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Kinetic analysis reveals potency of CD4$^+$ CD25$^{bright+}$ regulatory T-cells in kidney transplant patients

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Abstract

Donor-specific hyporesponsiveness as occurs after allogeneic kidney transplantation may be mediated by repression of effector cells by a specific subset of T-cells: the CD4$^+$ CD25$^{bright+}$ FoxP3$^+$ regulatory T-cells (Tregs). Here, we examined the suppressive capacity of Tregs isolated from the leukafereses product of 6 kidney transplant recipients, by reconstituting Tregs to responder T-cells at several time-points after initiation of proliferation. We show that Tregs derived from kidney transplant patients potently restrain proliferation to donor-antigens and 3rd party-antigens in classic reconstitution assays (i.e. addition of Tregs at the start of the co-incubation). However, when Tregs were added 5 days after initiation of proliferation, they were still capable of suppressing proliferation to donor-antigens (by 38%) but no longer to 3rd party-antigens. Thus, we conclude that the potency of Tregs to suppress reactivity to specific antigens should be determined by reconstitution to ongoing reactions.

Keywords: Regulatory T-cells; Kidney transplantation; Human

1. Introduction

Prevention of auto-immunity is partly mediated by regulation of auto-reactive effector T-cells in the periphery by specific regulatory subsets of T-cells [1,2], such as the naturally occurring CD4$^+$ CD25$^{bright+}$ FoxP3$^+$ regulatory T-cells (Tregs). Consequently, impaired immune regulation by Tregs results in various auto-immune diseases like type I diabetes and rheumatoid arthritis [3–5]. In addition, the involvement of these cells in controlling responses to allogeneic antigens is being extensively studied [6,7]. In experimental animal models low numbers of Tregs could already block allograft rejection [8,9]. In tolerant liver [10] but not kidney [11] transplantation recipients without immunosuppression, an increased frequency of CD4$^+$ CD25$^+$ Tregs compared with healthy volunteers was observed of which the *in vitro* suppression of allogeneic stimulated T-cell proliferation by Tregs was more specific for the donor than for a HLA mismatched control [12]. Also, in a small number of kidney transplant recipients free of immunosuppression, allograft acceptance was reported to be associated with immune regulation [13].

We and others found that in kidney transplant recipients treated with immunosuppression the peripheral anti-donor reactivity decreased over time [14–17]. This diminished reactivity to donor-antigens is not an *in vitro* artifact, since renal allograft recipients can be safely tapered in their immunosuppressive medication [18,19]. Recently, we showed that Tregs can mediate donor non-reactivity in patients long after clinical kidney transplantation [20]. Thus, T-cell mediated immunoregulation may be responsible for preserving a state of donor-specific non-responsiveness [21].

Therefore, we set out to study the capacity of CD4$^+$ CD25$^{bright+}$ FoxP3$^+$ Tregs of kidney transplant recipients to suppress proliferation in '*de novo*' and on-going reactions. To do so, large

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numbers of scarce Tregs were required and thus this study was performed on material obtained during a leukafereses procedure. Our data indicate that Tregs from kidney transplant patients more potently restrain proliferation to donor-antigens than to 3rd party-antigens when reconstituted after initiation of the allo-reactivity.

2. Objective

The objective of this study was to determine the capacity of $CD4^+$ $CD25^{bright+}$ $FoxP3^+$ regulatory T-cells to suppress ongoing anti-donor and anti-3rd party-reactivity and to assess differences therein.

3. Material and methods

3.1. Patients and leukafereses

During routine visits to the outpatient clinic of the Erasmus MC, patients were asked to participate in the study. Inclusion criteria for entry into the study were (1) time after transplantation ≥6 months, (2) stable graft function and glomerular filtration rate ≥50 ml/min, (3) no acute rejection episodes in the 3 months before the start of the study, (4) unchanged immunosuppressive medication in the 3 months before the start of the study, (5) recipients of a first or second kidney transplant from a living (un)related donor, (6) no active infections at the time of entry into the study.

The study was performed in accordance with the declaration of Helsinki and its amendments. The study was approved by the local ethics committee of the Erasmus MC and all patients gave written informed consent. The characteristics of the patients are described in Table 1.

A single 6-L apheresis procedure was performed by use of a Cobe Spectra (Cobe laboratories, Lakewood, CO) apheresis device. Venous access for the apheresis procedure was by peripheral vein-to-peripheral vein technique. Anticoagulant citrate dextrose solution formula (ACD-A; Baxter, Deerfield, Ill. USA) was used to prevent coagulation.

3.2. Isolation of T-cells and regulatory T-cells

First, peripheral blood mononuclear cells (PBMC) were isolated from the leukafereses product by density gradient centrifugation using Ficoll–Paque [density 1.077 g/ml] (Amersham, Uppsala, Sweden). The PBMC were collected from the interphase, washed twice in RPMI 1640 (Biowithaker, Verviers, Belgium) and resuspended in RPMI 1640-Dutch Modification (Gibco BRL, Paisley, UK) supplemented with 2 mM L-glutamine, 100 IU/ml penicillin, 100 μg/ml streptomycin (all Gibco) and 10% heat inactivated pooled human serum. Subsequently, T-cells (serving as responders) and Tregs were isolated. T-cells were isolated using the pan T-cell isolation kit II according to manufacturers (Miltenyi Biotec, Bergisch Gladbach, Germany) protocol, resulting in ≥95% $CD3^+$ cells. To isolate $CD4^+$ $CD25^{bright+}$ regulatory T-cells, PBMCs were resuspended in 45 μl MACS-buffer/10×10^6 PBMC prepared according to manufacturers (Miltenyi Biotec) protocol and supplemented with 5 μl CD25-microbeads (directed against epitope A of the CD25-molecule; Miltenyi Biotec). Subsequently, the "posseld"-separation protocol was used on the autoMACS (Miltenyi Biotec) which led to a pure positively separated fraction. This separation routinely results in ≥95% $CD4^+$ $CD25^{bright+}$ cells of which >80% expresses FoxP3.

3.3. Mixed lymphocyte reactions

5×10^4 Viable (as determined by trypan-blue exclusion) purified T-cells were incubated with 5×10^4irradiated thawed stimulator cells (37 Gy) from the donor or 5×10^4irradiated PBMC from a 3rd party (fully HLA-A, B and DR mismatched with the recipient and the donor) in RPMI 1640 supplemented with 10% heat inactivated pooled human serum, 2 mM L-glutamine and 100 U/ml and 100 μg/ml penicillin–streptomycin. All experiments were performed in triplicate in 96 well plates (NUNC, Roskilde, Denmark) At the end of day 6, 0.5 μCi ^{3}H-thymidine was added to the culture. Sixteen hours later, the samples were harvested and counted using a β-counter (PerkinElmer, Oosterhout, The Netherlands).

3.4. Maintenance of regulatory T-cells

$CD4^+$ $CD25^{bright+}$ regulatory T-cells were isolated as described above. Since we wanted to reconstitute these Tregs at several time-points after the start of the experiment, we maintained the Tregs in RPMI 1640 supplemented with 10% heat inactivated pooled human serum, 2 mM L-glutamine and 100 U/ml and 100 μg/ml penicillin–streptomycin in the presence of 50 U/ml human recombinant interleukin-2 (Chiron, Amsterdam, The Netherlands). At the time-point of reconstitution (day 0, day 2 and day 5), the cells remained ≥95% viable. For the expression of CD25 and FoxP3, see Fig. 2; as 98% of the cells were $CD4^+$, the cells shown were gated on CD4.

3.5. Flow cytometry

3.5.1. Purity

To check the purity of the isolated fractions, the T-cells and Tregs were incubated with anti-CD25-PE (clone M-A251; directed against epitope B of the CD25 molecule), anti-CD3-PerCP and anti-CD4-FITC (Becton Dickinson, San Jose, USA). Additionally, the expression of FoxP3 was determined in the Tregs at the three different time-points of reconstitution using the APC-anti human FoxP3 staining set (clone PCH101, eBioscience, San Diego, USA). The samples were measured on the four color FACScalibur (Becton Dickinson) and analyzed using Cell Quest software.

3.5.2. Activation markers

To determine the activation status of donor- and 3rd party-activated responder T-cells, the stimulator cells were labeled with PKH-67 dye prior to stimulation (Sigma Aldrich, St Louis, MO, USA) according to manufacturer's protocol. Briefly, the stimulator cells were washed twice with serum-free medium, resuspended in loading buffer and incubated for 5 min with PKH-67 (1 mM in ethanol, Sigma Aldrich) at room temperature after which the reaction was stopped by addition of fetal calf serum. After several washing steps, the cells were irradiated and used as stimulators in an MLR-reaction. After 2 and 5 days, the cells in the MLR were stained with CD25-PE or CD69-PE or HLA-DR-PE

Table 1
Patient characteristics

Patient	Sex	Primary kidney disease	Tx No.	Tx type	Age at leukapheresis	Months after transplantation	HLA MM	Immunosuppression	Serum creatinine μmol/L
1	M	Reflux nephropathy	First	LR	50	80	1-1-1	MMF, Pred	109
2	M	IgA nephropathy	First	LUR	51	8	2-2-2	MMF, Tac, Pred	162
3	M	Reflux nephropathy	First	LR	27	8	1-1-1	MMF, Tac, Pred	131
4	M	Glomerulonephritis	First	LR	20	7	1-1-1	MMF, Tac, Pred	167
5	M	Hypertension	First	LR	39	42	1-1-1	MMF, Tac, Pred	128
6	M	Polycystic kidney disease	First	LUR	46	47	1-2-2	MMF, Tac, Pred	127

LR = Living Related; LUR = Living UnRelated.