Stem cell transplation - Experimental

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HUMAN CORD BLOOD CD45+ CELLS PROTECT MICE BRAIN AFTER CLOSED HEAD INJURY

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Background: Traumatic brain injury is a major health care problem and a significant socioeconomic challenge worldwide, causing death and disability for at least 1.4 million patients in the United States alone each year. Regrettably, no therapeutic neuroprotective agents are clinically available to date. Cell therapy by umbilical cord blood (CB) transplantation has been lately suggested for the treatment of brain trauma, but although CB contains different stem cell populations, only the potential of mesenchymal stem cells was evaluated for this aim.
Aims: To explore the protective potential of CB CD45$^+$cells for the treatment of brain trauma.
Methods: In the present study, we used a closed head injury (CHI) mice model, in which a focal blunt injury over an intact skull is being induced by a standardized weight-drop device. The resulting mechanical impact triggers a profound neuroinflammatory response within the brain with high consistency and reproducibility, leading to neurological and cognitive impairment and breakdown of the blood–brain barrier. CB-cells were separated according to their CD45 expression and the derived populations were than transplanted into CHI mice. Animals were evaluated for their neurologic severity score (NSS) (motor ability, balancing, alertness) and under rotarod test (motor deficits and ataxia).
Results: We demonstrated that CB derived mononuclear cells (MNC) and CD45$^+$, but not CD45$^-$ -cell subset reduced the neurobehavioral and motor deficits which typically occur in a mouse model of CHI. Transplantation of CB-cells was given at day 1 or 7 post-trauma and their therapeutic effect was observed up to 35 days. A significant reduction in brain anatomical damage and head wound area were measured in treated mice from 2 to 7 days after cell transplantation, as evaluated both *ex vivo* and by non-invasive near-infrared measurement of the hemorrhage surface. CB cells which were administered either intracerebroventricularly or intravenously (iv) displayed similar efficacy. Transplanted cells, labeled with near-infrared dye and infused iv were detected at the site of injury, indicating their homing properties. Head *in vivo*and brain *ex vivo* imaging, taken at short times after cell transplantation indicated a fast increase in brains fluorescence 1.5 h after iv cells transplantation, which was reduced shortly thereafter (5 h). Acute (2 days) and chronic (35 days) after transplantation, differential levels of the BDNF, NGF and VEGF were measured in the ipsilateral and contralateral hemispheres. Finally, anti CD45 antibodies blocked the beneficial effects of the cord blood derived MNC, strengthening the notion that CD45$^+$ cells are responsible for the protective effect.
Summary and Conclusions: Altogether, these findings demonstrate the potential of the wide therapeutic window and protective properties of CB derived CD45$^+$ cell fraction in animal model of brain trauma. Based on the minimal manipulation for CD45$^+$ cells isolation from CB, their ability to reduce neurological deficits even when transplanted 7 days after the insult and their ability of fast homing to the brain, we propose that CD45$^+$ cells should be considered for translational therapy in treating patients with brain trauma.

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PYROSEQUENCING IS A NEW PROMISING APPROACH TO PERFORM HLA TYPING IN A QUICKLY, SIMPLE AND ACCURATE WAY

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Background: The HLA system is characterized by an high number of alleles and haplotypes because the HLA genes are the most polymorphic in the human genome. A variety of methodologies have been developed for HLA typing at the protein and nucleic acid level, but ambiguity can affect the possibility of the right call for each HLA-locus. Particularly phase ambiguities arise from the incomplete genomic coverage or the contemporary Sanger sequencing of two heterozygous alleles that determines different haplotypes. The new generation sequencing (NGS) technologies have the potential to perform HLA-typing in a rapid and accurate way without phase ambiguities.
Aims: A simple and reliable method to implement the knowledge of HLA compatibility between donor and recipient would be necessary to obtain a rapid and accurate HLA-typing and to reduce phase ambiguities. To this purpose in this study we evaluated the feasibility, reliability and robustness of the HLA-typing by NGS in 40 samples.
Methods: We performed high-resolution HLA-typing using pyrosequencing and subsequent bioinformatic analysis. Fourtheen amplicons for sample were synthesized using two custom assay. The output file was then uploaded into JSI SeqPilot software to align all sequences with the reference database (ref 3.9 2012).
Results: Using the method of Multiplex Identifier (MID) tag, we can pooling amplicons from different samples; so we have pooled 5 different samples into 8 sequencing runs (total 40 samples). The PCR reactions generate 560 amplicons who correspond to HLA-A/B/C exons2, 3 and4, DQB1 exons 2 and 3 and DPB1, DQA1, DRB1/3/4/5 exon 2. We have obtained over of 150 reads for most amplicons. The assignment of unambiguous genotype was possible on 45.5% of alleles. The ambiguities were related to the assay design (above all for class-II). Notably, some ambiguities on the locus B and C have a little biological importance because both alleles coding for the same peptide binding domain, instead the genomic differences between the two alleles were located on the transmembrane domain coding region. Ten cases analyzed in this study were also genotyped using conventional strategies, for the most part ssp with a concordance of 100%.
Summary and Conclusions: Clonal amplification and pyrosequencing strategy is a feasible and reliable method to perform the HLA-typing. This method discriminates very well the alleles that determines differences on the peptide binding domain. Using a NGS technique we have obtained a high-resolution HLA-typing for the most important loci of the samples, quickly and without phase ambiguities. This work was supported by Lions Club " Bassa Bresciana", BCC Pompiano e Franciacorta Founds and by Roche.

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A RANDOMIZED DOUBLE BLIND CONTROL TRIAL COMPARING FILGRASTIM AND PEGFILGRASTIM IN CYCLOPHOSPHAMIDE PERIPHERAL BLOOD HAEMATOPOIETIC STEM CELL MOBILIZATION–PRELIMINARY ANALYSIS

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Background: There were very few randomized trials published in full article comparing filgrastim and pegfilgrastim in peripheral blood haematopooietic stem cell mobilization (PBHSCM). None of them to our knowledge examined cyclophosphamide as single chemotherapy (CT) agent, which is the commonest CT mobilization in our centre.
Aims: Hence, we conducted a trial to compare filgrastim (F) and pegfilgrastim (PF) in cyclophosphamide PBHSCM. (NMRR ID: NMRR-10-755-6906)
Methods: This was a randomized double blind control trial. Only patient without previous history of apheresis was eligible. Cyclophosphamide 2g/m2 was given on Day (D) 1 On D3 onwards, patient received a daily subcutaneous injection at 6pm, which was either Arm 1: F 5μg/kg till completed apheresis, Arm 2: PF 6mg on D3, normal saline (NS) D4-10, or Arm 3: PF 6mg on D7, NS D3-6 & D8-10. Peripheral blood CD34+ cell count (PB34)(/μL) was checked in the early morning on D8,11, and onwards till stop apheresis. Only if PB34 was ≥10, apheresis was done and repeated until CD34+ cell collection of ≥2 × 10^6/kg.
Results: There was 153 patients enrolled between 1st September 2009 till 31st December 2012 (Arm 1 49 (32%), Arm 2 50 (33%), Arm 3 54 (35%). Basic characterics: M:F=75:78, Malay:Chinese:Indian:Others=88:49:10:6; diagnosis were acute leukemia, myeloma, and lymphoma=23 (15%), 33 (22%), and 97 (63%), respectively; mean (SD) weight (kg) 60.4 (16.6); median age (range) 41 (12-66). There was no significant difference of sex, ethnicity, diagnosis, status of disease (new, relapse, relapse ≥2), and response of disease during PBHSCM (CR, PR, primary refractory, relapse refractory), weight and age between the 3 arms. The successful mobilization rate for Arm 3 was 1.5 times higher than Arm 2 (RR=1.51, 95% CI=1.09, 2.09). Arm 1 seems to give 1.3 times higher than Arm 2 but the result was not significant (RR=1.29, 95% CI=0.90, 1.85). In comparison to Arm1, Arm 3 seems to have a higher mobilization rate but the result was not significant (RR=1.17, 95% CI=0.90, 1.53).
Summary and Conclusions: Pegfilgrastim 6mg given on D7 resulted in more successful PBHSCM than D3 probably because of the surge of neutrophil resulting in degradation of PF when given in D3. There was a favourable trend towards PF 6mg D7 compared to F 5μg/kg from D3 onwards.

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DIFFERENT DOSE OF ATG INDUCTION THERAPY IN HAPLOIDENTICAL HEMATOPOIETIC STEM CELL TRANSPLANTATION: LONG TERM EFFECT ON TH17 AND CONVENTIONAL T CELLS, NOT ON NK RESPONSES

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Background: Anti–T lymphocyte globulin (ATG) have been demonstrated to play important roles in graft-*versus*-host disease (GVHD) prophylaxis after myeloablative unrelated or HLA-mismatched transplantation resulting in a reduction of GVHD without compromising anti-leukemia activity. However, the risk of infectious complications and post-transplantation Lymphoproliferative disorder (PTLD) were shown to be significantly elevated. Therefore, to further explore the effect of ATG on immune responses would be help to explore the optimal dose of ATG induction in HLA-mismatched transplantation.
Aims: Based on the open-label, prospective, randomized trial to compare two different total doses of ATG (6 mg/kg, ATG-6 group) *vs.* (10 mg/kg, ATG-10 group) in patients receiving myeloablative conditioning prior to allo-HSCT from haploidentical donors in our center, weevaluated the effect of the two different doses of ATG in conditioning regimen on immune reconstitution.
Methods: Using flow cytometry, we prospectively collected 29 standard-risk patients (15 received 6mg/kg ATG and 14 received 10mg/kg ATG) every 1 months until half-year post-transplant continuously to investigate the reconstitution kinetics of NK cells and T cells subsets as well as Treg cells, T cells functions such as CD4 helper activity, *in vitro* cytokine responses ie IL-17 and IFN-γ.
Results: The reconstituted kinetics of CD3+, CD4+ T-cell and CD56+ NK-cell abolute numbers in peripheral blood were similar between patients in ATG-6 group and ATG-10 group during half-year after transplantation. There were no differences in the expression of KIR, NKG2C, NKP30, and CD57 within the CD3-CD56+ NK lymphocyte compartment between the patients in ATG-6 group and ATG-10 group no matter at day15, day 30, day 60, day 100 or day 180 after transplantation. Meanwhile, the cytotoxic function or the IFN-γ secretion of NK cells reconstitution between the patients in ATG-6 group and in the ATG-10 group were similar during half-year post-transplant. However, patients in ATG-6 group showed faster Th17 cells and conventional T cells reconstitution at day15, day 30, day 60, day 100 or day 180 after transplantation. The proportions of Treg cells tended to be high in patients of ATG-10 group compared to those of ATG-6 group.
Summary and Conclusions: ATG 10mg/kg applied in conditioning regimen showed impared Th17 cells and conventional T cells reconstitution, therefore would reduce the GVHD occurrence compared to ATG 6mg/kg after transplantation.

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CIRCULATING ENDOTHELIAL CELLS (CEC) ARE A RELIABLE AND DYNAMIC BIOMARKER OF ACUTE GVHD (AGVHD) IN PATIENTS UNDERGOING ALLOGENEIC STEM CELLS TRANSPLANTATION.

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Background: Acute GVHD (aGVHD) is one of the major cause of morbility and mortality in allogeneic stem cell transplantation (allo-SCT). Clinical and physiopathological evidences have shown that vascular endothelium could be a target of aGVHD in very early phase; therefore markers of endothelial damage are warranted as valuable support in aGVHD diagnosis and to monitor its response to immunosuppressive treatments.
Aims: The primary endpoint of our study was to investigate the values of CEC count in peripheral blood to diagnose and predict aGVHD in patients submitted to allo-SCT.
Methods: We conducted an explorative and prospective study to evaluate CEC count at different time points: before and at the end of the conditioning regimen, at engraftment, at aGVHD onset and at 1 and/or 2 weeks after corticosteroids therapy administration. The CellSearch System® was used to capture and enumerate CEC. Magnetic particles conjugated to anti-CD146 are used to capture CEC from 4.0 mL of peripheral blood. Enriched cells are stained with DAPI and anti-CD105-PE antibody. APC conjugated anti-CD45 is used to exclude leukocytes. Enriched and stained cells are dispensed into a MagNest® cartridge for magnetic mounting. The cartridge is scanned and individual images of cells are presented for review and scored as CEC, based on CD146+, CD105+, DAPI+ and CD45- phenotype and cell morphology.
Results: We studied 10 healthy subjects (controls) and 41 patients with hematologic neoplastic diseases (7 HL, 1 NHL, 2 AL, 12 AML, 5 ALL, 8 MM, 3 CLL, 1 CML, 2 SAA) undergoing allo-SCT from either HLA-matched familial (n=11) or unrelated donor (n=30). The median count in controls was2,5 CEC/mL (range 1-14). The median CEC/mL pre-allo-SCT was 20 (n=33, range 4-718, P<0.0001 compared to controls), going up to 33 CEC/mL (n=39, range 3-648, P=NS compared to pre-allo-SCT) at the end of the conditioning regimen. At aGVHD onset the median CEC/mL was 58 (n=17, range 16-299, P=0.0009 compared to pre-allo-SCT), while at aGVHD response the median CEC/mL decreased to 31 (n=12, range 6-107, P=0,0079 compared to aGVHD onset) after 1 week of steroids therapy, and to 10 (n=5, range 5-29, P=0,0017 compared to aGVHD onset) after 2 weeks of steroids therapy (Figure 1).

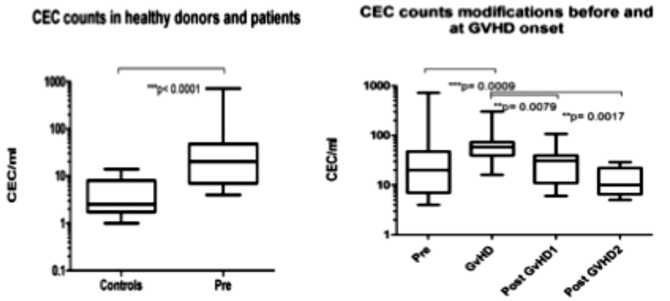

Figure 1.

Summary and Conclusions: Circulating endothelial cells can represent a promising marker to monitor endothelial damage in patients undergoing allo-SCT. We have showed a statistical significant increase in CEC numbers at aGVHD onset with a normalization at treatment response. CEC count helped us to settle between aGVHD and other transplant complications such as infections or autoimmune diseases leading to endothelial injuries: *e.g.* in a patient with diarrhea and low number of CEC, colon biopsy diagnosed infective colitis. The confirmation of the clinical utility of CEC counts, together with the use of a semiautomatic, standardized and reproducible technology, will allow a valuable help in the diagnostic definition of aGVHD in early phase and an information on the response to treatment.
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EFFECT OF GRANULOCYTE COLONY-STIMULATING FACTOR MOBILIZATION ON THE EXPRESSION OF REGULATORY GAMMA DELTA T CELLS

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Background: The immune modulatory effect of granulocyte colony-stimulating factor (G-CSF) on T cells resulted in an unexpected low incidence of graft-*versus*-host disease (GVHD) in allogeneic peripheral blood stem cell transplantation. Our previous studies demonstrated that G-CSF mobilization influenced the distribution and clonality of TRGV and TRDV repertoire (T cell receptors of gamma delta [GD]Tcells), and significant positive correlation was observed between the invariable clonality of TRDV1 gene repertoire after G-CSF mobilization and low incidence of GVHD in recipients (P=0.015, OR=0.047) (Li Xuan *et al.* Journal of Translational Medicine 2011). Regulatory GD T cells (GD Tregs), which express Foxp3 and primarily belong to $CD27^+CD25^{high}$phenotype, are a novel subset of cells with immunosuppressive function (Xiaoyan Li *et al.* Journal of Immunology 2012). However, whether G-CSF could influence the expression of GD Tregs remains unknown.
Aims: To investigate the effect of G-CSF mobilization on the expression of GD Tregs.
Methods: The immunophenotyping of GD Tregs was analyzed in peripheral blood mononuclear cells (PBMCs) from 20 donors before and after G-CSF mobilization, using flow cytometry.
Results: Compared with that before mobilization, the proportions of Vdelta 1 and $CD25^+$ subsets were significantly increased (P=0.012, P=0.032), whereas the Vdelta 2 proportion was significantly decreased after G-CSF mobilization (P=0.002). The proportions of total GD T cells, $CD27^+$ and $Foxp3^+$ subsets were similar between the two groups (P=0.133, P=0.110, P=0.780, respectively). In addition, there was a significant increase in the proportions of $Foxp3^+$Vdelta 1 and $CD25^+Foxp3^+$subsets (P=0.038, P=0.013), and a significant decrease in the proportions of $CD27^+$Vdelta 2 and $CD25^+$Vdelta 2 subsets after G-CSF mobilization (P=0.013, P=0.022). The proportions of $CD27^+$GD T, $CD25^+$GD T, $Foxp3^+$GD T, $CD25^+CD27^+$, $CD27^+Foxp3^+$, $CD27^+$Vdelta1, $CD25^+$Vdelta 1 and $Foxp3^+$Vdelta 2 subsets were similar before and after G-CSF mobilization (P=0.422, P=0.342, P=0.724, P=0.070, P=0.503, P=0.053, P=0.386 and P=0.097, respectively). We then compared the Foxp3, CD27 and CD25 phenotypes in total GD T cells, Vdelta 1 and Vdelta 2 subsets. We observed a significant increase in the proportion of $CD27^+Foxp3^+$Vdelta 1 subsets after G-CSF mobilization (P=0.036). The proportion of $CD27^+Foxp3^+$GD T and $CD27^+Foxp3^+$Vdelta 2 subsets before mobilization were similar to that after mobilization (P=0.539, P=0.507). The proportion of $CD25^+Foxp3^+$GD T, $CD25^+Foxp3^+$Vdelta1, $CD25^+Foxp3^+$Vdelta2, $CD25^+CD27^+$ GD T, $CD25^+CD27^+$Vdelta 1 and $CD25^+CD27^+$Vdelta 2 subsets were also similar between the two groups (P=0.249, P=0.539, P=0.507, P=0.934, P=0.209 and P=0.061, respectively).
Summary and Conclusions: G-CSF mobilization significantly increased the proportions of Vdelta 1 subsets, including $Foxp3^+$Vdelta 1 and $CD27^+Foxp3^+$Vdelta 1 subsets, whereas decreased the Vdelta 2 proportion.

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ENUMERATION OF RECENT THYMIC EMIGRANTS AFETER AUTOLOGOUS HSCT ACCORDING TO THE TYPE OF CONDITIONING: IRRADIATION VERSUS CHEMOTHERAPY

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Background: Stem cells present n the graft are predominant source of T cell recovery after HSCT. The process, however requires proper function of the thymus, which may be negatively affected by many factors including toxicity of the conditioning regimen. Experimental data suggest that irradiation may be more harmful to the thymus compared to myeloablative chemotherapy.
Aims: The goal of the current analyusis was to verify this hypothesis in a clinical setting of autologous HSCT.
Methods: The thymic function was evaluated by enumeration of circulating recent thymic emigrants (RTEs) determined by flow cytometry as CD4+CD31+CD45RA+CD62L+ cells. It was done before start of conditioning and on day +100 after autoHSCT. A total of 57 patients with hematologic malignancies 9mm, n=32, NHL, n=22; other, n=3) were included in the study. Median age was 57 (22-66) years. Patients were divided in 4 groups according to the type of conditioning; total body irradiation (TBI, 12Gy) monotherapy (n=20), TBI+CHT (n=11), total marrow irradiation (TMI) monotherapy (n=11) and chemotherapy alone (BEAM or high-dose melphalan, n=15). The groups did not differ in terms of age.CD34+ cell was significantly higher for TBI+CHT compared with remaining groups.
Results: RTEs could be detected in peripheral blood of all subjects prior to HSCT (median 21, range 2-261cells/uL) with no significant differences between groups (Kruskall-Wallis test, P=0,95). On day +100 after autoHSCT the highest number of circulating RTEs was observed for TBI alone (9,2-36/uL) followed by TMI alone (7,2-15/uL), CHT alone (5,2-24/uL) and TBI+CHT (3,0-14/uL) (Kruskall-Wallis test, P=0,03). In the post-hoc analysis (Umann-Whitney test) the values for TBI alone were significantly higher compared to TBI+CHT (P=0,01) and tended to be higher compared to CHT alone (P=0,11). Analysis of lymphocyte subsets on day +100 revealed no differences between groups for the number of circulating T CD4+ cells (P=0,41), T CD8+cells (P=0,52) and B cells (P=0,24). The number of Treg cells tended to be lower for TBI+CHT compared to other groups (P=0,07) and the number of NK cells was lower for TBI alone than for other groups(P=0,003).
Summary and Conclusions: We conclude that conditioning regimens have variable impact on thymic function after autoHSCT. As opposed to previously published data from animal models we demonstrated that TBI itself is not more harmful to the thymus than high-dose chemotherapy. In contrast, the addition of chemotherapy to irradiation increases significantly the toxicity.

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SERUM MICRORNAS SIGNATURES IN ACUTE GRAFT VERSUS HOST DISEASE (AGVHD) AFTER ALLOGENEIC HEMATOPOIETIC STEM CELL TRANSPLANTATION (ALLO HSCT)

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Background: aGVHD is one of the most frequent and lethal complications after allo HSCT, underscoring the need to develop novel therapies. To achieve this goal, aGVHD mechanisms needs to be further elucidated. Our group recently reported aberrant miRNA expression in donor T cells from animals and patients with aGVHD. Further studies focusing on miR-155 showed that this miRNA is modulating aGVHD. Emerging data also indicate that miRNAs are also present in the human serum and regulate immune responses. Here, we hypothesize that serum miRNAs expression is deregulated in aGVHD and could play a role in aGVHD pathogenesis or serve as biomarkers for the disease.
Aims: To identify miRNAs associated with aGVHD by performing serum miRNA expression analysis using deeP-sequencing in allo HSCT recipients at the time of clinical suspicion of aGVHD.
Methods: Peripheral blood (PB) samples were collected weekly until day 100+ and at the time of clinical diagnosis of aGVHD from allo HSCT patients enrolled into OSU11002 (a biorepositorium trial). After serum separation, total RNA was extracted using Trizol. Libraries were constructed using the small RNA profiling kit and sequenced on the Solid analyzer. A mouse model of aGVHD (B6 mice donor splenocytes and bone marrow cells transplanted to lethally irradiated F1 recipients) was used to assess serum miRNA expression in animals with aGVHD after transplant.
Results: In this study we included 10 patients with aGVHD (bowel aGVHD n=2; skin aGVHD (n=5) and both skin and bowel aGVHD (n=3). Median age was 51.9, conditioning regimens were myeloablative (n=1) and non-myeloablative (n=9) and the type of donors used were unrelated (n=9) and related (n=1). PB samples were obtained from these patients at the time of clinical suspicion of aGVHD. PB samples from allo HSCT patients with no aGVHD and matched for age, disease, conditioning regimen, donor and timing of sample collection were obtained and used as controls. Sequence alignment was performed using miRBase. The average reads count per sample was 875,000. Normalization as reads per million was followed by quantiles. First we compared miRNA expression between all patients with aGVHD (n=10) and controls (n=7) using BRB tools and class comparison. We found 7 miRNAs uP-regulated (miR-146a, miR-323-b, miR-34c, miR-363, miR-4245, miR-29a, miR-181a*) and 3 miRNAs down-regulated (miR-3168, miR-662, miR-550a) (Fold change (FC) >2, P<0.01). Next, we compared miRNA expression of patients with bowel aGVHD (n=5) *vs.* controls (n=7). We found 3 miRNAs uP-regulated (miR-146a, miR-4295 and miR-181a*) and 4 miRNAs down-regulated (miR-3168, miR-582, miR-193a and miR-662) (FC>2, P<0.01). Last we compared miRNA expression between patients with skin only aGVHD (n=5) and controls (n=7). We found 5 miRNAs uP-regulated (miR-323b, miR-34c, miR-3940, miR-3674, miR-4258) and 2 down-regulated (miR-3168 and miR-3678) (FC>2,P<0.01). MiR-3940 and miR-4258 are expressed exclusively in the skin (miRBAse). Remarkably, miR-146a, a miRNA associated with innate immunity, was found uP-regulated as well on serum samples from mice with aGVHD (n=6) compared with controls (n=6) (FC>2,P<0.01). Both, miR-181a and miR-29a, which are known to regulate immunity, were found deregulated in serum samples from patients with aGVHD. Ongoing experiments are being performed to dissect the function of those miRNAs.
Summary and Conclusions: miRNAs related to skin and immune system regulation were found to be de-regulated in the serum of allo HSCT recipients with aGVHD suggesting that they may play a role in the modulation of this process.

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PARALEL STUDY OF CHIMERISM AFTER ALLOGENIC HAEMTOPOIETIC STEM CELL TRANSPLANTATION MONITORED BY TWO DIFFERENT MOLECULAR METHODS

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Background: Over the past decades, allogeneic stem cell transplantation (allo-SCT) has gained increasing importance as a treatment option for patients with both malignant and non-malignant life threatening disorders. Surveillance of chimerism after allo-SCT seems an indispensable tool for the clinical management of transplant recipients. There are many genetic methods used for this purpose. The FISH analysis of chromosome X and Y is applicable only in sex mismatched transplantations. The most widespread method for testing of chimerism is the PCR-based analyses of highly polymorphic short tandem repeats (STR). Real-time PCR of SNP or NPs isn´t used so frequently.
Aims: To compare patient´s chimerism after allo-SCT measured using relative quantification (RQ PCR) of SYBR green-based real-time PCR of SNP or NPs or using PCR-based analysis of STR markers by fragment analysis on an automated genetic analyzer.
Methods: Whole peripheral blood samples were collected for DNA extraction from both the donor and recipient before transplantation in order to determine an informative marker. The blood samples of patients after allo-SCT (N=65 pairs) were collected at regular intervals at the Department of Hematology and Transfusion, Comenius University Medical School, Bratislava, Slovakia and provided for chimerism testing. RQ-PCR was performed by the real-time PCR system using SYBR green and 12 pairs of specific primers for two allelic variants of DNA polymorphism and GAPDH as endogenous gene control. The STR analysis was performed using of commercially available STR multiplex amplification kits with fluorescently labeled PCR primers. The quantification of donor and recipient signal was done by comparing the fluorescence intensity given by the peak area of analyzed fragments.
Results: We screened 65 related and unrelated donor/recipient pairs by both methods and we found at least one informative marker. The quantification of informative markers was provided at the same time by both methods in parallel and estimated chimerisms were compared. We found that our results were identical only in 2% and the discrepancy was noticed also in 2% between the two methods used. In the case of 1-50% mixed chimerism (MC) similar results were obtained. However, complete chimerism (CC) estimated by the fragment analysis was evaluated as mixed chimerism (MC) by the real-time PCR in 94% patients, mainly in the first half of a year of the post-transplantation monitoring. The example of the parallel monitoring of one patient is shown in Figure1. Figure 1 Monitoring of chimerism after allogeneic stem cell transplantation (allo-SCT) by analysis of STR markers by fragment analysis and by relative quantification (RQ PCR) of SYBR green-based real-time PCR of SNP or NPs.

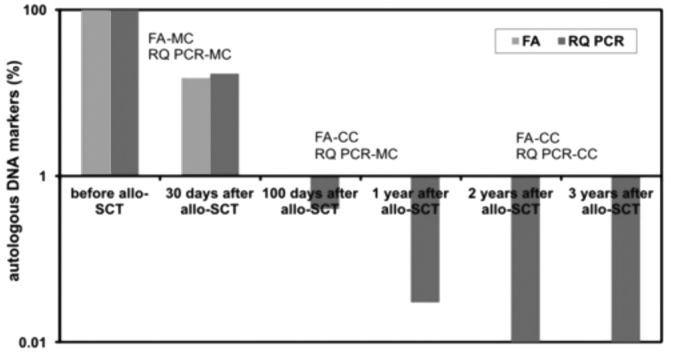

Figure 1.

Summary and Conclusions: Both methods compared above are suitable for chimerism assessment after the allogeneic SCT. Complete chimerism detected by the fragment analysis (FA) and mixed chimerism (MC) detected by the real-time PCR was due by the different sensitivity of two methods used. RQ PCR had the higher sensitivity (<1%) for the detection of the autolologous DNA markers than FA, so it is better for earlier revealing of eventual relaps. On the other hand the quantification of donor´s DNA markers is more precise estimated by the FA.

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VENO-OCCLUSIVE DISEASE MAY DEVELOP IN SECONDARY IRON OVERLOADED MICE AFTER TOTAL BODY IRRADIATION.

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Background: The outcome of hematopoietic stem cell transplantation (HSCT) is poor in patients with secondary iron overload (SIO). High incidence of veno-occlusive disease (VOD), acute GVHD, and infection were observed in SIO patients treated with HSCT.
Aims: We evaluated the relation between SIO and VOD in an animal model of HSCT.
Methods: We used 6 week-old female BDF1 (H-$2^{b/d}$) as recipient and male C57/BL6 (H-2^{b}) as donor. Recipient mice were injected intraperitoneally with 10mg of iron dextran according to experimental design (cumulative dose: 50mg, 100mg, and 200mg). All mice were treated with HSCT including total body irradiation designed by dose. Also, some mice without SIO were treated with allogeneic or syngeneic HSCT. We obtained peripheral blood for alanine aminotransferase (ALT) and liver for pathologic findings, lipid hyperoxide (LH), and liver iron content (LIC) as reactive oxygen species on post-HSCT day 1 and day 7. The score for VOD was assessed by pathologic findings (Hepatology, 1999;29:1779-91).
Results: All mice with SIO died within 10 days of HSCT. ALT level was increased depending on cumulative iron dose, with a significant difference between day 1 and day 7 for 200mg iron group (P<0.01). LH level was significantly increased in the 200mg iron group than in other groups (P<0.01). For the 100mg iron group, the LH level depended on radiation dose (P<0.01). There was a statistically significant relation among ALT, LH and LIC parameters (ALT *vs.* LH; r^2=0.911, ALT *vs.* LIC; r^2=0.548, LIC *vs.* LH; r^2=0.564). Also, the pathologic scores for VOD correlated with LIC (P<0.01, r^2=0.597).
Summary and Conclusions: The liver with SIO showed a high level of ROS that depended on cumulative iron dose, which also showed correlations with elevated liver enzyme and LIC. The pathologic score for VOD was associated with LIC. Our results suggest that SIO may induce VOD after HSCT done with irradiation. Iron chelation may improve outcome in SIO model of HSCT.

P885

ASSESSMENT OF CMV SPECIFIC IMMUNITY BY FLOW CYTOMETRY USING A COMBINED APPROACH OF SPECIFIC CMV CELL ENUMERATION AND FUNCTIONAL T CELL ANALYSIS

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Background: CMV reactivation is one of the most frequent infectious complications following allogeneic stem cell transplantation (SCT). CMV specific memory CD8+ cells (CMV CD8+ Tly) play crucial role in the regulation of CMV infection. In practice, CMV specific response can be monitored by detection and quantification of CMV CD8+ Tly using tetramer technology and/or *ex-vivo* assessment of CMV induced T cell function.
Aims: To study the recovery of CMV specific immunity by analysing the functional activity of CMV stimulated CD8+ Tly in terms of simultaneous production of IL-2 and IFNg in correlation with CMV CD8+ Tly enumeration by CMV tetramer test.
Methods: In 37 patients after SCT, we determined the presence of CMV CD8+ Tly using the CMV tetramer test and simultaneously evaluated the functional status of CMV stimulated CD8+ Tly by evaluation of intracytoplasmic presence of both IL-2 and IFN-g using multiparameter flow cytometry (MPFCM).
Results: During the last 4 months, 37 patients were analyzed on day +60, +150, +240, +360 following SCT. In 10 (27%) patients, we demonstrated the presence of CD8+IL-2+INFg+ double positive cells, indicating sufficient CMV specific response of Tly in agreement with literary data. In 7 of 10 cases (70%), we proved the presence of CMV CD8+Tly: 203 c/µl (min. 51 c/µl, max. 785 c/µl). All of these 10 patients didn't experience any CMV complications. In 3 of 37 patients (8%) suffered from severe CMV infection and neither CMV CD8+ Tly, nor CMV CD8+IL-2+,IFNg+ Tly were identified.
Summary and Conclusions: Preliminary results suggest possible correlation between *ex-vivo* functional activities of CMV stimulated Tly and the presence of tetramer positive CMV CD8+Tly in concentration >10 c/µl. Further analysis is necessary to confirm this observation and implement both techniques in clinical practice for laboratory evaluation of CMV specific immunity recovery and optimal timing of anti-CMV therapy.

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SERUM FREE LIGHT CHAIN (SFLC) AN EARLY PROGNOSTIC MARKER OF OUT-COME IN LYMPHOMA AND LEUKEMIA PATIENTS POST ALLOGENEIC TRANSPLANTATION

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Background: Early markers of allogeneic transplant engraftment and graft *versus* host disease (GVHD) may aid patient management. Previously a small study of 47 multiple myeloma patients undergoing allogeneic transplant identified changes in serum free light chain (FLC) production (of the non-clonal isotype) as markers of allogeneic transplant outcome.
Aims: Here we present data looking at the delta changes of FLC compared to baseline and comment on their role as markers of immune-reconstitution in a group of leukemia and lymphoma patients.
Methods: Serum from 19 lymphoma (13 NHL, 6 HL), 73 leukemia (53 AML, 7 CML, 6 ALL, 4 PLL, 3 CLL) and 8 patients with other hematological disorders (3 MDS, 2 MF, 2 aneuploidy, and 1 ATLL) taken at baseline (median 7 days before transplant (range 1-19 days) and 5 weeks post-transplant were analysed retrospectively (male/female ratio 61/39, median age 51 years, range 19-71). FLC kappa and lambda were measured using automated immunoassays nephelometrically and results summated (κFLC+λFLC) to give a combined polyclonal FLC measurement (cFLC). Delta cFLC were characterised as increased, stable or decreased. Intact immunoglobulin (IgG, IgA, IgM) measurements were analysed in a similar fashion. All statistics were performed on SPSS v19.0.
Results: Median cFLC values at baseline and at week 5 post-transplant were 25 mg/L (range 2.21-371) and 15.63 mg/L (range 0.54-56.73), respectively. At week5, the absolute values of cFLC increased in 25/100 (median increase 7.19 mg/L, range 0.18-38.15) and decreased in 75/100 patients (median decrease 13 mg/L, range 0.02-335.51). Overall survival (OS) was better in patients with increased delta cFLC and these patients were more likely to have acute GVHD (aGVHD) compared to patients with decreased delta cFLC (75%ile survival rate: not reached v. 231 days, respectively, P<0.001; 50%ile rate of aGVHD free survival: 55 days v. not reached, respectively, P=0.004). Due to assay analytical variation, subtle changes in the delta cFLC may not represent increase or decrease in cFLC levels. A cut-off of ±8 mg/L was used to define delta cFLC in to 3 groups: 1) increased, 2) stable, or 3) decreased. Patients with increased delta cFLC (n=10) had a better OS compared to patients with stable (n=36) or decreased (n=54) delta cFLC and patients with stable delta cFLC had a better OS compared to patients with decreased delta cFLC (75%ile survival rate: not reached v. 533 days v. 206 days, respectively; P=0.001). In comparison, changes in intact immunoglobulin measurements did not offer significant information on the outcome of these patients (p value for IgG P=0.34; IgA P=0.15; and IgM P=0.057).
Summary and Conclusions: Delta increases in FLC correspond to better OS and an increase in GVHD, suggesting they may be early markers of engraftment and immune reconstitution. Changes in intact immunoglobulins did not offer the same information, possibly reflecting longer serum half-lives which may buffer subtle changes in concentrations.

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STUDY OF SOLUBLE HLA-G LEVEL DYNAMICS IN HEMATOLOGICAL PATIENTS UNDERGOING AUTOLOGOUS OR ALLOGENEIC STEM CELL TRANSPLANTATION

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Background: Human leukocyte antigen G (HLA-G) is a non-classical major histocompatibility complex (MHC) class I molecule with immune-modulatory properties. HLA-G expression is highly tissue restricted and can be elevated by a variety of inflammatory cytokines including IL-10 and INF-γ, heat shock, radiation, oxidative stress and hypoxia. Aberrant HLA-G expression was found in a variety of pathologic situations, including solid as well as hematologic malignancies (AML, CLL, NHL and HL). Soluble HLA-G (sHLA-G) is generated by proteolytic cleavage of HLA-G1 surface antigen. Increased sHLA-G plasma levels has been suggested as disease specific markers in several solid tumors, with higher levels correlating with an advanced disease status. HLA-G molecules have an inhibitory effect on cells of the immune system including NK, T cells and dendritic cells, suggesting that they may have a role in immune surveillance and transplantation. In hematological malignancies both tumor and the BM stroma may contribute to plasma sHLA-G. We hypothesized that sHLA-G is increased in patients (pts) with hematological disorders and may have a biological significance in patients undergoing either autologous (Auto) or allogeneic (Allo) SCT.
Aims: To study plasma sHLA-G levels and dynamics in pts with hematological disorders undergoing SCT.
Methods: Patients undergoing SCT between 08/2006 and 08/2009 were included in this study. sHLA-G was determined in ACD plasma collected before conditioning and at 2 days intervals until engraftment. Concentrations were determined by ELISA using MEM-G and HRP-antiB2-microglobulin as capture and detection antibodies, respectively, and are expressed as ng/mL. sHLA-G levels determined during conditioning were compared with baseline values and are expressed as fold changes (level at a specific day/baseline).
Results: 106 pts, median age 50 years (range 1-72), F/M 44/62 undergoing either Auto (n=27) or Allo (n=79) SCT were included in the study. Their baseline characteristics are shown in Table 1. Mean sHLA-G level prior to SCT was 40.7 ng/mL (range 5-148) in the entire cohort and was significantly different according to diagnosis (mean±SEM); 71±17 ng/mL in HL, 56±12 ng/mL in ALL, 55±16 ng/mL in severe aplastic anemia, 55±5 ng/mL in CML, 52±9 ng/mL in myelofibrosis, 48±4 ng/mL in CLL, 40±8 ng/mL in MDS, 37±8 ng/mL in NHL, 32±3 ng/mL in AML and 23.50±5 ng/mL in MM (one way ANOVA, P=0.0123). Mean sHLA-G levels were similar in pts transplanted in CR1 or 2 (42±7 ng/mL), active disease (PR+PD) (40±6 ng/mL) and in pts with no prior therapy (39±7ng/mL). sHLA-G levels gradually increased in Allo SCT pts, achieving maximal levels at engraftment±5 days, with mean fold changes from baseline of 2.6±0.4. In contrast, sHLA-G level in Auto SCT pts remained relatively stable with mean fold change at engraftment of 0.8±0.2 compared to baseline levels. Pre-transplant sHLA-G levels in Allo SCT pts developing acute GVHD (n=24, 30%) were not significantly different from pts with no GVHD (43±6 ng/mL and 44±5 ng/mL, respectively), however in AML pts, pre-transplant higher sHLA-G levels were observed in pts with acute GVHD (41±10 ng/mL) compared to those without acute GVHD (31±4 ng/mL), P=0.3.

Table 1. Clinical characteristics of study patients.

	N = 106
Age (years) median (range)	50 (1-72)
Gender F / M	44 / 62
Diagnosis N (%) Acute leukemia- AML / ALL Multiple myeloma MDS Lymphoma – HL / NHL Severe aplastic anemia Myelofibrosis CML CLL Unknown	 24 (23%) /10 (9%) 13 (12%) 10 (9%) 6 (6%) / 16 (15%) 6 (6%) 3 (3%) 3 (3%) 5 (5%) 10 (9%)
SCT-N (%)- Allo / Auto	79 (75%) / 27(25%)
Disease status at SCT N (%) CR1 / CR2 PR / Progressive disease Untreated Unknown	 11 (10%) / 13 (12%) 20 (19%) / 18 (17%) 18 (17%) 26 (25%)

Summary and Conclusions: sHLA-G levels in patients with hematological disorders are quite variable with the highest values found in HL pts. Serial plasma sHLA-G measurements in SCT pts demonstrate different dynamics in Allo SCT compared to Auto SCT. Further investigation into the clinical significance of these findings is warranted.