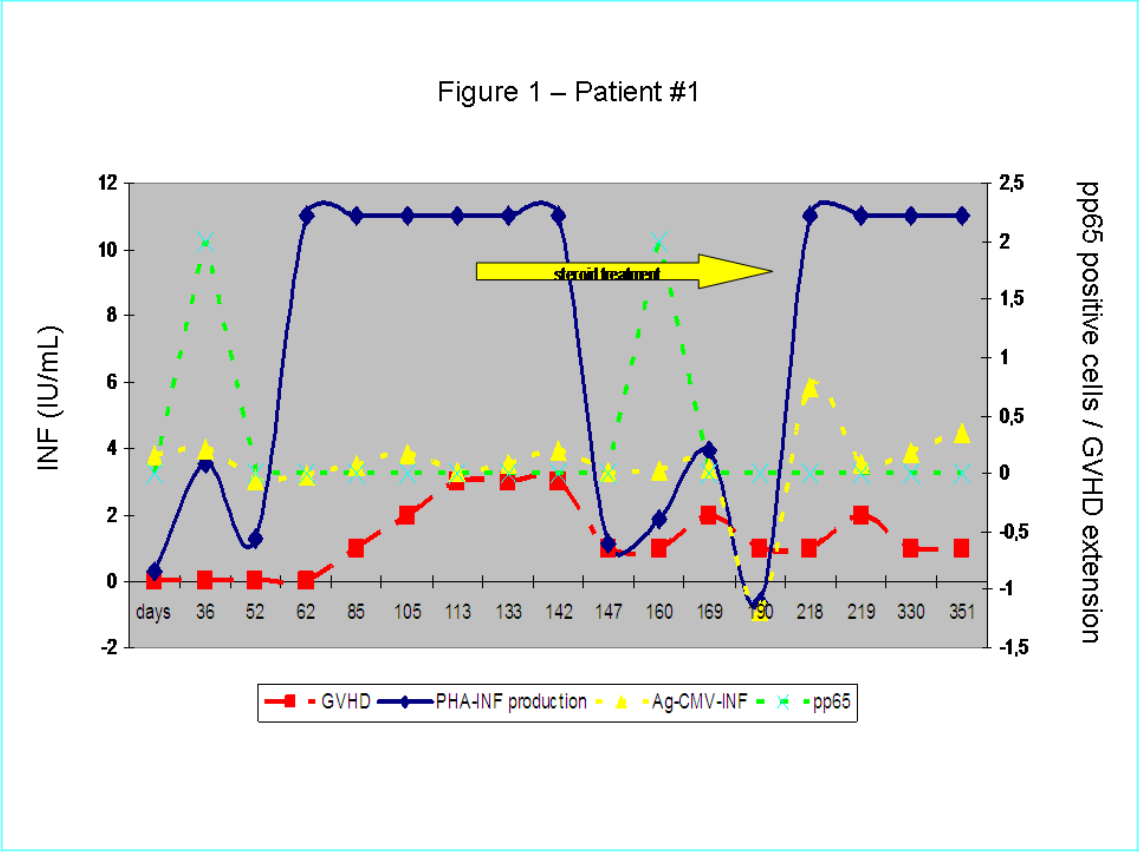
- MUD (Matched unrelated donor) p=0.031
- source p=0.015

Second step:

- CMV infection p=0.004
- increasing levels of PHA-induced INF-γ at first month p=0.000
- increasing levels of PHA-induced INF-γ at third month p=0.008
- Tapering of immune-suppression during the first 4 months p=0.000

All the patients treated with at least 2 immune-suppressive drugs for chronic GvHD experienced a marked reduction of PHA-induced INF-γ as displayed in the case reported below (Figure 4). One patient develops drug-resistan cGvHD and PHA-induced INF-γ values remains elevated during the follow-up.

Figure 4 – Example of immune-monitoring of a patient



Comment: in this figure we observe firstly an increase in PHA-induced INF-γ (blue line) and a subsequent increase in GvHD organ involvement (red line). The steroid treatment causes a reduction in PHA-induced INF-γ and subsequent immune-suppression (CMV infection, yellow line).

Discussion and conclusions

As more and more patients undergoing allogeneic hematopoietic SCT (HSCT) survive the early post-transplant period, the number of individuals at risk for chronic GvHD has grown. Treatment for established cGvHD remains unsatisfactory causing mortality, morbidity and impairs quality of life. Timing of Immune-modulation is a crucial issue in the management of the balance between tolerance and allo-reactivity. Evenmore, the lack of predictive markers for the onset of cGvHD and able to monitor response to therapy, is a major obstacle to this difficult management.

We proved that the increment of PHA-induced INF-γ within 4 months from alloHSCT is an useful tool to predict chronic GvHD during immune-suppressors tapering.

The strong association between the increment of PHA-induced INF-γ in the first month after alloHSCT could be explained since at this time the conditioning damage, the major responsible of the onset of subsequent acute GvHD (Ferrara 2009), could have sensitized the immunesystem versus a more reactive status. Furthermore the high rate of infections in this neutropenic phase (Young 2008)could act as a trigger of a more reactive status; finally the engraftment of NK cells, occurring after neutrophils engraftment in the first 40 days, could activate immune-system and INF-γ production (Pegram 2011).

As mentioned above, Hirayama et al. (Hirayama 2006) demonstrate that infections "per se" do not induce INF-γ production in the setting of alloHSCT, whereas acute GvHD induces an increase in INF-γ production, supporting the use of PHA induced INF-γ as early marker of GvHD.

The strong association between the increment of PHA-induced INF-γ in third month instead, could be explained since in the third month usually, if GvHD is absent, tapering of immune-suppression take place and onset of GvHD during tapering of Cyclosporin A is common: the tapering itself can be the reason for the more reactive status of immune-system (Soiffer 2008, Filipovich 2008).

If GvHD does not develop, the PHA-induced INF-γ could represent a "rough" test to monitor the "ability to react" of the immune-system against tumor alloantigens. In fact, all the patients in continuous complete remission have high levels of PHA-induced INF-γ. This data is in accordance with what was exstensively demonstrated infact, seceral groups demonstrated that INF-γ is a cornerstone cytokine for the graft versus malignancy effect (Holler 2002, Yang 2005, Laurin 2010).

PHA-induced INF-γ could be finally a reliable test in order to monitor response to immune-suppressive treatment of GvHD. Decrease of PHA-induced INF-γ was observed in all but

one patients treated with at least 2 immune-suppressive drugs and subsequently GvHD resolved. One patient developed drug resistant GvHD and its PHA-induced INF-γ remains elevated despite aggressive immune-suppression.

To our knowledge this is the first report showing this in vivo observation.

Although the multivariate analysis identifies PHA-induced INF-γ as independent risk factor for GvHD, the heterogeneity of this cohort of patiens could limit the reproducibility of the results and a larger cohort of patients should be analysed.

Unlikely, in our experience, the QuantiFERON-CMV® test was not accurate to identify patients at risk for CMV-infection. An high rate of Indeterminate and negative results were registered in the first 2 months, but no association with CMV infection was proved. During the follow-up instead no CMV disease was observed so it would be impossible to conclude anything about the predictivity of the test in a late timepoint after transplant. As a case report we can say that one patient (excluded from the analysis for GvHD) developed CMV enteritis after prolonged immune-suppression and repeated negative values of the test. Similar data were observed by Fleming et al. (Fleming 2010).

This test should be evaluated in a larger cohort of patients and possibly in different subsets of patients in order to detect cutoff values able to identify patients at risk of CMV infection or disease. At present the more sensitive immune-monitoring tests for CMV-specific response remain the tetramer based flow cytometric assays (Gratama 2010).

From our data we proved that the production of IFNgamma is a reliable marker of GvHD, with a high sensitivity (97%) at the time of the sampling. Although the AUC is low (0,61), the single protein used in the model of Ferrara's group (Paczesny2009) have a comparable AUC. For this reason, integrating this model with other biomarkers (haptoglobin for example as reported in McGuirk 2009) could improve the specificity of the test.

In conclusion, according to the NCI criteria for biomarkers in chronic GvHD, the limits and the possibilities of this simple method are:

- predicting response to therapy: a reduction in PHA-induced INF-γ is associated to a response to immune-suppressive therapy, but the number of treated patients was too little;
- measuring disease activity and distinguishing irreversible damage from continued disease activity: the test is too “rough” to define irreversible organ damage. Is is notably that prolonged inactivation of immune-system is associated with an higher TRM; the follow-up of these patients was limited to 365 days and is not able whether the damage of involved organ could be associated with elevated levels of PHA-

induced INF-γ.

- predicting the risk of developing chronic GvHD: the increase in the values of the test in the first 3 months after alloHSCT are associated with chronic GvHD. These results should be validated by larger prospective trials.
- diagnosing chronic GvHD: although the AUC was low, the test was highly sensitive to exclude GvHD. Higher levels of PHA-induced INF-γ are associated with cGVHD but the difference was not proved significant.
- predicting the prognosis of chronic GvHD: the aim of this study do not contemplate this outcome.
- evaluating the balance between GvHD and graft-versus-leukemia effects (graft-versus-leukemia or GVT): prolonged high levels of PHA-induced INF-γ are associated with a good outcome, but the GVT effect could be not separated from the GvHD.
- serving as a surrogate end point for therapeutic response: as mentioned above, although the number of patients treated for a cGVHD was low, there was a drop in the PHA-induced INF-γ values after treatment, preceding the clinical response. This test could help in the management of cGVHD if there is the need to avoid excessive immune-suppression in patients with an high risk of relapse. On the other hand, patients with a reduced risk of relapse (for example Severe Aplastic Anemia, or thalassemia) could be managed in order to avoid excessive "ability to react" of the immune-system.

This is the first demonstration of PHA-induced INF-γ as predictive marker of GvHD.

This is a very simple test that could help the physician in the management of immune-supppression after HSCT. The correct balance between risk of relapse and "ability to react" of the immune-system is the goal of new studies. New studies should be designed in order to improve specifity of the test and to validate the test in the different subsets of transplanted patients.

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