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Isolation, expansion and functional assessment of CD4+CD25+FoxP3+ regulatory T cells and Tr1 cells from uremic patients awaiting kidney transplantation

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ABSTRACT

Background: The immunosuppressive properties of regulatory T cells have emerged as an attractive tool for the development of immunotherapies in various disease contexts, e.g. to treat transplantation induced immune reactions. This paper focuses on the process of obtaining and functionally characterizing CD4+CD25+FoxP3+ regulatory T cells and Tr1 cells from uremic patients awaiting kidney transplantation.
Methods: From October 2010 to March 2011 uremic patients awaiting living donor kidney transplantation, and their corresponding kidney donors, were enrolled in the study. A total of seven pairs were included. Isolation of CD4+CD25+FoxP3+ regulatory T cells was performed by magnetic activated cell sorting of peripheral blood mononuclear cells obtained from the uremic patients. Donor specific Tr1 cells were differentiated by repetitive stimulation of immature CD4+ T cells with immature dendritic cells, with the T cells coming from the future kidney recipients and the dendritic cells from the corresponding kidney donors. Cells were then expanded and functionally characterized by the one-way mixed leukocyte reaction and assessment of IL-10 production. Phenotypic analysis was performed by flow cytometry.
Results: The fraction of CD4+CD25+FoxP3+ regulatory T cells after expansion varied from 39.1 to 50.4% and the cells retained their ability to substantially suppress the mixed leukocyte reaction in all but one patient (3.8–19.2% of the baseline stimulated leukocyte activity, $p<0.05$). Tr1 cells were successfully differentiated from all but one patient and produced high levels of IL-10 when stimulated with immature dendritic cells (1,275–11,038% of the baseline IL-10 secretion, $p<0.05$).
Conclusion: It is practically feasible to obtain and subsequently expand CD4+CD25+FoxP3+ regulatory T cells and Tr1 cells from uremic patients without loss of function as assessed by in vitro analyses. This forms a base for adoptive regulatory T cell therapy in the setting of living donor kidney transplantation.

1. Introduction

Regulatory T cells (Tregs[1]) play a critical role in controlling immune responses in various settings such as autoimmune diseases [1,2], infectious diseases [3,4], antitumor immunity [5], maternal tolerance to a fetus [6,7] and allograft transplantation [8–11]. This feat is accomplished by several mechanisms, some of which are yet to be characterized in greater detail [12,13]. Several Treg subsets have been identified [14,15] and among the CD4+ Tregs two major subgroups have been identified: the CD4+CD25+FoxP3+ Tregs, also known as natural Tregs (nTregs) because they develop in the thymus and are present from birth [16], and the inducible Tregs (iTregs) that are generated in the periphery from naïve T cells under tolerogenic conditions [17]. The best studied form of iTregs is the Tr1 cells, which were originally described in the mid 1990s [18,19]. It has recently been confirmed that nTregs and Tr1 cells are indeed distinct subsets of cells with regulatory activity [20]. In the last decade the immunosuppressive properties of regulatory T cells have emerged as an attractive tool for the development of immunotherapies in a variety of disease contexts.

Here we focus on the potential use of Tregs in adoptive cell therapy to treat transplantation induced immune reactions in the setting of kidney transplantation in humans, in particular living donor kidney transplantation. Previous studies have shown higher levels of nTregs in operationally tolerant transplant patients [21–23] and several preclinical models indicate that transplantation tolerance can be induced by the transfer of nTregs [9,24–29]. Furthermore, the importance of nTregs to maintain

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[1] Abbreviations: Tregs, regulatory T cells; nTregs, natural regulatory T cells; iTregs, inducible regulatory T cells; GMP, good manufacturing practice; PBMCs, peripheral blood mononuclear cells; MACS, magnetic-activated cell sorting; iDCs, immature dendritic cells; CM, complete media; rhIL-2, recombinant human interleukin-2; rhIL-4, recombinant human interleukin-4; rhGM-CSF, recombinant human granulocyte macrophage colony stimulating factor; IL-10, interleukin-10; GVHD, graft-versus-host disease.

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peripheral tolerance is evident in patients with the IPEX syndrome (immunodysregulation, polyendocrinopathy and enteropathy, X-linked syndrome), where mutations in the FoxP3 gene cause varying degrees of enteritis and endocrinopathies [30–33]. The inducible Tr1 cells also show promise for induction of transplantation tolerance [17,18,29,34].

The immunosuppressive drugs used today efficiently inhibit the immune system, making solid organ transplantation possible. Unfortunately, they are associated with an increased risk of infection and certain malignancies as well as numerous drug specific side effects [35]. Albeit decreasing the risk of graft loss due to acute rejection, the number of patients experiencing chronic graft dysfunction is still high [36]. Previous studies have shown that the vasculopathy can be avoided in tolerant animals [37]; therefore, it is possible that chronic graft dysfunction can be decreased or even ameliorated with a Treg based therapy.

2. Objective

To make autologous adoptive Treg cell therapy a reality for controlling transplantation induced immune reactions Tregs need to be isolated from transplant candidates and expanded ex vivo, without loss of function. This paper focuses on the process of obtaining and functionally characterizing CD4+CD25+FoxP3+ Tregs and Tr1 cells from uremic patients awaiting kidney transplantation.

3. Materials and methods

3.1. Patients

From October 2010 to March 2011 uremic patients awaiting living donor kidney transplantation, and their corresponding kidney donors, were enrolled in the study. All living kidney donor/recipient pairs were asked to participate and were given written information about the study. All patients and donors showed interest to participate, but the only pairs included were those where the transplantation date matched the capacity of available laboratory resources. This was considered a random collection.

All patients had a scheduled date for transplantation, making planning in advance possible. A total of seven living donor pairs were included (Table 1) with a mean age of 50.1 ± 15.1 years for the uremic patients (all male) and 49.1 ± 9.1 years for the kidney donors (5 females and 2 males). Five of the uremic patients were on renal replacement therapy at the time of inclusion in the study, three with hemodialysis and two with peritoneal dialysis. One had been transplanted previously. All had given their written informed consent prior to phlebotomy or leukapheresis, and peripheral blood mononuclear cells (PBMCs) were obtained both from the future kidney recipients and the kidney donors. Ethical approval for the project was obtained from the Uppsala regional ethical review board (Dnr 2010-069).

Table 1
Demographics of the future kidney recipients and their corresponding donors. Donor A donated a kidney to recipient 1, donor B to recipient 2, and so on.

	Age	Gender	Cause of uremia	Tx previously
Recipient 1	61	Male[a]	Diabetes, hypertension	No
Recipient 2	44	Male[b]	IgA nephropathy	No
Recipient 3	70	Male[b]	Thrombotic microangiopathy	No
Recipient 4	64	Male[c]	Lithium nephropathy	No
Recipient 5	38	Male[a]	Primary hyperparathyroidism	No
Recipient 6	29	Male[c]	IgA nephropathy	No
Recipient 7	45	Male[b]	Diabetes	Yes[d]
Donor A	62	Female[e]	–	
Donor B	41	Female[e]	–	
Donor C	46	Female[f]	–	
Donor D	35	Female[e]	–	
Donor E	35	Female[g]	–	
Donor F	52	Male[h]	–	
Donor G	53	Male[g]	–	

[a] On renal replacement therapy with peritoneal dialysis at the time of inclusion.
[b] On renal replacement therapy with hemodialysis at the time of inclusion.
[c] Has not yet started renal replacement therapy at the time of inclusion.
[d] Underwent combined kidney and pancreas transplantation 8 years earlier, and is currently not taking any immunosuppressive medications.
[e] The donor is the wife of the recipient.
[f] The donor is the daughter of the recipient.
[g] The donor is the sibling of the recipient.
[h] The donor is the father of the recipient.

3.2. Isolation of CD4+CD25+FoxP3+ Tregs (nTregs)

The isolation, culturing and expansion of nTregs were performed at the Clinical Immunology Division, Rudbeck Laboratory, Uppsala. At least 30 days prior to transplantation future kidney recipients visited the Uppsala University Hospital's blood bank where 450 mL of whole blood was drawn. The whole blood, contained within a sterile standard container for whole blood with 63 mL of citrate–phosphate–dextrose (CPD) solution, was centrifuged for 13 min at a rate of 4000 rpm and then mechanically squeezed to roughly separate erythrocytes and plasma from a buffy coat consisting mainly of leukocytes and platelets. If the hemoglobin level was too low to allow for phlebotomy, or if the patient requested, leukapheresis was performed for 60 to 90 min. The process of leukapheresis produced a buffy coat without having to further separate it from erythrocytes and plasma.

Individual buffy coats were subsequently brought to the laboratory where the PBMCs were obtained by centrifugation at 1500 rpm for 5 min over a Ficoll-Paque gradient (GE Healthcare, Uppsala). Next, CD4+ T cells were magnetic-activated cell sorting (MACS) separated using the CD4+ T Cell Isolation Kit II (Miltenyi Biotec) where CD4+ T cells are isolated by depletion of non-CD4+ T cells (negative selection) using a cocktail of biotin-conjugated monoclonal antibodies and anti-biotin monoclonal antibodies conjugated to MicroBeads. Finally, selection of CD4+CD25+FoxP3+ T cells was performed using the CD25+ CD49d-regulatory T Cell Isolation Kit (Miltenyi Biotec) where cells are negatively selected for CD8 and CD49d and positively selected for CD25 [38].

The buffer used for MACS separation contained phosphate-buffered saline (PBS), pH 7.2, 0.5% human serum albumin (HSA), and 2 mM EDTA; and was degassed and kept cold (2–8 °C) before use.

3.3. Differentiation of Tr1 cells

Tolerogenic donor specific Tr1 cells were produced by repetitive stimulation of peripheral blood naïve CD4+ T cells with allogeneic immature dendritic cells (iDCs), similar to what has been described previously [39].

Briefly, PBMCs from the future kidney donors were obtained at least 30 days prior to kidney donation by phlebotomy followed by centrifugation over a Ficoll-Paque (as described above). Monocytes were isolated as the adherent fraction after incubation for 2 h at 37 °C in complete media (CM) consisting of RPMI-1640 supplemented with 10% pooled human AB serum (pooled, sterile filtered, and heat inactivated AB serum from 15 to 20 healthy blood donors; tested for pathogenic contamination according to hospital standards), 1% penicillin–streptomycin, 1% 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 0.5% L-Glutamine, and 0.04% β-mercaptoethanol. Adherent monocytes were subsequently differentiated into immature dendritic cells (iDCs) by culture in 25 ng/mL recombinant human IL-4 (rhIL-4) and 50 ng/mL recombinant human granulocyte macrophage colony stimulating factor (rhGM-CSF). After 2 and 4 days of culturing half the media was replaced and rhIL-4 and rhGM-CSF were again added, in the same concentrations as above. On day 6, iDCs were collected and used to differentiate Tr1 cells.

Next, CD4+ T cells were purified from the corresponding future kidney recipients by negative selection using the CD4+ T cell Isolation Kit II (Miltenyi Biotec), according to the manufacturer's instructions. Some of the resultant CD4+ T cells were cryopreserved (as described below) and the remainder were used to differentiate Tr1