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Increase in FOXP3$^+$ Regulatory T Cells in GVHD Skin Biopsies Is Associated with Lower Disease Severity and Treatment Response

Cristina Fondi,[1] Chiara Nozzoli,[2] Silvia Benemei,[3] Gianna Baroni,[1] Riccardo Saccardi,[2] Stefano Guidi,[2] Paola Nicoletti,[3] Benedetta Bartolozzi,[2] Nicola Pimpinelli,[4] Marco Santucci,[1] Alberto Bosi,[2] Daniela Massi[1]

In animal models, CD4$^+$/CD25$^+$ T-regulatory cells (Tregs) have been reported to prevent/delay the onset of graft-versus-host disease (GVHD). Recently, an insufficient upregulation of Tregs was found in target organ (intestinal) biopsies from patients with GVHD. We have analyzed by immunohistochemistry the number of CD3$^+$ T lymphocytes and FOXP3$^+$ Tregs in skin biopsies from (1) recipients of allogeneic hematopoietic stem cell transplantation (HSCT, n = 26), (2) nontransplanted patients diagnosed with cutaneous drug reaction (n = 12), and (3) healthy donors (n = 10). Infiltrating CD3$^+$ cells were significantly higher in both transplanted patients showing acute GVHD (aGVHD) and drug reaction when compared to healthy donors and patients without GVHD. Tregs number in aGVHD was higher than in patients without GVHD or healthy subjects and lower than in drug reaction. Interestingly, the number of infiltrating FOXP3$^+$ Tregs was significantly higher in patients responding to GVHD treatment and with a low GVHD grade. Increase in FOXP3$^+$ Tregs in GVHD skin biopsies correlates with less severe GVHD and is associated with response to GVHD treatment. Larger studies are required to confirm that evaluation of Tregs in minimally invasive skin biopsies assists the diagnosis and prognosis of GVHD patients.

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INTRODUCTION

Allogeneic hematopoietic stem cell transplantation (allo-HSCT) is successfully applied to the treatment of hematopoietic malignancies as well as genetic disorders. However, the application of allo-HSTC is limited because of severe life-threatening complications such as graft-versus-host disease (GVHD) [1,2]. Reduction in conditioning regimen-related toxicity, which results in a wider application of allo-HSCT, does not appear to decrease the incidence of GVHD [3,4]. Depending on the different type of transplantation, immunosuppressive treatment and underlying diseases, between 35% and 70% of patients develop GVHD after allo-HSCT, with >35% of these patients requiring specific immunosuppressive therapy [5].

In the development of GVHD, donor T lymphocytes responding to the allogeneic antigens undergo clonal expansion, developing cytotoxic reactivity against recipient histocompatibility antigens. Recently, much attention has been directed to regulatory T (Treg) cells and to their potential to attenuate GVHD in animal models [6,7]. In particular, CD4$^+$/CD25$^+$ Tregs cells have been proposed to play a key role because they inhibit activation of reactive T cells in an antigen-specific and cell contact-dependent manner [8-11]. This effect may delay the onset or prevent the occurrence of GVHD [12-14].

In humans, the impact of Treg cell count in the prognosis of GVHD is controversial, and discrepancies may result from differences in surface phenotyping techniques used to characterize these cells [15-22]. In most studies Treg phenotype has been obtained by CD4 and CD25 coexpression. However, in humans, CD25 (interleukin-2 receptor α chain) is also expressed by recently activated T cells. FOXP3, which encodes a forkhead-winged-helix transcription

From the [1]Department of Human Pathology and Oncology, University of Florence, Florence, Italy; [2]Department of Haematology, Bone Marrow Transplantation Unit, University of Florence, Florence, Italy; [3]Department of Preclinical and Clinical Pharmacology, University of Florence, Florence, Italy; and [4]Department of Dermatological Sciences, University of Florence, Florence, Italy.

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Correspondence and reprint requests to: Daniela Massi, MD, Department of Human Pathology and Oncology, University of Florence, Viale G.B. Morgagni 85, I-50134, Firenze, Italia (email: daniela.massi@unifi.it).

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factor designated Scurfin [23], is currently considered the more specific marker for Tregs [24,25]. An additional confounding factor derives from the fact that evaluation of Tregs has been performed in peripheral blood, a compartment that may not reflect their number in target organs of GVHD, such as the gut, the liver, and the skin.

Recently, $FOXP3^+$ Tregs were found to be expanded in intestinal biopsies of allografted patients without GVHD and selectively downregulated in biopsies of patients with GVHD, thus suggesting that an insufficient upregulation of Tregs in the target organ may contribute to the underlying mechanism of the disease [26]. However, it is not known if Tregs downregulation is a unique feature of intestinal GVHD or if it can be found in other tissues and organs affected by GVHD, including the liver and the skin. In addition, skin rashes of GVHD show overlapping features with various cutaneous erythematous drug reactions, not infrequently observed in posttransplantation period, thus raising a challenging diagnosis with relevant therapeutic consequences [27].

In the present investigation, we have compared the number of $CD4^+/CD25^+/FOXP3^+$ Tregs in skin biopsies taken under different clinical circumstances, including, (1) lesional skin of acute GVHD (aGVHD) or chronic GVHD (cGVHD) patients, (2) in HSCT recipients without GVHD showing mild to moderate erythema (non-GVHD rash), (3) lesional skin from nontransplanted patients with cutaneous drug reactions, and (4) healthy skin from patients undergoing plastic surgery. An increased number in Tregs was associated to a favorable outcome of both aGVHD and cGVHD. Thus, present results support the hypothesis that $FOXP3^+$ Tregs exert a protective role in cutaneous GVHD.

PATIENTS, MATERIALS, AND METHODS

Patients and Samples

A retrospective analysis of 48 paraffin-embedded tissue samples obtained from the Department of Human Pathology and Oncology, University of Florence, was performed. Biopsies were taken from: (1) normal healthy skin (n = 10) obtained from residual specimens discarded after plastic surgery of the breast or abdomen; (2) skin biopsies taken on cutaneous erythemas (n = 6) from patients undergoing allogeneic stem cell transplantation (SCT; biopsied from 14 to 71 days upon transplantation, median 27 days), who subsequently did not develop clinical GVHD (non-GVHD rash category); (3) skin biopsies taken on erythematous macular and/or papular rash (n = 20) in allografted patients with clinical signs of (suspect) GVHD, graded according to standard criteria; and (4) skin biopsies from nonallografted patients with clinically and histopathologically confirmed drug reactions (n = 12). Specifically, the non-GVHD rash group included transplanted patients experiencing erythematous rashes in the posttransplant period. Biopsies were performed in these patients to clarify the nature (toxic versus drug-induced versus viral versus GVHD) of the skin rash. To be included into the non-GVHD rash group, patients should not have developed any clinical manifestation related to GVHD involving either the skin or other organs and experienced

Table 1. Characteristics of Patients Undergoing Allogeneic Stem Cell Transplantation (n = 26)

	GVHD (n = 20)	no GVHD (n = 6)
Median age, years	47	45
Sex		
Male	12	4
Female	8	2
Diagnosis		
Chronic myeloproliferative syndrome	3	1
Acute lymphoblastic leukemia	2	1
Acute myelogenous leukemia	4	2
Chronic lymphocytic leukemia	1	0
Myelodysplastic syndrome	1	0
Multiple myeloma	4	2
Non-Hodgkin lymphoma	3	0
Hodgkin lymphoma	1	0
Aplastic anemia	1	0
Conditioning regimen		
Myeloablative	6	4
Reduced-intensity	14	2
Stem cell source		
Bone marrow	5	5
Peripheral blood	14	1
Cord blood	1	0
Donor		
Related	11	5
Unrelated	9	1
GVHD reactions		
Acute	14	
Chronic	9	
Acute GVHD grade		
Grade I	4	
Grade II-IV	10	
Median time after transplantation (range), days	22 (10-40)	
Chronic GVHD grade		
Limited	3	
Extensive	6	
Chronic GVHD		
De novo	5	
Progressive	4	
Median time after transplantation (range), days	188 (120-468)	
Prophylaxis		
CsA + MTX	13	5
CsA + MMF	7	1
T cell depletion (ATG)		
Yes	6	1
No	0	5
Response to GVHD therapy		
Refractory*	7	
Responsive	13	

GVHD indicates graft-versus-host disease; ATG, antithymocyte globulin; CSA, cyclosporine; MTX, methotrexate; MMF, mycophenolate mofetil; *Patients refractory to therapy were defined as patients experiencing progression after 3 days or no change after 7 days or incomplete response after 14 days of specific GVHD therapy.

rash resolution in absence of specific GVHD treatments. Among 6 non-GVHD rash patients, 3 patients experienced HHV6 or cytomegalovirus (CMV) reactivation and the rash disappeared upon antiviral treatment. Three additional patients were interpreted as probable drug-induced rashes. For patients with clinical grade >I GVHD, the diagnosis was made on the basis of concomitant and/or subsequent involvement of other organs (eg, gastrointestinal, liver) and/or decrease of performance status. Patients with clinical grade I GVHD had skin rashes involving 25% to 50% of body surface, and were not on drug therapy potentially inducing cutaneous reaction (trimethoprim/sulfamethoxylol or allopurinol). Diagnostic biopsies in GVHD patients were taken before any specific GVHD treatments were initiated. The study was reviewed and approved by the institutional review board of the University of Florence.

Details of patients undergoing allogeneic bone marrow transplantation are reported in Table 1. Ten patients received a conventional myeloablative conditioning regimen and 16 patients received reduced-intensity conditioning (RIC) regimen. Specifically, within the myeloablative (MA), 4 were total body irradiation containing cyclophosphamide (TBI/Cy) and 6 chemotherapy based on busulfan and Cy (Bu/Cy); within the nonmyeloablative (NMA), 8 were TBI containing fludarabine and [Fill in what "M" is] (TBI/Flu ± M) and 8 chemotherapy based (TTCY). All patients were treated with standard prophylaxis for GVHD (cyclosporing [CsA] + methotrexate [MTX] in 18 patients and CSA + MMF in 8 cases) and 7 cases of transplantation from unrelated donor received additional T cell depletion with antithymocyte globulin (ATG). The majority (16 patients) received transplants from matched related donors and 10 from unrelated donors. Stem cell source was bone marrow (BM) in 10 cases, peripheral blood (PB) in 15 cases, and cord blood (CB) in 1 case. HLA matching was 6/6 in all identical sibling and unrelated donor (PB and BM) transplants, the cord blood transplant (CBT) was performed with 2 CB units at least 4/6 matched. Fourteen patients experienced aGVHD (Grade I in 4 cases, Grade >I in 10 cases) and 9 patients cGVHD (de novo in 5 patients and progressive in 4 patients). cGVHD was limited in 3 patients and extensive in 6 patients. GVHD first line treatment consisted in methylprednisolone at the dose of 2 mg/kg in GVHD and 1 mg/kg in cGVHD. Patients refractory to therapy were defined as patients experiencing progression after 3 days or no change after 7 days or incomplete response after 14 days of specific GVHD therapy.

Immunohistochemical Analysis

Immunohistochemical staining was performed on 3 μm-thick serial sections cut from formalin-fixed and paraffin-embedded tissues. All tissue sections were placed on Ventana automated stainer BenchMark XT™ ICH system, deparaffined, reydrated, and processed blocking endogenous peroxidase and epitope retrieval. The following primary antibodies were used: anti-CD3 (CD3 polyclonal antibody; Cell Marque; CA, ready to use); anti-CD4 (CD4 monoclonal antibody, [mAb] clone 1F6, Diapath, Italy; dilution 1:10); anti-CD25 (CD25 mAb clone 4C9, Novocastra, UK, dilution 1:20). Primary antibodies were placed on tissue sections and incubated for 32 minutes at 37°C using as rivelation system iVIEW DAB Detection Kit. After completion of the staining run, sections were counterstained with hematoxylin. Additional sections from the same specimens were deparaffined in Bio-Clear (Bio-Optica, Milan, Italy) hydrated with grade ethanol concentration until distilled water and placed in 3% hydrogen peroxide/H_2O_2. for blocking endogenous peroxidase. Antigen retrieval by microwave pretreatment in Citrate buffer 10 mM pH 6.0 for 30 minutes was followed by incubation with the mAb anti-FOX-P3 (FOXP3 mAb, Abcam, DBA, Milan, Italy; dilution 1:40). Bound antibody was evaluated using streptavidin-biotin-peroxidase complex technique and 3,3′ diaminobenzidine (liquid DAB, DAKO) as chromogen. The sections were lightly counterstained with Mayer's hematoxylin. A negative control was included with each run by omitting the primary antibody and yielded no signal. As positive control, a tonsil tissue specimen was used. The control sections were treated in parallel with the samples in the same run. Quantification of the frequency of immunostained cells was performed in the single-stained serial sections. The number of

Table 2. Phenotype of the Lymphocytic Infiltrate in 48 Skin Biopsies of Healthy Skin, Non-GVHD rash, aGVHD and cGVHD in Transplanted Patients and Control Drug Reactions

Cell Type	Healthy Skin (n = 10)	Non-GVHD rash (n = 6)	aGVHD (n = 13)	cGVHD (n = 7)	Drug Reactions (n = 12)
$FOXP3^+$	1.43 ± 0.22	0.23 ± 0.16	6.09 ± 1.57	4.66 ± 1.65	20.35 ± 4.39
$CD4^+$	5.08 ± 0.25	4.37 ± 2.80	16.12 ± 3.56	8.17 ± 3.64	47.54 ± 7.756
$CD25^+$	5.90 ± 0.43	4.20 ± 1.43	15.52 ± 3.02	9.06 ± 2.56	22.09 ± 1.60
$CD3^+$	10.94 ± 1.12	11.20 ± 6.24	86.46 ± 20.53	49.77 ± 13.29	91.90 ± 10.66

GVHD indicates graft-versus-host disease; cGVHD, chronic graft-versus-host disease; aGVHD, acute graft-versus-host disease; HFP, high power field. Mean number of $CD3^+$, $CD4^+$, $CD25^+$, and $FOXP3^+$ lymphocytes per HPF, calculated in 5 HPF. Results are expressed as mean ± SEM.

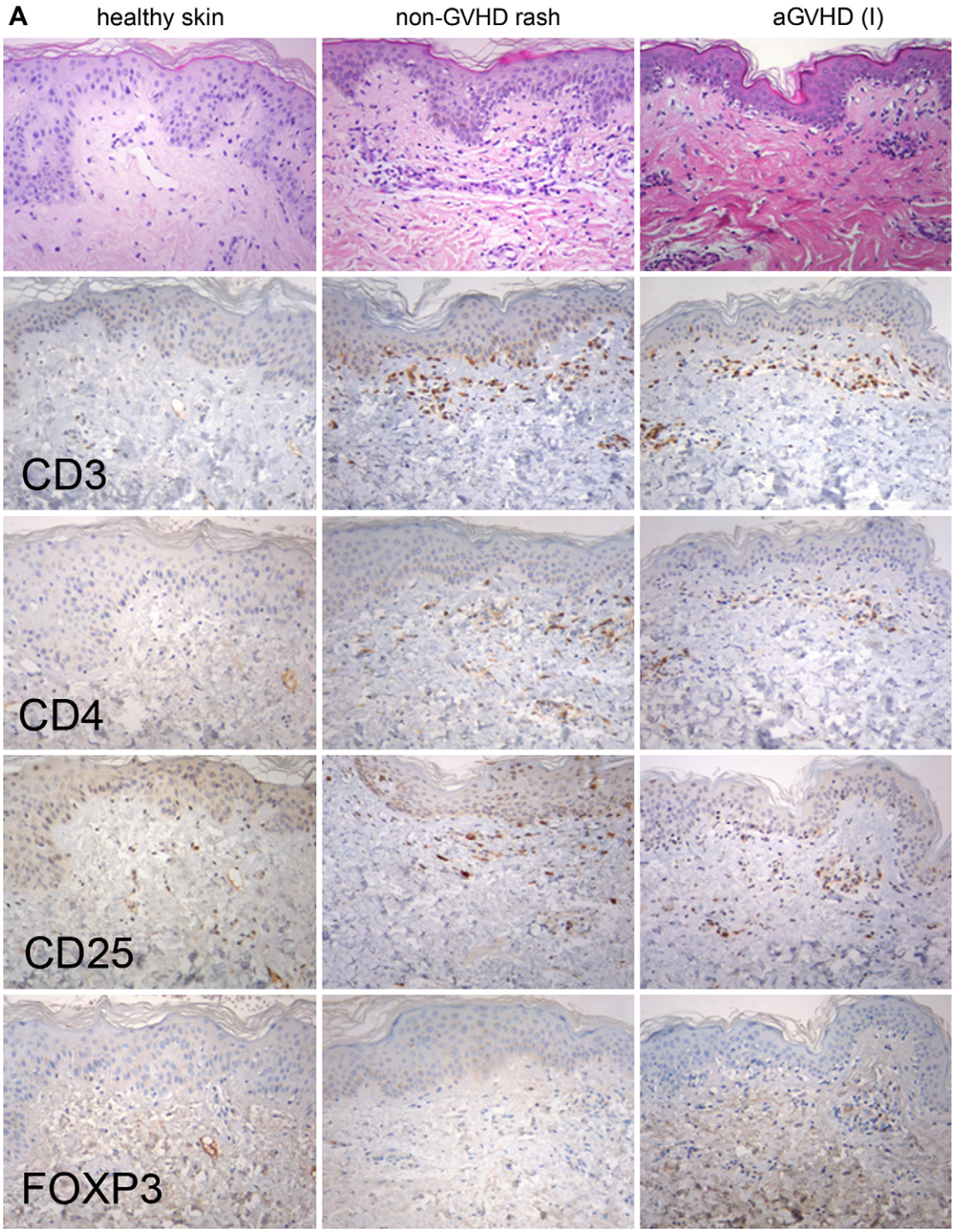

Figure 1. Histology and immunohistochemistry for CD3, CD4, CD25, and FOXP3$^+$ T cells of representative skin biopsies from normal skin, allografted patients with or without GVHD, and drug reactions. A small amount of CD3$^+$ T cells and a low number of regulatory T FOXP3 is observed in the healthy skin, (A) whereas skin biopsies of patients with GVHD shows a high number of CD3$^+$ and an intermediate amount of FOXP3$^+$ T cells mainly observed in the dermoepidermal junction and superficial dermis. In contrast, skin biopsies taken from drug reactions (B) show an elevated number of CD3$^+$ and FOXP3$^+$ cells at the dermoepidermal junctions and superficial dermis especially around keratinocytes displaying many characteristic apoptotic bodies (original magnification $\times$20). Stains: hematoxylin and eosin (H&E, top row); APAAP and immunoperoxide.

FOXP3$^+$ Tregs was quantified (mean number/high power field (HPF) calculated in 5 HPF) and related to the number of CD3$^+$ T lymphocytes (FOXP3/CD3 ratio).

To determine whether, in our present conditions, FOXP3$^+$ antibody effectively identified a subset of selective CD4$^+$/CD25$^+$ cells, a double-labeling of FOXP3$^-$CD25 and FOXP3$^-$CD4 was performed in

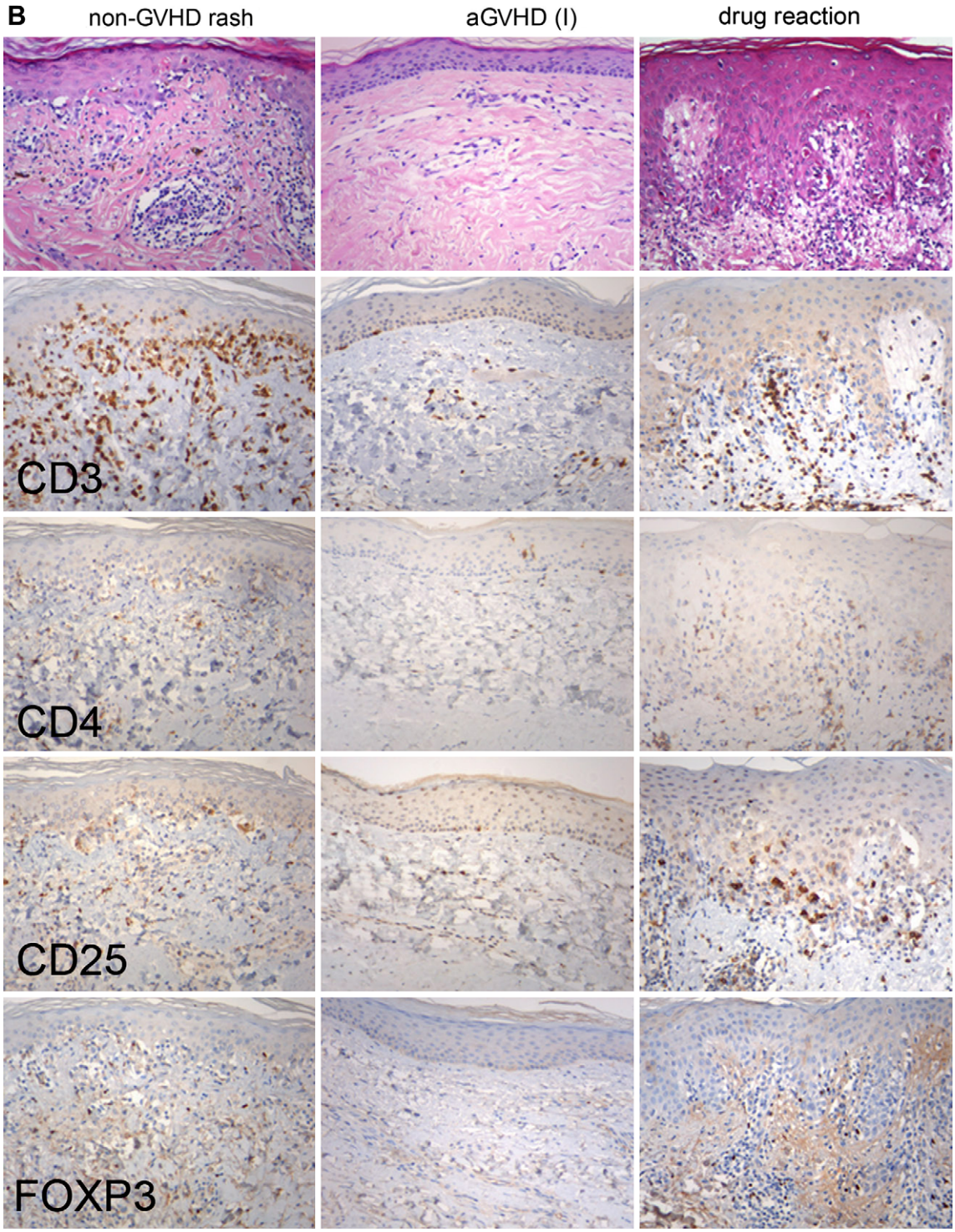

Figure 1. (*continued*).

a series of 12 different patients representing all groups of cases investigated in the present study. Anti-CD25 and anti-CD4 antibodies were used with the above-described conditions. For anti-FoxP3 antibody streptavidin-biotin-phosphatase as revelation system and nitro blue tetrazolium as chromogen were employed. Double labeling invariably confirmed the expression of CD25 and CD4 in $FOXP3^+$ cells.

Statistical Analysis

Results are expressed as mean ± SEM. Statistical analysis was performed by the 2-tailed Student's *t*-test

or the analysis of variance followed by the Bonferroni's post hoc test. A value of $p < .05$ was considered significant.

RESULTS

Histopathologic Examination of Skin Biopsies

Histopathologic examination of biopsies taken from erythema in patients that did not develop GVHD (non-GVHD rash category) showed focal or diffuse vacuolar degeneration of the basal epidermal cells (4 cases) and a perivascular and interstitial mixed inflammatory infiltrate with scattered eosinophils and mild spongiosis (urticarial-like changes) in 2 cases. Biopsies from erythematous macular and/or papular rashes in GVHD patients, taken before day 100 (13 cases) showed signs of aGVHD (Lerner et al [28] grade 1: n = 2, grade 2: n = 2, grade 3: n = 9) and after day 100 in 7 cases displayed evidence for possible cGVHD in 4 cases and for consistent with cGVHD in 3 cases, according to currently accepted criteria [29]. Histopathologically, all drug reactions showed an interface dermatitis characterized by diffuse vacuolar degeneration of the basal epidermal cells, colloid bodies, and a lichenoid and/or dermal perivascular mixed inflammatory infiltrate, with scattered eosinophils.

Increased T Cell Infiltration in the Skin of Patients with aGVHD and with Drug Reactions in Nonallografted Patients

By conventional immunohistochemical analysis, we demonstrated that $CD3^+$ T cell number was markedly and similarly elevated in aGVHD and drug reactions compared to the small and similar number found in both healthy skin and in erythematous skin in non-GVHD rashes (Table 2). In cGVHD, the number of $CD3^+$ cells was intermediate between those observed in aGVHD and drug reaction, but without reaching the statistically significant level (Table 2; Figure 3, upper panel).

The numbers of $FOXP3^+$ Tregs in aGVHD Is Intermediate between Non-GVHD Rashes and Cutaneous Drug Reactions

Despite similar T cell numbers, the pattern of the skin-infiltrating T lymphocytes differed remarkably between patients with GVHD and patients with drug reactions (Figure 1). Double immunohistochemical staining showed that all $FOXP3^+$ cells were $CD3^+$ and coexpressed CD4 and CD25 (Figure 2). In skin specimens from non-GVHD rashes, we found a low number of $FOXP3^+$ Tregs similar to that observed in healthy skin. In aGVHD, $FOXP3^+$ cells were intermediate between the low level observed in non-

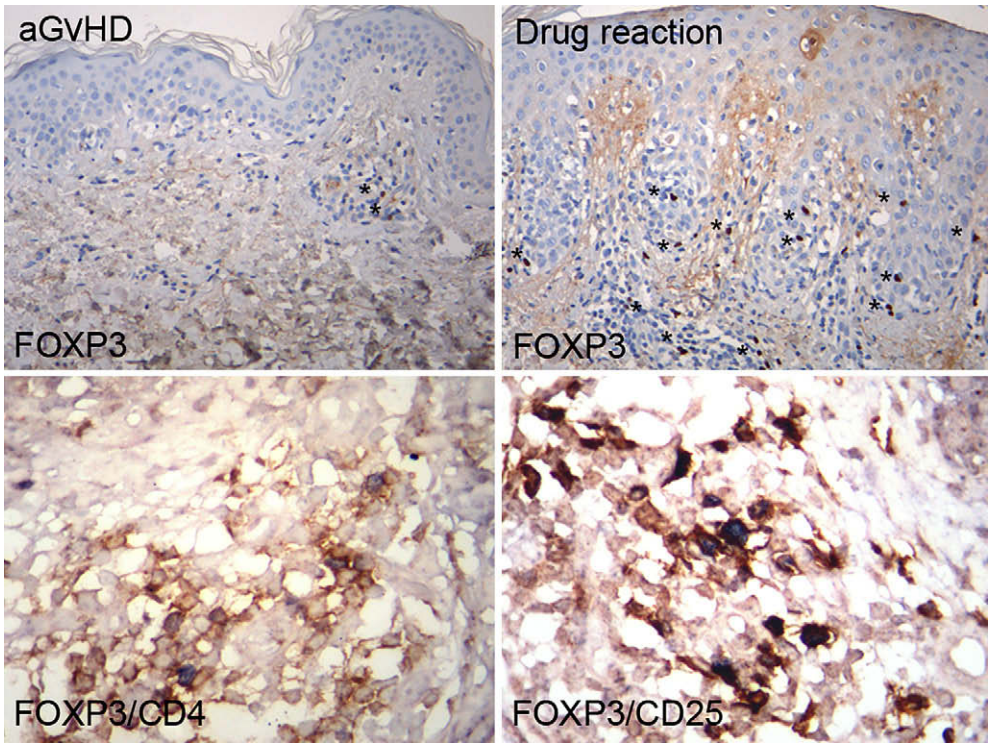

Figure 2. FOXP3 brown nuclear staining shows a few $FOXP3^+$ Tregs cells (asterisks) in aGVHD skin biopsy in comparison with drug reaction (original magnification ×20). Double immunohistochemical staining shows that all $FOXP3^+$ cells coexpress CD4 (FOXP3 in blue nuclear staining, CD4 in brown membranous staining, left panel, original magnification ×40) and all $FOXP3^+$ cells express CD25 (FOXP3 in blue nuclear staining, CD25 in brown membranous staining, right panel, original magnification ×40).

GVHD rashes and the elevated number found in cutaneous drug reactions (Table 2; Figure 3, mid panel). In cGVHD the number of FOXP3$^+$ cells was not statistically different from the values obtained in other clinical conditions (Table 2; Figure 3, mid panel). The FOXP3$^+$ Tregs/CD3$^+$ ratio (Figure 3, lower panel) was similarly high in healthy skin and in skin from drug reactions, indicating that in this latter condition the 2 lymphocyte subsets show a parallel increase in their respective absolute number. The statistically higher ratio found in these 2 conditions compared to skin biopsies from non-GVHD rashes, indicated that, in this latter circumstance, there is a selective deficient recruitment in the skin of the skin. In both aGVHD and cGVHD the FOXP3$^+$ Tregs/CD3$^+$ ratio was intermediate, but not statistically different from those observed in the other conditions.

Decreased Number of FOXP3$^+$ Tregs Is Associated with the Severity of GVHD and Treatment Response

In patients submitted to allo-HSCT, the number of CD3$^+$ cells and FOXP3$^+$ Tregs was similar in standard MA and RIC transplantation (Figure 4A and B). In patients with aGVHD, the number of FOXP3$^+$ Tregs was significantly reduced in skin biopsies of patients classified as clinical grade >I in comparison with patients classified as grade I (Figure 4D). The number of CD3$^+$ cells in grade >I showed a tendency to a reduction that, however, did not reach the significance level (Figure 4C).

In patients affected by either aGVHD or cGVHD, skin biopsies from subjects refractory to the therapeutic intervention showed a significantly lower number of FOXP3$^+$ Tregs in comparison with the more elevated number of FOXP3$^+$ Tregs found in skin specimens from responsive subjects (Figure 4F). The number of CD3$^+$ cells in responders appeared also to be increased, but the difference was not statistically different (Figure 4E). In the category of aGVHD patients only, skin biopsies from patients refractory to therapy had lower Tregs numbers in comparison with skin biopsies from responsive patients ($P = .03$). Finally, examining all transplanted patients (with or without GVHD), the number of FOXP3$^+$ Tregs in biopsies taken before day 100 compared to biopsies obtained after day 100 from transplantation (Figure 4 G and H) did not show any significant difference.

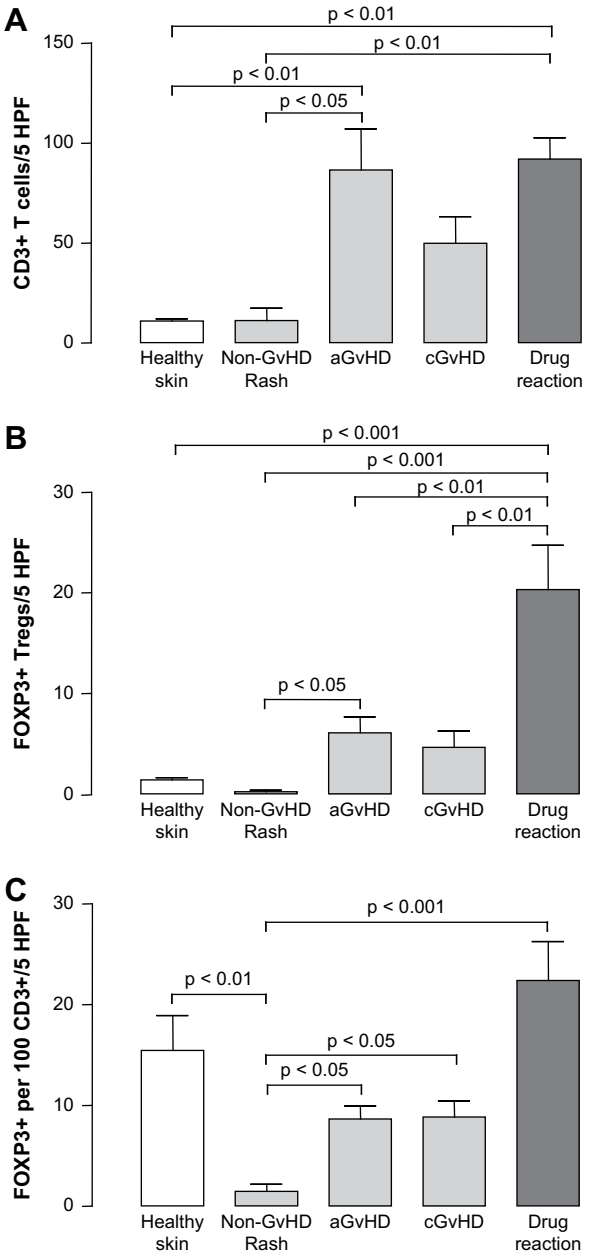

Figure 3. Number of CD3$^+$ T cells (A), FOXP3$^+$ Tregs (B), or ratio of FOXP3$^+$ per 100 CD3$^+$ cells in paraffin-embedded biopsies of healthy skin (C) (healthy skin, n = 10) or of skin with erythema of allografted patients without GVHD (non-GVHD rash, n = 6), of skin of allografted patients with acute GVHD (aGVHD, n = 13) or chronic GVHD (cGVHD, n = 7) and of inflamed skin of nontransplanted patients with cutaneous drug reactions (drug reaction, n = 12). Results are expressed as mean ± SEM.

DISCUSSION

A number of studies in murine models convincingly demonstrated that donor CD4$^+$/CD25$^+$ T cells prevent GVHD without loss of donor T cell-mediated graft versus leukemia effect [12-14]. However, in humans, the role of these cells in preventing GVHD after allo-HSCT has not been clearly defined. *Foxp3* expression was significantly decreased in patients with GVHD, the lowest values being found in the most severe cases [15]. In addition, active cGVHD correlates with decreased CD4$^+$/CD25$^+$ Treg and reduced *Foxp3* gene expression [19]. In addition, the lower the number of FOXP3$^+$/CD4$^+$ T cells, the greater the risk of developing GVHD [22]. However,

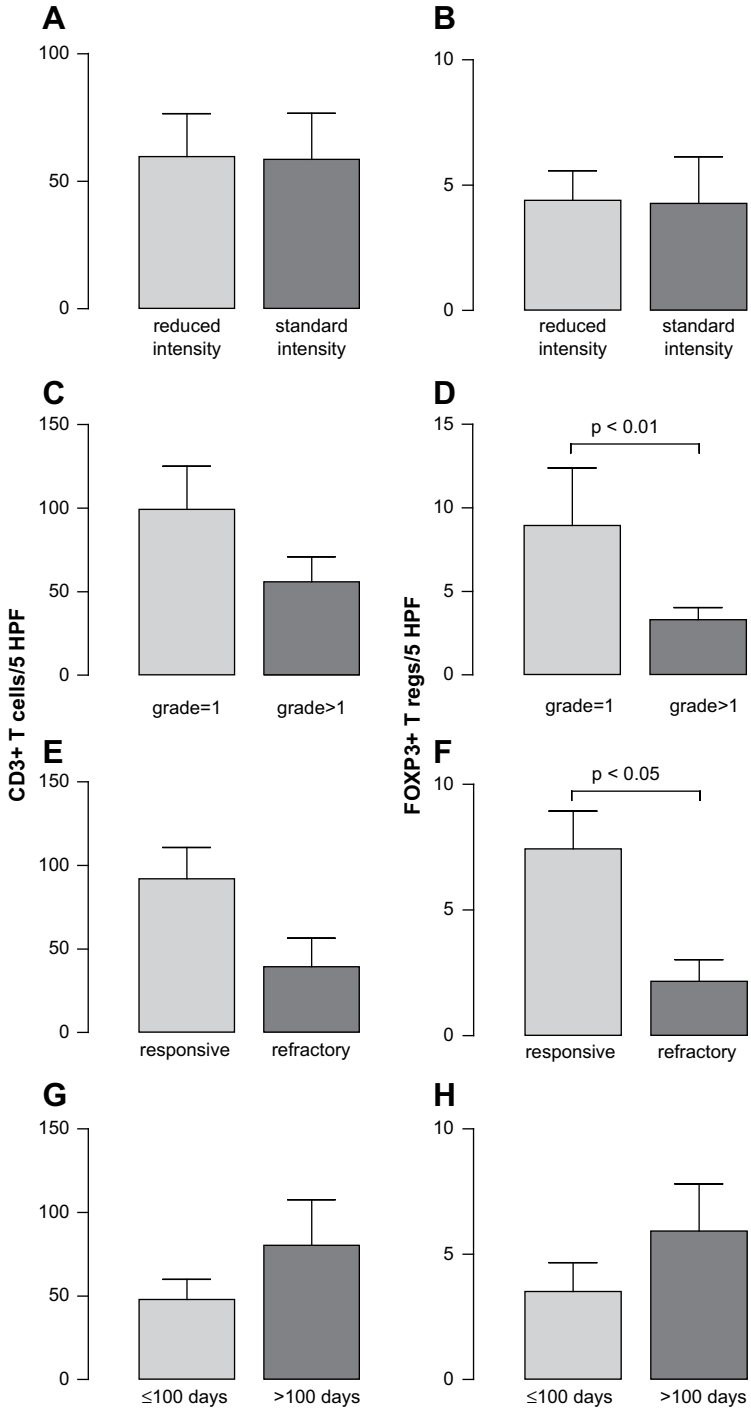

Figure 4. (A, B) Number of $CD3^+$ T cells and $FOXP3^+$ Tregs per 5 HPF in skin biopsies of patients who received RIC versus myeloablative conditioning regimen pretransplant (n = 26). (C, D) Number of $CD3^+$ T cells and $FOXP3^+$ Tregs per 5 HPF in skin biopsies of patients affected by aGVHD with clinical grade I versus grade >I (n = 13). (E, F) Number of $CD3^+$ T cells and $FOXP3^+$ Tregs per 5 HPF in patients in responsive versus refractory patients (n = 20). (G, H) Number of $CD3^+$ T cells and $FOXP3^+$ Tregs per 5 HPF in skin biopsies taken before and after 100 days from transplantation (n = 26). Results are expressed as mean ± SEM.

other studies could not confirm the protective role of Tregs in GVHD. A long-lasting reduction in $CD4^+$ T cell reconstitution, affecting similarly regulatory or nonregulatory cells, was shown following allo-HSCT [20]. No significant difference between absolute numbers of $CD4^+CD25^{high}$ T cells in patients with and without GVHD has been reported [30]. Similarly, the ratio of PB $FOXP3^+$ per total leukocytes or T cells was not found to predict the occurrence of GVHD [31]. Conversely, patients with cGVHD were shown to have markedly elevated numbers of $CD4^+CD25^{high}$ T cells in the PB compared to patients without GVHD [16]. Conflicting results most likely reflect the difficulty to obtain a reliable

identification of Tregs in PB, and the shortcomings associated to the use of CD4 and CD25 as selective markers of Tregs phenotype. Robust evidence indicates that Foxp3 immunostaining is a more suitable tool to precisely identifying the Treg phenotype and recent findings showed that it is possible to localize Tregs in biopsies of GVHD target organs, as the intestine [26].

A major finding of the present study is that the number of $FOXP3^+$ Tregs in skin specimens from aGVHD was higher than in cutaneous biopsies of non-GVHD rashes, but lower than that of cutaneous drug reactions. To our knowledge, only 1 study [26] has directly investigated the Treg population at the site of GVHD reactivity. This study reported that in intestinal biopsies the number of $CD8^+$ T cells was similarly elevated in patients with either GVHD or intestinal CMV infection and diverticulitis compared to the low T cell levels observed in specimens from healthy controls (normal mucosa from patients screened for colorectal cancer). In contrast, a negligible level of $FOXP3^+$ Tregs, similar to that seen in healthy controls, was found in the intestinal mucosa of patients with aGVHD compared to the elevated values observed in CMV infection and diverticulitis [26]. In the same study, specimens obtained from allografted patients with gastrointestinal (GI) symptoms, but without the histologic signs of GVHD, showed a numbers of $FOXP3^+$ Tregs, significantly higher than in GVHD patients and similar to patients with CMV infection of diverticulitis. $CD8^+$ were uniformly increased in GVHD and CMV invention and diverticulitis compared to healthy controls.

In our present study, $CD3^+$ T cell number was similarly elevated in GVHD and skin drug reactions, whereas a negligible amount of inflammatory cells were recorded in skin specimens from allografted patients with erythema without subsequent GVHD or from healthy skin. Interestingly, within cutaneous $CD4^+$ T cells, 30% were $FOXP3^+$ among aGVHD patients and 50% were $FOXP3^+$ in both cGVHD and drug reactions. Thus, the proportion of skin $FOXP3^+$ cells within $CD4^+$ cells was remarkably increased in comparison to peripheral blood and this could be explained by FOXP3 induction after activation or preferential recruitment of natural Tregs. In this context, it should be underlined that previous studies have demonstrated that the main changes in GVHD seem to affect the $CD8^+$ T cell compartment.

Present findings confirm at the cutaneous level, as previously found in the intestinal mucosa [26], that both $CD8^+$ or $CD3^+$ cells, which drive the inflammatory response, are markedly increased in target tissues of GVHD as in other inflammatory conditions. However, whereas biopsies, from intestinal GVHD were characterized by a low level of Tregs compared to the elevated Tregs in non-GVHD rash specimens, in skin biopsies, the number of Tregs in non-GVHD rash specimens was negligible and similar to that found in healthy skin. One possible explanation for the different results between the present and previous article [26] may be from a difference in the response to the allograft procedure between the intestine and the skin. In the intestine increases in resistance to depletion or in migration to the tissue by Tregs may occur, whereas these phenomena may be absent or less pronounced in the skin.

A relevant result of the present investigation is the finding that cutaneous GVHD patients with a better response to therapy showed a higher number of $FOXP3^+$ Tregs than patients with a poor response. Although we found that the number of Tregs was reduced in patients with higher clinical GVHD grades, we underline that, because of the relatively small sample size, our results should we interpreted with caution. However, present data obtained in cutaneous GVHD are consistent with the previous hypothesis [26] that an insufficient upregulation of $FOXP3^+$ Tregs in the target tissue contributes to the disease severity and to the resistance to drug therapy.

The need for a valuable prognostic factor is of particular importance in the setting of allo-HSCT from unrelated donors and the use of PBSC as graft source. These conditions have modified the clinical pattern of GVHD [32,33] and represent today a relevant subset of the activity. Indeed new, more aggressive GVHD prophylaxis strategies have further mixed up the clinical onset of low-grade GVHD, resulting for the first-line treatment in a challenging dilemma. Ideally, timing and intensity of the treatment should be adjusted according to a reliable prognostic index. Skin biopsy is an easy, minimally invasive diagnostic tool that is now proposed also as a prognostic factor to be integrated with more established parameters. Further investigation on a large cohort of patients is needed to assess the role of skin biopsy within this context.

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