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ORIGINAL ARTICLE

The extent of HLA-DR expression on HLA-DR$^+$ Tregs allows the identification of patients with clinically relevant borderline rejection

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Summary

Regulatory T cells (Tregs) were shown to be involved into the pathogenesis of acute rejection after transplantation. The suppressive activity of the total regulatory T cell pool depends on its percentage of highly suppressive HLA-DR$^+$-Treg cells. Therefore, both the suppressive activity of the total Treg pool and the extent of HLA-DR expression of HLA-DR$^+$-Tregs (MFI HLA-DR) were estimated in non transplanted volunteers, patients with end-stage renal failure (ESRF), healthy renal transplant patients with suspicion on rejection, due to sole histological Bord-R or sole acute renal failure (ARF), and patients with clinically relevant borderline rejection (Bord-R and ARF). Compared to patients with only Bord-R or only ARF, the suppressive activity of the total Treg cell pool was exclusively reduced in patients with clinically relevant Bord-R. In parallel, the HLA-DR MFI of the DR$^+$-Treg subset was significantly decreased in these patients, due to a significantly lower proportion of DR^{high+}-Tregs, which were shown to have the highest suppressive capacity within the total Treg pool. Our findings clearly demonstrate that the determination of the HLA-DR MFI of the HLA-DR$^+$-Treg subset allows a highly sensitive, specific and non-invasive discrimination between patients with clinically relevant Bord-R (Bord and ARF) and patients with subclinical rejection or other causes of transplant failure.

Introduction

Acute rejection represents an important risk factor for the development of chronic allograft dysfunction following kidney transplantation [1]. In the BANFF classification histopathological graft lesions below a defined threshold are designated as borderline rejection (Bord-R) [2, 3]. A high proportion of such histological lesions are detected in patients without impaired graft function [4, 5]. Meanwhile, numerous studies, evaluating the predictive value of such subclinical rejection processes for graft outcome were carried out, and led to contradictory results [6]. Nevertheless, borderline rejections combined with acute graft impairment may have potential effects on long-term graft survival. Their consequences are very often underestimated, as previous studies showed that their occurrence is associated with a doubled incidence of acute clinical rejection versus those without these changes [7], an impaired graft function 5 years after transplantation [8] and a higher risk of the development of interstitial fibrosis and tubular atrophy [9]. Therefore, a non-invasive test that leads to the detection of clinically relevant

Bord-R would be helpful for the early diagnosis and treatment of acute rejection.

There is reliable evidence that regulatory T cells (Tregs), which were shown to prevent the activation and proliferation of both auto-reactive and allo-reactive T cells, are involved in the pathogenesis of Bord-R via partial inhibition of the graft-harmful influence of cytotoxic T cells [10]. Tregs are characterized by a constitutive, strong IL-2 receptor alpha-chain- (CD25)-expression and a low to negative Interleukin 7 receptor alpha chain (CD127)-expression [11]. Furthermore, these cells express the transcription factor FoxP3, which is fundamentally important for the evolution and function of these cells [12]. Vondran *et al.* demonstrated that the level of pre-transplant FoxP3 expression within the $CD4^+$ $CD25^+$ $CD127^{low}$ population correlates with the patient's risk for acute graft rejection [13]. Furthermore, the expression of FoxP3 mRNA in urinary cells was shown to predict independently both the reversal of acute rejection and graft failure [14]. However, two recently published studies revealed that the quantification of FoxP3$^+$-Tregs within the graft is neither useful for the detection of rejection, nor for the prediction of future graft function [15, 16]. Human studies, investigating Foxp3 expression in peripheral blood cells also did not confirm significantly altered levels or cell numbers in correlation to graft rejection or graft function [17]. However, one could assume that changes in the functional activity of the Treg cells may have the most reliable predictive value concerning the occurrence of graft rejection. As the total Treg cell pool contains different Treg subset with different suppressive activity, the most promising approach seems to be the determination of the most suppressive Treg subset within the total Treg cell pool.

The total Treg pool includes a considerable proportion of cells that are positive for HLA-DR expression. The HLA-DR$^+$-Tregs are supposed to be highly differentiated and expressed higher levels of FoxP3 and induced a more vigorous rapid T-cell suppression [18, 19]. Herein, we demonstrate that the suppressive activity of the total Treg cell pool was potentially influenced by its percentage of Treg cells expressing the HLA-DR marker very strongly and that the extent of HLA-DR on the surface of HLA-DR$^+$-Tregs could be a suitable non-invasive marker to discriminate between patients with clinically relevant rejection (Bord-R in combination with ARF) and patients with subclinical rejection or ARF due to other causes such as CNI toxicity or vascular renal failure.

Patients and methods

Study population

The study included 20 healthy volunteers, 22 patients with end-stage renal disease and 114 kidney transplant patients. Blood samples for Treg measurement were obtained on the same day when kidney transplant recipients admitted to the Department of Nephrology for graft biopsy. The study was approved by the local Ethics Committee. All patients were fully informed of the aim of the study and written informed consent was obtained from all participants. Kidney biopsies were classified according to the BANFF-classification [2]. BANFF scores were evaluated by two independent pathologists (LB, RW). The pathologists were blinded to the Treg measurements. Only kidney biopsies with 10 or more glomeruli were included in the study. In this study we focused on borderline rejections (Bord-R), high-grade cellular rejections were excluded. Peritubular capillaritis (ptc) was scored as described by Sis *et al.* [20]. Interstitial fibrosis and tubular atrophy (IF/TA) was graded as mild, moderate and severe. We summarized signs of acute graft inflammation as a single numeric score, which is the acute graft inflammation sum score = i-score + t-score + ptc-score (0–9). Biopsies with C4d deposition, glomerular pathologies and patients with donor specific antibodies were also excluded. Acute renal failure was defined in this study as rising creatinine over 30% above the last three measurements. All transplant patients were divided into five groups (Table 1). The first group contained healthy non transplanted volunteers (Healthy Controls; group 1). The second group were patients with end-stage renal failure (ESRF; group 2). The third group consisted of transplant recipients with no signs of rejection in renal biopsy and without acute renal failure (no Bord-R, no ARF; group 3). The fourth group contained transplanted patients with suspicion on rejection. Depending on the clinical picture, this group was subdivided into patients without any signs of rejection in renal biopsy but who had acute renal failure, for example patients with pre- or postrenal, vascular damage or drug - induced kidney failure (no Bord-R, ARF; group 4a). The fourth group further contained transplanted recipients with borderline rejection but who had stable graft function (Bord-R, no ARF; group 4b). The fifth group were transplanted patients with borderline rejection and acute renal failure (Bord-R, ARF; group 5). All 114 transplant patients received an immunosuppressive therapy which is summarized in Table 1.

Fluorescence-activated cell sorter (FACS) staining

Whole peripheral blood mononuclear cells (PBMCs) were isolated as described previously [21]. PBMCs (4×10^6 cells) were surface-stained with 10 μl PerCP-conjugated-anti-CD4 (BD Bioscience, San Jose, CA, USA), PE-conjugated anti-CD127 (eBioscience, San Diego, CA, USA) and PE-Cy7-conjugated anti-HLA-DR (BD Bioscience) antibodies, and analyzed by four colour flow cytometric analysis.

Table 1. Clinical characteristics of patients. The data are presented as median values together with their range (minimum-maximum).

	HC *n* = 20 Group 1	ESRF *n* = 22 Group 2	No Bord-R & no ARF *n* = 31 Group 3	No Bord-R & ARF *n* = 14 Group 4a	Bord-R & no ARF *n* = 27 Group 4b	Bord-R & ARF *n* = 42 Group 5
Recipient sex-no. fem (%)	9 (45%)	7 (32%)	12 (39%)	7 (50%)	5 (19%)	16 (38%)
Recipient age	42 (21–88)	47 (22–86)	45 (21–75)	58 (23–71)	52 (21–74)	51 (16–74)
Primary disease						
Diabetes		3 (14%)	8 (25%)	1 (7%)	3 (11%)	2 (5%)
Hypertension		4 (18%)	3 (10%)		1 (4%)	6 (14%)
GN/vasculitis		11 (50%)	12 (39%)	8 (57%)	13 (48%)	18 (43%)
Interstitial nephritis		1 (4,5%)	3 (10%)	2 (14%)	3 (11%)	4 (10%)
ADPKD		1 (4,5%)	1 (3%)		3 (11%)	3 (7%)
Others			1 (3%)		1 (4%)	3 (7%)
Unknown		2 (9%)	3 (10%)	3 (22%)	3 (11%)	6 (14%)
Donor-Living-no. (%)			8 (26%)	4 (29%)	7 (26%)	15 (36%)
Number of transplantations						
First transplant			28 (90%)	12 (86%)	26 (96%)	40 (95%)
Retransplant			3 (10%)	2 (14%)	1 (4%)	2 (5%)
Panel reactive antibodies						
0–10%			30 (97%)	14 (100%)	27 (100%)	41 (98%)
11–25%						1 (2%)
>25%			1 (3%)			
HLA mismatches			2.5 (0–6)	3.0 (0–6)	2.5 (0–6)	3 (0–6)
Time posttransplant (days)			99 [5–7575]	489 [17–5311]	618 [58–4155]	714 [6–6938]
Reason for biopsy						
Protocol biopsy			7 (23%)	3 (21%)	5 (19%)	7 (17%)
Indication biopsy			24 (77%)	11 (79%)	22 (81%)	35 (83%)
Creatinine (mg/dl) at Treg measurement	0.8 [0.5–1.1]	6.8 [2.8–17.7]	1.9 [1.0–4.0]	2.6 [1.5–4.6]	1.8 [1.0–3.7]	2.4 [1.2–7.1]
Immunosuppression*						
Tac + MPA			8 (26%)	7 (50%)	11 (41%)	17 (40%)
CsA + MPA			17 (55%)	6 (43%)	11 (41%)	21 (50%)
mTor + MPA			2 (6%)	1 (7%)	3 (11%)	1 (2,5%)
mTor + CsA + MPA			1 (3%)		1 (4%)	2 (5%)
MPA			2 (6%)		1 (4%)	
Aza			1 (3%)			1 (2,5%)

*All patients were treated with low dose steroids.

Positive selection and staining of $CD4^+CD127^{low+/-}CD25^+$-Treg cells

$CD4^+CD127^{low+/-}CD25^+$-Treg cells were selected and stained as described previously [21].

Co-culture suppression assay

In this study the inhibitory effect of the Treg cells was calculated by special suppression assays, described previously [22]. To compare the suppressive capacity of the isolated $CD4^+CD127^{low+/-}CD25^+$-Tregs between the different patient groups, we calculated the maximum suppressive activity (ratio of Treg cells to responder T cells 1:1) and the minimum ratio of Treg cells to responder cells, with which a suppression of more than 15% could be achieved.

Sorting and functional testing of the DR^{high}-Treg cell subset

For fluorescence activated cell sorting of the DR^{high}-Treg cell subset, $CD4^+CD127^{low+/-}CD25^+$-Treg cells were purified using the $CD4^+CD127^{low+/-}CD25^+$-Regulatory T cell Isolation Kit II (Miltenyi Biotec, Bergisch Gladbach, Germany) as described above. Respectively, 5×10^5 cells of the isolated $CD4^+CD127^{low+/-}CD25^+$-Treg cells were stained with 20 µl FITC-conjugated anti-CD4 (BD Bioscience), 10 ml PE-conjugated anti-CD25 (BD Bioscience), and 20 µl PE-Cy7-conjugated anti-HLA-DR (BD Bioscience) antibodies. Contaminating non-$CD4^+$-T cells were excluded while the remaining cells were sorted using a FACS-VantageSE-Sorter (BD Bioscience). Thereby, the $CD4^+CD127^{low+/-}CD25^+$-Treg cells were divided into a Treg population consisting exclusively of DR^{high+}-Treg cells

and a Treg population consisting of the remaining DR^{low+}- and DR$^-$-Treg cells. Subsequently, the suppressive activity of both Treg populations was analyzed using the above described suppression assay.

Statistical analysis

Statistical comparison was done using the non-parametric H test of Kruskal and Wallis. Each H test was followed by a Dunn test. $P < 0.05$ was considered significant. The strength of linear dependence between two patient groups was measured with Pearson 's correlation coefficient. Cutt-off levels, sensitivity and specificity of the HLA-DR MFI oft the DR$^+$ -Treg subset were calculated with ROC analysis.

Results

After transplantation, the composition of the DR$^+$-Treg subset with DR^{high+}-Tregs and DR^{low+}-Tregs changes characteristically with time and shows differences between patients with stable graft function and patients with acute Bord-R

The percentage of DR$^+$-Tregs within the total Treg pool, the percentage of DR^{low+}- and DR^{high+}-Tregs within total DR$^+$-Tregs and the HLA-DR MFI of the DR$^+$-Tregs were determined in the circulation of healthy controls (HC, group 1), patients with end-stage renal failure (ESRF, group 2), transplant patients without any signs of Bord-R or ARF (group 3), transplant patients with suspicion on rejection (group 4), due to sole ARF without any signs of rejection (group 4a) or subclinical Bord-R in the absence of ARF (group 4b) and transplant patients with Bord-R combined with ARF (group 5) (Table 1). To this end, PBMCs from each participant were stained with anti-CD4, anti-CD127, anti-FoxP3 and anti-HLA-DR monoclonal antibodies and analyzed by four colour flow cytometric analysis. Figure 1 shows the gating strategy for these measurements. First, PBMCs were analyzed by fluorescence intensity of CD4 versus side light scatter (SSC), (Fig. 1a). The CD4$^+$ T-cells (P1) were gated and analyzed by fluorescence intensity of FoxP3 versus CD127, (Fig. 1b). The CD4$^+$CD127$^{low+/-}$FoxP3$^+$-Tregs were gated (P2) and analyzed by their expression of HLA-DR (Fig. 1c). The percentage of DR$^+$ Tregs within the total Treg cell pool (P3) and the percentages of DR^{low+}-Tregs (Fig. 1c, P4) and DR^{high+}-Tregs (Fig. 1c, P5) of DR$^+$-Tregs (P3) were estimated for all participants. As DR^{low+}-Tregs and DR^{high+}-Tregs could hardly be separated from each other we additionally estimated the HLA-DR MFI of the DR$^+$-Treg

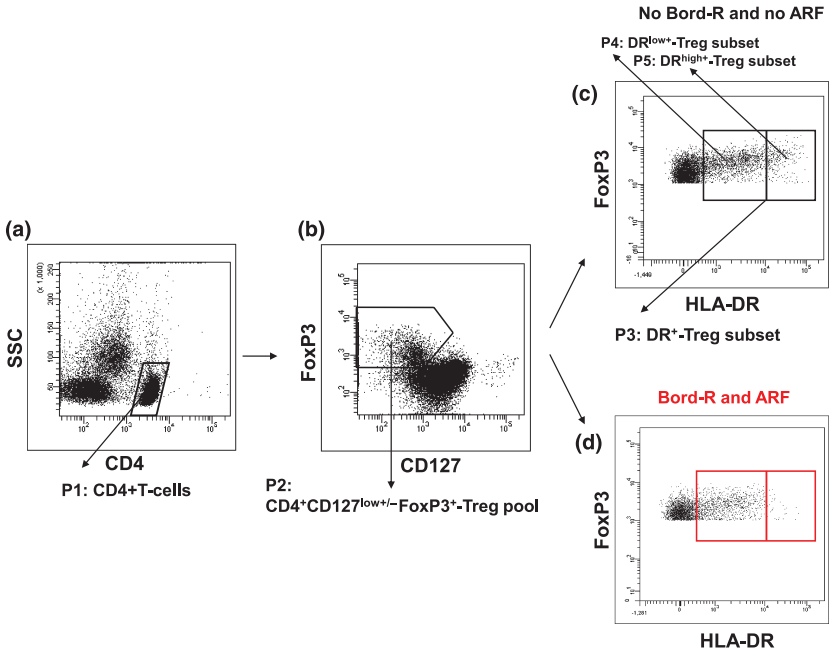


Figure 1 Gating strategy for four colour flow cytometric detection of the total CD4$^+$CD127$^{low+/-}$FoxP3$^+$ -Treg cell pool, its percentage of DR$^+$-Tregs, the percentage of DR^{low+}- and DR^{high+}-Tregs of DR$^+$-Tregs and the HLA-DR MFI of the DR$^+$-Tregs. (a): CD4$^+$-T cells (P1) were gated by fluorescence intensity of CD4 versus side light scatter (SSC). (b): CD4$^+$CD127$^{low+/-}$FoxP3$^+$-Treg cells were gated by fluorescence intensity of FoxP3 versus CD127 (P2). C and D: The percentage of the DR$^+$-Tregs (P3) of the total CD4$^+$CD127$^{low+/-}$FoxP3$^+$-Treg cell pool, the percentages of DR^{low+}- (P4) and DR^{high+}-Tregs (P5) within the DR$^+$-Treg subset and the HLA-DR MFI of the DR$^+$-Treg subset (P3) were estimated for all participants. Illustrated is a representative experiment for a healthy kidney transplant patients in the absence of Bord-R and ARF (c) and a kidney transplant patient in the presence of Bord-R and ARF (d). Bord-R = biopsy proven borderline rejection; ARF = acute renal failure.

subset (P3). Figure 1c depicts an example for a transplant patient with stable graft function without any signs of rejection (group 3) in comparison to a transplant patient with Bord-R and ARF (group 5; Fig. 1d).

Figure 2 shows the changes in the percentage of DR$^+$ Tregs within the total Treg pool (Fig. 2a), their HLA-DR MFI (Fig. 2b) and their composition with DR^{low+}- versus DR^{high+}-Tregs (Fig. 2c and d) during the time after the transplantation. In transplant patients, the percentage of DR$^+$-Tregs within the total Treg pool showed a minimum value at about 300 days after surgery. Afterwards, it increased again and reached levels comparable with non-transplanted controls. We did not find any significant differences in the percentage of DR$^+$-Treg cells between the different transplanted patients groups (group 3–5, Fig. 2a).

Compared to non-transplanted controls (group 1–2), the HLA-DR MFI of the DR$^+$-Treg cells decreased strongly soon after surgery in transplanted patients (groups 3–5) (Fig. 2b). Subsequently, the HLA-DR MFI of the DR$^+$-Treg subset increased continuously over time for all transplanted patient groups. The calculation of the regression lines for all transplanted patient groups revealed that there was a significantly lower increase in transplanted patients with Bord-R combined with ARF (group 5) in contrast to transplanted patients with stable graft function (group 3), $P < 0.001$) and also in contrast to transplanted patients who had only a suspicion on rejection (groups 4a and 4b), ($P < 0.01$). Furthermore, the percentage of the DR^{low+}-Tregs within the DR$^+$-Tregs decreased continuously, while the percentage of the DR^{high+}-Tregs increased continuously during the time after transplantation (Fig. 2c and d). The calculation of the regression lines for the different transplanted patient groups confirmed that there is a significantly lower decrease of the DR^{low+}-Tregs and a significantly lower increase of the DR^{high+}-Tregs in patients with Bord-R combined with ARF (group 5) compared to patients without Bord-R and stable graft function (group 3) and also compared to patients suspected on rejection (groups 4a and 4b). Since the regression lines have significantly different slopes, our data suggest that there may be a certain time point after

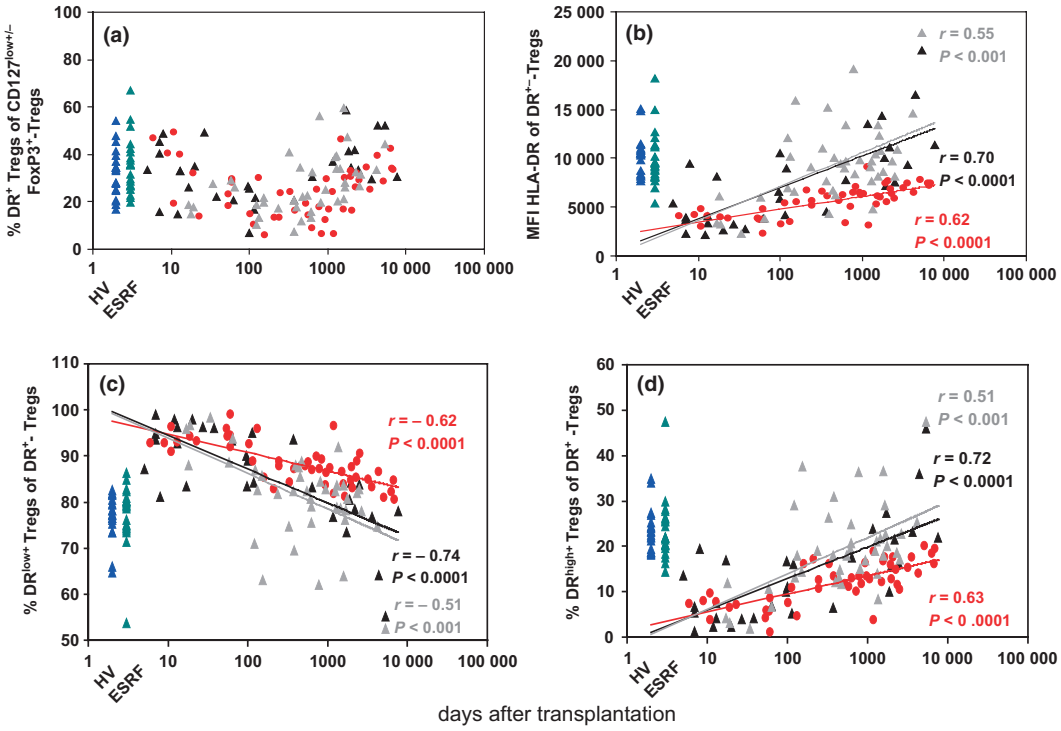


Figure 2 Detection of the percentage of DR$^+$-Treg subset within the total DR$^+$-Treg cell pool, its HLA-DR MFI and its percentage of DR^{low+}- and DR^{high+}-Tregs in renal transplant patients. The percentage of DR$^+$-Tregs within the total CD4$^+$CD127$^{low+/-}$FoxP3$^+$ -Treg cell pool (a), the HLA-DR MFI of the DR$^+$-Treg subset (b) and its percentage of DR^{low+}- (c) and DR^{high+}- (d) Tregs was estimated in healthy non-transplanted volunteers (▲), patients with ESRF (▲), healthy kidney transplant patients (group 3, ▲), kidney transplant patients with suspicion on rejection (group 4a and 4b, ▲) and kidney transplant patients with acute Bord-R (group 5, ●) during the time after transplantation. For kidney transplant patients, there were significant correlations between the HLA-DR MFI (b), the percentages of DR^{low+}- (c) and DR^{high+}-Tregs (d) and the time after transplantation. The figure shows the regression lines which differed significantly between healthy transplant patients (group 3) or transplant patients suspected on rejection (groups 4a and 4b) and those with acute Bord-R (group 5).

surgery from which on the detection of the HLA-DR MFI allows a significant discrimination between patients with acute rejection and those without rejection.

In order to examine whether the different immunosuppressive regimes had an effect on these findings, we compared the HLA-DR MFI of the transplanted patients without clinically relevant Bord-R (group 3–4) treated with tacrolimus (Tac), cyclosporine (CsA) and other regimes at various time points (<90 days: Tac vs. CsA, $P = 0.15$; 90–365 days: Tac vs. CsA, $P = 0.20$; 365–1095 days: Tac vs. CsA, $P = 0.31$; >1095 days: Tac vs CsA, $P = 0.64$). Significant differences between these immunosuppressive regimes were not found.

The suppressive activity of the CD4$^+$CD127$^{low+/-}$CD25$^+$-Treg cell pool is significantly reduced in patients with Bord-R and ARF

To evaluate the suppressive capacity of CD4$^+$CD127$^{low+/-}$CD25$^+$-Tregs, obtained from the different patient groups, we calculated their maximum suppressive activity (Fig. 3a) and determined the ratio of Treg cells to Tresp

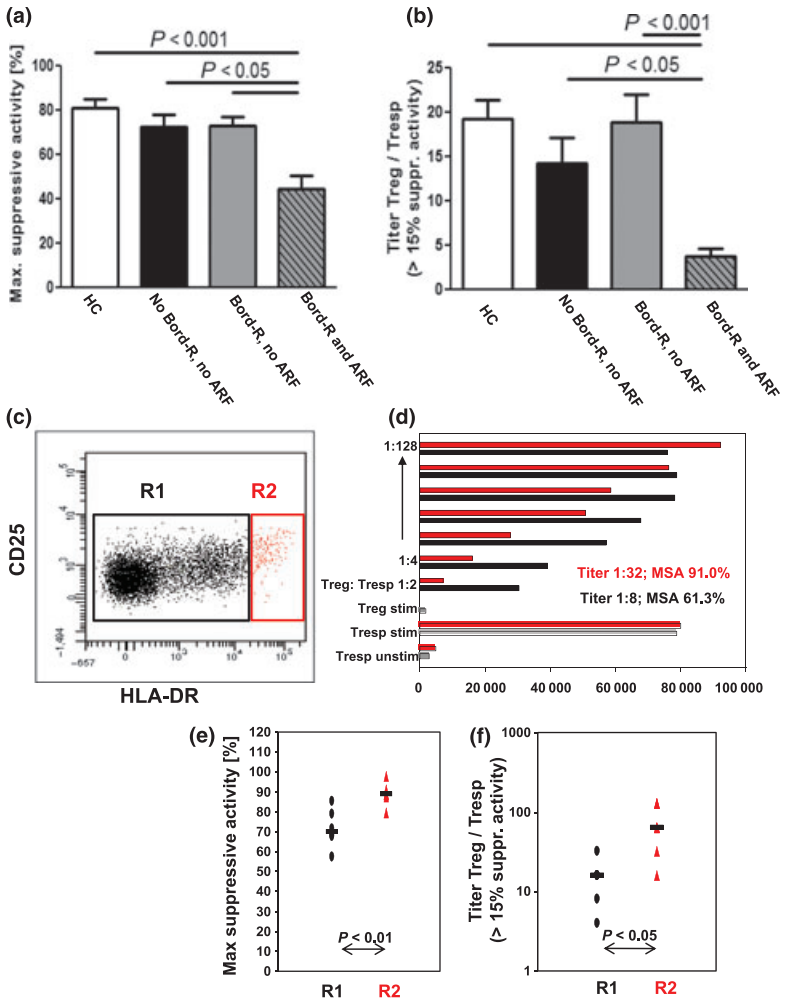


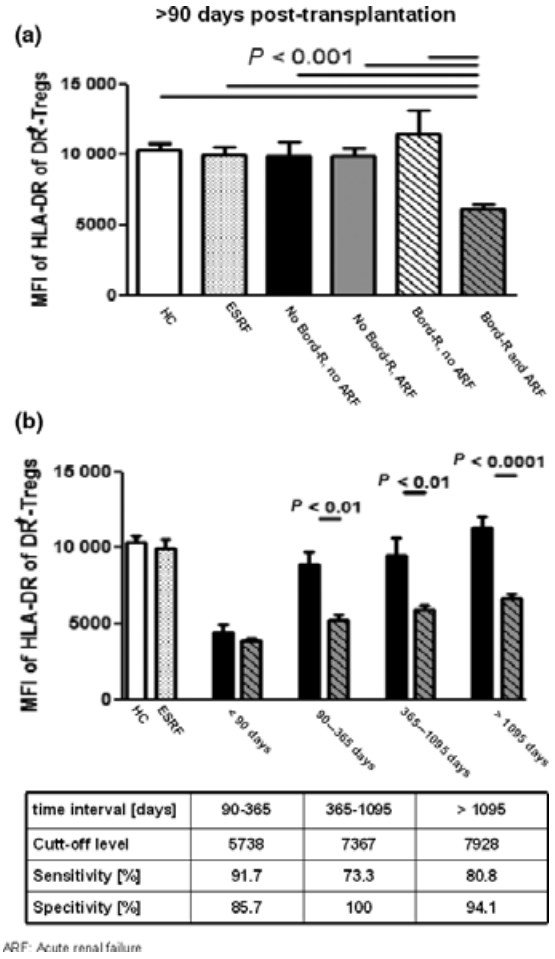
Figure 3 Evaluation of the suppressive activity of CD4$^+$CD127$^{low+/-}$CD25$^+$-Treg cells in renal transplant patients. a–b: CD4$^+$CD127$^{low+/-}$CD25$^+$-Treg cells were isolated by the MACS technique and their suppressive activity was examined using suppression assays (see methods). The figure shows the mean values with its SEM of the maximum suppressive activity (Treg/Tresp = 1/1) (a) and the titer up which the Tregs could be diluted to achieve a minimum suppressive activity of more than 15% (b). (c): Magnetically isolated CD4$^+$CD127$^{low+/-}$CD25$^+$-Tregs obtained from healthy non-transplanted volunteers were stained with anti-CD4, anti-CD25 and anti-HLA-DR specific antibodies and sorted into a population of a population consisting of DR$^-$- and DR^{low+}-Tregs (R1) and into a population of DR^{high+}-Tregs (R2). (d): Subsequently, both Treg populations were analyzed concerning their suppressive activity.The figure shows the results of a suppression assay performed for one representative subject. Figures (e) and (f) summarize the results concerning the maximum suppressive activity (Treg/Tresp = 1/1) (e) and the minimum ratio of Treg/Tresp (titer) with at least 15% suppression (f) obtained for six different healthy non-transplanted volunteers.

cells (Fig. 3b), with which a minimum suppression of more than 15% could be achieved. We found that both parameters did not differ between healthy controls (group 1) and transplanted recipients without Bord-R and stable graft function (group 3). In addition, both parameters were also in the same range for health controls (group 1) and for transplanted patients with Bord-R, but stable graft function (group 4b). In contrast, both parameters were significantly reduced in patients with Bord-R and ARF (group 5), compared to healthy controls (group 1) and compared to stable transplant patients with suspicion on rejection due to sole Bord-R or sole ARF (group 4a and 4b), (Fig. 3a and b).

Our data demonstrate that both the level of HLA-DR expression of the DR$^+$-Treg cell subset and the suppressive activity of the total Treg cell pool are significantly reduced in transplanted patients with Bord-R and ARF. These findings gave rise to the assumption that the DR^{high+}-Treg subset has the highest suppressive activity within the total Treg pool. Treg cells were sorted into a population consisting of DR^{low+}- and DR$^-$ Tregs (Fig. 3c, R1) and a population consisting of DR^{high+}-Tregs (Fig. 3c, R2). Subsequently, both Treg populations were analyzed concerning their suppressive activity. Figure 3d shows one representative experiment which was performed with Tregs obtained from six different healthy controls. Both the maximum suppressive activity (Fig. 3e) and the titer (Treg/Tresp) with which a minimum suppressive activity of 15% could be achieved (Fig. 3f) is strongly increased for the positively sorted DR^{high+}-Tregs (R2) compared to the remaining Treg population (R1). Thus, the DR^{high+}-Treg subset exhibits the highest suppressive activity of the total CD4$^+$CD127$^{low+/-}$CD25$^+$-Treg cell pool.

The HLA-DR MFI of DR$^+$ -Treg subset is a very specific and sensitive marker to detect patients with Bord-R and ARF

Figure 4a shows the mean values of the HLA-DR MFI of the DR$^+$-Treg subset in non-transplanted controls (groups 1–2) and in the different groups of transplanted patients (groups 3–5) after 90 days post surgery. The level of the HLA-DR MFI of the transplanted patients with stable graft function (group 3), that of the transplanted patients with suspicion on rejection due to ARF in the absence of Bord-R (group 4a) and that of the transplanted patients with suspicion on rejection due to Bord-R in the absence of ARF (group 4b) was in the same range than that of healthy volunteers and patients with ESRF. Only the group of patients with Bord-R and ARF showed a highly significant decrease of the HLA-DR MFI, approximately a reduction of 50% (Fig. 4a). Therefore, we summarized the patients from group 3, 4a and 4b as non-rejecting transplant patients and compared their data concerning the HLA-DR$^+$ MFI of the



time interval [days]	90-365	365-1095	> 1095
Cutt-off level	5738	7367	7928
Sensitivity [%]	91.7	73.3	80.8
Specitivity [%]	85.7	100	94.1

ARF: Acute renal failure

Figure 4 Determination of cut-off levels, sensitivity and specificity of the HLA-DR MFI of DR$^+$-Treg cells as a marker for acute Bord-R. (a): The HLA-DR MFI of the DR$^+$-Tregs was estimated for HC (group1), patients with ESRF (group 2) and for healthy transplant patients (group 3), transplant patients with suspicion rejection due to ARF in the absence of Bord-R (group 4a), transplant patients with suspicion on rejection due to Bord-R in the absence of ARF (group 4b) and for patients with acute Bord-R in the presence of ARF (group 5) after a period of at least 90 days post surgery. (b): As significant differences of the HLA-DR MFI of DR$^+$-Tregs were ascertained between patients with acute Bord-R (group 5) and all other groups (groups 1–4), the patients of group 3, 4a and 4b were combined and the cutt-off levels, the sensitivity and the specificity of the HLA-DR MFI was estimated separately for different consecutive periods after transplantation.

DR$^+$-Treg-subset with those of patients with acute clinical rejection (Bord-R and ARF, group 5). As the HLA-DR MFI increased over the time after transplantation (Fig. 2), we calculated its cut-off level, its specificity and its sensitivity for four consecutive periods after the transplantation. Figure 4b demonstrates that before 90 days post surgery the HLA-DR MFI is strongly reduced in both non-rejecting and acute rejecting patients. Therefore, determining the HL-DR MFI does not allow the differentiation between these two

patient groups. After 90 days post transplantation the HLA-DR MFI was significantly lower in transplanted patients with Bord-R and ARF during all three periods in comparison to non-rejecting patients, (Fig. 4b). ROC analysis was performed to examine whether the determination of the HLA-DR MFI of the DR$^+$-Treg subset could be a useful marker for the the discrimination between rejecting and non-rejecting transplant patients. Figure 4b comprises the cut-off levels of the HLA-DR MFIs determined for the renal transplant patients during three different time intervals and identifies this parameter as a marker with both high specificity and high sensitivity for a non-invasive prediction of rejection during the time after transplantation.

The HLA-DR MFI of DR$^+$ -Treg subset is significantly decreased in transplanted patients with acute graft inflammation

The extent of HLA-DR on the surface of HLA-DR$^+$-Tregs was significantly reduced in transplanted patients with signs of acute inflammation in renal biopsy. Patients with increased acute graft inflammation sum score showed a significant decrease of the HLA-DR MFI (Fig. 5a). In contrast, the HLA-DR MFI was not different in regard to signs of chronic allograft nephrophathy. Mild, moderate or severe interstitial fibrosis and tubular atrophy (IF/TA) did not correlate to the expression of HLA-DR on the surface of HLA-DR$^+$-Tregs (Fig. 5b).

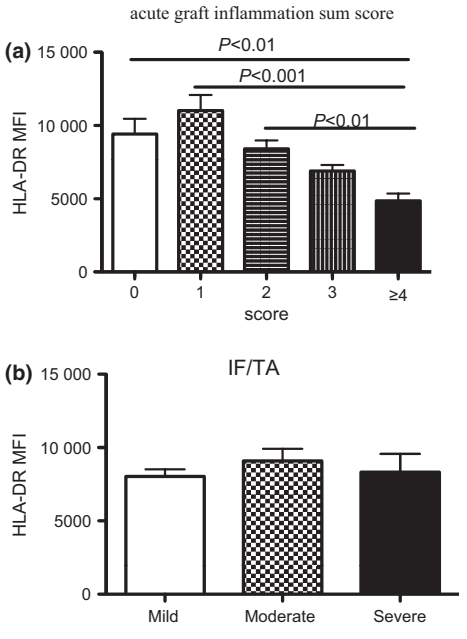


Figure 5 The HLA-DR MFI and histopathological findings for acute and chronic graft nephropathy. (a): The HLA-DR MFI of DR$^+$-Tregs was decreased in transplant biopsies with increased acute graft inflammation sum score (BANFF-"i"+"t"+"ptc"). (b): The extent of interstitial fibrosis and tubular atrophy (IF/TA) showed no correlation to the HLA-DR MFI.

Discussion

The aim of this study was to examine whether there is a correlation between the extent of HLA-DR expression of HLA-DR$^+$-Tregs and the clinical status of renal transplant patients. Thereby, we assessed whether the HLA-DR MFI of this Treg subset could be a suitable marker to discriminate between patients with clinically relevant borderline rejections and patients with subclinical rejection or other causes of graft impairment. Borderline rejections are often diagnosed in transplant biopsies, but their clinical relevance is discussed controversially in the literature [5, 6, 10, 23]. Normally, tubulointerstitial renal graft rejection is a continuously evolving process starting with the infiltration of few mononuclear cells. At this stage, the organ's function is not impaired and such histological signs of rejection are declared as subclinical borderline rejections [24]. The BANFF classification refers exclusively to the influx of mononuclear cells. However, the simple counting of mononuclear cells does not allow to recognize the different phenotypes of mononuclear cells, which may have been recruited to fulfil completely different tasks, ranging from inflammatory response mechanisms and replacement of damaged and apoptotic cells, to tissue remodelling and immune-regulation [25–27]. In this context, T-cell mediated immune-regulation may have the most important role for the induction of allograft tolerance [28–30].

Meanwhile it was shown, that even the quantitative detection of FoxP3$^+$-Treg infiltrates did not have the capacity to discriminate between a fatal immunological process predicting imminent rejection or harmless immune responses [16, 31]. Obviously, besides the extent of Treg cell infiltration, the suppressive potency of the infiltrating Treg cells has also a decisive effect on silencing immunological rejection processes within the transplanted organ. As the total Treg pool consists of different Treg subsets which presumably show differences concerning their suppressive activity, it is certainly a promising strategy to examine whether the Treg cells of the transplanted patient have deficiencies concerning their suppressive activity.

In a recently published study, our group showed that the suppressive activity of Treg cells obtained from renal transplant patients with acute graft rejection was strongly reduced compared to stable patients [21]. We could verify that only clinical relevant borderline rejection but not borderline rejection only or acute renal failure only are related with decreased HLA-DR expression of DR$^+$-Tregs. Our results confirmed the findings of Dijke and colleagues who demonstrated that Tregs of heart transplanted patients with acute rejection also showed a reduced suppressive

function compared to those obtained from non-rejecting patients [32].

In addition, we demonstrated that the suppressive activity of the total Treg pool positively correlates with the expression of HLA-DR on HLA-DR$^+$-Tregs. The HLA-DR MFI of these cells showed characteristic changes during the time after transplantation, due to characteristic changes in the composition of the total Treg pool with DR^{low+}- and DR^{high+}-Treg cells. These changes comprise rising HLA-DR MFIs with progressing time after transplantation and significantly reduced HLA-DR MFIs, exclusively in transplant patients affected by clinically relevant Bord-R. These findings may be explained by the fact that HLA-DR$^+$-Tregs are known to represent a highly differentiated and therefore a highly suppressive Treg subset [18, 19, 33]. Our findings demonstrated that positive selection and evaluation of the suppressive activity of such extremely matured DR^{high+}-Treg cells, identified these particular cells as Treg cells with the highest suppressive activity within the total Treg pool. As these cells rapidly disappeared after surgery, while the DR^{low+}-Treg cells increased within the DR$^+$-Treg subset, our results propose that the Treg pool may have the lowest suppressive capacity soon after surgery. However, with progressing time after transplantation, the maturation of such DR^{high+}-Tregs seems to increase and presumably potentiates the suppressive activity of the total Treg pool with time. Such findings may explain why transplant outcome studies have shown markedly increased rejection rates in the first 6 months and reduced rejection rates after 3 years post surgery [34, 35].

As we observed on the one hand a higher proportion of DR^{low+}-Tregs and on the other hand a lower proportion of DR^{high+}-Tregs in patients with clinically relevant Bord-R, one could assume that these patients could have deficiencies in the maturation of highly differentiated DR^{high+}-Tregs. In a recently published study, a similar effect on the suppressive activity of the total Treg pool was ascertained for pregnancies affected by preterm labour necessitating preterm delivery [33]. In these cases, the HLA-DR MFI of the DR$^+$-Treg subset was decreased due to an increased percentage of DR^{low+}-Tregs. These findings indicate that the immunological mechanisms leading to preterm labour show parallels to those leading to allograft rejection after transplantation. The lower proportion of HLA-DR$^+$ -Treg cells in the circulation of neonates compared to the circulation of adults proposes that HLA-DR expression may be increased during the process of ontogenesis [36]. Therefore, it seems that rejecting patients may have deficiencies in development and recruitment of HLA-DR^{high+}- Tregs.

Our findings clearly demonstrate that there is a relationship between the level of HLA-DR expression of the DR$^+$-Treg cell subset and the suppressive activity of the Treg pool. Since the HLA-DR MFI allows a precise evaluation of the ratio between DR^{high+}-Tregs and DR^{low+}-Tregs within the DR$^+$-Treg cell subset, our findings demonstrate that this marker, which can be determined non-invasively in the circulation, has the capability to discriminate between rejecting and non-rejecting renal transplant patients. It is noteworthy that only patients with borderline rejections and acute graft failure showed a reduction of HLA-DR MFI and not patients with borderline rejection and stable graft function. Furthermore, we demonstrate that patients with typical mild signs of acute inflammation in graft biopsy showed a lower extent of HLA-DR expression of HLA-DR$^+$-Tregs. But the HLA-DR MFI is not a sufficient marker to detect chronic allograft nephropathy. Non immunological alterations have no influence on the HLA-DR MFI. We think that the BANFF borderline definition only is not sufficient to discriminate between clinically relevant and harmless borderline rejection. Additionally borderline biopsies are often misleading because of sampling errors. Moreover, the HLA-DR MFI of HLA-DR$^+$-Tregs even seems to be a suitable, highly sensitive and highly specific marker to distinguish between clinically relevant acute Bord-R and harmless Bord-R without impaired graft function. Prospective monitoring of the HLA-DR MFI of DR$^+$-Treg cells may therefore be a useful screening marker to detect early occurring rejection processes.

Authorship

MS, ES, AS, SM and MZ: designed the study. MS, NS, FH, AS, CS and RW: performed the study. MZ, AS, SM and CS: contributed important reagents. MS and FH: collected data. MS, NS, RW, LB, SS and AS: analysed the data. MS and AS: wrote the paper.

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