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Steroid free immunosuppression is associated with enhanced Th1 transcripts in kidney transplantation

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ABSTRACT

Background: Steroid avoidance in immunosuppression in kidney transplantation offers several metabolic advantages, however it is associated with higher early acute rejection rate. Cellular and molecular mechanisms of this phenomenon remain poorly understood.

Methods: In this single center observational study, low-risk kidney transplant recipients randomized into large multicenter prospective ADVANCE trial with steroid avoidance/early withdrawal and center standard of care treated patients were monitored for 12 months. The expressions of 28 transcripts, associated with alloimmune response and operational tolerance, were evaluated in the peripheral blood using RT-qPCR at 0, 7, 14, 90 and 365 postoperative days (POD) and in the protocol graft biopsy at 3 months while lymphocyte subpopulations were analyzed by flow-cytometry within the follow-up.

Results: Both steroid avoidance and withdrawal regimens were associated with significantly higher granzyme B (*GZMB*) transcript at POD 14 and perforin 1 (*PRF1*) transcript at POD 7. The higher interleukin 2 (*IL-2*) expression at POD 7 was detected only in the steroid avoidance group. Initial steroids decreased the expression *SH2D1B* transcript at POD14 and there were no further differences in other operational tolerance transcripts among groups. The statistically significant decrease in absolute numbers of peripheral NK cells in the first 14 days was observed in the standard of care group only. There were no differences in analyzed intrarenal transcripts in 3-month biopsies among groups.

Conclusions: The enhanced expression of some of Th1 associated transcripts and limited effects on NK cells of steroid avoidance immunosuppression suggest higher susceptibility for early acute rejection.

1. Introduction

Corticosteroids are routinely used as a component of maintenance immunosuppression in kidney transplant recipients. Since corticosteroids have several side effects, many attempts were done to withdraw corticosteroids at a specified time post-transplant [1–3] or avoid them at all [4]. Most published data have shown that steroid avoidance at transplantation or early withdrawal of steroids after transplantation is associated with increased risk of acute rejection but without adversely affecting patient or graft survival [2,5–7] while some studies did not find any significant difference in acute rejections or graft loss [8]. ADVANCE, a large, prospective, multicenter trial with > 1000 enrolled kidney transplant recipients, has very recently shown that steroid avoidance is associated with plus 5% ($p = 0.001$) more acute rejection as compared to 10-day steroid withdrawal arm while all patients received basiliximab induction and tacrolimus/MMF long term immunosuppression [9].

The mechanisms of how steroids prevent early acute rejection development remain ill-defined and *in vivo* data are lacking. *In vitro* steroids exert their immunosuppressive effects by inhibiting Th1 cytokine production and by enhancing the production of Th2 cytokines [10,11]. Many rejection-associated markers were described so far, including *GZMB, PRF1* [12,13], *C3orf23, MAN1A1* [14], *FOXP3* [15] and inflammatory cytokines *TGFB1, IFNG* and *IL-2*. Very recently,

Abbreviations: HLA, human leukocyte antigen; PBMCs, peripheral blood mononuclear cells; POD, post-operative day; PRA, historical panel reactive antibodies; TCMR, T cell mediated rejection

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several B-cell related markers of operational tolerance were also identified [16,17] and these markers were shown to play a role also in rejection free patients [18].

In this single center observational study we evaluated molecular and cellular markers associated with rejection and operational tolerance in both ADVANCE trial arms [9] of locally enrolled patients and compared them with markers measured in the standard of care treated patients.

2. Materials and methods

2.1. Study design and patients

Twenty three kidney transplant recipients who received kidney transplantation between 2011 and 2013 at the Institute for Clinical and Experimental Medicine in Prague, Czech Republic and participated on large randomized, open-label, prospective, multicenter clinical study (ADVANCE, ClinicalTrials.gov; NCT01304836) were randomly assigned to the steroid 10-day withdrawal or steroid avoidance arm with only perioperative steroids bolus [9]. Fourteen patients were randomized into steroid avoidance arm and 9 patients into steroid 10-days withdrawal arm. As a control group, patients who received center standard of care immunosuppression with basiliximab induction, tacrolimus once daily, mycophenolate mofetil and long term steroids were selected based on identical inclusion criteria as for ADVANCE study ($n = 10$) [9]. All patients participated in local prospective immune-monitoring and biobank study.

Immune monitoring of peripheral blood was performed prospectively in all patients regularly before transplantation and then in POD 7, 14, 90 and 365. Intragraft gene expression was measured at 3-month protocol biopsies in a patient subgroup (steroid avoidance, $n = 6$; steroid withdrawal, $n = 8$ and standard of care, $n = 5$).

Main pre-transplant donor's and recipient's characteristics were not significantly different among steroid avoidance, steroid 10-day withdrawal and standard of care treated group (Table 1).

2.1.1. Inform consent

All patients signed informed consent with the participation in local immune monitoring study (ethic approval no. G 10-04-11) and patients who were enrolled also in ADVANCE study (ethic approval no.1771/10) signed both informed consents.

2.1.2. Biopsies

Renal biopsies were obtained under ultrasound guidance (Toshiba, Power Vision 6000) using a 14-gauge Tru-Cut needle (Uni-Cut Nadeln, Angiomed, Germany). Most of the renal tissue was processed for conventional histology. Histological examination was interpreted according to the 2013 Banff working classification criteria [19]. The residual portion (2 mm) of the cortical zone of the renal tissue was immediately placed in RNA later (Ambion Corporation, Austin, TX), snap frozen and stored at − 80 °C until RNA extraction. Intragraft gene expression was measured in patients on steroid free regimens (steroid avoidance, $n = 6$ and steroid withdrawal, $n = 7$) and steroid-based immunosuppression (standard of care, $n = 5$).

2.1.3. Flow cytometry and isolation of peripheral blood mononuclear cells

Peripheral blood mononuclear cells (PBMCs) were isolated by density gradient centrifugation over Lymphoprep™ (Axis-Shield, Oslo, Norway) from peripheral blood anticoagulated with ethylenediaminetetraacetic acid (EDTA).

PBMCs (100 µl approximately 1×10^6) were labeled with fluorochrome-conjugated (FITC–fluorescein isothiocyanate, PE-phycoerythrin, ECD-Phycoerythrin-Texas-Red, APC-Allophycocyanin, PC5-Phycoerythrin-Cyanin 5, PC7-Phycoerythrin-Cyanin 7) monoclonal antibodies diluted in phosphate-buffered saline-bovine serum albumin buffer for 20 min at room temperature in the dark. The specific antibody panels used consisted of anti-CD4-PC7 (clone: SFC112T4D11) anti-CD25-PC5 (clone: B1.49.9) anti-CD127-PE (clone: R34.34) (Beckman Coulter, Brea, CA). Intracellular FoxP3 staining of Tregs was performed as described by the manufacturer (Human Regulatory T Cell Staining Kit, eBioscience, San Diego, CA). Extracellular staining of freshly prepared and isolated PBMCs was carried out using CD4-FITC (clone: RPA-T4), CD25-APC (clone: BC96), antibodies prior to intracellular staining with anti-Foxp3-PE (clone: PCH101). NK cells were labeled with CD45-FITC (clone: B3821F4A), CD16-PE(clone: 3G8), CD56-PE (clone: N901), CD3- PC5 (clone: UCHT1), T lymphocytes with CD45-FITC (clone: B3821F4A), CD3-PC5 (clone: UCHT1) and B lymphocytes with CD45-FITC (clone: B3821F4A), CD19-ECD (clone: J3–119), CD3-PC5 (clone: UCHT1) (Beckman Coulter, Brea, CA).

Following staining procedure, samples were analyzed using an FC 500 flow cytometer (Beckman Coulter) and the data were processed by C × P and Kaluza software (Beckman Coulter, Brea, CA). Individual subsets were defined as follows: Tregs as $CD4^+ CD25^+ FoxP3^+$ and $CD4^+ CD25^+ CD127^-$, NK cells as CD45 + CD3-CD16/$CD56^+$, T lymphocytes as CD45 + $CD3^+$ and B lymphocytes as $CD45^+ CD19^+ CD3^-$. Flow cytometric analyses were performed with at least 100 gated events.

2.1.4. RT-qPCR analysis

For gene expression analysis RNA was isolated from peripheral blood, collected at POD 0, 7, 14, 90 and 365 using the PAXgene Blood RNA kit (Qiagen, Hilden, Germany) or from 3-month protocol biopsies using the RNeasy Fibrous Tissue Mini Kit (Qiagen, Hilden, Germany) according to manufacturer's instructions. RNA was quantified by measuring absorbance at 260 nm on the ND-1000 Spectrophotometer (NanoDrop Technologies).

RNA was reverse transcribed using Superscript Reverse transcriptase II (Invitrogen). The synthetized cDNA was subjected to RT-PCR analysis. Quantitative RT-PCR was performed using a custom-made

Table 1
Patient's characteristics.

	Steroid avoidance ($n = 14$)	Steroid withdrawal ($n = 9$)	Standard of care ($n = 10$)	p
Donor's characteristic				
Age, years[a]	44 [20; 65]	46 [18; 63]	56 [28; 67]	0.231
Gender (male/female), n	10/4	6/3	8/2	0.800
Recipient's characteristics				
Age, years[a]	46.7 [36.6; 64.2]	53.2 [40.9; 68.2]	61.9 [42.4; 69.1]	0.057
Gender (male/female)	12/2	7/2	7/3	0.869
PreTx BMI[a]	27.5 [23.8; 36.7]	28.8 [21.1; 33.9]	27.9[20.7; 33.9]	0.926
HLA mismatch[a]	2.5 [1; 5]	2 [0; 5]	3.5 [2; 5]	0.142
PRA, %[a]	2 [0; 16]	0 [0; 8]	6 [0; 25]	0.098
Dialysis vintage, days[a]	795.5 [156; 2746]	760.5 [209; 1225]	572 [161; 1257]	0.579
Cold ischemia, hours[a]	17.1 [14.7; 22.9]	16.1 [13.5; 24]	19.0 [15.9; 25.6]	0.043
Hemodialysis/ peritoneal dialysis, n	13/1	8/1	8/2	0.632
Tacrolimus blood level (µg/L)				
At POD 7	16.5 ± 5.9	16.1 ± 6.6	11.3 ± 4	0.075
At POD 14	13.6 ± 6.4	13.2 ± 5.5	14.7 ± 5.8	0.270
At POD 90	9.15 ± 2.8	8.3 ± 2.6	10.2 ± 2.3	0.227
Serum creatinine (µmol/L)				
At 3 months[a]	143.7 [86.6; 289.2]	122.7 [79.4; 235.7]	132.5 [90.6; 168.2]	0.988
At 1 year[a]	137,6 [72.3; 283.5]	125.2 [72.5; 311.5]	112.2 [85.8; 204.3]	0.722

[a] Data are presented as medians [min; max] or as means ± SD.