A molecular and computational diagnostic approach identifies FOXP3, ICOS, CD52 and CASP1 as the most informative biomarkers in acute graft-*versus*-host disease

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ABSTRACT

Background
Acute graft-*versus*-host disease is a severe complication of allogeneic stem cell transplantation in which the functional immune cells of the donor recognize the recipient as foreign and mount an immunological attack. There is an urgent need for better diagnostic instruments for the assessment of acute graft-*versus*-host disease. In the present study, a novel bioinformatics framework was used to identify gene expression patterns associated with acute graft-*versus*-host disease in patients undergoing allogeneic hematopoietic stem cell transplantation.

Design and Methods
Peripheral blood cells were collected prospectively from patients who did develop acute graft-*versus*-host disease (YES) and from those who did not (NO). Gene expression profiling was performed using a panel of 47 candidate genes potentially involved in alloreactive responses. The entire population of YES/NO acute graft-*versus*-host disease patients formed the experimental validation set. Personalized modeling based on a gene selection technique was applied to identify the most significant mRNA transcripts, which were then used to profile individual data samples for training and testing the classification/prediction framework.

Results
A leave-one-out cross-validation procedure was performed to investigate the robustness of the classification framework producing the following results: 100% on the training dataset and 97% on the testing dataset. According to our integrated methodology, transcripts for *FOXP3*, *ICOS*, *CD52* and *CASP1*, genes involved in immune alloreactive responses and participating in immune cell interactions, were identified as the most informative biomarkers in allogeneic stem cell transplant recipients experiencing acute graft-*versus*-host disease.

Conclusions
This study demonstrates that the integrated methodology proposed is useful for the selection of valid gene targets for the diagnosis of acute graft-*versus*-host disease, producing satisfactory accuracy over independent clinical features of the allogeneic transplanted population.

Key words: stem cell transplantation, graft-*versus*-host-disease, computational method, gene expression profile.

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Introduction

The establishment of the donor's immune system in an antigenically distinct recipient undergoing allogeneic hematopoietic stem cell transplantation (HSCT) confers a desired therapeutic graft-*versus*-tumor effect. It can, however, also result in detrimental acute graft-*versus*-host disease (aGvHD) associated with a high rate of transplant-related mortality. The aGvHD phenomenon occurs classically within 100 days after transplantation and is mainly associated with the clinical consequences of an immune-mediated attack or "cytokine storm" of target tissues including the gastrointestinal tract, liver and skin.

More specifically, aGvHD is a complex disease resulting from donor T-cell recognition of a genetically disparate recipient that is unable to reject donor cells following allogeneic HSCT.[1] In fact, the induction of aGvHD is dependent on the presentation of host alloantigens by antigen-presenting cells to naïve donor T cells.[2] Unfortunately, the clinical diagnosis of aGvHD is not always straightforward or easy to establish, and skin or intestinal biopsies are required to confirm the diagnosis. Consequently, therapeutic strategies based on the suppression of critical molecular pathways involved in T-cell activation and the clinical manifestation of aGvHD may lead to unnecessary over-treatment by clinicians. Extensive research is currently underway to identify blood-based biomarkers of aGvHD using novel tools including proteomic and molecular methods.[3,4] In this respect, proteomic analysis has identified several serum proteins as potential biomarkers of multi-organ aGvHD. One promising biomarker, Elafin protein, has been proposed to have significant diagnostic and prognostic value specifically for skin GvHD.[5] Recently, a strong association was observed between soluble tumor necrosis factor receptor 1 (sTNFR1) levels and the development of aGvHD, although the low sensitivity of sTNFR1 determination reduces the clinical utility of this biomarker.[6] Donor gene-expression profiles (GEP) can also be used to predict the occurrence of chronic GvHD in the recipient.[7] Although only a pilot study, Buzzeo *et al.* identified a GEP signature of aGvHD, independent of tissue localization, in patients undergoing allogeneic HSCT.[8]

In the present study, we evaluated the gene expression levels of a complex panel of 47 functional candidate genes hypothesized to be involved in immune alloreactive responses in aGvHD.[9] We examined the applicability of GEP analysis for supporting the diagnosis of aGvHD at presentation of symptoms with multiple targets and severity.

Although transcriptional profiles may be obtained from many human tissues, the status of the immune system can be best monitored by profiling transcripts in the blood. We, therefore, chose to use unselected peripheral blood cell (PBC) samples, which have the advantage of being easily accessible and offering a non-invasive way of monitoring molecular changes safely.

The major objective of our study was to determine whether GEP could be used to discriminate, at the onset of a clinical suspicion of aGvHD, those allogeneic HSCT recipients who will develop aGvHD from those who will not. A novel computational framework previously presented in the technical literature[10,11] was applied in this study.

Design and Methods

Patients and graft characteristics

In this single center study we included 59 consecutive patients who received allogeneic transplants between 2007 and 2010. Hematopoietic progenitor cells were collected by apheresis and used as stem cells in 44 cases, whereas in 15 transplants the source of stem cells was bone marrow. The characteristics of the patients, transplants and grafts are presented in Table 1.

Any GvHD before day100 was classified as acute. The severity was categorized as grade I-IV according to modified Glucksberg criteria (A-D by the International Bone Marrow Transplant Registry index).[12,13] The diagnosis of aGvHD was confirmed by skin or gastrointestinal biopsy in all cases. Prophylaxis for aGvHD consisted mainly of cyclosporine A (2 mg/kg on day -1, 1 mg/kg from day 0) combined with a short-term course of methotrexate: 15 mg/m^2 on day +1, and 10 mg/m^2 on days +3 and +6 and also on day +11 in the case of mild-moderate mucositis. In the case of an alternative donor, anti-lymphocytic serum at a total variable dose ranging from 4.5-15 mg/kg was added to the conditioning regimens for *in vivo* T-cell depletion. In the case of total lymph-node irradiation plus antithymocyte globulin, methotrexate was replaced by mycophenolate mofetil at a maximum dose of 2 g daily. For patients undergoing haploidentical transplantation, aGvHD prophylaxis consisted of cyclophosphamide 50 mg/kg on days +3 and +4 followed by tacrolimus and mycophenolate mofetil.

Experimental design and sampling

The study was approved by the local Ethics Committee and the procedures followed were in accordance with the World Medical Association's Helsinki Declaration. PBC were collected from all patients +15, +30, +45, +60, +90 and +120 days after HSCT and at each event compatible with suspected aGvHD. Overall, 400 samples were collected. Because the experimental design plays a crucial role in the search for useful biomarkers, the first step was to choose the most informative specimens. To this end, we selected those samples obtained upon observation of the first clinical symptoms and which were confirmed by biopsy. Positive cases of aGvHD (YES) and controls negative for aGvHD (NO) were matched to avoid bias due to immunosuppressive treatment. This matching was best achieved using a database containing high-quality samples linked to quality controlled clinical information. We used 23 samples from aGvHD (YES) patients obtained at diagnosis and before any anti-aGvHD therapy and we selected 36 samples from patients who did not experience aGvHD (NO). Recipients were regarded as not having developed aGvHD when they remained at least 120 days without presenting any aGvHD events. The entire group of YES/NO patients formed the validation population for subsequent modeling studies.

Biological samples RNA extraction, low density gene expression assay

Total RNA was extracted from PBC samples using the RNAeasy Mini Kit (Qiagen) according to the manufacturer's instructions. Purified RNA was reverse transcribed using the High Capacity cDNA Archive kit with random primers (Applied Biosystems), and a multigene expression assay was carried out with a TaqMan® Low Density Array Fluidic card (TLDA-card) on the PRISM Real-Time 7900HT Sequence Detection system platform (Applied Biosystems), according to the manufacturer's instructions. For the TLDA-card, we selected 47 candidate genes[9] (*Online Supplementary Table S1*) involved in the immune network and in the pathogenesis of inflammation; *18S* was con-

sidered the endogenous reference gene. A pool of PBC from ten healthy volunteers was used to obtain cDNA to be used as the calibrator for the assay. Expression of each gene transcript was measured in triplicate and then normalized to the calibrator sample.

Quantitative real-time polymerase chain reaction, single assay

Total RNA was extracted from PBC samples or healthy donors and cDNA was obtained as described above. The levels of expression of *FOX-P3* (ID 00203958_A1) and *ICOS* (ID 00359999_A1) were determined using primers and probes from Applied Biosystems (ABI Assays on Demand; *http://www.appliedbiosystems.com/*). The *18S* pre-developed TaqMan assay (99999901_S1) was used as the endogenous control. All gene expression assays were performed on the ABI PRISM Real-Time 7900HT Sequence Detection system (Applied Biosystems). The relative expression level of target genes was determined using the ΔΔCT method.

Immunohistochemical analysis

Immunohistochemical staining was performed on 3 μm thick serial sections cut from formalin-fixed and paraffin-embedded tissues. Conditions were optimized for each primary antibody: anti-FOXP3 (mouse monoclonal antibody, Abcam) and anti-ICOS (rabbit monoclonal antibody, Spring Bioscence). Antigen retrieval was performed in a temperature-controlled water bath at 98 °C with EDTA pH 9/citrate buffer pH 6 and antibody binding was visualized using an EnVision™ Detection System, Peroxidase (DAKO) with DAB as the chromogen. Tonsil and lymph node were used as positive controls for FOXP3 and ICOS, respectively. The negative control was included with each run by substituting the primary antibody with non-immune rabbit serum. The control sections were treated in parallel with the samples.

Personalized modeling for identification of target genes in acute graft-versus-host disease

With the aim of identifying the most informative genes able to detect aGvHD at the onset of clinical signs, we proceeded to better define our dataset by pre-processing experimental variables such as race, disease, stem cell source, HLA tissue typing, number of mismatched loci between donor/recipient, donor/recipient age, donor/recipient sex, conditioning intensity, and donor/recipient blood group. In this study, we used a novel personalized modeling based gene selection (PMGS) method,[11] previously developed for GEP data tailored to the diagnosis/classification of aGvHD. Personalized modeling is a relatively new method in bioinformatics research and published information based on this method is still fairly sparse. Representative works have been published by Song and Kasabov.[14,15]

The main objective of personalized modeling is to create a model for each patient (sample), which is able to reveal the most important information specifically for each sample, focusing attention on the individual patient (sample) rather than simply on the global problem space.[16,17]

Previous works have reported that personalized modeling can produce better classification results than those obtained from classical global modeling,[14,16,18] making it more appropriate to build clinical decision support systems for new patients. The framework proposed by Fiasché *et al.*[11,19] used a "personalized" wrapping method, a PMGS described in detail in recent papers[20,21] for gene expression data analysis, integrating new data with the existing models; the block diagram is reported in Figure 1. We used weighted-weighted distance K-nearest neighbor (WWKNN) as the classification algorithm[15] given that the simple weighted distance K-nearest neighbor (WKNN) algorithm had returned better results than other classification techniques in previous experiments (an interesting comparison is reported by Fiasché's group).[11,19,20]

Finally, a compact genetic algorithm[22] was used to optimize the learning function during the training process making framework performances useful in clinical applications.

Statistical analysis

Results of quantitative relative real-time polymerase chain reaction, performed as single assays, were used to calculate the area under the curve (AUC) by receiver operating characteristic (ROC) analysis for *FOXP3* and *ICOS* expression levels. Statistical analyses were performed using the SPSS software package for Windows, release v13.0, 2004 (SPSS, UK).

Table 1. Main hematologic characteristics of the HSCT recipients, transplant properties, conditioning regimens, prophylaxis and treatments of aGvHD.

Number of patients	59
Sex (male/female)	31/28
Median age (range)	43 (14-70)
Diagnosis	
Acute myeloid leukemia/acute lymphoblastic leukemia	29/9
Chronic myeloid leukemia/myelodysplastic syndrome	5/1
Non-Hodgkin's lymphoma/Hodgkin's lymphoma	3/2
Chronic lymphocytic leukemia	6
Myelofibrosis/chronic myelomonocytic leukemia	1/1
Paroxysmal nocturnal hemoglobinuria/multiple myeloma	1/1
Disease status at allogeneic HSCT	
CR/PR/PD	19/39/1
Donor type	
Median age (range)	43 (14-70)
Sex (male/female)	38/21
Sibling HLA-identical	43
Alternative HLA- identical	8
Alternative HLA- mismatched	2
Haploidentical	6
Conditioning regimens	
Myeloablative conditioning	27
Reduced intensity conditioning	32
aGvHD YES/NO	23/36
Type: skin/gut	20/3
Grade I/ II/ III	5/14/4
aGvHD prophylaxis	59
Cyclosporine A + methotrexate	36
Cyclosporine A + mycophenolate	19
Tacrolimus + mycophenolate	3
None	1
Graft-*versus*-host disease treatments	
Prednisone	17
Prednisone + extracorporeal photoapheresis	5
Prednisone + infliximab	1
Duration; days (range)	44.7 (16-75)

CR: complete remission; PR: partial remission; PD: progressive disease.

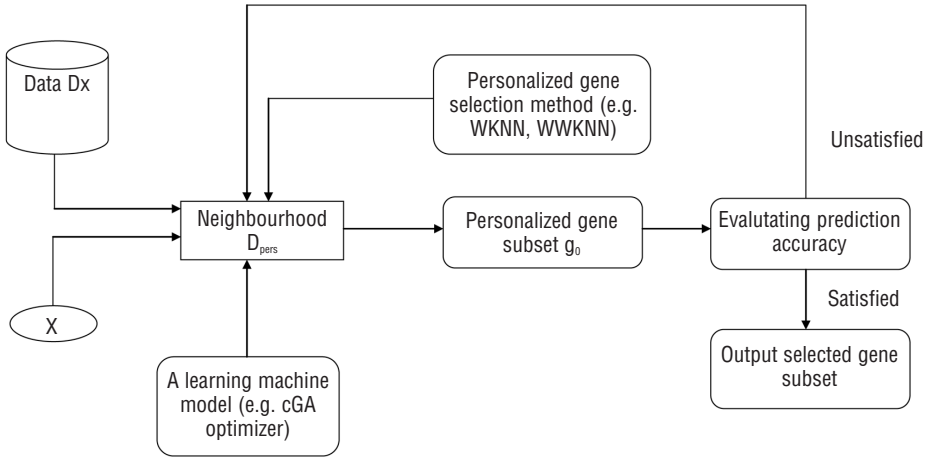

Figure 1. A block diagram representing the PMGS algorithm.

Results

Personalized modeling-based gene selection identified FOXP3, ICOS, CD52 *and* CASP1 *as the most informative gene transcripts associated with acute graft-*versus*-host disease*

In our attempt to determine a gene expression signature that is diagnostic of aGvHD, we first correlated global GEP data obtained from the TLDA-card with the occurrence of aGvHD in allogeneic HSCT recipients at the onset of clinical signs. A heat-map of differentially expressed genes is shown in *Online Supplementary Figure S1*. Since simple statistical analysis or classical data-mining techniques failed to identify a specific gene pattern, we used computational intelligence methods according to previously published algorithms.[17,19,20] The PMGS approach was applied to the data obtained from the TLDA-card to identify a group of genes to represent the data set. In our framework the most important genes selected for each patient may differ (personalized subset), but during our analysis, it was evident that for each run, four genes were always present in the entire subset of patients who did develop aGvHD. The following genes were always identified with other clinical variables: *FOXP3* (a member of the forkhead transcriptional factor family), *ICOS* (a CD28 homolog termed inducible co-stimulator), *CD52* and *CASP1* (caspase 1 or apoptosis-related cysteine peptidase). Notably, the leave-one-out cross-validation (LOOCV) testing procedure system had an accuracy of 97%, specificity of 0.96, and sensitivity of 1.

*Application of the personalized modeling-based gene selection method identified the characteristics of patients with acute graft-*versus*-host disease*

The new model, created for each step, was trained (*D0tr*) with the personalized model for 59 runs and a LOOCV (97% for the integrated method) was calculated for the new dataset *D*, obtained by adding a new sample for each new run. A scenario of personalized feature weights is shown in Table 2. It is evident that HLA tissue typing, number of mismatched loci and age are relevant characteristics in the personalized approach making it possible to select a very low number of transcript genes with good classification results. Another important observation was made from our experimental computational analysis, as shown in the scenario in Table 2 for two samples representative of the whole dataset, which, however, differed in our personalized modeling for the sex variable. In our personalized analysis, age had a different weight in male and female subjects. Indeed, in female subjects, "age at transplantation" correlated with the features "donor age" and "blood group", which were more important (higher weight) in female subjects than in male ones. Finally, we must highlight that the gene transcript subgroups: (*FOXP3*, *CASP1*) and (*ICOS*, *CASP1*), selected with the clinical variables above, returned a classification result with an accuracy of 86%.

Table 2. A scenario of "personalized" models for two different subgroups, female (F) and male (M), comparing with weight of variables and genes obtained with global modeling.

Input variables	Subject 1 (F) weights of input variables	Subject 2 (M) weights of input variables	Global weights/ importance (F + M)
Age (years)	0.9101	0.7025	0.7127
HLA type (g/L)	0.8521	0.8447	0.8429
Number of mismatched loci	0.9507	0.8452	0.8769
Conditioning regimen	0.7478	0.752	0.8104
FOXP3	0.9617	0.9269	0.9254
ICOS	0.7295	0.8641	0.8228
CASP1	0.8651	0.7459	0.8096
CD52	0.8797	0.8802	0.9009
Actual output			
Predicted output with PMGS	0.95	1.01	

An example demonstrating how PMGS can present the analysis results from a data sample is provided in Figure 2. For four sample-patients, the PMGS method selected three genes as the best subgroup (*CASP1*, *FOXP3*, *ICOS*) and (*FOXP3*, *ICOS*, *CD52*) and the classifier successfully predicted corresponding samples as diseased.[19] It is easy to appreciate that a sample is more likely to be in the diseased group, since most of its nearest neighbors belong to the diseased group.

Gene expression assay confirmed low expression of FOXP3 *and* ICOS *in acute graft-*versus*-host disease*

All patients' samples identified as aGvHD (YES) or (NO) included in the validation setting were simultaneously quantified by gene expression assays for *FOXP3* and *ICOS*

transcripts. ROC curve analysis was used to identify the best informative transcripts associated with aGvHD resulting from the computational algorithm (Figure 3). Since we demonstrated that the levels of expression of *FOXP3* and *ICOS* were very different between aGvHD YES and NO subsets, we also determined the minimum expression level that indicated the aGvHD NO condition. Specifically, the cut-off value of the relative expression level of target gene was 1.40 for *FOXP3* (AUC=0.745, *P*=0.002) and 1.44 for *ICOS* (AUC=0.944, *P*<0.0001).

Immunohistochemical analysis confirmed the presence of low levels of FOXP3 and ICOS proteins in cutaneous biopsies from patients with acute graft-versus-host disease.

In a subset of ten patients with histologically confirmed aGvHD we evaluated FOXP3 and ICOS protein expression by immunohistochemistry in formalin-fixed, paraffin-embedded skin tissue biopsies. In all cases, rare FOXP3$^+$ lymphocytes and scattered ICOS$^+$ activated lymphocytes were observed at the dermo-epidermal junction, in the superficial dermis and around the adnexal follicular epithelium (Figure 4).

Discussion

In our study we used dynamic genomic markers derived from peripheral blood gene expression profiling to develop an instrument for assessing aGvHD quickly and non-invasively. Not only is blood easier to obtain than tissue, but it also contains a number of circulating cell types that are mechanistically associated with immunological disease processes.[23]

It is interesting that in addition to the more well-known mechanisms involved in aGvHD,[24] novel players have recently been discovered, such as *CASP1* and *FOXP3*, supported by an intense body of research which has provided compelling evidence for their correlation with aGvHD.[9,25] For example, a humanized monoclonal antibody against CD52 has been widely used for preventing aGvHD in allogeneic HSCT.[26] Notably, CD52 signaling is correlated with induction of CD4$^+$ regulatory T cells,[27] and *FOXP3* is the major gene transcript representative of CD4$^+$/CD25$^+$/CD127$^-$ Treg. In our study, *FOXP3* and *ICOS* were the most informative genes selected by PMGS. These findings were then validated by single gene expression assays, which showed significant AUC values for *FOXP3* and *ICOS* and cut-off values suggestive of the occurrence of aGvHD. Notably, immunohistochemical analyses, performed on cutaneous biopsy specimens from aGvHD (YES) patients, showed small amounts of FOXP3 and ICOS proteins, in accordance with the observed low expression of their molecular transcripts.

In clinical practice the low expression levels of these genes could be helpful for discriminating patients who are developing aGvHD from those who are not. In addition, we observed that, in patients with aGvHD who underwent anti-aGvHD treatments, the number of copies of *FOXP3* and *ICOS* increased in patients responding to treatment (*data not shown*). This phenomenon also occurred for genes regulated by ICOS, including T-helper (Th)2 cytokines (i.e.IL-4, STAT-6, IL-18). This finding is supported by the fact that Th2 cell therapy can rapidly improve severe aGvHD via IL-4- and IL-10-mediated mechanisms.[28] Thus, determining the levels of *FOXP3* and *ICOS* gene expression may represent a molecular

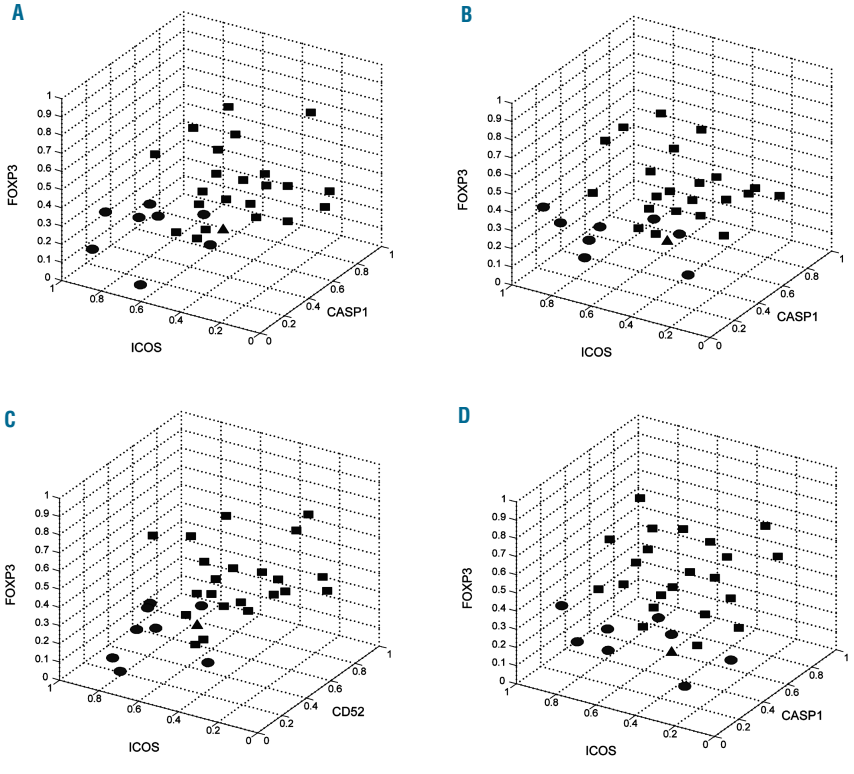

Figure 2. Visualization of the results of the decision making framework with three genes: triangular point, actual value of this sample; upward square points, healthy; downward circle points, diseased. Examples of incremental-personalized-method during the execution of the run: (A) sample with ID 8, (B) sample with ID 20, (C) sample with ID 3, (D) sample with ID 14. Note: the values on x and y axes are normalized to 0 and 1.

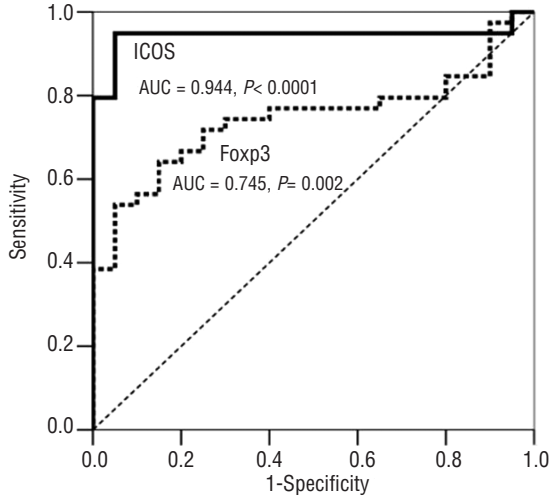

Figure 3. ROC curve analysis identifying the most suitable cut-off of relative expression for *FOXP3* (1.40) and *ICOS* (1.44) target genes for patients experiencing acute graft-*versus*-host disease

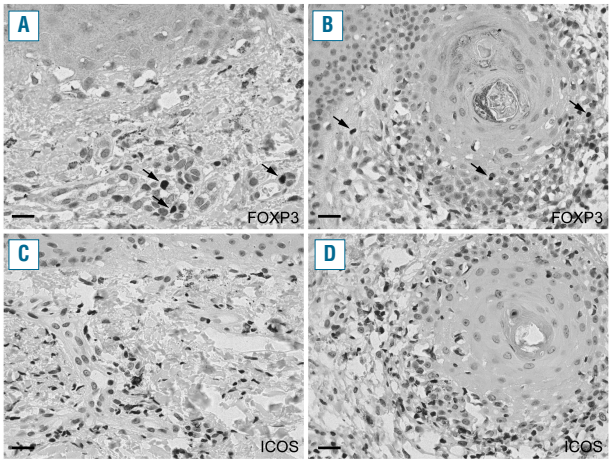

Figure 4. Immunohistochemical analysis on formalin-fixed and paraffin-embedded specimens from cutaneous GvHD. (A-B) Rare FOXP3$^+$ lymphocytes (arrows) can be seen in the superficial dermis and in the adnexal follicular epithelium. (C-D) Scattered ICOS$^+$ activated lymphocytes can be seen around dermal capillaries of the superficial plexus and around the adnexal follicular epithelium. Lymphocytes are intermingled with numerous melanophages (brown granular staining). (Original magnification x40, scale bar=20 μm).

approach to monitoring the efficacy of therapy in allogeneic HSCT recipients with aGVHD. This could be of clinical value, since the method used in this study may identify patients with a deteriorating acute syndrome, despite the administration of corticosteroids. We found that the *ICOS* gene was down-regulated during aGvHD in allogeneic HSCT recipients, which is a novel observation in humans. Previously, Buzzeo *et al.*[8] observed that the human ICOS ligand was down-regulated during the onset of aGvHD, but changes in *ICOS* expression have never been reported.

Great attention is placed on the role of ICOS in the pathophysiology of aGvHD. ICOS is a CD28/cytotoxic T lymphocyte antigen 4 (CTLA-4) family member expressed on activated T cells.[29] The role of ICOS in aGvHD is controversial. In experimental mice models, ICOS exacerbated Th1-mediated aGvHD[30] while another animal study demonstrated that ICOS blockade had positive effects on inhibiting aGvHD. In contrast, Witsch showed that the ICOS signal inhibits the development of aGvHD mediated by CD8$^+$ effector cells in myeloablative bone marrow transplantation.[31,32]

From a clinical standpoint, the best transcript gene targets prioritized by computational systems for the assessment of immunological alloreactions in HSCT patients are also very robust since they exhibit the same behavior even in the presence of confounding factors related to sex or HLA donor-disparity, transplant conditioning and co-morbidity such as viral infection. In fact, the expression levels of the most informative genes were not influenced in the subset of patients who were positive for DNA cytomegalovirus (*data not shown*). We observed that this model was applicable to all cases of aGvHD including four cases of grade III aGvHD. Moreover, the level of expression of the four most informative genes showed a slight trend in correlating with non-relapse related mortality (*data not shown*).

The transferability of the aGvHD-linked gene-expression profiles might suggest a major epigenetic influence rather than an environmental one. However, higher-order predictive variable combinations do require the support of many more samples in order to prevent over-fitting of the model. Convincing assessment of this issue will, therefore, require expression profiling of the genes identified here in larger cohorts of participants and validation by other centers before it can be widely used to guide clinical decision-making processes.

In conclusion, our results could lay the basis for a novel approach in transplantation medicine by contributing to the differentiation of an immune reaction from other morbidities and to tailoring immunosuppressive regimens.

Authorship and Disclosures

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