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Extracellular Vesicles as Potential Biomarkers of Acute Graft-*vs*.-Host-Disease

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Abstract

Acute Graft-vs.-Host Disease (GVHD) is a serious complication after allografting. We carried out an exploratory study to investigate a potential correlation of surface antigens on Extracellular Vesicles (EVs) and acute GVHD. EVs were extracted from serum samples from 41 multiple myeloma patients who underwent allografting. EVs were characterized by flow-cytometry using a panel of 13 antibodies against specific membrane proteins which were reported to be predictive of acute GVHD. We observed a correlation between 3 potential biomarkers expressed on EVs surface and acute GVHD onset by both logistic regression analysis and Cox proportional hazard model. In our study, CD146 (MCAM-1) was correlated with an increased risk - by almost 60% - of developing GVHD, whereas CD31 and CD140-α (PECAM-1 and PDGFR-α) with a decreased risk - by almost 40% and 60%, respectively -. These biomarkers also showed a significant change in signal level from baseline to the onset of acute GVHD. Our novel study encourages future investigations into the potential correlation between EVs and acute GVHD. Larger prospective multi-center studies are currently in progress.

Introduction

Acute and chronic Graft-*vs*.-Host Disease (GVHD) remain major causes of transplant-related toxicity and mortality after allografting[1–3]. These two syndromes differ in clinical characteristics and time of onset. Distinct T cell subsets and cytokines are involved in their pathogenesis. Acute GVHD has been associated with increased early mortality. The discovery of reliable, non-invasive, prognostic biomarkers of acute GVHD may be a major advance to improve clinical outcomes. Several potential biomarkers such as single nucleotide polymorphism (SNPs) [4,5], miRNAs[6–9], and cytokines, chemokines and their receptors[10,11], have so far been investigated. Overall, biomarkers may be classified in systemic ones, which change their level in response to systemic injury, and organ-specific ones which are associated with targeted tissues. Among others, the former include miRNA such as miR-423, miR-199a-3p, miR-93, and miR-377; ST2 (suppression of tumorigenicity 2); and several biomarkers of immune-activation[12]; while the latter include REG3a, CK-18, S100, and TIM-3 for gastro-intestinal GVHD, HGF for liver-GVHD, and elafin for skin-GVHD[12]. Several investigational studies showed correlations between single or combinations of biomarkers and acute GVHD outcomes. However, no current validated test has reliably become available to predict the onset of acute GVHD and/or its response to treatment. Extracellular Vesicles (EVs) have recently emerged as a promising new category of biological biomarkers in different scenarios. Secreted by many cell types, EVs are membrane-enclosed structures which include exosomes, shedding vesicles or microvesicles and apoptotic bodies. EVs play an important role in the intercellular crosstalk and in direct cell-to-cell interactions, in the interplay between cells and the extracellular matrix - juxtacrine signaling -, in the secretion of soluble factors such as cytokines, chemokines, growth factors, and hormones[13–15]. EVs carry proteins, bioactive molecules, DNA and miRNA, and EVs composition may greatly differ depending on patient status and cell of origin[16]. High concentrations of EVs can be found in blood and urine. Notably, EVs can non-invasively be extracted from these body fluids. Cells release EVs under a variety of physiological and pathological conditions. Over the past few years, many studies have shown that EVs contain nucleic acids and proteins implicated in cancer and in many conditions such as neurodegenerative, metabolic, and infectious diseases[16–20].

In the light of these findings, we carried out an exploratory study to characterize EVs surface antigens by flow cytometry and to investigate the potential correlation of specific membrane proteins and acute GvHD in a series of multiple myeloma patients who underwent allografting[21–26].

Materials and methods

Patients, transplant characteristics, and graft-vs.-host disease

Forty-one multiple myeloma patients who underwent an allograft at our Center were included. Patient and transplant characteristics are reported in Table 1. Overall clinical outcomes were reported in previous reports[27–32]. All patients received G-CSF mobilized peripheral blood (PB) stem cells as stem cell source and 37/41 (90%) were prepared for transplant with a non-myeloablative/reduced intensity conditioning. Non-myeloablative/reduced intensity regimens consisted of low dose 200 cGy total body irradiation (TBI) with/without fludarabine (no.=33/41, 80%) or with fludarabine/melphalan with/without low dose TBI (no.=4/41, 10%). During neutropenia, patients received prophylactic cephalosporins. Long-term prophylaxis against Herpes Virus and Pneumocystis jirovecii was performed in all patients. Pre-emptive antiviral therapy was initiated for positive Cytomegalovirus (CMV) antigenemia and/or for CMV DNA viral load >10.000 copies/ml in peripheral blood. Irradiated red blood cell units and buffy-coat derived platelet units were transfused for hemoglobin levels of < 8 mg/dl and platelet counts < 20.000 / ul respectively or as clinically indicated. Acute GVHD was diagnosed according to Glucksberg score and clinical diagnosis of chronic GVHD was scored following the NIH indications [33,34]. The study was conducted after obtaining informed consent and according to the Declaration of Helsinki.

Serum samples collection

Serum samples were obtained from peripheral blood draws. After collection, samples were kept at room temperature (RT) for 30 minutes to allow clotting. Serum was than obtained by centrifuging at 1,500 x g for 13 minutes in a refrigerated centrifuge and stored at −80 °C until use. For each patient, serum samples were collected before transplant, and at the following post-transplant days (median): +28; +58; +92; +119; +147; and +179, or at disease relapse.

Extracellular Vesicles extraction

Patient serum samples (1 ml each) were thawed on ice. EVs were then precipitated by adding 250 μl of precipitation solution [composition for 20 ml of solution: 5 g of Poly Ethylene Glycol (PEG 35,000; Merck KGaA, Darmstadt, Germany) and 0,25 g of Protamine-(P) (Sigma-Aldrich, St. Louis, MO) resuspended in deionized water][35]. Samples were vortexed for 10 seconds and incubated at 4°C for at least 1h and centrifuged at 1500

x g at RT for 30 minutes. Supernatant was removed and samples centrifuged at 1500 x g at RT for 5 minutes. EVs pellets were re-suspended with 150 μl of Roswell Park Memorial Institute (RPMI) medium supplemented with antibiotics and antimycotic (Penicillin, Streptomycin, Amphotericin b), plus 10 % of DMSO, and stored at -80 °C. EV size was characterized by Nano-particle tracking (NTA) analysis. Briefly, EV preparations were diluted (1:100-1000) in sterile saline solution 0.9% and analyzed by using a NanosightLM10 instrument (NanoSightLtd., MintonPark, United Kingdom) equipped with the nanoparticle tracking analyses 2.0 analytic software[36].

Samples of 3 ml from buffy-coat derived platelet Units were centrifuged at 1500 x g at RT for 30 minutes to pellet white blood cells. Supernatant was then centrifuged 3 times at 2000 x g at RT for 30 minutes to precipitate platelets. Complete depletion of platelets was verified using a Sysmex XS-1000i Dasit cell counter before EVs precipitation.

Extracellular vesicles preparation for flow cytometry analysis

EVs were characterized by flow cytometry using Fluorescein Isothiocyanate (FITC) or Phycoerythrin (PE) conjugated antibodies. Initial screening was carried out on EVs extracted from 9 healthy donors and 2 transplant patients, one with GVHD and one without, and included a panel of mouse antibodies against the following 23 markers: CD44, CD138, CD146, CD120-α, CD8, CD81, CD63, CD25, CD31, CD144, CD14, CD15, CD42b, CD9, CD3, CD86, CD45, CD40, CD105, CD30, CD106 (all antibodies from Miltenyi Biotech, Bergisch Gladbach, Germany), CD140-α (BioLegend, San Diego, CA), KRT18 (Abnova,Taiwan). Mouse non-immune isotypic FITC or PE IgGs (Miltenyi Biotech, Bergisch Gladbach, Germany) were used as negative controls. Hundred μl of FACS flow dilution buffer (Becton Dickinson Biosciences, San Jose, CA), filtered using a 0,1 μm syringe, was added to 1-4 μl of EVs, and incubated at RT with antibodies in the dark for 20 minutes. Reactions were stopped by adding filtered FACS flow dilution buffer (400 μl in control samples with non-immune isotypic IgGs, and 300 μl in study samples with antibodies), and immediately acquired using a Guava Instrument (GUAVA easyCyte™ 8, Millipore).

In the light of expression levels between donors and patients (Supplementary Figure 2-5), a second panel of 13 potentially informative GVHD biomarkers was selected as study panel: CD44, CD138, CD146, KRT18, CD120-α, CD8, CD30, CD106, CD25, CD31, CD144, CD86, and CD140-α.

FACS measurements and analysis

The Millipore GUAVA easyCyte™ 8 was cleaned using the Guava® Instrument Cleaning Fluid (EMD Millipore, Billiberica, MA, USA) and rinsed several times with nuclease free water (AMBION) before each measurement. A filtered FACS Flow solution (with a 0.1 μm syringe filter) was acquired to set gates and to discriminate true EVs events from background noise. Signals from EVs samples incubated with non-immune isotypic IgG controls were used to differentiate specific from non-specific antibody binding. A total of 5,000 events were acquired each time at low speed for each marker to determine a) fluorescence mean value; b) percentage of positive EVs for a given marker; c) total EVs concentration; d) and concentration of positive EVs for a given marker. Data were then analyzed using the GuavaSoft ImCyt 2.5 program.

Statistical analyses

Cumulative incidences of acute and chronic GVHD were calculated from the date of transplant to the date of onset of GVHD. The estimations were performed considering death from any cause as competing event according to the method by Gooley et al.[37]. Patients alive without GVHD were censored at the date last known to be alive. Correlations between biomarkers were evaluated by Spearman's rho correlation coefficient. Effects of repeated measurements of each marker on incidence of acute were analyzed dividing the follow up of each patient in period of 30 days. Patients were classified by presence/absence of GVHD (0=absent, 1=present) during each period. In case of more than one measurement for a given marker in the same 30-day periods, the closest to the date of transplant was considered. Thus, the probability of developing GVHD in each period with respect to marker levels, evaluated as both absolute measure and as change from pre-transplant values, was calculated by logistic regression model. Effects on GVHD incidence were reported as standardized Odds Ratio (OR), reporting the effect for a 1- standard deviation (SD) increase for a given variable per 1-point increase (relative increase of 100%) and corresponding p value for statistical significance. Given that analyses were based on repeated measurements on the same patient, ORs were estimated controlling the standard errors with the Huber-White Sandwich Estimator[38]. Sensitivity analyses were performed estimating the ORs after an imputation of missing values in each 30-day period using the last observation carried out for each individual patient. Moreover, for sensitivity analysis, Cox proportional hazard models for acute GVHD were estimated using EVs parameters at each time-point as a time varying covariate and

reporting the Hazard Ratios (HRs) for 1-SD increase. All reported p-values were two-sided. All statistical analyses were performed using STATA 11.2 (Stata Corp LP).

RESULTS

Clinical Outcomes

Acute GVHD requiring systemic therapy was observed in 23/41 (56%) of patients (Table 1). Skin and gastrointestinal tract were the most frequently involved sites. Median day of onset (range) was day +40 (+22-+145). Though the analysis of chronic GVHD was out of the scope of this study, chronic GVHD was observed in 29/40 (72%) of evaluable patients (one patient died within 100 days post-transplant). Cumulative incidences of acute GVHD and chronic GVHD at day +100 and +400 respectively were 56.25% and 70.7% (Figure 1). Twenty/41 (49%) patients experienced CMV reactivation, but no CMV disease within 100 days post-transplant. Median day of CMV reactivation was day +48 (range 27-81). No other viral infections were documented. Overall, 12/41 (29.2%) patients received buffy-coat derived platelet transfusions, only 5/41 (12%) within the 7 days preceding sample collection for EVs measurements.

EVs Characterization

The initial characterization of EVs surface was carried out with the first panel of 23 biomarkers previously correlated with GVHD (Supplementary Table 1 and Supplementary Figures 2-5). Mean fluorescence measurement and percentage of positive EVs were obtained for each marker. Thirteen potentially informative biomarkers were selected for our study population in the light of expression levels and their variations over time, and the differences between patients and donors (Table 2). Figure 2 illustrates representative examples of flow cytometry physical parameter dot plots and of fluorescence distribution of EVs after incubation with anti-CD8-FITC, anti-CD31-FITC, anti-CD146-FITC, anti-CD140-α-PE and corresponding negative controls. The difference between the isotype and biomarker mean fluorescence distributions represents the biomarker expression level on EVs surface. Mean fluorescence, percentage of EVs labelled with anti-CD140-α-PE and total EVs concentration are plotted in Figure 3. Similar plots were obtained for each biomarker (Supplementary Figure 1).

Correlation between biomarkers and onset of acute GVHD

Table 3 illustrates the association between variation or absolute levels of each marker and onset of acute GVHD. Three biomarkers, CD146, CD31 and CD140-α, were significantly associated with the onset of acute GVHD by both logistic regression analysis and by Cox proportional hazard model. CD146 fluorescence was associated with an increased risk of developing acute GVHD (OR 1.57, p=0.040 by logistic regression analysis, and HR 1.60, p=0.031 by Cox model). Furthermore, CD31 fluorescence (OR 0.55, p=0.052 by logistic regression analysis, and HR 0.67, p=0.089 by Cox model), CD140-α percentage (by LR: OR 0.30, p=0.003 and by CM: HR=0.77, p=0.014) and CD140-α EVs concentration (OR=0.40, p=0.063 by logistic regression analysis, and HR=0.68, p=0.058 by Cox model) were associated with a decreased risk of acute GVHD. Two biomarkers, CD8 and CD25, showed ORs ranging from 0.91 to 1.27 indicating a very minimal effect even though a statistically significant p value, p<0.001, was observed. Co-expression of CD31, CD140-α and CD146 on EVs surface by 2-color flow cytometry showed a rather high association of CD146 with CD31 (65%) and with CD140-α (Supplementary Figure 6).

A monotonic association between biomarkers was observed by Spearman's rank-order correlation (Table 4, Supplementary Table 2-4). CD146 fluorescence, percentage concentration and relative concentration showed a significant correlation with CD44 (r_s=0.60; r_s=0.58, r_s=0.70, p=0.001), KRT18 (r_s=0.54, r_s= 0.49, r_s= 0.65, p=0.001), CD106 (r_s=0.61, r_s=0.58, r_s=0.68, p=0.001), CD31 (r_s=0.58, r_s=0.53, r_s=0.68 p=0.001). CD31 fluorescence, percentage concentration and relative concentration showed a significant correlation with CD44 (r_s=0.67, r_s=0.60, r_s=0.82 p=0.001), KRT18 (r_s=0.63, r_s=0.52, r_s=0.76 p=0.001) and CD106 (r_s=0.74, r_s=0.68, r_s=0.91, p=0.001). No significant correlations were observed between CD140-α fluorescence and percentage concentration with other biomarkers, whereas CD140-α absolute concentration was associated with CD44 (r_s=0.67, p=0.001), CD146 (r_s= 0.50, p=0.001), CD106 (r_s=0.85, p=0.001) and CD31 (r_s=0.82, p=0.001).

Finally, CD146, CD31 and CD140-α also showed a significant change in signal level before the onset of acute GVHD: an increase in CD146 and a reduction in CD31 and CD140-α respectively (Figure 4).

The impact of potential confounding factors such as viral infections and/or contamination of EVs from platelet transfusions was also investigated. By logistic and Cox regression models, CMV reactivation did not appear a confounding factor for the correlation between EVs and the onset of aGVHD (Supplementary Table 5). Moreover,

mean EVs concentration was 10 times lower in samples from 5 buffy-coat derived platelet Units as compared with that of all 41 patients: $6.13x10^9$ EVs/ml in platelet buffy-coats and $6.73x10^{10}$ EVs/ml in patient serum samples. Importantly, results of our statistical analyses were also confirmed when EVs measurements from samples obtained during the 7 days following platelet transfusion were not included (Supplementary table 6).

DISCUSSION

An ideal biomarker should specifically and sensitively predict the onset of a pathological condition and its course, including response to treatment and clinical outcomes. Moreover, it should be obtained through non-invasive procedures and evaluated through inexpensive standardized assays. Acute GVHD incidence varies from 30% to 60%[39] and it remains a major cause of transplant related morbidity and mortality despite improvements in HLA-typing, donor selection, and GVHD prophylaxis. Several studies on its pathogenesis and potential biomarkers have been published. However, predictive biomarkers are still lacking and diagnosis and treatment response rely on clinical signs and symptoms, and tissue biopsies.

We designed a novel study to explore the potential correlation and roles of EVs and acute GVHD. Many factors may involve EVs in acute GVHD including their cells of origin and their roles in inflammatory processes. Furthermore, EVs can be easily extracted from biological fluids such as blood and urine, making them very attractive for diagnostic applications. EVs are also characterised by higher stability under various storage conditions as compared with soluble molecules. Moreover, we tried to select a homogeneous patient population with the same diagnosis of multiple myeloma, transplanted with the same stem cell source (mobilized peripheral blood) and prepared in the large majority (90%) with a non-myeloablative/reduced intensity conditioning regimen. In fact, biomarkers may potentially be influenced by several factors including age, disease, conditioning, GVHD prophylaxis, and all other causes of tissue and/or endothelial inflammation. Importantly, we ruled out a potential confounding role of CMV reactivation and/or other viral infections in our analysis (Supplementary table 5). Moreover, no patient with GVHD was treated with mesenchymal cells which express CD146.

A strong potential and statistically significant correlation of three biomarkers expressed on the EVs surface of our patients who developed acute GVHD as compared with those who did not clearly emerged from this study. While awaiting results from

prospective studies currently in progress, our preliminary findings are intriguing and highly encourage further investigations into the roles of EVs in GVHD.

CD146 was associated with an approximately 60% increased risk (Table 3) of acute GVHD. This membrane protein, also known as melanoma cell adhesion molecule (MCAM) or cell surface glycoprotein MUC18, is used as a marker of endothelial cells, and it is also expressed on a subset of CD4+ T cells, and follicular dendritic cells. In 2014, Li et al.[44] investigated the expression profiles on blood cells from 214 recipients of an allograft at the onset of acute GVHD. The frequency of Th17-prone CD146+CCR5+ T cells was significantly increased in patients with gastrointestinal GVHD and it was higher as early as 14 days after transplant in patients who would later develop gastro-intestinal GVHD[40,41]. Moreover, an increase of this T cell subset has also been observed in many autoimmune diseases and in inflammation driven by autoimmunity[42,43].

Considering that endothelial damage and neovascularization represent early steps in acute GVHD pathogenesis, the use of endothelial markers may be considered a helpful support to confirm GVHD diagnosis. Almici et al.[44] recently showed that the count of $CD146^{+}CD106^{+}CD45^{-}$ circulating endothelial cells (CECs) significantly increased in patients who develop acute GVHD. Given that EVs membrane composition relies on the cell of origin, CD146(+) EVs may have been shed by a reactive Th17 prone T cell population and/or by endothelial cells damaged by cytokine storm and inflammation. Interestingly, we also found that CD146 levels were associated with other endothelial biomarkers such as CD44 by Spearman's rank-order correlation. CD44, cell-surface receptor for hyaluronan (H-CAM) has been correlated with an inhibitory role in angiogenesis, endothelial cell vitality and proliferation[45]. In our cohort, higher levels of CD44+ EVs were observed in patients with acute GVHD. This may be explained with a reduction of angiogenesis and impaired endothelial protection. Interestingly, we also observed a certain degree of co-expression (>65%) of CD146 and CD31 suggesting that, in our patients, EVs may have shared the same endothelial origin (Supplementary figure 6). These findings should however be confirmed prospectively.

CD31 was correlated with an approximately 40% (see Table 3) decreased risk of acute GVHD. CD31 is a membrane protein, also known as platelet endothelial cell adhesion molecule or PECAM-1, normally found on several cell types including, macrophages and Kupffer cells, granulocytes, T cells/ NK cells, megakaryocytes, osteoclasts, neutrophils. Platelets also express CD31. We ruled out a potentially significant contamination of EVs expressing CD31 from transfused platelet Units. Life-span of

transfused platelets ranges from 3 up to 7 days [46].Moreover, a clearance time of only a few hours for transfused platelet derived EVs has been reported [47]. Only 5 (12%) patients received buffy-coat derived platelet Units during the 7 days before EVs evaluation. Our statistical analyses were invariably confirmed when EVs measurements obtained during the 7 days following platelet transfusions were not included (Supplementary table 6). Moreover, mean EVs concentration of samples from buffy-coat derived platelet Units is 10 times lower than that of our patients.

PECAM-1 is a member of the Ig gene superfamily expressed at high density on the endothelial cell borders and, at a lower density, on hematopoietic cells. CD31 has been involved in leukocyte trans-endothelial migration both *in vitro* and *in vivo*. Cheung et al. showed that CD31-induced-signaling pathway plays a key role in preventing inflammation-induced endothelial cell death[48]. CD31 immuno-regulatory role is however not limited to endothelial cells as both T cells and antigen-presenting cells can express it [49,50]. When CD31 signaling pathways are triggered, a partial inhibition of T-cell receptor signalling and a reduced production of inflammatory cytokines in dendritic cells may follow. CD31 prevents lymphocyte hyper-reactivity by increasing the activation threshold of T-cell receptor signalling, thus enhancing peripheral tolerance. Moreover, excessive immune-reactivity and susceptibility to cytotoxic killing was associated with a loss of CD31 function. CD31 deficient mice displayed accelerated and pronounced tumour rejection, suggesting an immune regulatory role of CD31[49,51]. Notably, some studies showed that CD31 gene polymorphisms in donor-derived leukocytes were significantly associated with the pathogenesis of acute GVHD[52–54]. We clearly observed a reduction of CD31 levels in our patients who developed acute GVHD. By contrast, CD31 expression may have had a somewhat protective role against inflammation and immunity damage in patients without GVHD, in keeping with previous studies.

Finally, CD140-α was also associated with a decreased risk of acute GVHD (Table 3). This membrane protein is also known as Platelets Derived Growth Factor Receptor alpha (PDGFRα). While the beta form of the PDGFR is essential for pericytes recruitment, blood vessels maturation and angiogenesis[55], the alfa variant is important for fibroblast migration and wound healing[56]. We observed a reduction of CD140-α levels in patients with acute GVHD. Zhang et al.[57] showed that TNF-α decreases the expression of PDGFR-α after fibroblast injury. Pro-inflammatory TNF-α has an important role in both initiating acute GVHD as well as amplifying the disease process once established[58]. As TNF-α levels are significantly higher in patient with acute GVHD[59], high levels of TNF-α could

decrease PDGFR-α reducing fibroblast activation and tissue recovery [60]. Overall, this may result in increased antigen exposure and alloreactivity as seen during acute GVHD.

In summary, our preliminary findings underline a potential role of EVs surface proteins as biomarkers of acute GVHD. Larger prospective multi-center studies are currently in progress. Moreover, the characterization of EVs and their "biological" content may shed new light on the pathogenesis of several inflammatory complications after allografting.

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AUTHOR CONTRIBUTIONS

B. Bruno, G. Camussi designed the study. G. Lia, L. Brunello, B. Bruno wrote the report. B. Bruno supervised the conduction of the study and data analyses. S. Bruno, B. Bruno, P. Omedè supervised the laboratory procedures. M. Festuccia, E. Maffini, L. Giaccone, M. Arpinati, M. Boccadoro supervised data collection, analyzed data, and reviewed and assisted in writing the manuscript. L. Lia, L. Tosti, S. Bruno, A. Carpanetto undertook the experimental procedures. G. Ciccone, A. Evangelista did the statistical analysis.

AUTHOR DISCLOSURES OF POTENTIAL CONFLICTS OF INTEREST

The Authors declare that they have no conflicts of interest pertinent to this study.

SUPPLEMENTARY INFORMATION

Supplementary Information accompanies this paper on the Leukemia's website

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Figure Legends

Figure 1. Cumulative incidence of acute GVHD and chronic GVHD.

a) Day 100-Cumulative incidence of acute GVHD (56.25%; 95% CI, 40.7–71.8%) b). 24 month-Cumulative incidence of chronic GVHD (70.7%; 95% CI, 56.3–85.2%).

Figure 2. Extracellular vesicles (EVs) characterization by light scattering and fluorescence.

Physical parameter dot plots of EVs analyzed after incubation with non-immune isotypic FITC and PE-IgG (negative controls) by A) light scattering (forward *versus* side scattering) and by B) side scattering *versus* fluorescence.

C) (from top to bottom) Physical parameter dot plots (side *versus* forward scattering, and side scattering *versus* fluorescence), and EVs fluorescence distribution after incubation with (from left to right) anti-CD8-FITC, anti-CD31-FITC, anti-CD146-FITC and anti-CD140-α-PE. R1 and R2: gate regions; dashed lines: threshold to discriminate background from positive fluorescence signal. In fluorescence distribution panels, the white area represents the fluorescence distribution of EVs incubated with non-immune isotypic FITC or PE-IgG (negative controls), while the grey area represents the fluorescence distribution of EVs labelled with FITC or PE-antibodies.

Figure 3. EVs Mean Fluorescence, percentage of positive EVs and total EVs concentration.

a) EVs Mean fluorescence level b) Percentage of CD140-α positive EVs c) Total EVs concentration. Red dots are EVs measurements in patients with acute GVHD, while blue dots in patients without acute GVHD.

Figure 4. Signal level before GVHD onset

a) CD146 and CD31 Fluorescence levels as relative variation from pre-transplant baseline values and onset of acute GVHD onset

b) CD140-α Concentration of positive EVs as absolute levels

Evs Measurements in patients with acute GVHD (red) and in patients without acute GVHD (blue)

Table 1. Patient and Transplant Charateristics

	Number (%)
Patients	41
Median age, years (range)	53 (range 34-65)
Male	27 (66%)
Multiple Myeloma	41 (100%)
Myeloablative Conditioning	4 (10%)
Bu-Mel	2/4
Cy-TBI 12Gy	2/4
Non Myeloablative Conditioning	32 (78%)
TBI 2Gy	23/32
Flu-TBI 2Gy	9/32
Reduced Intensity Conditioning	5 (12%)
Flu-Mel	1/5
Flu-Mel-TBI 2 Gy	4/5
Donors	
Matched Unrelated Donor	7 (17%)
HLA-identical Sibling	34 (83%)
Stem Cell Source	
PBSC	41 (100%)
Graft-versus-host-disease prophylaxis	
CyA+MMF	34 (83%)
CyA+MTX	2 (5%)
CyA+MTX+ATG	3 (7.3%)
Tacrolimus+MMF	2 (5%)
Acute GVHD grade II-IV	23 (56%)
Median day of onset (range)	40 (22-145)
Acute GVHD Grade III-IV	4 (9%)
No Acute GVHD	18 (44%)
Chronic GVHD	29/40 (72%)
Median day of onset (range)	187 (77-649)
Severe Chronic GVHD	10 (34%)
CMV Reactivation (day 0-100 post transplant)	20/41 (49%)
Median day of CMV Reactivation (range)	48 (27-81).

CR= Complete Remission; Bu=Busulfan; Mel=Melphalan; Cy=Cyclophosphamide; TBI= Total Body Irradiation; Flu= Fludarabine; PBSC= Peripheral Blood Stem Cells; ATG= Antithymocyte Globulin; CyA= Cyclosporine A; MMF= Mycophenolate Mofetil; CMV= Citomegalovirus

Table 2. Panel of potential biomarkers (Guava experiments): antigen, alternative definition, ligand and distribution, references on potential correlation with acute GVHD.

Antigen	Alternative Definition	Ligand/Receptor	Distribution	Reference
CD44	H-CAM, Pgp-1, Hermes antigen, ECMRIII	Hyaluronin,osteopontin, fibronectin	Leukocytes, endothelial, epithelial	cGVHD[22]
CD138	Syndecan-1	Extra Cellular Matrix	Plasma cells, pre-B, basolateral surface of epithelium, neurons	aGVHD[26]
CD146	MUC18, S-endo, MCAM, Mel-CAM		Endothelial, Melanoma, FDC, T^{act}	aGVHD[41]
KRT18	Keratin 18 Type I, Cytokeratin-18, CYK18	C-Cbl, TRADD, Collagen, type XVII, alpha 1, DNAJB6, Pinin	Epithelial,	aGVHD[26]
CD120-α	TNFR-1, TNFRSF1A	TNFα, TNFβ	Nucleated cells	aGVHD[21]
CD8	T8, Leu-2	MHC class I	T subset, Thimocytes subset	aGVHD[26]
CD30	Ki-1, Ber-H2	CD153	B^{act}, T and NK cells, Reed-Sternberg cells, anaplastic large cell lymphoma	aGVHD[26]
CD106	VCAM-1, INCAM-110	CD49d/CD29, CD49d/β7	$Endothelial^{act}$, FDC	aGVHD
CD25	Tac, p55, IL-2Ra	IL-2	T^{act}, B^{act}, Treg, Lymphoid progenitors	aGVHD[21]
CD31	PECAM-1, endocam, GPIIa	CD31, CD138	Leukocytes, Platelets, Endothelial	cGVHD[22]
CD144	VE-Cadherin, Cadherin-5	CD144, β-Catenin	Endothelial, stem cells	aGVHD
CD86	B70, B7-2	CD28, CD152	Monocytes, DC, B^{act}, and T^{act}	cGVHD[22]
CD140-α	PDGFRA, PDGFRa	PDGF-A, PDGF-B, PDGF-C	Mesenchymal, Fibroblasts, Glial cells, Monocytes, Endothelial	cGVHD[22]

Table 3. Association between marker levels and acute GVHD. Marker analysis by 30-day time periods (logistic regression analysis) with/without imputation of previous values in case of missing data, and by a time varying approach (Cox model-proportional hazard model). Significant odd and hazard ratios (OR and HR respectively) are highlighted in grey.

		Logistic regression analysis								Cox Model			
		Without Imputation				With Imputation							
		Change		Absolute		Change		Absolute		Change		Absolute	
Marker	Type	OR	p	OR	p	OR	p	OR	p	HR	p	HR	p
Total EVs concentration		0.64	0.196	0.49	0.179	0.74	0.207	0.65	0.199	1.15	0.717	0.81	0.318
CD44 HCAM	Fluo	1.08	0.821	1.74	0.058	0.92	0.777	1.33	0.242	0.92	0.684	0.93	0.739
	Pos %	0.98	0.948	1.72	0.066	0.86	0.535	1.24	0.367	0.80	0.333	0.83	0.370
	Pos Conc	0.76	0.518	1.20	0.618	0.71	0.268	1.01	0.983	0.71	0.327	0.74	0.212
CD138 Syndecan-1	Fluo	0.76	0.402	0.81	0.462	0.68	0.107	0.88	0.567	0.76	0.426	1.02	0.922
	Pos %	0.86	0.650	0.78	0.349	0.77	0.271	0.77	0.179	0.82	0.517	0.85	0.520
	Pos Conc	0.60	0.150	0.50	0.160	0.67	0.102	0.63	0.167	1.11	0.810	0.79	0.279
CD146 MCAM	Fluo	1.35	0.375	1.40	0.183	**1.57**	**0.040**	1.44	0.095	**1.60**	**0.031**	1.32	0.147
	Pos %	1.17	0.635	1.37	0.308	1.41	0.142	1.37	0.176	1.27	0.230	1.19	0.404
	Pos Conc	0.87	0.659	0.96	0.924	1.11	0.648	1.08	0.801	1.21	0.400	1.04	0.842
KRT18 Cytokeratin-18	Fluo	0.65	0.233	0.86	0.596	0.72	0.183	1.08	0.795	0.75	0.163	1.07	0.729
	Pos %	0.65	0.240	0.88	0.656	0.73	0.210	1.02	0.946	0.67	0.084	1.01	0.948
	Pos Conc	0.55	0.141	0.66	0.315	0.67	0.135	0.93	0.848	0.79	0.465	1.04	0.841
CD120-α TNFR-1	Fluo	1.00	0.992	1.09	0.762	0.78	0.457	1.06	0.811	0.71	0.168	0.92	0.700
	Pos %	1.06	0.892	1.11	0.756	0.84	0.560	0.92	0.724	0.72	0.211	0.80	0.298
	Pos Conc	0.63	0.166	0.53	0.173	0.69	0.106	0.66	0.197	0.92	0.844	0.79	0.264
CD8	Fluo	0.91	0.000	1.09	0.779	0.92	0.000	1.09	0.717	2.48	0.633	1.03	0.904
	Pos %	1.56	0.070	1.37	0.275	1.21	0.181	1.21	0.460	1.20	0.592	1.09	0.714
	Pos Conc	1.48	0.063	1.22	0.515	1.18	0.196	1.26	0.312	1.21	0.648	1.17	0.438
CD30 Ki-1	Fluo	0.76	0.472	1.10	0.729	0.84	0.517	1.08	0.729	0.65	0.202	0.91	0.636
	Pos %	0.80	0.534	1.23	0.452	0.92	0.748	1.07	0.766	0.54	0.104	0.85	0.464
	Pos Conc	0.64	0.258	0.89	0.696	0.84	0.518	0.89	0.642	0.57	0.177	0.86	0.462
CD106 VCAM-1	Fluo	0.68	0.352	0.84	0.592	0.79	0.395	1.07	0.807	0.68	0.222	0.97	0.915
	Pos %	0.64	0.124	0.85	0.593	0.78	0.246	0.95	0.833	0.72	0.166	0.85	0.481
	Pos Conc	0.51	0.059	0.51	0.129	0.65	0.077	0.69	0.242	0.72	0.432	0.79	0.297
CD25 IL-2Ralpha	Fluo	0.85	0.563	1.08	0.819	1.27	0.000	1.25	0.392	1.07	0.440	0.97	0.898
	Pos %	1.08	0.757	1.17	0.588	1.34	0.169	1.14	0.553	0.94	0.764	0.84	0.431
	Pos Conc	0.98	0.935	1.00	0.991	1.32	0.176	1.07	0.783	0.96	0.882	0.86	0.491
CD31 PECAM-1	Fluo	0.67	0.311	1.05	0.867	**0.55**	**0.052**	0.93	0.759	**0.67**	**0.089**	0.86	0.498
	Pos %	0.78	0.415	1.10	0.724	0.69	0.130	0.89	0.594	0.64	0.068	0.78	0.250
	Pos Conc	0.60	0.158	0.61	0.238	**0.65**	**0.067**	0.68	0.205	0.71	0.415	0.77	0.219
CD144 VE-Cadherin	Fluo	0.72	0.331	0.79	0.488	1.01	0.960	0.94	0.811	1.12	0.602	0.95	0.802
	Pos %	0.70	0.283	0.68	0.132	0.94	0.838	0.88	0.577	1.15	0.552	1.01	0.969
	Pos Conc	0.54	0.105	0.49	0.171	0.77	0.398	0.70	0.300	1.18	0.542	0.88	0.541
CD86 B7-2	Fluo	1.46	0.015	1.28	0.309	1.22	0.249	0.87	0.571	0.92	0.713	0.82	0.350
	Pos %	1.09	0.724	1.34	0.359	0.92	0.717	0.94	0.832	0.63	0.233	0.96	0.858
	Pos Conc	0.78	0.456	0.67	0.098	0.68	0.231	0.56	0.022	0.65	0.219	0.77	0.263
CD140-α PDGFRa	Fluo	1.25	0.477	**0.30**	**0.003**	1.19	0.475	**0.54**	**0.035**	1.03	0.926	0.75	0.208
	Pos %	1.40	0.154	**0.43**	**0.012**	1.30	0.169	**0.58**	**0.037**	1.18	0.826	**0.77**	**0.014**
	Pos Conc	0.78	0.580	**0.40**	**0.063**	0.85	0.587	0.58	0.094	1.23	0.654	**0.68**	**0.058**

Table 4. Spearman Correlation

(A. EVs Fluorescence, B. EVs Percentage Concentration, C. EVs Concentration)

A.	**CD44**	**CD146**	**KRT18**	**CD106**	**CD31**	**CD140-α**
CD44	1					
CD146	0.60*	1				
KRT18	0.61*	0.54*	1			
CD106	0.62*	0.61*	0.64*	1		
CD31	0.67*	0.58*	0.63*	0.74*	1	
CD140-α	0.10	-0.001	0.19	0.21	0.14	1
B.	**CD44**	**CD146**	**KRT18**	**CD106**	**CD31**	**CD140-α**
CD44	1					
CD146	0.58*	1				
KRT18	0.52*	0.49*	1			
CD106	0.56*	0.58*	0.52*	1		
CD31	0.60*	0.53*	0.52*	0.68*	1	
CD140-α	0.03	-0.07	-0.01	0.17	0.13	1
C.	**CD44**	**CD146**	**KRT18**	**CD106**	**CD31**	**CD140-α**
CD44	**1**					
CD146	0.70*	1				
KRT18	0.75*	0.65*	1			
CD106	0.80*	0.68*	0.75*	1		
CD31	0.82*	0.68*	0.76*	0.91*	1	
CD140-α	0.67*	0.50*	0.61*	0.85*	0.82*	1

* correlation significant at 0.001 level.

Figure 1

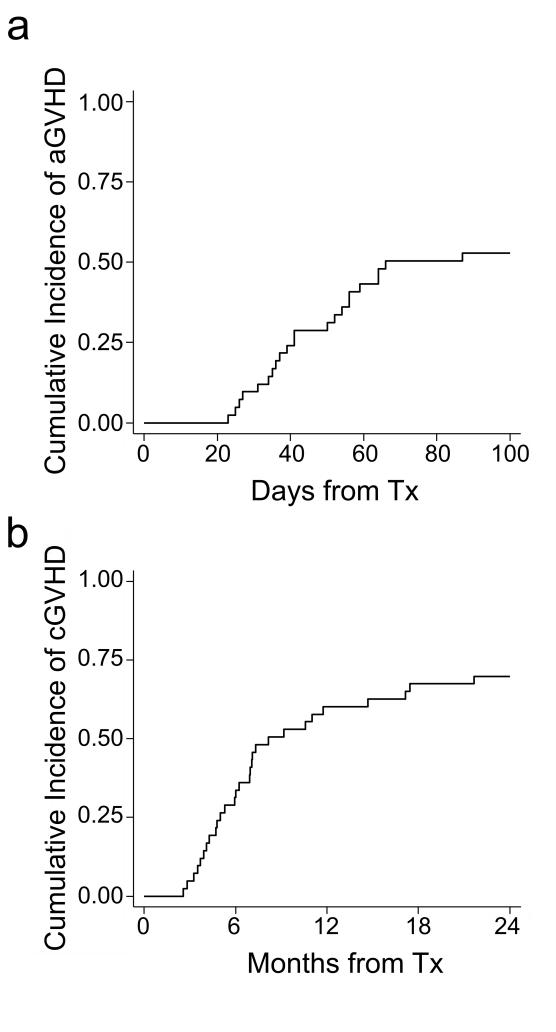

Figure 2

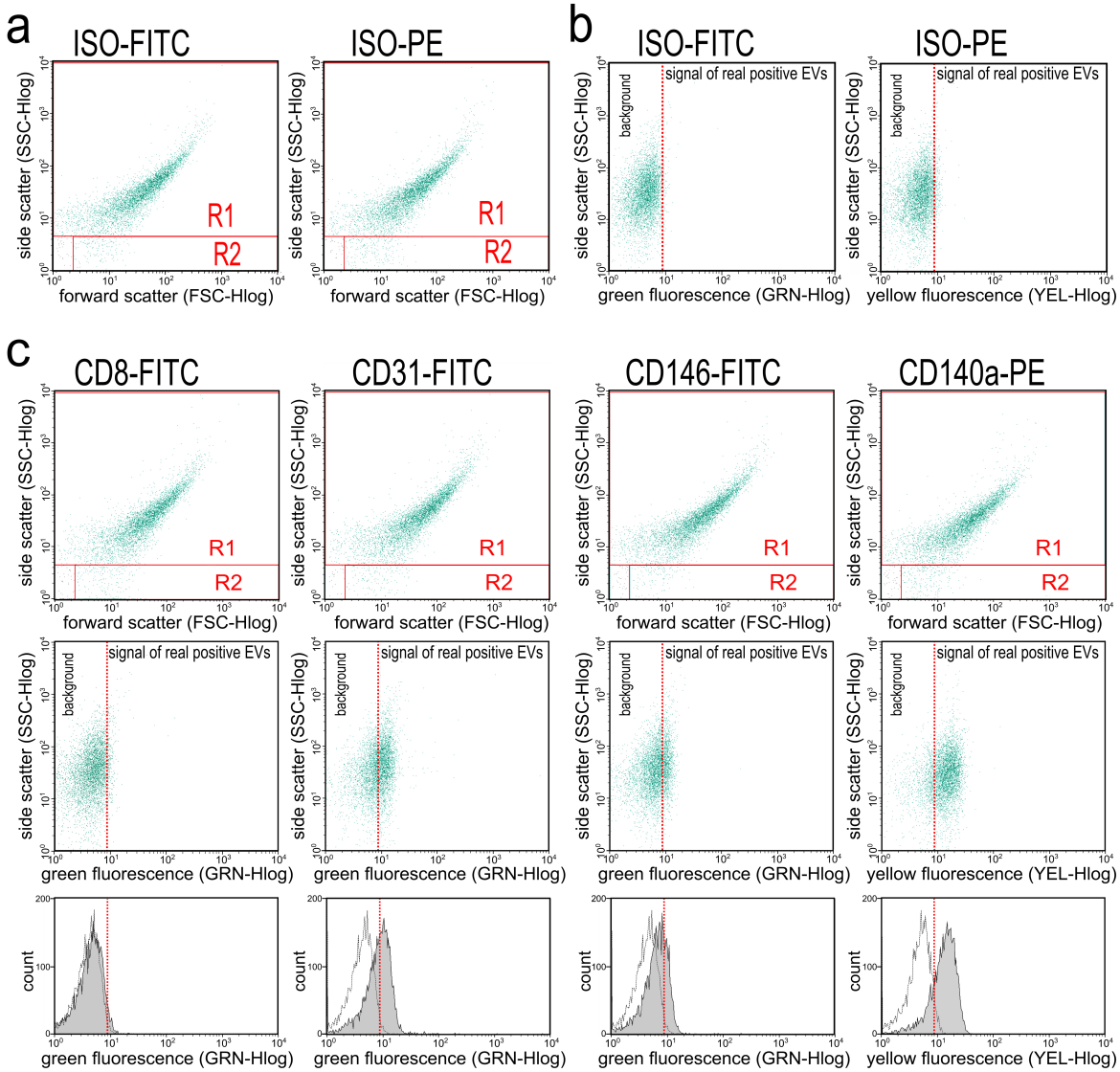

Figure 3

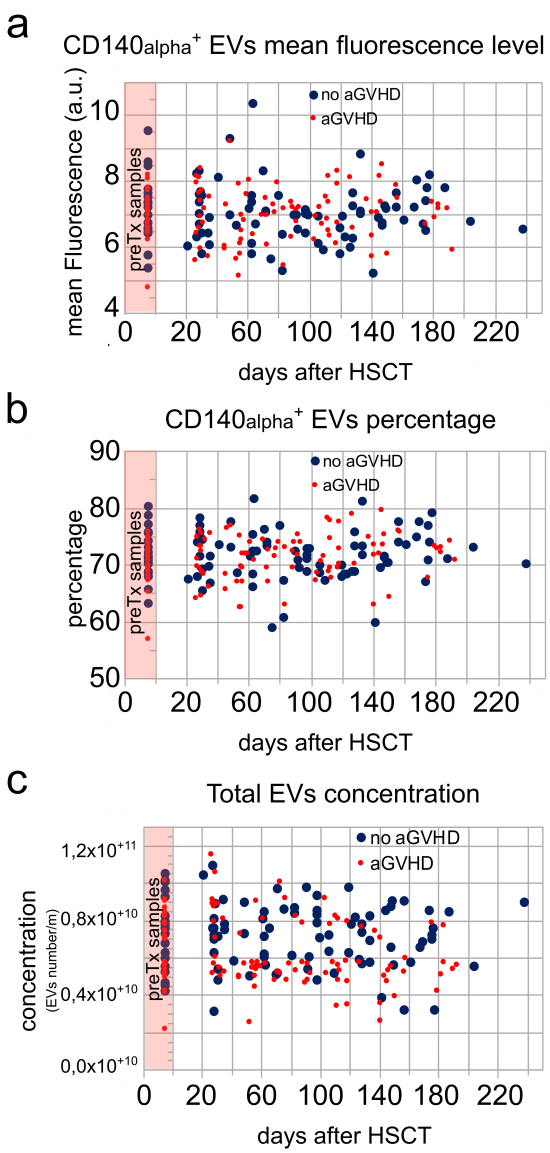

Figure 4