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Thrombospondin-1, Platelet Factor 4, and Galectin-1 are Associated with Engraftment in Patients with Sickle Cell Disease Who Underwent Haploidentical HSCT

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Abstract

Background—Sickle cell disease (SCD) is an inherited red blood cell disorder that leads to significant morbidity and early mortality. The most widely available curative approach remains allogeneic hematopoietic stem cell transplantation (HSCT). HLA-haploidentical (haplo) HSCT expands the donor pool considerably and is a practical alternative for these patients, but traditionally with an increased risk of allograft rejection. Biomarkers in patient plasma could potentially help predict HSCT outcome and allow treatment at an early stage to reverse or prevent graft rejection. Reliable, non-invasive methods to predict engraftment or rejection early after HSCT are needed.

Objectives—We sought to detect variations in the plasma proteomes of engrafted patients compared to those who rejected their grafts.

Study design—We used a mass spectrometry-based proteomics approach to identify candidate biomarkers associated with engraftment and rejection by comparing plasma samples obtained from nine engrafted patients with ten patients who rejected their graft.

Results—A total of 1378 proteins were identified, and 45 proteins were differentially expressed in the engrafted group compared with the rejected group. According to bioinformatics analysis results, information from the literature, and immunoassay availability, seven proteins, namely

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thrombospondin-1 (Tsp-1), platelet factor 4 (Pf-4), talin-1, moesin, cell division control protein 42 homolog (CDC42), galectin-1 (Gal-1), and CD9 were selected for further analysis. We compared these protein concentrations among 35 plasma samples (engrafted n=9, rejected n=10, healthy volunteers n=8 and non-transplanted SCD, n=8). Further, ELISA analysis confirmed the significant up-regulation of Tsp-1, Pf-4, and Gal-1 in plasma samples from engrafted patients compared to rejected, African American healthy volunteers and non-transplant SCD group ($p < 0.01$). By receiver operating characteristic analysis, these three proteins distinguished engrafted patients from other groups (area under the curve, AUC > 0.8 and $p < 0.05$).Next, we evaluated the concentration of these three proteins in samples collected pre-transplant and at days 30, 60,100, and 180 post-HSCT. The results demonstrate that Tsp-1 and Pf-4 stratified engrafted patients as early as day 60 post-HSCT ($p < 0.01$). Gal-1 was significantly higher in engrafted patients as early as day 30 post-HSCT ($p < 0.01$). We also divided the rejected group into those who experienced primary (n=5) and secondary graft rejection (n=5). We found Tsp-1 levels were significantly higher in engrafted patients than those who developed primary graft rejection at days 60 and 100 PT; Pf-4 levels were also higher at day 100 PT $p <0.05$. Further, Tsp-1 levels were significantly higher at PT Day 60 and day 100 and Pf-4 at PT Day 100 in engrafted patients than those who experienced secondary graft rejection.

Conclusions—While others have demonstrated Tsp-1, Pf-4, and Gal-1 have been associated with improved outcome in other transplant settings, here, we have shown for the first time that these proteins are associated with engraftment in patients with SCD who undergo haplo HSCT. As the proteins were not higher until day 60 PT, these proteins could be more helpful in diagnosing patients with secondary graft rejection. Earlier time points may be valuable to assess whether a patient may experience primary graft rejection. Increased concentrations of plasma Gal-1, Tsp-1 and Pf-4 could reflect increased T regulatory cells, IL-10 and TGF-β, which are essential players in the initiation of immunological tolerance. These biomarkers may provide opportunities for preemptive intervention to minimize the incidence of graft rejection.

Keywords

Sickle Cell Disease; Haplo-HSCT; Rejection; Engraftment; Proteomics; Biomarkers

Introduction

Sickle cell disease (SCD) is an inherited blood disorder that affects millions of people worldwide and leads to significant morbidity and early mortality. SCD is caused by a single amino acid substitution (valine for glutamic acid) at the sixth position of the β-globin chains of hemoglobin, resulting in mutated hemoglobin tetramers and the sickling of the erythrocytes in patients with SCD [1–4].

Allogeneic hematopoietic stem cell transplantation (HSCT) remains the most widely available curative approach for patients with SCD. Human leukocyte antigen (HLA)-matched sibling donor HSCT offers excellent long-term survival for SCD [1, 5]. Globally, approximately 2,000 patients with SCD have undergone HLA-matched allogeneic HSCT; the survival exceeds 90% in the United States and European studies [1]. However, as <18 % of SCD patients have HLA-matched sibling donors [6, 7], HLA-matched sibling HSCT is a treatment choice available for very few patients [8, 9]. In the United States, less than 1% of the

SCD population has received a transplant [10]. As a result, other transplant sources are needed to expand the donor pool. HLA-haploidentical (haplo) donors expand the donor pool with at least 90% of patients having a haplo-donor [11], making haplo-HSCT a practical alternative for patients with SCD who lack HLA-matched sibling donors [12].

Recently we reported the results of 21 patients with SCD who received a haplo-HSCT. The conditioning regimen consisted of alemtuzumab, 400 cGy total body irradiation (TBI), sirolimus, and a dose-escalation of post-transplant cyclophosphamide (PT-Cy).

Event-free survival improved from 0/3 (0%) with no PT-Cy to 6/12 (50%) with 100 mg/kg PT-Cy; however, the graft rejection rate remained high[12]. Patients who rejected their grafts either experienced primary graft failure or secondary graft failure with graft loss between 60 and 100 days post-HSCT. There was no grade 2 to 4 acute or chronic extensive graft-versus-host disease (GVHD).

Therefore, the major disadvantage of our previous nonmyeloablative haplo-HSCT protocol for patients with SCD and severe organ damage was the high incidence of graft rejection [12]. Allograft rejection remains a significant cause of graft loss of function in the haploidentical setting [13]. Historically, graft rejection was a major limitation of haplo-HSCT, which occurs by an immune response directed against the transplanted tissue due to the HLA mismatch between the recipient and the donor [14].

The mechanisms of graft rejection and tolerance induction are multifactorial, resulting from the combination of several cell types and their cytokine production [15, 16]. In our experience, graft rejection has been impossible to reverse, even with several immunosuppressive therapies, possibly due to the prolonged time needed to differentiate graft rejection from other transplant-related complications such as infection and drug toxicity. The graft rejection diagnosis is currently based initially on the presence of nonspecific clinical symptoms, including reduced blood counts and fever, and these may generate many false positives [17]. Unfortunately, donor chimerism levels do not decrease until late in the rejection process, when the process has already become irreversible.

Hence, currently available approaches to assess transplanted organs are insufficient to evaluate the possibility of drug toxicity and predict acute rejection and engraftment in a timely fashion [18, 19] and there is a unmet need for sensitive and specific noninvasive biomarkers that could diagnose the allograft status. Biomarkers of HSCT outcome would ideally be measurable in the blood (plasma or serum) and would allow physicians to diagnose and evaluate graft outcomes (rejection or engraftment) early in the process when treatment may still be effective.

The current advances in technology have led to fast growth in the application of “omics” in medical research. Mass spectrometry (MS) has been very powerful in identifying plasma biomarker proteins for diagnosis and providing information about the pathology of many diseases [20, 21]. Proteomics has been a successful approach to identify the activity, treatment response, diagnosis, and severity of acute and chronic graft versus host disease (GVHD) [22–29].

To date, diagnostic and prognostic plasma biomarker proteins associated with engraftment and rejection in patients with SCD who underwent haplo-HSCT have not been evaluated. A detailed understanding of the changes in regulatory and inflammatory protein expression in the plasma may provide a more in-depth understanding of engraftment and graft rejection in SCD patients who underwent haplo-HSCT. We hypothesized that comparing plasma proteomic profiles between engrafted and rejected patients could identify diagnostic and prognostic proteins associated with engraftment or rejection. A protein signature may help us improve our understanding of successful engraftment and graft rejection and could help us to predict the HSCT outcome, allow treatment of graft rejection early, and aid in developing novel conditioning approaches.

In this study, we sought to explore alterations in plasma proteome in transplanted patients. Furthermore, a series of bioinformatics approaches were applied to explore the mechanisms of HSCT outcome. In addition, we sought to validate several potential biomarkers that could distinguish engrafted patients from both rejected patients, healthy volunteers, and patients with SCD who did not undergo HSCT. We also measured the differentially expressed proteins in plasma samples collected pre-transplant and serially post-transplant (PT) in patients with SCD who underwent haplo-HSCT. We identified three candidates, galectin-1 (Gal-1), thrombospondin-1 (Tsp-1) and platelet activating factor-4 (Pf4) in the present study, which can aid clinical biomarker discovery for engraftment.

Material and Methods

Human Plasma samples

A study was conducted with samples received from nineteen adult patients suffering from severe SCD who underwent nonmyeloablative haploidentical HSCT at the National Institutes of Health (NIH) from March 2010 through September 2015 (ClinicalTrials.gov Identifier NCT00977691). The study was approved in 2009 by the NIH National Heart, Lung, and Blood Institute (NHLBI) Institutional Review Board, monitored by an independent data and safety monitoring board and all patients gave their respective written informed consent. Patients were grouped based on their engraftment status)engrafted (n=9) or rejected (n=10)(. Patients were conditioned with alemtuzumab, 400cGy TBI, sirolimus and PT-Cy. Patients enrolled in cohort 1 received 0 mg/kg, cohort 2 50 mg /kg and cohort 3 100 mg/kg PT-Cy. Sirolimus was given 1 day before transplant in cohort 1 patients and in the first 6 cohort 2 patients and 1 day after PT-Cy in the remaining cohort 2 patients (PT-day 4) and in all cohort 3 patients (PT-day 5, Table 1A and B) [12]. Blood samples from healthy controls (n=8) and non-transplanted SCD patients (n=8) were obtained at the NIH. Donor engraftment was defined as a sufficient percentage of donor chimerism at PT-day 180 to maintain donor-type hemoglobin and prevent acute SCD complications. Peripheral blood samples were collected pre-HSCT and at serial times PT (days 30, 60, 100, and 180). Plasma was extracted from EDTA tubes (Becton Dickinson, San Jose, CA, USA) within 2 hours (hrs) of the collection and then stored at −80°C until further analysis.

Protein extraction and digestion

Plasma proteins from each patient sample were quantified using the Bradford Assay Kit according to the manufacturer's instructions. 100 μg of protein from each sample was denatured in 8M urea buffer, followed by reduction using dithiothreitol (DTT), alkylation by iodoacetamide, and then digestion by recombinant lysine-c (rlys-c) (enzyme to substrate ratio of 1:40) performed for 3 hrs at 37°C. The 8M urea buffer was diluted to <1M, and then the sample was digested overnight. Later, digestion was performed by trypsin enzyme (enzyme-to-substrate ratio of 1:12.5), resulting in the peptide solution for mass spectrometry analysis. The rlys-C enzyme cleaves the proteins in lysine amino acid residues, whereas trypsin cleaves proteins at lysine and arginine amino acid residues, yielding tryptic peptides.

TMT10-plex labeling and High pH Reverse-Phase Liquid Chromatography

Fractionation—Plasma samples were divided into two Tandem Mass Tag (TMT) kits because the TMT test has a maximum capacity to assay ten samples at one time. In each TMT, an equal amount of plasma from five engrafted and five rejected patients were analyzed, and the samples were age-, gender-, and PT-day matched. One engrafted plasma sample was tested in both TMT Kits to ensure reproducibility.

The 10-plex TMT labeling was carried out according to the manufacturer's protocol (ThermoFisher Scientific, San Jose, CA). Each of the TMT 10-plex label reagents was reconstituted in acetonitrile (ACN). Further, the digested peptides from each sample were incubated with a specific label provided in the kit (126, 127N, 127C, 128N, 128C, 129N, 129C, 130N, 130C, and 131) for a duration of approximately 1 hr at room temperature. The TMT labeled samples were then combined and fractionated using off-Line high pH-basic Reverse-Phase Fractionation to decrease the complexity of the sample and simultaneously increase the number of peptides being identified into a total of 24 fractions, which were later analyzed using liquid chromatography-mass spectrometry (LC-MS/MS). The labeled tryptic peptides from each of the 24-fractions were used for global proteomic analysis by the LC-MS/MS analysis.

Liquid Chromatography-Tandem MS Analysis—Protein identification with the LC-MS/MS analysis of peptides was performed using an Orbitrap Fusion Lumos Tribrid Mass Spectrometer (ThermoFisher Scientific) interfaced with an Ultimate 3000 Nano-HPLC apparatus (ThermoFisher Scientific). The peptides were fractionated by the EASY-Spray PepMAP RPLC C18 column (2 μm, 100A, 75 μm x 50 cm) using a 120-min linear gradient of 5–35% ACN in 0.1% formic acid, at a flow rate of 300 nl/min. The instrument was operated in a data dependent acquisition mode with the help of fourier transform mass analyzer for one MS survey scan for selecting precursor ions, followed by the top 3-second data-dependent higher energy c-trap dissociation-MS/MS scans for precursor peptides numbering 2–7 charged ions above a threshold ion count of 10,000, with a normalized collision energy of 37%. Survey scans of peptide precursors from 300 to 2000 m/z were performed at 120k resolution, and MS/MS scans were acquired at 50,000 resolutions with a mass range of 100–2000 m/z.

Protein identification and data analysis—All MS and MS/MS raw spectra of the TMT experiments from each set were processed and searched using the SEQUEST-HT algorithm within the proteome discoverer 2.2 (PD2.2) software (ThermoFischer Scientific). The settings for the precursor mass tolerance were set at 12 ppm, fragment ion mass tolerance to 0.05 Da, trypsin enzyme with two missed cleavages with carbamidomethylation of cysteine, TMT6-plex (lysine), TMT6-plex (peptide N-term) as fixed modification and deamidation of glutamine and asparagine, and oxidation of methionine as variable modifications. The human sequence database from Swissprot was used for the database search. For peptide and protein identification, the false discovery rate (FDR) was set to 1%. Identified peptides were used for the Percolator algorithm in PD 2.2, along with an additional peptide confidence set to high. Only peptide spectra, containing all reporter ions, were designated as "quantifiable spectra" and used for peptide/protein quantitation. The final lists of protein identification/quantitation were grouped by PD 2.2. The quantitative protein ratios were weighted and normalized by the median ratio for all quantifiable spectra of the peptides pertaining to the total protein identified. The raw data obtained for each experiment (TMT1 and TMT2) after MS analysis were filtered separately using two criteria, the fold change difference and p-value between engrafted and rejected patients. The cutoff values of 1.25-fold for upregulated and 0.75-fold for downregulated proteins with $p < 0.05$ were established as statistically differentially expressed proteins between the two groups of patients[30, 31]. We used the proteins identified by this procedure in both experiments, TMT 1 and TMT 2, for further analysis.

Bioinformatics analysis—The differentially expressed proteins were entered in the database for annotation, visualization, and integrated discovery (DAVID, http://david.abcc.ncifcrf.gov/) database for pathway analysis. To visualize the PPI (protein-protein interaction) network, the set of differentially expressed proteins was mapped using the online Search Tool for the Retrieval of Interacting Genes (STRING) database (http://string-db.org). Gene Ontology (GO) functional enrichment analysis of proteins in the PPI network was directly performed online to retrieve the GO-terms assigned to a set of proteins in the GO categories of molecular function, biological process, and cell component with an FDR cutoff of < 0.05 on the whole genome background. The PPI networks associated with these proteins were retrieved from a web-based tool (lens for enrichment and network studies of proteins, LENS: http://severus.dbmi.pitt.edu/LENS). The interacted gene network was analyzed by the weblink at GenMANIA (http://genemania.org).

ELISA assay—The expression levels of selected biomarkers were measured from plasma samples of nine engrafted patients, ten rejected patients, eight healthy controls, and eight non-transplant SCD patients using ELISA quantitation kits. The following ELISA kits were procured: Talin-1 (Tln-1) and cell division control protein 42 homolog (CDC42 MyBioSource), Tsp-1 (ThermoFisher Scientific), Pf-4 (Abcam), Gal-1 (R&D Systems), and CD9 and Moesin (LifeSpan BioSciences). The experimental methods of ELISA were carried out according to the manufacturer's instructions.

Statistical Analysis—All statistical analyses of this study were carried out using GraphPad prism (GraphPad Software, version 7, Inc. La Jolla, CA). The protein

concentration of individual samples was compared using unpaired Student's t-test. $p < 0.05$ was considered statistically significant and represented as * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, and **** $p < 0.0001$. Data were presented as mean ± standard error of the mean (SEM). Receiver operating characteristic (ROC) curves were constructed to assess the diagnostic values of the candidate proteins by using GraphPad prism. If the area under the curve (AUC) was larger than 0.7, then the selected proteins were considered informative biomarkers [31]. The correlation between Gal-1, Tsp-1, Pf-4, and donor myeloid chimerism (DMC) level or Plasma Levels of TGF-β was determined by GraphPad prism 7. A cluster analysis was performed to detect if clustering occurs based upon engrafted and rejected patients. We first standardized the three proteins (Gal-1, Tsp-1, and Pf-4) to make the scales more similar by subtracting the mean and dividing them by standard deviations. We then used K means clustering (R package "cluster") to assess the engrafted and rejected patient groups.

Results

Patient Characteristics

The demographic and clinical characteristics of patients in this study are provided in Table 1A. The engrafted group comprised of 4 men and 5 women, while the rejected group consisted of 7 men and 3 women. The mean age in the engrafted group was 35.5 ± 6.1years and in the rejected group 34.2 ± 12.2 years. Of the ten patients who rejected their grafts, 5 patients experienced primary graft rejection and 5 secondary graft rejection between days 60 and 100 post-HSCT (Table 1B). More donors were female in both engrafted and rejected groups, 7/9 (78%) and 8/10 (80%), respectively. There were no significant differences between the recipient's or donor's age, gender, race, HLA matching, and cell numbers infused between the engrafted and rejected groups (Table 1A).

TMT comparative proteomic analysis

We first performed a discovery proteomic analysis comparing plasma samples from 9 engrafted patients to 10 patients who rejected their grafts (Table 1B). Because TMT reagent kits can only analyze up to 10 samples at a time, plasma samples from 19 patients were divided into two TMT experiments. Each experiment had plasma samples from five successfully transplanted patients and five patients who rejected their grafts. One sample was included in both TMT experiments as a reference. The experimental workflow is illustrated in Figure 1.

In this study, plasma samples were subjected to LC-MS/MS analysis following tryptic digestion and TMT labeling. By TMT analysis, 1144 plasma proteins from TMT 1 and 1090 plasma proteins from TMT 2 were successfully identified (Table S1 and Table S2). First, we excluded proteins with < 2 quantified peptides[32, 33]. We also filtered the TMT data set using the following criteria: $p < 0.05$ with a fold change of > 1.25 for upregulated proteins and fold change of < 0.75 for downregulated proteins. Subsequently, 195 and 63 proteins were identified as significantly differentially expressed between engrafted and rejected patients in TMT 1 and TMT 2, respectively. The list of the differentially expressed proteins is highlighted in Table S1 and Table S2. In TMT 1, 183 of 195 differentially expressed proteins

were upregulated in the engrafted group while the remaining 12 were downregulated. In TMT 2, 59 out of 63 differentially expressed proteins were upregulated in the engrafted group, while four were downregulated.

A Volcano plot of the p-values generated by the protein ratio fold change obtained by comparison of engrafted and rejected patients is depicted in Figure 2A. Among the two groups, 45 differentially expressed proteins were common across all samples by these two TMT experiments (Table S3). Venn diagrams displayed overlapping differential proteins in 2 TMT, as shown in Figure 2B. The cluster map of differentially expressed proteins that are identified commonly in the two groups is shown in Figure 2C. All the 45 proteins were significantly higher in engrafted patients compared to rejected patients.

Bioinformatics analysis of the differentially expressed proteins

Different databases were used to understand the biological significance of these differentially expressed proteins, and one such analysis was DAVID, which suggested that 41 pathways were significantly enriched ($p < 0.05$). The top 20 significantly enriched pathways are presented in Figure 3A. Pathway enrichment analysis revealed that the differentially expressed proteins mainly were related to Rap1 signaling pathway (10 proteins), focal adhesion (9 proteins), regulation of actin cytoskeleton (8 proteins), platelet activation (6 proteins), oocyte meiosis (5 proteins), leukocyte transendothelial migration (5 proteins), proteoglycans in cancer (6 proteins), shigellosis (4 proteins), and phagosome (5 proteins). All enriched pathways are listed in Table S4. The results implied that these pathways might be more critical in the pathogenesis of engraftment and rejection in SCD patients who underwent haplo-HSCT.

The molecular function, cellular component, and biological process were analyzed for 45 proteins by searching Gene Ontology (GO) database (Figure 3B, Table S5-S7). Forty-five differentially expressed proteins were involved in 268 significant functional terms in the ‘Biological Process’ category. As presented in Figure 3B, few of the top 10 significantly enriched GO terms in the ‘Biological Process’ category include platelet degranulation, regulated exocytosis, vesical-mediated transport, and response to stress. Furthermore, the GO analysis indicated that differentially expressed proteins were involved in various aspects of the immune system, such as the positive regulation of the immune system process (10 proteins), regulation of the immune system process (11 proteins), immune response-activating cell surface receptor signaling pathway (6 proteins), immune system process (12 proteins), and immune response (9 proteins). A complete list of all biological processes is listed in Table S5. These results suggest that the differentially expressed proteins can participate in several biological processes including immune system. The scattering of proteins for the ‘Molecular Function’ category showed the proteins involved in actin binding, cytoskeletal protein binding, integrin binding, cell adhesion molecule binding, receptor binding, protein binding, enzyme binding, and structural constituent of the cytoskeleton. Therefore, the primary function group of differentially expressed proteins in this category was binding activity. A complete list of all molecular functions is listed in Table S6. In addition, the identified proteins were distributed among numerous cellular components, with 65 GO terms in the ‘Cellular Component’ category being significantly

enriched, including cytoplasmic vesicle lumen, secretory granules, cytoskeleton, platelet alpha granule, and cytoplasm. A complete list of all cellular components is listed in Table S7.

A PPI network based on LENS software was generated to provide a clear picture of the functional relationships among the differentially expressed proteins. 33 out of 45 differentially expressed proteins were found to be interacting with each other in a protein interaction network (Figure S1A). The other PPI networks were generated by using differentially expressed proteins as candidates and graft rejection-related genes as target genes (Figure S1B). Thirty-three proteins and 11 graft function-related genes, which include cytotoxic T-lymphocyte-associated protein 4 (CTLA4), angiotensin l-converting enzyme (ACE), HLA histocompatibility complex II DM alpha chain (HLA-DMA), intracellular adhesion molecule 1 (ICAM1), interferon gamma (IFN-γ), interleukin 10 (IL-10), integrin beta 3 (ITGβ3), transforming growth factor beta (TGFβ1), TGFβ2, TGFbβ3, and tumor necrosis factor (TNF) were enriched in the interaction networks. Of these, ITGβ3 was found to be a common between candidate and target genes. When only candidate genes were used, the network statistics related to the LENS analysis showed that the minimum shortest path length, average shortest path length, and the count of disconnected nodes were lower in candidate genes than in random genes. Also, when both candidate and target genes were used, the candidate to target was lower than the candidate to random genes. The results supported that these networks are well enriched.

Further, the interacted gene network analyzed by GenMANIA indicated that most genes encoding the differential proteins were co-expressed (73.44%), Figure S1C, suggesting that these proteins have additional connections among themselves than what would be estimated for a random set of proteins of the same size. Such enrichment indicates that the proteins are at least partially biologically connected as a group.

ELISA Validation

Based on the reports from the literature, availability of suitable ELISA kits, and the limited volume of patient samples, seven biologically relevant candidate proteins were selected for further analysis by ELISA, which included Tsp-1, Pf-4, Tln-1, Moesin, CDC42, Gal-1, and CD9 (Table 2).

In addition to the plasma samples from engrafted (n=9) patients and those who rejected their grafts (n=10), plasma samples from healthy African-American volunteers (n=8) and from patients with SCD who had not been transplanted (n=8) were included in the ELISA analysis to determine the baseline plasma concentration of the various proteins and also to exclude the association between differentially expressed proteins and SCD. ELISA results revealed that Tsp-1, Pf-4, and Gal-1 were significantly higher in the engrafted group than the rejected group, healthy African American healthy volunteer group, and non-transplanted SCD group with $p < 0.001$ to 0.03 (Figure 4). There was no significant difference in Tln-1 levels between the engrafted and rejected patients. On the other hand, the Tln-1 concentration was significantly higher in engrafted patients than healthy controls and non-transplanted SCD patients (Figure 4). None of the CDC42, moesin, and CD9 levels were significantly different among groups (Figure S2).

Since five of the rejected patients experienced primary graft rejection and five secondary graft rejection, we next evaluated Tsp-1, Pf-4, and Gal-1 levels in engrafted patients versus patients who experienced either primary or secondary graft rejection. Tsp-1 and Gal-1 but not Pf-4 were significantly higher in the engrafted group compared to those who experienced primary graft rejection with $p < 0.05$ (Figure S3A). Tsp-1 was significantly higher in the engrafted group than those who experienced secondary graft rejection.There was no significant difference in Pf-4, and Gal-1 levels between the engrafted patients and patients who experienced secondary graft rejection (Figure S3B). None of the Tsp-1, Pf-4, and Gal-1 plasma levels were significantly different between primary versus secondary graft rejection patients (data not shown).

We further evaluated the level of these proteins in the sub cohorts (no cyclophosphamide, n=2; low dose cyclophosphamide, n=5; and high dose cyclophosphamide, n=12). We found no significant differences among treatment groups (Figure S3C). Twelve of 19 patients who received high dose cyclophosphamide were grouped based on their engraftment statutes (engrafted, n=6 and rejected, n=6). The plasma levels of these proteins were significantly higher in the engrafted group than in the rejected group (Figure S3D). This result suggests immunosuppressive treatment does not affect the level of these markers.

ROC analysis (Figure S4) was performed to assess the biomarker potential of differentially expressed proteins. The diagnostic accuracy of each biomarker among disease groups is reported in Table S8 Those tests involving Pf-4 as a biomarker had AUCs of 0.8889, 0.8194, and 0.8, which discriminate between engrafted patients and healthy volunteers, engrafted and non-transplanted SCD patients, and engrafted and rejected groups, respectively. When Tsp-1 served as a biomarker, the AUCs were 0.9167, 0.8056, and 0.9, which discriminate between engrafted patients and healthy volunteers, engrafted and non-transplanted SCD patients, and engrafted and rejected groups, respectively. In addition, when Gal-1 was tested as a biomarker, then AUCs were 0.9861, 0.9306, and 0.8444, which discriminate between engrafted patients and healthy volunteers, engrafted and non-transplanted SCD patients, and engrafted and rejected groups, respectively. Overall, these results showed that Pf-4, Tsp-1, and Gal-1 exhibited the possibility of serving as engraftment biomarkers.

Prognostic biomarker panel for risk stratification of graft rejection and engraftment

With the same cohort of patients, we next tested the prognostic significance of Tsp-1, Pf-4, and Gal-1 by measuring protein concentration in samples taken before HSCT and at PT days 30, 60, 100, and 180 (Figure 5). At baseline, no difference was observed for Tsp-1 and Pf-4 between engrafted and rejected groups. Pf-4 and Tsp-1 concentrations in plasma largely showed a decline from their pre-HSCT levels after the HSCT in the rejected group during the entire PT period ($p < 0.01$). The plasma concentration of Pf-4 and Tsp-1 in the engrafted group decreased similarly at day 30 PT but then recovered close to the baseline level. These results suggest that the conditioning regimen may alter these protein levels. Alternatively, Pf-4 and Tsp-1 concentrations were significantly higher in the engrafted group from PT day 60 and day 100 compared to those of the rejected group with p-values ranging from $p < 0.01$ to < 0.05 and with AUCs between 0.92 and 0.87. On the other hand, the concentration of Gal-1 was lower in the rejected group during the entire pre- and PT period. Although

the difference was not statistically significant, we observed a trend towards a higher Gal-1 level in the engrafted group at baseline ($p = 0.08$, AUC = 0.72). Gal-1 concentrations were significantly higher at PT day 30 and day 60 compared to those of the rejected group with $p < 0.05$ and AUCs 0.8 and 0.84, respectively. We observed no significant difference between the two groups for Tsp-1, Pf-4, and Gal-1 at day 180.

Even though the sample size is modest, we split the rejected group into those who experienced primary (n=5) and secondary graft rejection (n=5) and then examined the prognostic significance of the three proteins at baseline and PT days 30, 60, 100, and 180. We found significant differences between engrafted patients and those who developed primary graft rejection for Tsp-1 at day 60 and 100 PT and Pf-4 at day 100 PT but not for Gal-1 with $p < 0.05$ (Figure S5A). We observed no significant difference between patients who engrafted and those who experienced secondary graft rejection for Pf-4 at baseline, day 30, 60, and 180 PT and Gal-1 at all time points. On the other hand, Tsp-1 levels were significantly higher at PT day 60 and day100 and Pf-4 at PT Day 100 in engrafted patients than those who experienced secondary graft rejection (Figure S5B). There was no significant difference in the three proteins between those who developed primary and secondary graft rejction (data not shown). We detected the most significant differences in the three protein levels between the engrafted and rejected groups at Day 60 PT, consistent with clinical findings of secondary graft rejection. Remarkably, Gal-1 was significantly increased in the engrafted group on Day 30 PT. These data illustrate that Tsp-1 and Pf-4 stratified engrafted patients as early as day 60 post-HSCT. On the other hand, Gal-1 was significantly higher in engrafted patients as early as day 30 post-HSCT.

Relationship between GAL-1, Tsp-1, Pf-4 and Myeloid Chimerism

Several assays have been used to evaluate chimerism after HSCT [34]. Differentiating between host and donor origin of bone marrow and blood cells is extremely critical for monitoring the engraftment process [35]. Chimerism testing (engraftment analysis) by DNA was performed by analyzing short tandem repeat loci in the patients in this study [12]. To test the ability of these proteins to predict the graft outcome, we performed a correlation analysis between our three differentially expressed proteins and DMC. Figure S6A shows the correlation between Gal-1, Tsp-1, Pf-4, and the percent of DMC of the 19 patients in the study. There was a significant positive correlation between Gal-1 ($r = 0.5565$, $p = 0.0133$) and Tsp-1 ($r = 0.542$, $p = 0.0165$) and DMC. However, no significant correlation was found between Pf-4 and DMC level ($r = 0.4155$, $p = 0.0769$). These results suggest that Gal-1 and Tsp-1 may be used to predict the graft outcome.

Relationship between Gal-1, Tsp-1, Pf-4, and plasma levels of TGF-β

Recently we found increased frequencies of Tregs and early myeloid-derived suppressor cells and increased plasma levels of TGF-β and IL-10 in engrafted patients with SCD who underwent haplo-HSCT compared to those who rejected their grafts[36]. The most significant differences in cytokine levels between the engrafted and rejected groups were at day 60 PT, which correlated with the clinical findings of graft rejection. We performed a correlation analysis between Tregs, TGF-β, and IL-10 and these proteins (Gal-1, Tsp-1, and Pf-4). We observed a positive correlation between Tsp-1 and TGF-β at PT-Day 60 and PT-Day 180

(r=0.62, P<0.009 and r=0.61, P<0.01, respectively). We also detected a positive correlation between Pf-4 and TGF-β at PT-Day 180 (r=0.56 and P<0.03, Figure S6B).

Cluster analysis based on Gal-1, Tsp-1, and Pf-4

Lastly, we performed a cluster analysis to detect if clustering occurs based upon engrafted and rejected patient groups. The plots show the K means cluster algorithm results for 2 clusters, plotted in the coordinate space spanned by the first two principal components. As shown in the plots (Figure S7), the two groups cluster very well concerning the three proteins. These results suggest that Gal-1, Tsp-1, and Pf4 differentiate well patients who engrafted compared to those who rejected their grafts.

Discussion

Currently, highly sensitive and minimally invasive biomarkers to predict graft rejection and engraftment are limited in general and do not exist for SCD patients who underwent haplo-HSCT. Further, there is a pressing need to understand the mechanisms of engraftment and graft rejection in SCD patients who underwent haplo-HSCT. Here, we present the use of an MS-based proteomics discovery approach to identify early biomarkers of engraftment and rejection in plasma samples collected from patients with SCD who underwent haplo-HSCT at the NIH.

The present study revealed that the regulatory proteins Gal-1, Tsp-1, and Pf-4 were significantly higher in engrafted patients than the patients who rejected their grafts, healthy individuals, and patients with SCD who did not undergo HSCT. Also, when we divided the rejected group into those who experienced primary versus secondary graft rejection, Tsp-1 and Gal-1 were significantly higher in the engrafted group than those who experienced primary graft rejection; Tsp-1 was significantly higher in the engrafted group than those who experienced secondary graft rejection. Additionally, we also observed that these protein levels were higher in the engrafted patients as compared to the rejected patients from 30 to 60 or 60 to 100 days PT. Subsequently, ROC statistical analysis indicated that these regulatory proteins bear good sensitivity and specificity in predicting engraftment. Importantly, when we tested the prognostic significance of these proteins, we found that Tsp-1 and Pf-4 stratified engrafted patients as early as day 60 post-HSCT, whereas Gal-1 stratified engrafted patients as early as day 30 post-HSCT. Of note, while the difference was not significant, Gal-1 was higher in the engrafted group at baseline (in addition to all subsequent time points post-HSCT), which makes it a potential candidate for use as a prognostic marker.

We also found significant differences between engrafted patients and those who developed primary graft rejection for Tsp-1 at days 60 and 100 PT and Pf-4 at day 100 PT. Similarly, Tsp-1 levels were significantly higher at PT Days 60 and day 100 and Pf-4 at PT day 100 in engrafted patients than those who experienced secondary graft rejection. Because Tsp-1 and Pf-4 levels were significantly higher at day 60 PT, which is around the time of secondary graft rejection, but not at day 30 PT, these proteins may be more helpful in evaluating secondary but not primary graft rejection. Future studies at earlier time points will be required to define the authenticity of these proteins in predicting primary graft rejection.

We also observed a significant positive correlation between Gal-1 and Tsp-1 concentrations and DMC. Analysis based on the three signature proteins revealed that engrafted and rejected patients can be differentiated into two distinct clusters, separated by the first principal component. All these features suggest that Gal-1, Tsp-1, and Pf-4 may be used to predict the graft outcome in SCD patients after haplo-HSCT. The biological functions and significance of these proteins are outlined below based on existing literature.

Gal-1 is a prototypical member of a family of β-galactose-binding proteins and is expressed in several organs, including the thymus, brain, spleen, liver, heart, muscle, skin, and immunologically privileged tissues such as the placenta, cornea, and testis [37–40]. Furthermore, Gal-1 is expressed by various immune cells, including activated T cells, regulatory T cells (Tregs), B cells, and macrophages [41, 42; 43–47]. Gal-1 controls various T cell processes, including T cell signaling, activation, apoptosis, cytokine production, and Treg expansion [48–50]. Gal-1 has also been shown to induce the apoptosis of T helper (Th) 1 and Th 17 cells [51]. Prior data suggest that Gal-1 controls the secretion of the pro-inflammatory cytokine, IFNγ, and anti-inflammatory cytokine, IL-10 [46, 52]. Of note, Gal-1 has been found to induce Treg and regulatory B cells function and stimulate the expansion of IL-10-producing regulatory type1 (Tr1) cells [49, 53–56].

Earlier studies identified various beneficial roles of Gal-1 in the treatment of several experimental autoimmune diseases, including experimental autoimmune encephalitis, collagen-induced arthritis, concanavalin A-induced hepatitis, nephrotoxic nephritis, autoimmune diabetes, experimental autoimmune uveitis, serum-induced nephritis, and inflammatory bowel disease [49]. Pertaining to transplantation, Gal-1 improves allograft survival and reduces the incidence of GVHD in murine allogeneic HSCT [38]. Furthermore, other studies showed that administration of Gal-1 in rats prolongs renal allograft survival by inhibiting the $CD8^+$ T cell response [40], and the absence of endogenous Gal-1 accelerated skin graft rejection in mice [57]. Similarly, Gal-1 increased the survival of mice liver allografts by decreasing the numbers of Th1 and Th17 cells, and at the same time, Gal-1 levels were elevated in stable liver transplant recipients versus rejected recipients and healthy individuals [58, 59].

The second protein that was significantly increased in engrafted patients is Tsp-1, which is encoded by the *Thbs1* gene [60]. Tsp-1 has a broad spectrum of biological activities on various cell types, and is secreted mainly by platelets, fibroblasts, endothelial cells, and antigen presenting cells (APCs) and B cells [61–66]. Tsp-1 possesses anti-inflammatory effects in various experimental disease models [61, 67]and comprises several domains that specifically interact with a variety of cellular receptors such as integrins, CD36, and CD47 that are differentially expressed by diverse cell types [67]. Tsp-1 is also known to cause angiogenesis, tumor cell growth, and promote apoptosis and is also involved in the activation of TGF-β in *in vivo* and *in vitro* conditions [61, 68, 69]. Previous studies further reported Tsp-1 acts as a strong inhibitor of T cell receptor (TCR)-mediated T cell activation and inhibits T cell production of IL-12, which promotes the cytotoxic activity of Th1 cells [70–73]. In addition, these effects are involved in the downregulation of TNF-α and IL-12, with concomitant upregulation of IL-10 by APCs [74, 75].

The biological role of Tsp-1 has been studied in several disease settings, as it has been show to inhibit the cutaneous delayed-type hypersensitivity reaction and plays a significant role in corneal transplants since Tsp-1 derived from APCs was able to suppress corneal graft rejection by reducing T cell sensitization[64]. In addition, the administration of Tsp-1 has induced tolerance in allergic disorders [64, 65, 76]. Tsp-1 expressing B cells exhibited immune regulatory properties and suppressed allergy-related mucosal inflammation by inducing $Foxp3^{+}$Tregs [65]. Furthermore, Tsp-1 activates the latent form of TGF-β in dendritic cells (DCs) and converts the DCs to TGF-β^{+} tolerogenic DCs in allergic environment [66].

Further, studies showed that Tsp-1 is involved in immune regulation, as it is associated with the production of IL-10 and TGF-β, as well as the induction of Tregs; moreover, TGF-β has been associated with maintenance of Foxp3 expression and Treg function [61, 77, 78].

Pf-4 (CXCL4) is a member of the CXC chemokine family and is released from the alpha granules of platelets upon activation. One of its primary functions is to regulate the coagulation processes through its anti-heparin activity [79, 80]. In addition, Pf-4 has also been associated with heparin-induced thrombocytopenia [81]. Pf-4 was shown to support the survival of hemopoietic stem and progenitor cells [82]. Furthermore, Pf-4 is involved in the activation or proliferation of many immune cells like neutrophils, monocytes, and NK cells, and in contrast, it has been shown to inhibit T cell proliferation through reduced IFN-γ and IL-2 production from T cells, while stimulating the proliferation of Tregs [83–88]. The role of Pf-4 has been studied in several transplant settings and has been reported to limit the development and differentiation of Th17 cells in cardiac and liver transplantation models. Recombinant Pf-4 administration protects kidney allografts [89–91].

We recently published reported 50 cytokines and ten immune cell subsets in the same cohort of patients. Similar to the increased regulatory proteins discovered in this study, we observed increased plasma levels of TGF-β and IL-10 in engrafted patients with SCD who underwent haplo-HSCT compared to those who rejected their grafts. We also found increased frequencies of Tregs early myeloid-derived suppressor cells in the engrafted group compared to the rejected group[36]. The most significant differences in cytokine levels between the engrafted and rejected groups were at day 60 PT, which correlated with the findings of this study, and is consistent with clinical outcomes of secondary graft rejection. These suppressive cytokines and regulatory cell populations support a tolerogenic environment. Therefore, our findings corroborate with the existing literature and suggest that Gal-1, Tsp-1, and Pf-4 help establish an immunosuppressive environment in engrafted patients with SCD after haplo-HSCT.

There were still several limitations to this study. First, the sample size is small, and it is from a single-center, reducing the study's statistical power. Another caveat reflects differences in immunosuppressive treatment during sample collection: some patients received sirolimus alone, and others received both sirolimus and 50–100mg/kg PT-Cy. To account for this limitation, we evaluated the sub cohorts (no cyclophosphamide, low cyclophosphamide, and high cyclophosphamide) and observed no significant differences among treatment groups. The HSCT protocol did not include a collection of blood samples between the start of conditioning and day 30 PT. As a result, we do not know whether our results indicate a

pre-rejection trend. Having samples at earlier time points would be critical to predict the upcoming graft outcome and better understand the pathophysiology of engraftment and graft rejection. In addition, post-transplant infection could be contributory factors; however, we could not perform correlative analyses due to the small sample size. As there was no moderate or severe acute or chronic GVHD in the study, we did not evaluate the correlation between these proteins and acute or chronic GVHD.

In summary, we performed a comparative proteomic study by using a TMT approach to analyze the differential protein expression in plasma samples obtained from engrafted patients and patients who rejected their grafts after haplo-HSCT for SCD. Various differentially expressed proteins and their signal transduction pathways are related to graft outcomes. This is the first study to show that Gal-1, Tsp-1, and Pf-4 regulatory proteins are significantly associated with engraftment in haplo-HSCT for SCD; they be more beneficial in diagnosing secondary graft rejection, though samples collected earlier post-transplant are needed. Our results suggest these regulatory proteins may play an immunosuppressive role in haplo-HSCT and, therefore, could serve as valuable biomarkers for engraftment in patients with SCD. However, additional studies are required with a larger cohort representing multiple institutions starting earlier post-HSCT to validate our results and evaluate the kinetics of our findings before clinical recommendations can be made and a clinical cut point can be defined.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- We aimed to identify the plasma proteins associated with engraftment and rejection after haplo-HSCT for SCD.
- Tsp-1, Pf-4, and Gal-1 distinguished the engrafted group from the rejected group.
- Tsp-1 and Pf-4 stratified the engrafted group as early as day 60, while Gal-1 at day 30 post-HSCT.
- These proteins may contribute to the pathogenesis of engraftment.

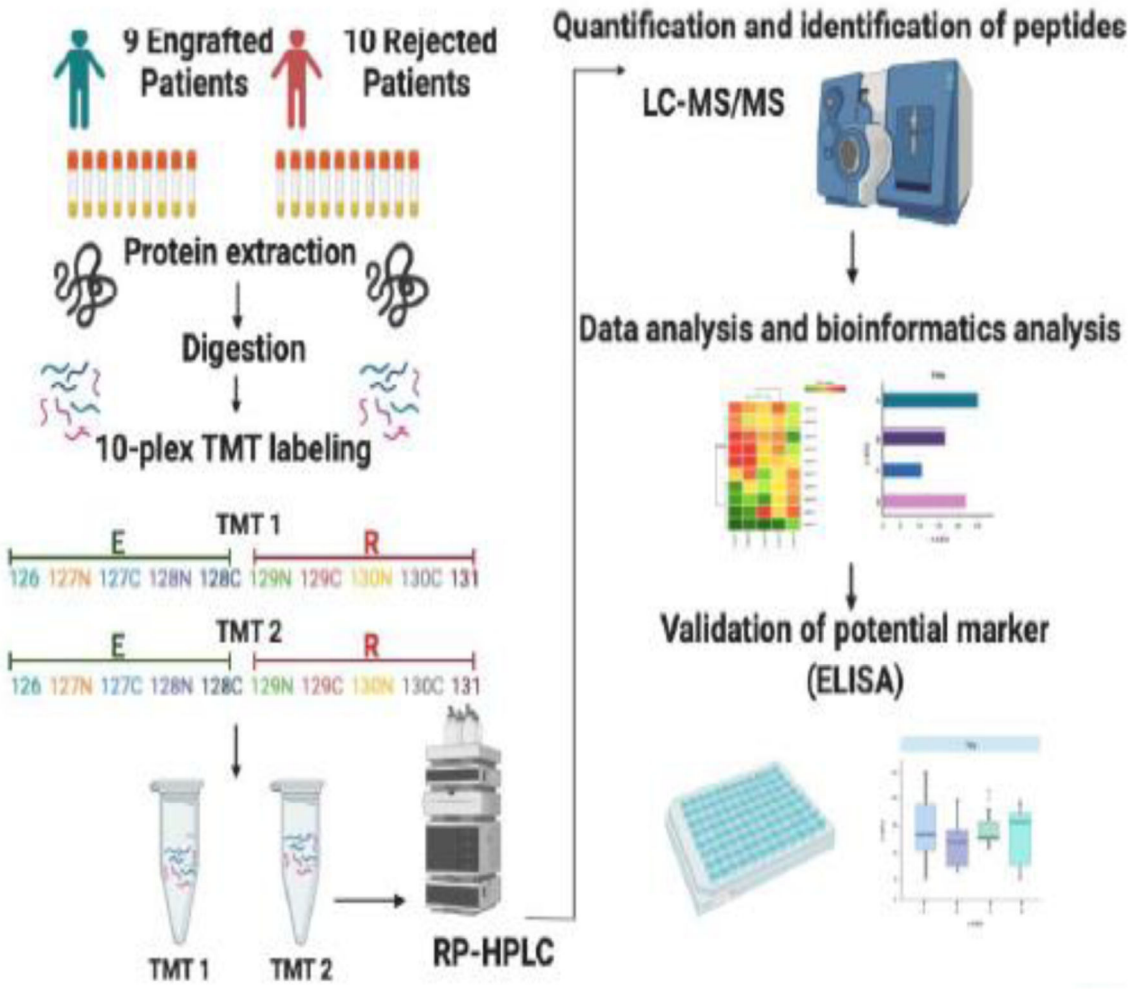

Figure 1.
Schematic workflow for the TMT-based proteomic study.

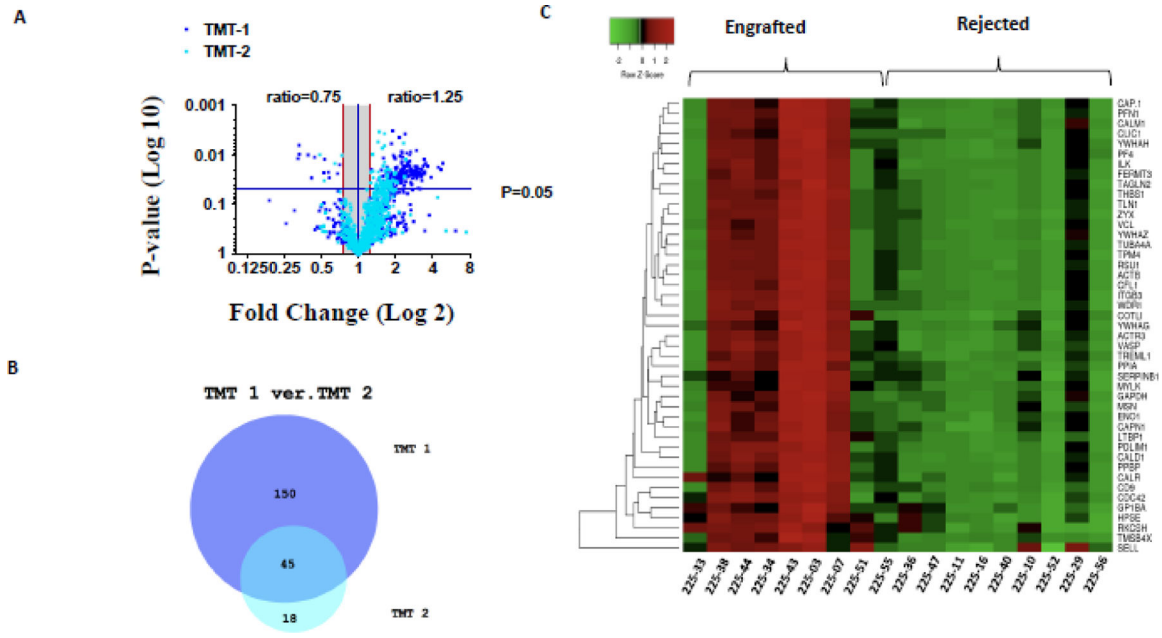

Figure 2.

Identification of the differentially expressed proteins. A) Volcano plot showing log2 fold change plotted against log10 adjusted p value for engrafted samples versus rejected samples. Data points in the upper right (ratio > 1.25) and upper left (ratio < 0.75) sections with p < 0.05 represent proteins that are significantly differentially expressed in engrafted patients according to the protein analysis of the 10-plex TMT labeled plasma samples after removing proteins with < 2 quantified peptides. The Volcano plot was generated using GraphPad Prism software version 7. B) Venn diagram showing the overlapping of proteins between TMT 1 and TMT 2. C) Heat map visualization of the differentially expressed proteins in 18 patients. green, downregulation, red, upregulation. Clustering proteomic data is based on differential proteins.

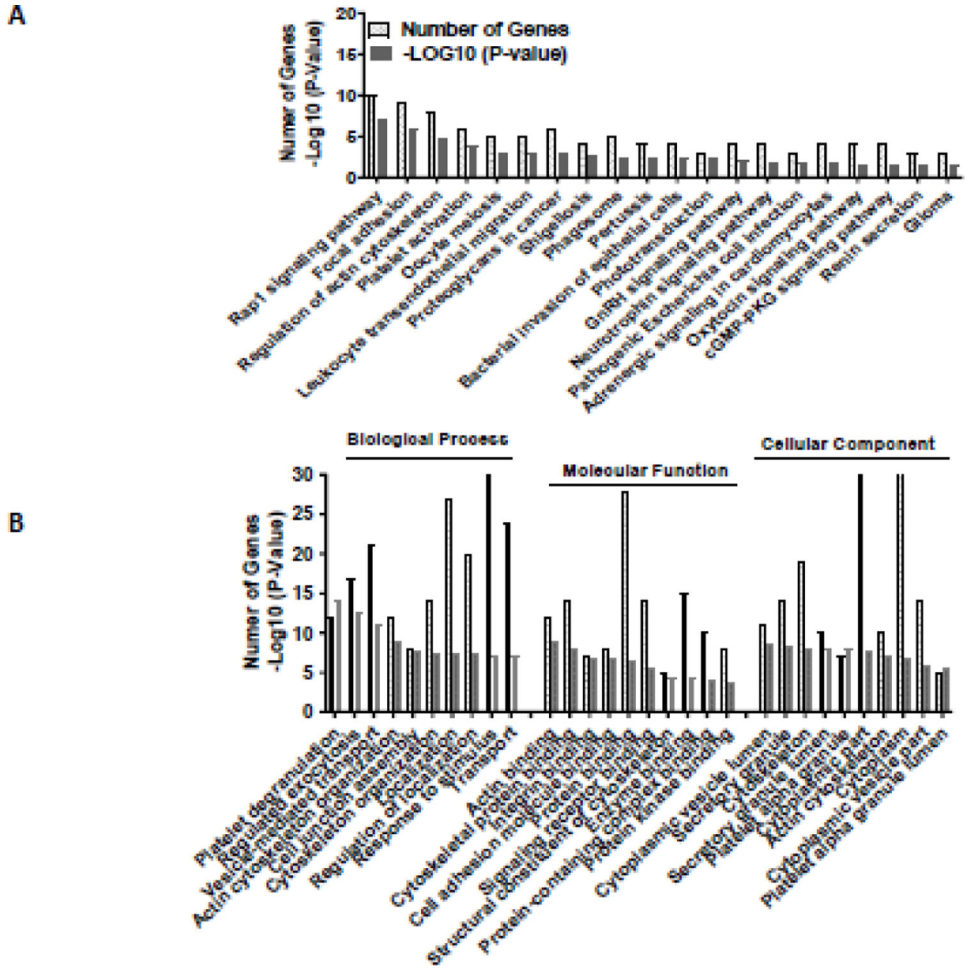

Figure 3.

Bioinformatics analysis of differentially expressed proteins in the plasma of engrafted and rejected patients. A) Pathway analysis of the differentially expressed proteins. Only the top 20 are presented with their p-value and number of genes in each pathway. B) Gene Ontology (GO) enrichment of differentially expressed proteins. The functional enrichment of proteins in the constructed interaction network was carried out online in the STRING database. Only the ten most significantly enriched GO terms in each GO category (Biological Process, Molecular Function, and Cellular Component) with their p-values are presented.

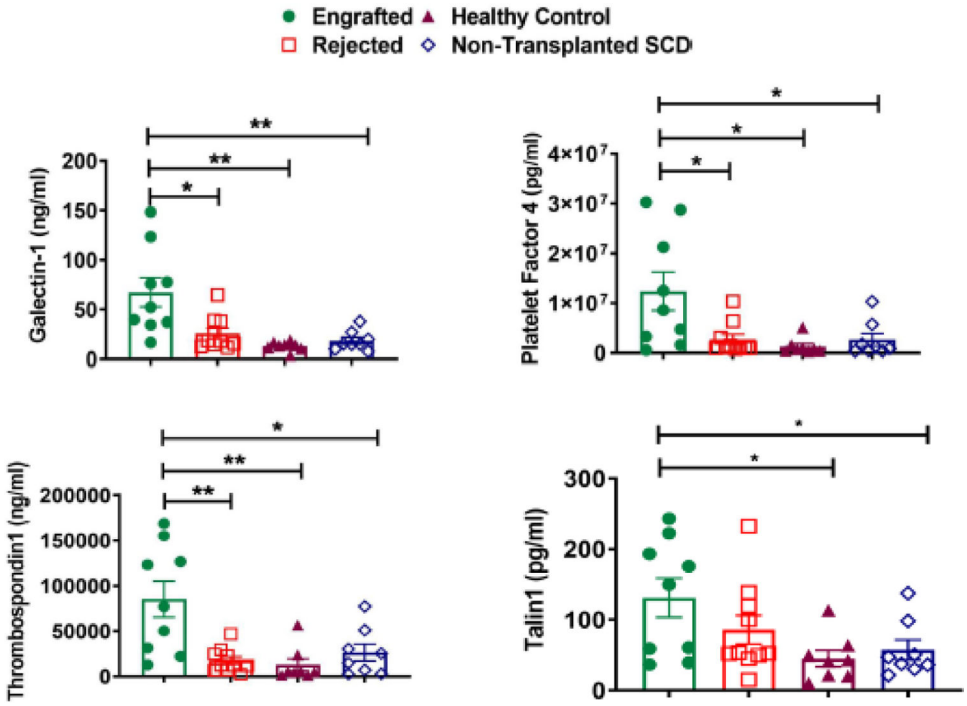

Figure 4.

Verification of the differentially expressed proteins by ELISA (Gal-1, Pf4, Tsp-1, and Tln-1). Protein expression was measured in the healthy volunteer group (n = 8), non-transplanted SCD group (n = 8), engrafted group (n = 9), and rejected group (n = 10). n, number of subjects. P-values were calculated with the unpaired Student's T test, *p < 0.05, and **p < 0.01, and data are presented as mean ± standard error of the mean (SEM).

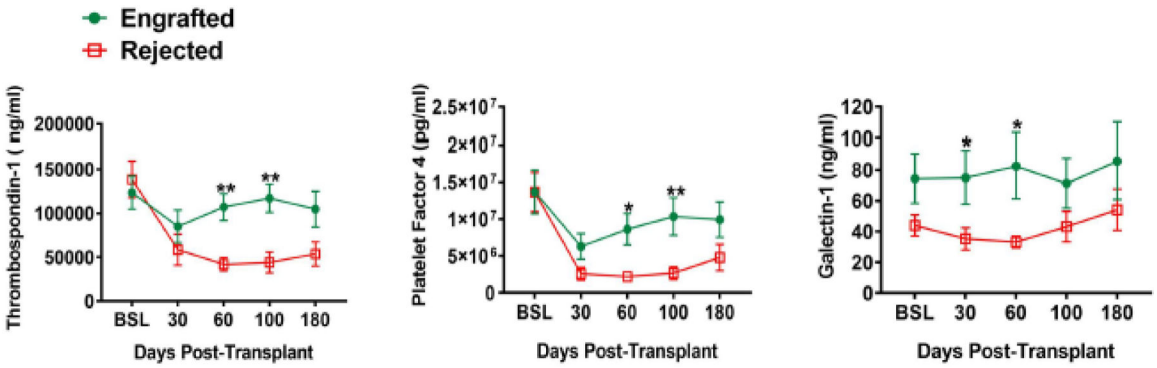

Figure 5.

Prognostic biomarkers of engraftment. Plasma biomarker concentrations measured by ELISA in engrafted and rejected patients at different days pre- and post-HSCT (Baseline and days 30, 60, 100, and 180 post-transplant). p values were calculated with the unpaired Student's T test, *$p < 0.05$, and **$p < 0.01$ and data are presented as mean ± standard error of the mean (SEM).

Table 1 A.

Demographic and clinical characteristics of the patients according to transplant outcome

		Engrafted N = 9 (47.4%)	Rejected N = 10 (52.6%)	Total N = 19 (100%)	P-value
Recipient	Age, Average years (SD)	35.5 (6.1)	34.2 (12.2)	34.8 (9.6)	0.768
	Sex, Male N (%)	4 (44.4)	7 (70.0)	11 (57.9)	0.255
	BMI, Average (SD)	22.2 (4.1)	24.1 (5.1)	23.2 (4.6)	0.395
	Race, N (SD)				0.474
	■ African-American	8 (88.9)	10 (100.0)	18 (94.7)	
	■ Caucasian	1 (11.1)	-	1 (5.3)	
Donor	Age, Average years (SD)	43.8 (16.5)	48.1 (12.5)	46.0 (14.3)	0.526
	Sex, Male N (%)	2 (22.2)	2 (20.0)	4 (21.0)	0.667
	Relation, N (%)				0.636
	■ Son	-	1 (10.0)	1 (5.3)	
	■ Sister	3 (33.3)	3 (30.0)	6 (31.6)	
	■ Father	-	1 (10.0)	1 (5.3)	
	■ Brother	2 (22.2)	-	2 (10.5)	
	■ Mother	4 (44.4)	5 (50.0)	9 (47.4)	
	Gender match				
	■ Same sex, parent	2 (22.2)	2 (20.0)	4 (21.0)	0.667
	■ Same sex, sibling	3 (33.3)	1 (10.0)	4 (21.0)	0.249
	■ Different sex, child	-	1 (10.0)	1 (5.3)	0.526
HLA mismatch	GVH direction				0.370
	■ 5–6	2 (22.2)	4 (40.0)	6 (31.6)	
	■ 7–9	7 (77.8)	6 (60.0)	13 (68.4)	
	HVG direction				0.115
	■ 5–6	2 (22.2)	6 (60.0)	8 (42.1)	
	■ 7–9	7 (77.8)	4 (40.0)	11 (57.9)	
Cell number	$CD34^+$ (SD) in 10^6	16.8 (6.2)	13.3 (5.9)	14.9 (6.2)	0.226
	$CD3^+$ (SD) in 10^8	4.8 (1.4)	4.2 (1.8)	4.5 (1.6)	0.473

SD, standard deviation; HLA, human leukocyte antigen; HVG, host versus graft; GVH, graft versus host.

Table 1B.

Categorization of patients by conditioning regimen, graft outcome, PT-Day for proteomic analysis and percentage of donor myeloid chimerism

Patient ID	Conditioning Regiment	E/R	PT-Day for proteomic analysis	DMC PT-Day 30	DMC PT-Day 60	DMC PT-Day 100	DMC PT-Day 180
225–19	Alemtuzumab, 400cGy TBI, Sirolimus, and 50 mg/kg PT-Cy	E	30	100	100	98	94
225–33	Alemtuzumab, 400cGy TBI, Sirolimus, and 100 mg/kg PT-Cy	E	60	100	96	79	78
225–38	Alemtuzumab, 400cGy TBI, Sirolimus, and 100 mg/kg PT-Cy	E	100	100	98	95	92
225–44	Alemtuzumab, 400cGy TBI, Sirolimus, and 100 mg/kg PT-Cy	E	100	98	99	97	97
225–34	Alemtuzumab, 400cGy TBI, Sirolimus, and 100 mg/kg PT-Cy	E	60	100	100	100	100
225–43	Alemtuzumab, 400cGy TBI, Sirolimus, and 100 mg/kg PT-Cy	E	30	99	98	97	98
225–03	Alemtuzumab, 400cGy TBI, Sirolimus, and 50 mg/kg PT-Cy	E	30	100	99	94	74
225–07	Alemtuzumab, 400cGy TBI, Sirolimus, and 50 mg/kg PT-Cy	E	30	100	66	26	18
225–51	Alemtuzumab, 400cGy TBI, Sirolimus, and 100 mg/kg PT-Cy	E	60	100	83	50	44
225–55	Alemtuzumab, 400cGy TBI, Sirolimus, and 100 mg/kg PT-Cy	PR	30	0	0	0	0
225–36	Alemtuzumab, 400cGy TBI, Sirolimus, and 100 mg/kg PT-Cy	SR	60	98	62	0	0
225–47	Alemtuzumab, 400cGy TBI,	SR	100	100	100	0	0
	Sirolimus, and 100 mg/kg PT-Cy						
225–11	Alemtuzumab, 400cGy TBI, and Sirolimus	PR	100	0	0	0	0
225–16	Alemtuzumab, 400cGy TBI, Sirolimus, and 50 mg/kg PT-Cy	SR	100	100	100	98	0
225–40	Alemtuzumab, 400cGy TBI, Sirolimus, and 100 mg/kg PT-Cy	SR	30	100	0	0	0
225–10	Alemtuzumab, 400cGy TBI, and Sirolimus	PR	30	0	0	0	0
225–52	Alemtuzumab, 400cGy TBI, Sirolimus, and 100 mg/kg PT-Cy	PR	30	0	0	0	0
225–29	Alemtuzumab, 400cGy TBI, Sirolimus, and 50 mg/kg PT-Cy	PR	60	6	8	0	0
225–56	Alemtuzumab, 400cGy TBI, Sirolimus, and 100 mg/kg PT-Cy	SR	30	64	17	8	1

TBI, total body irradiation; PT-Cy, post-transplant cyclophosphamide; E, engrafted; R, Rejected; P, primary; S, secondary; DMC, donor myeloid chimerism.

Table 2.

List of differentially expressed proteins selected for ELISA verification from discovery phase analysis.

Proteins	Fold change (Engrafted/ Rejected)	P-value	Function
Cell division control protein 42 homolog (CDC42)	1.8	0.0002	Involved in the TGF-b signaling pathway [92–94]
CD9 antigen	2.01	0.001	Functional marker for IL-10^{+} Breg [95]
Thrombospondin-1 (Tsp-1)	2.6	0.001	Major activator of TGF-b[69] , stimulates the production of IL-10 [96], stimulates Treg[61], decreases corneal rejection[64], and induces tolerance in an allergic environment[76].
Talin-1 (Tln1)	2.1	0.002	Controls Treg function and survival[97].
Platelet factor 4 (Pf-4)	2.5	0.002	Stimulates proliferation of Treg[88], Limits Th17 differentiation and IL-17 level[90], decreases kidney and cardiac rejection[90, 91].
Moesin	1.7	0.002	Mediates CD8^{+} Treg homeostasis and control of self-tolerance[98].
Galectin-1(Gal-1)	1.5	0.06	Promotes the expansion of IL-10-producing regulatory T (Tr1) cells[56 55], induces the apoptosis of Th1 and Th17 cells[51], induces the function of B regulatory cells[53], contributes to the suppressive activity of Tregs[42], promotes the generation of tolerogenic DCs[99], reduces the severity of GVHD, and prolongs renal graft survival[40]. Absence of Gal-1 accelerates skin graft rejection[57].