STIMULATORY AUTOANTIBODIES TO PDGF RECEPTOR IN PATIENTS WITH EXTENSIVE CHRONIC GRAFT-VERSUS-HOST DISEASE

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Short title: ANTI PDGF RECEPTOR ANTIBODIES IN eCGVHD

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ABSTRACT

Extensive chronic graft-versus-host disease (ecGVHD) is characterized by fibrosis similar to that of patients with systemic sclerosis (scleroderma). Since stimulatory autoantibodies against the PDGF receptor (PDGFR) have been found in patients with scleroderma and are responsible for the activation of skin fibroblasts, we tested the hypothesis that these autoantibodies are also present in patients affected by ecGVHD. Serum from 39 patients subjected to allogeneic stem cell transplantation for haematological malignancies (22 with ecGVHD and 17 without cGVHD) and 20 normal controls was assayed for the presence of stimulatory autoantibodies to the PDGFR by incubating purified IgG with mouse-embryo fibroblasts lacking PDGFR α or β chains or the same cells expressing PDGFR α. Stimulatory antibodies to the PDGFR were selectively found in all patients with ecGVHD but in none of the patients without cGVHD. Higher levels were detected in patients with generalized skin involvement and/or lung fibrosis. Antibodies recognized native PDGFR, induced tyrosine phosphorylation, accumulation of reactive oxygen species (ROS), stimulated type I collagen gene expression through the Ha-Ras-ERK1/2-ROS signalling pathway. The biological activity of these autoantibodies suggests a role in the development of fibrosis and argues for a common pathogenetic trait in ecGVDH and scleroderma phenotypes.
INTRODUCTION

Chronic graft-versus-host disease (cGVHD) is the most frequent long-term complication of allogeneic hematopoietic stem cell transplantation (HCT), occurring in 20% to 70% of subjects surviving more than 100 days. It represents the major cause of late death in this group of patients, and in survivors it is often associated with severe impairment of quality of life. 1

Chronic GVHD most commonly affects the skin, liver, eyes and mouth though other sites may also be involved. A clinical classification of cGVHD distinguishes patients with limited disease, in which only the skin or liver is involved, and patients with extensive cGVHD (ecGVHD) characterized by generalized or localized skin manifestations and/or hepatic dysfunction including involvement of other organs. 2 Skin lesions in ecGVHD are reminiscent of systemic sclerosis (scleroderma; SSc). Patients, developing extensive cGVHD with scleroderma-like skin manifestations, have also other clinical signs similar to those of patients with scleroderma. Fibrosis of the skin and of several visceral organs, such as lung, heart and esophagus, has been described in both diseases 3 including activation of immune system and cytokine abnormalities. 4-6 The hypothesis that fetal microchimerisms plays a role in the pathogenesis of scleroderma 7 further supports the notion of the similarity between scleroderma and cGVHD.

We have recently demonstrated that patients with scleroderma are characterized by serum stimulatory autoantibodies targeting the receptor of platelet-derived growth factor (PDGFR). 8 These antibodies trigger an intracellular loop, involving Ha-Ras-extracellular-signal-regulated kinases 1 and 2 (ERK 1/2) - reactive oxygen species (Ha-Ras-ERK 1/2 - ROS), which leads to increased collagen gene expression and myofibroblast phenotype conversion of normal human primary fibroblasts. 8-9 These
findings strongly argue for a pathogenetic role of anti PDGFR autoantibodies in the fibrosis in of scleroderma patients. Keeping in mind these data, we have evaluated patients with extensive cGVHD for the presence of stimulatory autoantibodies directed against the receptor of PDGF.
PATIENTS AND METHODS

Patients

Thirty-nine Caucasian patients (18 men and 21 women), median age 43 years (range, 17 to 70), who had received allogeneic stem cell transplantation for malignant haematological disorders, were studied. Twenty-two had extensive chronic GVHD, whereas 17 had not developed any sign of GVHD during the period of observation after stem cell transplantation. Diagnosis of ecGVHD was made according to established criteria. Skin disease was generalized in 9 of 22 patients and associated with lung fibrosis in four additional patients. Nine patients had minimal skin disease with either lung fibrosis (5 patients), sicca syndrome (3 patients), or hepatic and intestinal involvement (1 patient).

The median time interval between transplantation and the enrolment in the study was 23 months (range, 16 to 36) in the group of patients with ecGVHD and 42 months (range, 9 to 51) in patients without GVHD. The stem cell source was peripheral blood progenitor cells in 14 patients (12 in the ecGVHD group and 2 in those without GVHD), bone marrow in 24 patients (10 in the ecGVHD group and 14 in those without GVHD). Cord blood was used in 1 patient who did not develop GVHD. At the time of the investigation none of the 17 patients without GVHD were receiving immunosuppressive treatment, whereas all 22 patients with ecGVHD were receiving immunosuppressive drugs.

The demographic and clinical features of the study populations are presented in Table 1 and 2.

Control groups included 20 age-, sex-, and race- matched healthy volunteers.

The study was approved by the institutional ethics committee (Università Politecnica delle Marche, Ancona)
After oral and written informed consent, a blood sample was taken from patients and controls and spun in a refrigerated centrifuge after clot formation. The supernatants were collected and stored at -20°C until assayed, usually within 4 weeks.

**Bioassay for anti PDGFR autoantibodies**

As previously described, a functional bioassay was used to detect anti PDGFR autoantibodies. Briefly, mouse-embryo fibroblasts expressing PDGFR α subunit (Fα cells) were exposed in vitro to immunopurified IgG. Control cells were mouse-embryo fibroblasts devoid of PDGFR (F/- cells). Cells were plated in duplicate at a density of 20,000 cells in 1.83-cm² wells, cultured for 24 hours at 37°C in 0.2 percent foetal calf serum and incubated in the presence of 1 ml of normal or patient immunopurified IgG (200 µg/ml) for 15 minutes at 37 °C before ROS production determination.

Fluorimetric determination of intracellular ROS generated by adherent fibroblasts was carried out after loading the cells with 2',7'-dichlorofluorescein diacetate (DCFH-DA 10 µM, Molecular Probes, PoortGebouw, The Netherlands) as described. Each IgG sample was tested in duplicate and the average was recorded. The results were expressed as Stimulation Index (SI) which corresponds to (S-C)/(P-C), where S is the DCF fluorescence intensity of the test IgG, C is DCF fluorescence intensity of a negative control obtained by cell cultured without IgG, and P of a positive control obtained incubating the cells with PDGF (15 ng/ml for 15 minutes). The intraplate variation was less than 3 percent. The samples were recorded as positive if the SI was greater than 95th percentile of normal controls.

In selected experiments ROS generation was evaluated in cells exposed to the PDGF receptor tyrosine kinase inhibitor (AG 1296; 2 µM for 1-2 hours) and the
epidermal growth factor (EGF) receptor tyrosine kinase inhibitor (AG 1478; 2 µM for 2 hours) (Calbiochem, San Diego, CA, US).

**IgG isolation**

IgG fractions were purified by affinity chromatography on protein A/G-sepharose column (Pierce, Rockford, IL, US) from serum of normal subjects and ec patients with and without ecGVHD.

**Fibroblast cultures**

Human skin fibroblasts obtained from punch biopsies taken from the forearms of normal volunteers and the involved skin of patients who fulfilled the criteria for the diagnosis of ecGVHD were cultured as described. Fibroblasts between the fourth and the ninth subpassage were used for all experiments.

**Cell lysis and immunoblotting**

Cell culture plates were lysed with 0.3 ml of cold RIPA buffer (1x PBS, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 2 mM sodium orthovanadate, 2 µg/ml aprotinin, 1mM PMSF) and processed as described.

**RT-PCR Real Time for collagen**

Levels of α1 (I) and α2 (I) collagen RNA transcripts were quantitated by real-time PCR. Normal human fibroblasts were treated with normal and ecGVHD IgG (200 µg/ml for 15 minutes), total RNA from the samples were isolated using RNeasy (QIAGEN, Milan, Italy) and cDNA sequence derived from NCBI database (NCBI, http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?CMD=search&DB=nucleotide; accession numbers GAPDH:...
BC013310; COL α1(I): BC036531, COL α2(I): BC054498) was used to design the forward and reverse primers to the sequence using Beacon Designer software (PREMIER Biosoft International). The primers were as follows:

GAPDH FW: 5'-CCCTTCATTGACCTCAACTACATG-3';

GAPDH REV: 5'- TGGGATTTCCATTGATGACAAGC-3';

COL α1 (I) FW: 5’-AGGGCCAAGACGAACATC-3’;

COL α1 (I) REV: 5’-AGATCACGTCATCGCACAACA-3’;

COL α2 (I) FW: 5’-AGGTCAAACAGGAGCCCGTGGG-3’

COL α2 (I) REV: 5’-GCACCTGGGAAGCCTGGAGGG-3’

cDNA was reverse transcribed from 2 µg of total RNA each sample, using M-MLV RT kit (Gibco Invitrogen, Paisley, UK).Complementary DNAs were mixed with SYBR-Green PCR IQ Super mix (Bio-Rad, Rome, Italy) and primers, and real-time PCR were performed. GAPDH gene was used as internal control. For each single well amplification reaction, a threshold cycle (CT) was observed in the exponential phase of amplification, and the quantitation of relative expression levels was achieved using standard curves for both the target and endogenous controls. All assays were performed in triplicate.

**Statistical analysis**

Stimulation Index in the study groups is expressed as a median value and a range. Comparisons between the levels of anti PDGFR autoantibodies of scleroderma and control groups were performed with the non parametric Mann-Whitney test. All reported P-values are two-sided. Data were analyzed with the use of SAS software (SAS Institute).
RESULTS

Immunoglobulins from patients with extensive cGVHD induce ROS in fibroblasts

To detect antibodies stimulating PDGFR in patients with ecGVHD and eliminate the effects of PDGF or other cytokines present in serum, we purified total IgG from patients and controls and determined their biological activity by measuring the induction of ROS levels in a mouse-embryo cell line, expressing recombinant α PDGFR subunit (Fα cells). As negative control the immunoglobulins were tested on the same cell line lacking PDGFR genes and therefore unable to express any PDGFRs (F-/- cells). 

Figure 1 shows that the levels of ROS induced by IgG from patients with ecGVHD (median value 0.47; range, 0.15 to 1.71) were significantly higher (p < 0.001) than ROS generated by normal IgG (median value 0; range, 0 to 0.02), or IgG from transplanted patients without cGVHD (median value 0.01; range, 0 to 0.15) (p < 0.001). Using the 99th percentile as the upper limit of normal values, antibodies stimulating ROS levels were found in all patients with ecGVHD and in none of the controls.

IgG from patients with extensive cGVHD specifically recognize PDGFR

To determine the specificity of receptor activated by the IgG of ecGVDH patients, we pre-treated the cells with selective PDGFR or EGFR tyrosine kinase inhibitors, AG 1296 or AG 1478, respectively. PDGFR, not EGFR, inhibitor prevented ROS induction by IgG from ecGVHD patients (Fig 1A). Also, the ecGVHD IgG did not stimulate ROS in F-/- cells, that do not express PDGFR (Fig 1B). Finally, ecGVHD IgG, not IgG from the patients without GVHD, immunoprecipitated PDGFR α and β.
subunits from Fα cells (Fig. 2A). PDGFR interacting antibodies in ecGVHD IgG were completely removed by pre-absorption with Fα cells and not with F -/- cells. The depleted supernatant lost the ability to stimulate ROS production (data not shown). These data indicate that Fα cells were able to remove selectively from ecGVHD IgG, PDGFR-interacting antibodies (data not shown).

To determine unambiguously the stimulatory nature of antibody-receptor interaction, we challenged normal fibroblasts with increasing concentrations of IgG, derived from patients with ecGVHD, for 15 min and tested tyrosine phosphorylation of the PDGFR. IgG, derived from ecGVHD patients, induced tyrosine phosphorylation of the PDGFR in a dose-dependent manner (Fig. 2B). We also investigated a possible relationship between ROS-inducing activity of immunoglobulins and the clinical features of ecGVHD phenotype. Pooling the data together, we find that significantly higher levels of the antibodies, in terms of high biological activity, were present in 13 patients with generalized skin disease compared to the levels found in 9 patients with localized skin disease and organ involvement (p <0.03).

Biological activity of PDGFR stimulatory antibodies present in the serum of patients with chronic GVHD

To determine the biological effects induced by ecGVHD IgG, we assayed the expression of type I collagen genes in normal human fibroblasts exposed to ecGVHD IgG. Type I collagen α1 and α2 chain mRNAs were induced by ecGVHD IgG and not by IgG from patients without GVHD as shown by RT-PCR (Fig. 3). Also, PDGF receptor tyrosine kinase inhibitor (AG 1296 2 µM for 1 hour before treatment) inhibited type I collagen gene expression (Fig. 3). ecGVHD IgG induced-expression
of type I collagen was inhibited by N-acetyl cysteine, (20 mM for 1 hour before treatment), a potent ROS scavenger. Pre-treatment of the cells with FTI-277 (20 µM for 2 hours), a farnesyl protein transferase inhibitor, prevented ecGVHD IgG stimulation of collagen expression. These data indicate that both membrane-anchored Ha-Ras and ROS signals, similarly to scleroderma, are essential for PDGFR stimulatory activity of ecGVHD IgG (9).

To demonstrate the key role of the Ha-Ras-ERK 1/2 – ROS circuitry in the activation of fibroblasts, we isolated fibroblasts from patients with extensive cGVHD and assayed the levels of Ha-Ras, P-ERK 1/2 and reactive oxygen species. We have previously reported (8,9) that Ha Ras protein levels are stabilized by ROS-PDGFR activation. Fig.4 shows that fibroblasts derived from eGVHD patients, cultured in low serum, contained high levels of H-Ras, ROS and P-ERK1/2. The PDGFR specific inhibitor reduced significantly these markers to normal levels (Fig 4).

These data indicate that in cells, derived from ecGVHD patients, PDGFR signalling is constitutively stimulated. Constitutive signalling by ROS-Ha Ras-ERK1/2 by PDGFR is linked to overproduction of collagen and ultimately to fibrosis.
DISCUSSION

The present study shows the presence of antibodies directed against the receptor of PDGF in patients with extensive chronic GHVD. Their stimulatory activity is proved by the ability to stimulate: 1. tyrosine phosphorylation of PDGFR; 2. expression of type I collagen α1 and α2 gene; 3. Ha-Ras-ERK 1/2 – ROS signalling pathway. The presence of stimulatory antibodies in autoimmune diseases is rare. Insofar, only in scleroderma and Graves disease, agonistic antibodies to PDGFR and TSHR, respectively, have been reported. In fact, the bulk of antibodies found in autoimmune diseases is generally, represented by antibodies targeted towards several intra or extracellular components. These antibodies are able to bind their target, but do not stimulate selective intracellular signalling pathway. The findings reported here adds a new level of complexity to the scleroderma and ecGVHD phenotypes and suggest a common mechanism underlying the pathogenesis of these disorders. The similarity between the signals leading to fibrosis in scleroderma and extensive cGVHD, has been previously suggested; for a review see Artlett et al. For instance, several investigators consider ecGVHD a model of scleroderma. It is possible that in scleroderma and in extensive chronic GVHD the same mechanism may impair self tolerance towards PDGF receptor.

Autoantibodies against several antigens have been reported both in animal models of cGVDH and in clinical studies although it remains controversial at best whether they play an actual role in pathogenesis. The autoantibodies described here explain the fibrotic lesions in ecGVHD because 1. they stimulate type I collagen gene expression; 2. their levels correlate with the severity of fibrosis. Furthermore, there is evidence that cGVHD is a T-helper 2 disease, involving a sustained CD4+ T-cell help
for B cells, probably caused by derangement of negative selection process of autoreactive T lymphocytes.\textsuperscript{17}

Finally, our data confirm the pathogenetic relevance of the Ha-Ras - ERK 1/2 – ROS cascade in triggering and maintaining the activation of fibroblasts as already described.\textsuperscript{8,9} Targeting this circuitry and the inhibition of any of the molecules involved in the cascade (Ras-Erk1/2-ROS) are crucial for the development of a successful treatment of fibrosis in ecGVHD.\textsuperscript{18,19}

Although our data indicate a mechanistic explanation of the phenotype in ecGVHD fibroblasts, there are many unsolved questions to be answered. First, the precise epitope on PDGFR recognized by the autoantibodies: this is relevant for a more sensitive and less cumbersome diagnostic test, as well as for development of targeted therapy. Second, the presence of the antibodies we have described, suggests a pathogenetic link between scleroderma and ecGVDH, but does not provide information on the initial cause of both diseases. The mechanism by which PDGFR becomes self-antigen is not known. Autoimmunity may be induced by epitopes exposed as a result of a prolonged injury or viral infection or incompatibility between donor and recipient in terms of PDGF receptor genes.

The data reported in this study are to be considered preliminary, awaiting validation from larger clinical investigations, which should also establish the positive and negative predictive value of anti PDGF receptor autoantibodies for the development of GVHD.

The practical implication deriving from our findings is a possible targeted therapy based on inhibition of PDGFR- ROS and Ha Ras signalling. We speculate that blocking PDGFR activation with imatinib mesylate, a tyrosine kinase inhibitor, might
be of clinical benefit. However, our data indicate that inhibition of PDGFR in the presence of excess of ROS does not completely suppress the Ha Ras- ERK 1/2-ROS cascade \(^9\). For example, H2O2 generated in fibroblasts may diffuse and activate adjacent cells, irrespective of PDGFR activation.

We believe that targeting multiple steps of the Ha Ras- ERK- ROS circuitry is the only way to overcome the long term biological action of the autoantibodies to PDGFR and it will ultimately be clinically rewarding \(^{18,19}\).
ACKNOWLEDGMENTS

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AUTHORS’ CONTRIBUTIONS

Silvia Svegliati, Attilio Olivieri collaborated to the design of the study, performed the cellular studies, and contributed to writing the paper. Armando Gabrielli, Enrico V. Avvedimento designed the study, carried out a comprehensive analysis of the data and wrote the paper. Michele Luchetti, Gianluca Moroncini: performed the analysis of collagen gene expression (RT PCR). Pietro Leoni, Andrea Bacigalupo: collected the patient clinical and serological data; Nadia Campelli, Antonella Poloni, Silvia Trappolini performed the immunoprecipitation, immunohistological and immunofluorescence experiments.

The authors do not have conflicts of interest to declare.
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FIGURE LEGENDS

Fig 1 Antibodies against PDGF receptor in patients with extensive cGVHD

Levels of reactive oxygen species in mouse-embryo fibroblasts Fα (Panel A) and F-/- cells (Panel B) incubated with IgG (200 µg/ml for 15 minutes per 20000 cells) from normal subjects (N; n =20), extensive chronic GVHD patients (ecGVHD; n=22), and from patients who had not developed GVHD after transplantation (NoGVHD; n=17). Fα cells were also pre-incubated with the selective inhibitor of PDGFR tyrosine kinase (AG 1296, 2 µM for 2 hours), and of the epidermal growth factor (EGF) receptor tyrosine kinase inhibitor (AG 1478; 2 µM for 2 hours).

Fig 2 Immunoglobulins from patients with ecGVHD immunoprecipitate and phosphorylate PDGF receptor

Panel A Fα and F-/- lysates were immunoprecipitated with IgG purified from extensive cGVHD patients (ecGVHD) and patients without GVHD (No GVHD). The filters were developed with specific antibodies against the PDGFR α and β subunits (WB). Total lysates indicate immunoblots of total proteins. Representative results from 1 of 3 experiments are shown. Panel B. Normal human fibroblasts were stimulated with different concentrations of IgG from patients with ecGVHD (15 minutes), PDGF (15 ng/ml for 15 min) or grown in 0.2 percent foetal calf serum. Cell lysates were immunoprecipitated with a polyclonal antibody against PDGFR (subunit α) and the immunoblots were developed with a specific antibody against phosphorylated tyrosine. FCS indicates foetal calf serum.
Fig. 3 Stimulatory antibodies to PDGFR from patients with ecGVHD induce type I collagen

Type I collagen gene expression by normal human fibroblasts after incubation with 0.2 percent foetal calf serum for 48 hours, PDGF (15 ng/ml for 15 minutes), extensive cGVHD IgG (ecGVHD; 200 µg/ml for 15 minutes), IgG from patients without GVHD (No GVHD IgG; 200 µg/ml for 15 minutes) in the presence and absence of AG1296 (2 µM for 1 hour before treatment), N-Acetylcysteine (NAC; 20 mM for 1 hour before treatment), and farnesyl protein transferase inhibitor FTI-277 (20 µM for 2 hours before treatment). Real-time quantitative (RT)-PCR of the transcripts of genes encoding the α1 and α2 chain of type I collagen was performed as described in the Material and Methods section.

Fig 4 Ha-Ras-ERK 1/2-ROS signaling in fibroblasts from patients with cGVHD

Panel A Levels of reactive oxygen species, evaluated as DCF fluorescence intensity (arbitrary units) in three fibroblast cell lines from patients with and without GVHD (ecGVHD and No GVHD, respectively). Fibroblasts from patients with ecGVHD were also pre-incubated with selective inhibitors of EGFR and PDGFR (AG 1478 and AG 1296, respectively; 2 µM for 2 hours).

Panel B. Left part The data shown were obtained with 2 and 4 fibroblast cell lines from patients with and without GVHD (ecGVHD and No GVHD, respectively) cultured in 0.2% FCS for 48 hours before being harvested. Immunoblots were developed with specific antibodies against Ha-Ras on cell lysates immunoprecipitated with a monoclonal anti-pan-Ras antibody. Phosphorylated forms of ERK 1/2 were detected by immunoblotting with phosphospecific antibodies. The right part of the panel shows the densitometric analysis of the blots of the left part.
Tab 1: Clinical Characteristics of the 39 transplanted patients

<table>
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<tr>
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<th>Patients with ecGVHD</th>
<th>Patients without cGVHD</th>
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</thead>
<tbody>
<tr>
<td>Age recipients:</td>
<td>43 (17-70)</td>
<td>47 (22-70)</td>
</tr>
<tr>
<td>Sex recipients:</td>
<td>M/F 18/21</td>
<td>12/10</td>
</tr>
<tr>
<td>Age donors:</td>
<td>35 (11-71)</td>
<td>39.5 (24-69)</td>
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<tr>
<td>Sex donors:</td>
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<tr>
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<tr>
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<td>1</td>
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<td>Status before transplantation</td>
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<tr>
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<td>6/7</td>
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<tr>
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<td>1/8</td>
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</tr>
<tr>
<td>Relapse</td>
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ecGVHD= extensive chronic Graft-versus-Host Disease; ANLL= Acute Non Lymphoblastic Leucemia; ALL= Acute Lymphoblastic Leucemia; NHL= Non-Hodgkin’s Lymphoma; HL= Hodgkin’s Lymphoma; MM= Multiple Myeloma; CML= Chronic Myelogenous Leukemia; CLL= Chronic Lymphocytic Leukemia; MDS= Myelodysplastic syndrome; CR= Complete remission; PR= Partial remission; SD= Stable disease; PD= Progressive Disease; HLA-id. sibling= marrow from identical siblings; MUD= Matched-unrelated donor; CB= Cord Blood; HLA mismatch= mismatched marrow from parents/siblings; HLA haplo= marrow from haploidentical parents/siblings; PBSC= Peripheral Blood Stem Cell; BM= Bone Marrow.
<table>
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*MTX* = methotrexate; *MMF* = Mycophenolate mofetil; *CsA* = Cyclosporin A; *ATG* = antithymocyte globulin; *ECP* = photopheresis
Figure 1

A

Fα

B

F -/-

Stimulation index

N  ecGVHD  ecGVHD  ecGVHD  NoGVHD
+ AG 1296 + AG 1478

N  ecGVHD  ecGVHD  ecGVHD  NoGVHD
+ AG 1296 + AG 1478
Figure 2

A

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PDGF R α

PDGF R β

B

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<th>FCS</th>
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<th>ecGVHD IgG</th>
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<td>0.2%</td>
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pTyr

PDGF Rα
Figure 3

Col1 (α1)

Fold of increase

FCS 0.2%  PDGF  ecGVHD IgG  NAC  FTI  AG 1296  No GVHD

+ecGVHD IgG

Col1 (α2)

Fold of increase

FCS 0.2%  PDGF  ecGVHD IgG  NAC  FTI  AG 1296  No GVHD

+ecGVHD IgG
Figure 4

A

<table>
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<tr>
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DCF Fluorescence (arbitrary units)

B

Ha-Ras

pERK 1-2

β-actina

DU

DU

No GVHD ecGVHD

Ha-Ras

pERK 1-2
Stimulatory autoantibodies to PDGF receptor in patients with extensive chronic graft-versus-host disease

Silvia Svegliati, Attilio Olivieri, Nadia Campelli, Michele Luchetti, Antonella Poloni, Silvia Trappolini, Gianluca Moroncini, Andrea Bacigalupo, Pietro Leoni, Enrico V. Avvedimento and Armando Gabrielli