On Mesenchymal Stem Cells and Regulatory T Cells in Organ Transplantation

Anja U. Engela

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Mesenchymale Stamcellen en Regulatoire T Cellen bij Orgaan Transplantatie

Thesis

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Happiness is the consequence of personal effort.
Elizabeth Gilbert

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Chapter 1

General introduction and outline

Partly based on "On the interactions between MSC and Treg for immunomodulation in transplantation"

Anja U. Engela[1], Carla C. Baan[1], Frank M.J.F. Dor[2], Willem Weimar[1], Martin J. Hoogduijn[1]

[1]Department of Internal Medicine, Section Nephrology and Transplantation, [2]Department of Surgery, Erasmus University Medical Center, Rotterdam, the Netherlands

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ORGAN TRANSPLANTATION AND GRAFT REJECTION

In 2011, 860 kidneys, 135 livers, 68 lungs and 44 hearts were transplanted in the Netherlands [1]. The high number of kidney transplantations (KTx) compared to transplantations of other organs is a consequence of the possibility of living donation, successful campaigns to increase the awareness thereof and its less emotional nature compared to, for instance, heart transplantation. Further, over the past decades the risks for donors and recipients were reduced due to continuously improving surgical techniques and advances in post-transplantation care [2, 3]. Yet, despite all these efforts and more than 50% of the transplanted kidneys being donated from living individuals, the number of patients in need of an organ is constantly increasing [1, 4]. Progressive loss of kidney function is primarily caused by diabetic nephropathy, hypertension, glomerulonephritis and cystic kidney disease, and often leads to end-stage renal disease (ESRD). KTx is the most effective treatment for ESRD and significantly advances the patient's quality of life [4]. Graft survival in the early post-transplantation period improved over the past 15 years, while long-term graft survival rates remain largely unchanged [5, 6]. In addition, acute rejection still is experienced by approximately 15-30% of the kidney grafts depending on the population studied.

Allograft rejection is largely the result of alloreactivity and various insults to the organ which occur during organ donation, the transplantation procedure and post-transplantation. In addition, in the event of organ donation from a deceased donor, dramatic pathophysiological changes, inflicted upon brain death, cause a strong general inflammatory response [7]. In combination with cellular infiltration these changes affect the quality of the organ. Further, the time between organ procurement and grafting leads to additional tissue damage and enhanced inflammation due to ischemia. Consequently, upon graft reperfusion, the immune system of the recipient is exposed to a plethora of danger and stress molecules. While these inflammatory events pose a serious risk to graft dysfunction and graft loss, the biggest threat of graft rejection, short-term and long-term, is alloreactivity. After KTx between genetically non-identical individuals, alloreactivity occurs due to the recognition of donor antigens by the recipient's immune system. Antigen recognition occurs in a direct or indirect manner. During the direct process recipient T cells interact with human leukocyte antigens (HLA) molecules on donor cells. The indirect pathway is facilitated by T cell binding to processed allogeneic peptides bound to HLA molecules on recipient antigen-presenting cells (APC). In solid organ transplantation direct allorecognition is associated with acute rejection while indirect allorecognition mainly mediates chronic rejection [8]. Cell-mediated recognition of foreign antigens requires protein binding to HLA molecules, the simultaneous engagement of the HLA-peptide complex with the T cell receptor (TCR) and with co-stimulatory molecules on the T cell, such as CD28. These events lead to T cell activation and the

production of appropriate cytokines. When donor antigen is encountered by a $CD4^+$ T cell or $CD8^+$ T cell, each individual cell undergoes clonal expansion, i.e. activation, proliferation and differentiation into effector T cells. Fully-differentiated cytotoxic T lymphocytes (CTL) induce programmed cell death by apoptosis. $CD4^+$ effector T cells provide help to B cell differentiation and the resulting secreted antibodies cause complement activation and phagocytosis. The stimulation of T cells with allogeneic antigen therefore causes tissue damage and leads to rejection of the transplanted organ. While most T cells exert effector functions, some T cell populations promote immune tolerance after transplantation. The most studied immunosuppressive T cell subset are CD4+ regulatory T cells (Treg), but also other populations such as specific CD8+ T cells and $CD4^-CD8^-$ T cell subsets can also induce tolerance [9].

Progress in understanding the cellular interactions leading to graft rejection or immune tolerance and the molecular pathways involved opened up ways to augment T-cell and B-cell activation and to improve graft survival.

IMMUNOSUPPRESSIVE DRUGS

The knowledge gained from elucidating the mechanisms involved in transplant immunology contributed to the development of powerful immunosuppressive medication. Activation and function of the immune system, leading to alloreactivity and potentially causing graft failure, can be manipulated in different ways. Early attempts to prevent graft rejection involved the reduction of inflammation and the inhibition of effector T cell proliferation using corticosteroids and azathioprine (AZA). In 1978, the field of transplantation was revolutionized by the discovery of the immunosuppressive effect of cyclosporine A (CsA), a compound that dramatically reduced the occurrence of acute rejections [10]. CsA inhibits the protein phosphatase calcineurin. Calcineurin-mediated dephosphorylation of the nuclear factor of activated T cells (NFAT) activates signalling pathways required for T cell activation and cytokine production. The success of CsA and its effectiveness in kidney, heart and liver transplantation was continued by tacrolimus, another calcineurin inhibitor (CNI). Despite its similar mechanism of action, tacrolimus further reduced acute rejection incidents and is the most prescribed immunosuppressant in transplantation to date. Yet, the biggest drawback of CNI treatment is nephrotoxicity effect causing impaired tubular function, reduced glomerular filtration rates (GFR) and other undesired clinical symptoms such as hypertension and diabetes. This resulted in the exploration of other therapeutic approaches for the prevention of alloreactivity. Other small-molecule immunosuppressants preventing T cell and B cell proliferation include the mammalian target of rapamycin (mTOR) inhibitors sirolimus and everolimus, and the anti-metabolite drug mycophenolate mofetil (MMF), the ester prodrug of my-

cophenolic acid (MPA). While these drugs target intracellular molecules, other immunosuppressants have been developed which block extracellular proteins and receptor molecules crucial to cell activation and function. Basiliximab is a monoclonal anti-IL-2 receptor (IL-2R) antibody administered as induction therapy. Binding of basiliximab to the CD25 antigen, the α-chain of the IL-2R expressed on activated T cells, prevents T cell proliferation. Another agent used as induction therapy is anti-thymocyte globulin (ATG). ATG is prepared from serum of rabbits immunized with human thymocytes and contains a multitude of antibodies. It depletes circulating T cells through complement and cell-mediated cytotoxicity and is also used to treat steroid-resistant acute rejection. Although most drug research for graft rejection focuses on T cells, also monoclonal antibodies influencing B cell function have been developed. The monoclonal anti-CD20 antibody rituximab evokes B cell destruction and is used as induction therapy for highly sensitized transplant patients. Another agent targeting B cells is the IL-6 receptor antagonist tocilizumab. Tocilizumab interferes with B cell activation and differentiation, affects B cell hyperreactivity and reduces serum immunoglobulin levels [11]. In addition, tocilizumab induces and expands B cells with a regulatory phenotype [12].

Currently, the most commonly prescribed maintenance therapy in KTx consists of tacrolimus, MMF/MPA and steroids [4]. Due to the high nephrotoxicity of tacrolimus, transplantation professionals were excited about the FDA approval of belatacept in 2011. While increased occurrence of acute cellular rejections (ACR) was reported in belatacept-treated kidney recipients early after transplantation, belatacept proved less nephrotoxic than CNI and improved GFR [13]. Belatacept is a fusion protein consisting of the extracellular domain of cytotoxic T-lymphocyte antigen-4 (CTLA-4) and the Fc region of a human IgG1. By binding to CD80 and CD86 on APC belatacept blocks the interaction of CD80/86 with the T cell co-receptor CD28 and prevents the co-stimulatory signal required for T cell activation [14].

Experience gained from the past three decades of drug research to prevent graft rejection demonstrates that the development of immunosuppressive agents with good short-term and long-term graft survival remains challenging. Further research should also aim to reduce adverse side effects other than nephrotoxicity such as diabetes, dyslipidemia, hypertension, infections and malignancies. Particularly in terms of opportunistic infections, it will be important to strike a balance between suppression of the immune system to avert graft rejection and to maintain sufficient immune competence to avoid infections. In search of these immune-balancing anti-rejection therapies scientists are evaluating the potential of drug-alternative therapies. Due to the discovery of many cell populations promoting tolerogenic immune responses, cell-based therapies came into focus [9]. Such immunomodulatory cell populations include Treg, regulatory B cells and macrophages as well as mesenchymal stem cells.

CELLS WITH IMMUNOSUPPRESSIVE PROPERTIES

Mesenchymal stem cells

Mesenchymal stem cells (MSC) were discovered in bone marrow by Friedenstein et al. in 1970 [15]. Subsequently, researchers have been able to isolate these spindle-shaped and adherent cells from various postnatal and adult tissues, including adipose tissue, an easily accessible source [16-19]. MSC are self-renewing and capable of forming colonies while retaining their multilineage differentiation potential. They are able to differentiate into adipocytes, chondrocytes, osteoblasts and myocytes [17, 20]. These features represent part of the criteria that define MSC [21]. MSC are immunophenotypically characterized by the expression of the stromal markers CD73, CD90 and CD105, the absence of the haematopoietic markers CD14, CD34 and CD45, and lack of HLA-DR. Interest in MSC for their use in transplantation was fostered when it was first discovered that MSC possess immune-cell suppressive properties. These properties allow MSC to inhibit the activation and proliferation of T cells [22-24]. In addition, MSC are able to induce the arrest of T cell division [25]. Besides $CD4^+$ T cells and $CD8^+$ T cells, also activated B cells and natural killer (NK) cells are also susceptible to the suppressive activity of MSC [26, 27]. MSC also target dendritic cells (DC); they hamper DC differentiation and cause down-regulation of MHC class II molecules and co-stimulatory molecules on DC [28-30]. In addition to these immunosuppressive effects, MSC also have immunosupportive properties; by delaying the apoptosis of neutrophils they preserve them to be readily available for countering infections [31]. Further, MSC promote the survival of T cells in a quiescent state [32]. Immunomodulation by MSC is mediated by cell-cell contact and the production of paracrine factors. Important mediators are indoleamine-2,3-dioxygenase (IDO), transforming growth factor-beta (TGF-β), interleukin-10 (IL-10), prostaglandin E2 (PGE2), hepatocyte growth factor (HGF), human leukocyte antigen-G5 (HLA-G5) and nitric oxide (NO) [24, 33-39]. Up-regulated expression of these immunomodulatory mediators and full exertion of MSC's suppressive functions require MSC activation. MSC are activated in a pro-inflammatory microenvironment through cytokines such as interferon-gamma (IFN-γ), tumour necrosis factor-alpha (TNF-α), IL-1α and IL-1β [40]. The immunosuppressive properties of MSC have been confirmed in vivo as MSC prolonged the survival of skin and heart grafts in animal models [22, 41]. Intravenous administration of MSC in a rat kidney transplant model of prolonged cold ischemia reduced the cellular infiltration into the graft [42]. Further evidence of an organ protective function by MSC was observed in a swine model of acute myocardial infarction and in a rat lung disease model [43, 44]. In clinical studies MSC have successfully attenuated graft-versus-host-disease (GVHD) after haematopoietic stem cell transplantation (HSCT) [45, 46]. Currently, research groups strive to confirm the safety and feasibility of MSC therapy in solid organ transplantation [47-53].

Regulatory T cells

MSC have a potent inhibitory effect on T cells. This raises the question to what extent MSC affect T cells that possess immunomodulatory properties. Treg were first characterized by Hall et al. as activated $CD4^+$ T cells expressing CD25, the IL-2 receptor alpha-chain, which are involved in the maintenance of tolerance to self-antigens [54]. Further research revealed that the transcription factor forkhead box P3 (FOXP3) is a unique marker for Treg in mice [55]. In humans, however, FOXP3 expression is not Treg-specific. While a unique marker remains to be identified for human Treg, they are currently characterized as FOXP3 expressing $CD4^+CD25^+$ T cells that lack CD127, the α-chain of the IL-7 receptor [56, 57]; additional markers include the co-stimulatory molecule cytotoxic T lymphocyte antigen-4 (CTLA-4), glucocorticoid-induced TNF receptor-related protein (GITR), ectonucleotidase CD39 and L-selectin (CD62L) [58-64].

The importance of Treg in the maintenance of tolerance is highlighted in humans suffering from immunodysregulation polyendocrinopathy enteropathy X-linked syndrome (IPEX) [65, 66]. IPEX patients lack functional Treg as a result of a point mutation in the FOXP3 gene [67]. FOXP3 gene mutations are also associated with other autoimmune diseases [67-70]; the prevention of allergic rhinitis is related to a single nucleotide polymorphism in Chinese and Hungarian populations. Further, a $(GT)_n$ dinucleotide repeat polymorphism in the FOXP3 promoter region influences the promoter activity and its association with the onset of type 1 diabetes has been reported [71]. Despite these evidences the role of Treg or Treg-related mutations in the development of autoimmunity remains controversial [72].

Because of their immunomodulating nature Treg are interesting candidates for the induction of transplantation tolerance. Indeed, Treg have been reported to control allo-reactivity by inhibiting the functionality of cell types similar to those suppressed by MSC; Treg hamper the proliferation of $CD4^+$ T cells, $CD8^+$ T cells and DC [73-76]. Further, molecules associated with the tolerogenic and suppressive function of Treg partially overlap with those involved in MSC-mediated immunomodulation and include IL-10, TGF-β and heme oxygenase-1 (HO-1) [76]. In addition, it has been hypothesized that Treg are able to influence their target cells in a granzyme B-dependent and perforin-dependent manner [77, 78]. Results from different humanized mouse models and clinical studies in patients with GVHD encourage the use of freshly isolated or ex-vivo expanded Treg [79-81]. Despite the indication that Treg therapy is well tolerated in patients receiving haematopoietic stem-cell treatment [82, 83], information about the safety, feasibility and efficacy of Treg application in solid organ transplantation is still lacking. First results may become available from "The ONE study", a large multicentre phase I/II trial in living-donor kidney transplantation. The study group started recruiting patients in 2012 and aims to investigate the safety and feasibility of various regulatory and tolerogenic cell types including Treg.

Recent developments in Treg research revealed a heterogeneity of the Treg population. Apart from the well-described thymic-derived naturally occurring $CD4^+CD25^+CD127^-$

FOXP3$^+$ Treg (nTreg), other Treg subsets have been identified. Of these the most studied are induced Treg (iTreg). iTreg develop from naïve T cells in the periphery and their induction occurs upon T-cell receptor stimulation, CD28 co-stimulatory signalling, and in the presence of IL-2 and TGF-β. Phenotypically, iTreg resemble nTreg, yet, both populations can be distinguished by the methylation status of a specific locus within the FOXP3 gene [84, 85]. In nTreg this locus, the Treg-specific-demethylated-region (TSDR), is fully demethylated allowing for easy transcription of the FOXP3 gene, while the TSDR in iTreg is methylated. Further discrimination between nTreg and iTreg may be provided by Helios, an Icaros family transcription factor. It has been reported that nTreg express Helios while iTreg do not [86]. Opinion on this topic is divided, some groups claim that Helios only presents an additional activation marker and is mutually expressed by nTreg and iTreg [87, 88].

Both MSC and Treg are able to influence the innate and adaptive immune system by utilizing similar and distinct mechanisms. Therefore, it is essential to investigate whether upon interaction the overlapping mechanisms of MSC and Treg interfere with each other and influence the immunomodulatory function of both cell types.

AIM AND OUTLINE OF THE THESIS

Cellular therapy presents itself as a prospective alternative to current drug-based immunosuppressive regimens in the field of organ transplantation. Therefore it becomes essential to investigate its influence on immunomodulatory mechanisms operating in the graft recipient and to evaluate the potential efficacy of this novel treatment for the suppression of alloreactivity. The aim of this thesis is to study the interaction between MSC and nTreg, the effect of MSC on other T cell subsets and the influence of immunosuppressive drugs on MSC-mediated processes.

In **chapter 2** the adipose-tissue derived MSC used for the research presented in this thesis are characterized and their immunomodulatory capacity on different lymphocyte subsets is described. Further, the functionality of MSC and nTreg upon interaction is evaluated and the involved cytokines are assessed. **Chapter 3** focuses on the ability of MSC to induce *de novo* Treg. The newly formed Treg are characterized with respect to their suppressive function and immunophenotypical properties. In addition, the role of IL-2 in the generation of iTreg is explored by means of the anti-CD25 antibody basiliximab. The potential of another MSC-drug combination therapy for organ recipients is evaluated *in vitro* in **chapter 4**. As alloreactive CD8$^+$CD28$^-$ T cells remain unaffected by the CD28-CD80/86 co-stimulation blocking fusion protein belatacept, the effect of MSC on these cells is examined. **Chapter 5** describes a gene polymorphism association study to evaluate the effect of different FOXP3 gene variants on kidney graft survival.

In **chapter 6** the results obtained in the context of this thesis are summarized and appraised with respect to their translation to the clinical application of MSC therapy for organ transplantation.

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Chapter 2

Interactions between adipose tissue-derived mesenchymal stem cells and regulatory T cells

Anja U. Engela, Carla C. Baan, Annemiek M.A. Peeters,
Willem Weimar, Martin J. Hoogduijn

Department of Internal Medicine, Section Nephrology and Transplantation,
Erasmus University Medical Center, Rotterdam, the Netherlands

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ABSTRACT

Mesenchymal stem cells (MSCs) exhibit immunosuppressive capabilities, which have evoked interest in their application as cell therapy in transplant patients. So far it has been unclear whether allogeneic MSCs and host regulatory T cells (Tregs) functionally influence each other. We investigated the interaction between both cell types using perirenal adipose-tissue derived MSCs (ASCs) from kidney donors and Tregs from blood bank donors or kidney recipients 6 months after transplantation. The immunomodulatory capacity of ASCs was not prejudiced by both Tregs from healthy donors and Tregs from graft recipients, indicating that ASCs were not targeted by the inhibitory effects of Tregs and *vice versa*. In addition, Tregs supported ASC function as they did not alter the secretion of IFN-γ by immune cells and hence contributed to ASC activation and efficiency. ASCs exerted their suppressive role by expressing IDO, reducing levels of TNF-α, and by inducing the production of IL-10 in effector cells and Tregs. In conclusion, this study presents evidence that donor ASCs and acceptor Tregs do not impair each other's function and therefore encourages the use of MSC therapy for the prevention of graft rejection in solid organ transplantation.

INTRODUCTION

Increasing investigational efforts are undertaken to employ cell therapy for the advancement of graft survival in the field of transplantation. Mesenchymal stem cells (MSCs) are explantable from various sites including adipose tissue, bone marrow and amniotic fluid [1-4] and easily expandable. MSCs have the capacity to differentiate into multiple cell lineages such as osteoblasts, adipocytes, chondrocytes and myocytes [2, 5]. It has been demonstrated that MSCs mediate the repair of bone and cartilage [6-9], the improvement of cardiac muscle and kidney function [10-13] and the restoration of lung function [14]. The main characteristic of MSCs relevant to transplantation is their potent *in vitro* immunosuppressive effect on immune cell activation and proliferation [15, 16]; MSCs exhibit this feature upon stimulation by inflammatory conditions [17]. Soluble factors associated with the inhibitory effect of MSCs are transforming growth factor-beta (TGF-β), hepatocyte growth factor (HGF), interleukin-10 (IL-10), prostaglandins, human leukocyte antigen G5 (HLA-G5) and nitric oxide (NO) [15, 18-23]. In addition, MSCs create a local immunosuppressive microenvironment by production of indoleamine 2,3-dioxygenase (IDO), leading to tryptophan catabolism. Utilizing these mechanisms, MSCs promote heart and skin allograft survival in animal models [24, 25]. Further, there is clinical evidence that MSCs alleviate graft-versus-host disease (GVHD) after hematopoietic-stem-cell transplantation [26, 27]. Currently ongoing clinical studies aim to confirm the feasibility and safety of MSC therapy in solid organ transplantation [28, 29].

Regulatory T cells (Tregs), defined by the expression of CD4, CD25 and FOXP3 and the lack of CD127, possess immunomodulatory capacities. The influential role of Tregs on maintaining tolerance was observed in patients suffering from immunodysregulation polyendocrinopathy enteropathy X-linked syndrome (IPEX); these patients lack functioning Tregs due to a point mutation in the FOXP3 gene [30-32]. Tregs have been reported to control tissue injury and allo-reactivity [33-37] by functional inhibition of multiple cell types including $CD4^+$ helper T cells, $CD8^+$ cytotoxic T cells and antigen-presenting cells [38]. In clinical studies, *ex vivo* expanded Tregs diminished the symptoms and reduced the incidence of GVHD [39, 40]. Main factors involved in Treg function are the anti-inflammatory cytokines IL-10 and TGF-β [41-43]. In the transplantation setting, some immunosuppressants were identified to be permissive for Treg function including corticosteroids, anti-CD3 antibodies, anti-thymocyte globulin (ATG), anti-CD52 antibodies and mTOR inhibitors [44] whereas others, such as the calcineurin inhibitors cyclosporine A and tacrolimus, were found to negatively influence the development and the immunosuppressive activity of Tregs [45]. With MSCs emerging as a prospective therapy to prevent graft rejection after transplantation due to their potent inhibitory influence on effector T cells, the effect of MSCs on host Tregs remains to be investigated. Conversely, it is still unknown whether Tregs, as a consequence of their immunosuppressive capacities, inhibit the activation of

MSCs and consequently MSCs' repressive features; similar effects have been reported for other immunosuppressive agents such as rapamycin, tacrolimus or cyclosporine A [46, 47].

The present study investigated the impact of interaction between kidney donor perirenal adipose-tissue derived MSCs (ASCs) and Tregs, derived from either transplant recipients or healthy donors, on the suppression of allo-reactivity.

MATERIAL AND METHODS

Origin of adipose tissue

Perirenal adipose tissue was surgically removed from living kidney donors and collected in minimum essential medium Eagle alpha modification (MEM-α) (Sigma-Aldrich, St. Louis, MO, USA) supplemented with 2mM L-glutamine (Lonza, Verviers, Belgium), 1% penicillin/streptomycin solution (P/S; 100IU/mL penicillin, 100IU/mL streptomycin; Lonza). Samples were obtained with written informed consent as approved by the Medical Ethical Committee of the Erasmus University Medical Center Rotterdam (protocol no. MEC-2006-190).

Isolation and culture of human ASCs from perirenal adipose tissue

Perirenal adipose tissue was mechanically disrupted with a scalpel. The tissue was washed twice with 1x Dulbecco's phosphate buffered saline (PBS; PAA Laboratories, Pasching, Austria). Subsequently the tissue was enzymatically digested with sterile 0.5mg/mL collagenase type IV (Life Technologies, Paisley, UK) in Roswell Park Memorial Institute (RPMI) 1640 medium with GlutaMAX™-I (Life Technologies) and 1% P/S for 30min at 37°C. ASC culture medium consisting of MEM-α with 2mM L-glutamine, 1% P/S and 15% fetal bovine serum (FBS; Lonza) was added and the digested tissue was centrifuged at 1,200 x *g* for 10min. The cell pellet was resuspended in ASC culture medium and filtered through a 70µm cell strainer (BD Biosciences, San Jose, CA, USA). Finally, cells were transferred into a 175cm^2 cell culture flask (Greiner Bio-One, Alphen a/d Rijn, the Netherlands) and expanded in a humidified atmosphere with 5%CO_2 at 37°C. Non-adherent cells were removed after 3-4 days. Culture medium was refreshed twice weekly. Cells were removed from culture flasks at subconfluency using 0.05% trypsin-EDTA (Life Technologies) and reseeded at 1,000cells/cm^2 to ensure optimal proliferation. ASCs were used for experiments between passages 2 to 6. ASCs from these passages did not differ in their ability to differentiate or to exert their immunosuppressive functions.

Immunophenotyping of ASCs

Adherent ASCs were removed from culture flasks following incubation with 0.05% trypsin-EDTA at 37°C and washed twice with FACSFlow (BD Biosciences). Cells were incu-

bated with monoclonal antibodies (mAb) against CD14-phycoerythrin (PE), HLA-ABC-PE (all Serotec, Düsseldorf, Germany), CD90-allophycocyanin (APC), CD105-fluorescein isothiocyanate (FITC; all R&D systems, Abingdon, UK), CD34-APC, CD45-FITC, CD166-PE and HLA-DR-APC-Cy7 (all BD Biosciences) at room temperature and protected from light for 30min. Subsequently, samples were washed twice with FACSFlow and immunophenotypical analyses of ACSs were performed using the BD FACSCanto II flow cytometer, BD FACSDiva software (BD Biosciences) and FlowJo software (Tree Star, Ashland, OR, USA).

Differentiation assays

Adipogenic differentiation

Adipogenic differentiation was induced by culturing confluent ASCs in MEM-α supplemented with 2mM L-glutamine, 1% P/S, 15% heat-inactivated FBS (FBS-HI), 50µg/mL L-ascorbic acid, 500µM 3-isobutyl-1-methylxanthine (IBMX; Sigma-Aldrich), 60µM indomethacin (Sigma-Aldrich) and 10nM dexamethasone for 14 days in a humidified atmosphere with 5%CO_2 at 37°C. Lipid-filled vesicles were detected by Oil Red O staining. Cells were washed with PBS, fixed with 60% isopropanol for 1min and incubated with filtered 0.3% Oil Red O (Sigma-Aldrich; in 60% isopropanol) for 30min. Following three washes with PBS, the cells were photographed.

Osteogenic differentiation

Osteogenic differentiation was induced by culturing confluent ASCs in MEM-α supplemented with 2mM L-glutamine, 1% P/S, 15% FBS-HI, 5mM β-glycerophosphate (Sigma-Aldrich), 50µg/mL L-ascorbic acid (Sigma-Aldrich) and 10nM dexamethasone (Sigma-Aldrich) for 21 days in a humidified atmosphere with 5%CO_2 at 37°C. The deposition of calcified nodules was identified using von Kossa staining. Cells were washed with PBS and fixed with cold 4% paraformaldehyde for 5min. Following an additional wash step with PBS and two wash steps with distilled water, cells were incubated with 1% silver nitrate (in water) on a light box until blackening occurred. Cells were washed three times with water, incubated with 2.5% sodium thiosulphate (in water) for 5min, washed again twice with water, and photographed.

Isolation of peripheral blood mononuclear cells

Buffy coats from healthy blood donors were obtained from Sanquin Blood Bank, Rotterdam, the Netherlands. Peripheral blood samples from living kidney donors were collected prior to the donation procedure and from kidney recipients 6 months after transplantation. Kidney transplant patients received a triple immunosuppressive therapy consisting of tacrolimus, mycophenolate mofetil (MMF) and prednisolone. Peripheral blood mononuclear cells (PBMCs) were isolated from buffy coats or heparinized

peripheral blood samples by density gradient centrifugation using Ficoll-Paque PLUS (density 1.077g/mL; GE Healthcare, Uppsala, Sweden). Cells were washed with RPMI 1640 supplemented with 1% P/S and frozen in RPMI 1640 + GlutaMAX-I with 1% P/S, 10% human serum (Sanquin) and 10% dimethyl sulfoxide (DMSO; Merck, Hohenbrunn, Germany). PBMCs were stored at -150°C until further use.

Isolation of Tregs and effector cells from PBMCs

PBMCs were thawed and washed twice with RPMI 1640 supplemented with 20% FBS-HI, 1% P/S and 0.01mg/mL DNase I (Roche Diagnostics, Mannheim, Germany). PBMCs were resuspended in PBMC culture medium (PCM) consisting of MEM-α supplemented with 2mM L-glutamine, 1% P/S and 10% heat-inactivated human serum. The $CD25^{bright}$ cells (Tregs) were isolated by means of CD25 MicroBeads II (Miltenyi Biotec, Bergisch Gladbach, Germany). Cells and beads were incubated for 15min at 4°C in autoMACS rinsing solution (Miltenyi Biotec) supplemented with 0.5% bovine serum albumin (BSA; Sigma-Aldrich). Cells were centrifuged for 10min at 2000rpm, resuspended in buffer and subjected to a positive selection (Posseld program) on the autoMACS Pro separator (Miltenyi Biotec). The residual fraction ($CD25^{-/dim}$ cells; effector cell fraction) and the Tregs were washed and resuspended in PCM for functional assays. Cell fraction purity was determined by flow cytometry using mAb against CD3-AmCyan, CD4-Pacific Blue, CD25-PE-Cy7 (epitope B), CD127-PE (all BD Biosciences) and FOXP3-APC (eBioscience, San Diego, CA, USA). The characterization of both fractions from a representative separation example is depicted in Figure 1. Intracellular FOXP3 staining was carried out according to the manufacturer's instructions using the Anti-Human FOXP3 Staining Set APC (eBioscience). Flow cytometric analyses were performed using the BD FACSCanto II flow cytometer and BD FACSDiva software.

Mixed lymphocyte reaction and suppression assay

Mixed lymphocyte reactions (MLRs) consisted of $5x10^4$ $CD25^{-/dim}$ effector cells (residual fraction from Treg isolation) stimulated with $5x10^4$ γ-irradiated (40 Gy) allogeneic PBMCs in PCM in round-bottom 96-well plates (Nunc, Roskilde, Denmark). Effector-stimulator cell combinations were chosen based on a minimum of four HLA mismatches. In suppression assays, the immunomodulatory capacities of various concentrations of ASCs and Tregs on the MLRs were determined. Following an 8-hour incubation period, [^{3}H]-thymidine incorporation (0.25μCi/well; PerkinElmer, Groningen, the Netherlands) was measured on day 7 using the Wallac 1450 MicroBeta TriLux Liquid Scintillation Counter & Luminometer (PerkinElmer). Proliferation capacity of $CD25^{-/dim}$ effector cells was determined by stimulating $5x10^4$ cells with 1μg/mL phytohaemagglutinin-L (PHA; Roche Diagnostics, Mannheim, Germany). After 3 days, proliferation was measured by [^{3}H]-thymidine incorporation. When MLRs were performed in microtiter plates with different

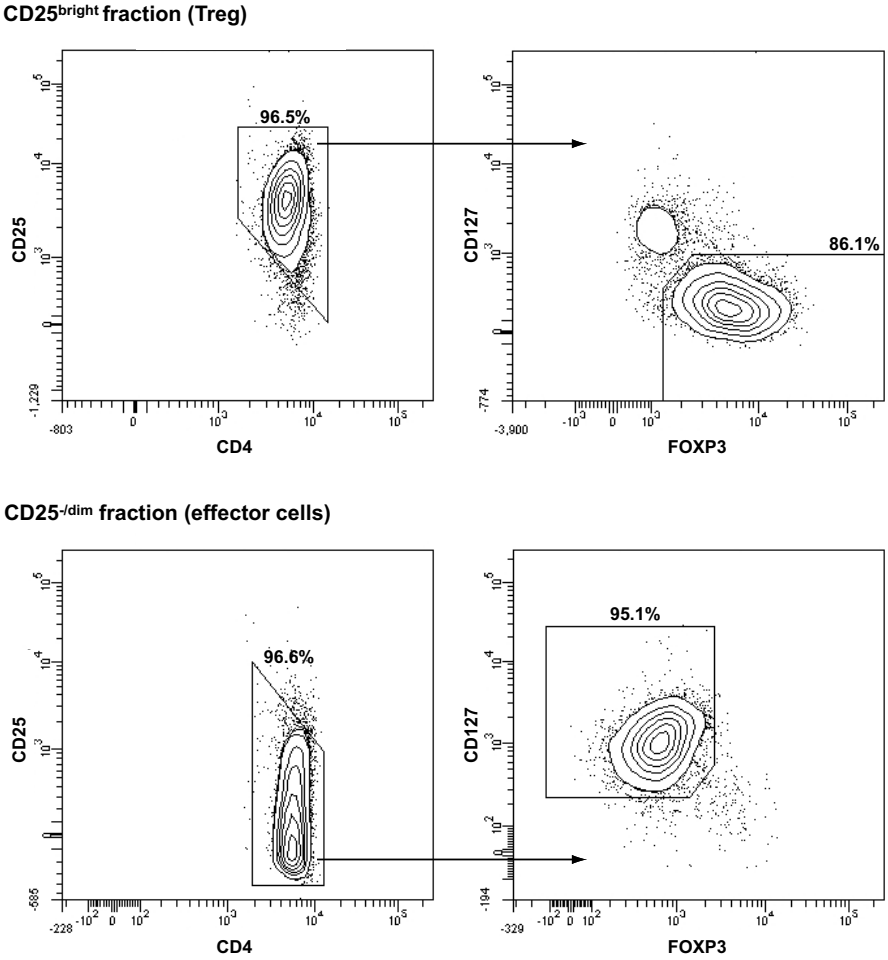

Figure 1. Characterization of Tregs and CD25$^{-/dim}$ effector cells. The CD25^{bright} cells (Tregs) and CD25$^{-/dim}$ effector cells were isolated from PBMCs by incubation with CD25 MicroBeads II followed by a positive selection. Both cell fractions were characterized using flow cytometry. (A) CD25^{bright} fraction: The CD25^{bright} cells are gated within the CD3$^+$CD4$^+$ cell population. The percentage CD127$^-$FOXP3$^+$ cells within the CD4$^+$CD25^{bright} T cells are shown. (B) CD25$^{-/dim}$ fraction: The CD25$^{-/dim}$ cells are gated within the CD3$^+$CD4$^+$ cell population. The percentage CD127$^+$FOXP3$^-$ cells within the CD4$^+$CD25$^{-/dim}$ T cells are shown.

well sizes, the number of cells was adjusted accordingly. When applicable, 50µL cell culture supernatant was harvested prior to the addition of [^{3}H]-thymidine and frozen until further use at -80°C.

PKH labeling and flow cytometric analysis

In order to study the effects of ASCs and Tregs on the proliferative capacities of various lymphocyte subsets, CD25$^{-/dim}$ effector cells were labeled using the PKH67 Green Fluorescent Cell Linker Kit (Sigma-Aldrich). PKH67 dilution due to proliferation was

measured by flow cytometry. Tregs and allogeneic stimulator PBMCs were discriminated from the effector cell population by labeling with PKH26 (PKH26 Red Fluorescent Cell Linker Kit, Sigma-Aldrich) or PKH26/67 double-labeling, respectively. Labeling was performed prior to the MLR set-up and according to the manufacturer's instructions. After 7 days, cells were harvested and washed twice with FACSFlow. Cells were stained with mAb against CD3-AmCyan, CD4-peridinin chlorophyll protein (PerCP), CD8-APC-Cy7, CD25-PE-Cy7 (all BD Biosciences) and CD45RO-Pacific Blue (BioLegend, San Diego, CA, USA). Following a 30min incubation period at room temperature and protected from light, cells were washed twice and subjected to flow cytometric analysis using the BD FACSCanto II flow cytometer and BD FACSDiva software.

mRNA expression analysis

ASCs were cultured alone or co-cultured with MLRs (1:5; ratio ASCs/effector cells) for 7 days. In co-cultures, MLRs were separated from ASCs by cell culture inserts with permeable membrane supports (0.4µm pore size; Greiner Bio-One). ASCs were harvested and washed twice with PBS-DEPC. Cells were either handled immediately or snap frozen in liquid nitrogen and stored at -80°C. Total RNA was purified using the High Pure RNA Isolation Kit (Roche Diagnostics) according to the manufacturer's instructions. Complementary DNA (cDNA) was synthesized by reverse transcription using random primers. Quantitative real-time PCR (qPCR) was performed using 500ng cDNA, the StepOnePlus™ Real-Time PCR System, TaqMan Universal PCR Master Mix and the assay-on-demand primer/probes for indoleamine 2,3-dioxygenase (INDO; Hs00158027.m1), interferon-gamma (IFN-γ; Hs00174143.m1), interleukin-6 (IL-6; Hs00174131.m1), IL-10 (Hs00174086.m1), transforming growth factor-beta (TGF-β; Hs00171257.m1), tumor necrosis factor-alpha (TNF-α; Hs99999043.m1) (all Applied Biosystems, Foster City, CA, USA). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA served as endogenous control for normalization (Hs99999905.m1; Applied Biosystems). Intra- and interassay variations were determined by negative controls and positive reference samples. Changes in expression of the target genes relative to the GAPDH gene were quantified using the comparative C_T method [48]. Fold changes of less than 3 were considered insignificant.

Cytometric bead array (CBA) and ELISA

Cell culture supernatants were obtained from suppression assays using Tregs (1:10; Tregs/effector cells), ASCs (1:40; ASCs/effector cells) or Tregs and ASCs in combination after a 7-day incubation period. Secreted IL-10, IFN-γ and TNF-α were measured in cell culture supernatants by means of the BD Cytometric Bead Array Human Th1/Th2/Th17 Cytokine Kit (BD Biosciences). Secreted IL-6 was determined by ELISA (U-CyTech biosciences, Utrecht, the Netherlands). Both methods were carried out according to the manufacturer's instructions.

Statistical analysis

Statistical analyses were performed by means of 1-way analysis of variance (ANOVA), Bonferroni multiple comparison test and (un)paired t-tests using GraphPad Prism 5 software (GraphPad Software, San Diego, CA, USA). A p-value lower than 0.05 was considered statistically significant. One-tailed p-values are stated.

RESULTS

Characterization of ASCs

ASCs were isolated from perirenal adipose tissue of living kidney donors. The cells adhered to the plastic surface of culture flasks and had a spindle-shaped morphology. Immunophenotyping of ASCs by flow cytometry demonstrated that the cells expressed low levels of CD34, and the cell surface markers CD90, CD105, CD166 and HLA-ABC. ASCs did not express CD14, the leukocyte marker CD45 or HLA-DR (Figure 2A). The culture of undifferentiated ASCs under adipogenic conditions for 14 days induced the development of lipid-filled vesicles. These vesicles, characteristic for cells of adipogenic lineage, were detected by Oil Red O staining (Figure 2B). Osteogenic differentiation of ASCs for 21 days resulted in the deposition of calcified nodules which were blackened by von Kossa staining (Figure 2B). The ability of the isolated cells to induce these morphological changes confirmed that they were ASCs.

ASCs and Tregs dose-dependently inhibit the proliferation of $CD25^{-/dim}$ effector cells

The effect of ASCs and Tregs on the proliferation of $CD25^{-/dim}$ effector cells was examined by means of [^{3}H]-thymidine incorporation. Stimulation of $CD25^{-/dim}$ effector cells with γ-irradiated allogeneic PBMCs for 7 days resulted in a strong proliferative activity of these cells. Co-culture with 3rd party ASCs (Figure 3A) or autologous Tregs (Figure 3B) suppressed the effector cell proliferation in a dose-dependent manner (both $p < 0.0001$). ASCs significantly reduced the proliferation from a concentration of 1:160 (ratio ASCs/effector cells). Tregs significantly suppressed the proliferative capacity of $CD25^{-/dim}$ effector cells from a concentration of 1:10 (ratio Tregs/effector cells).

ASCs and Tregs do not impair each other's immunosuppressive functions

The influence of ASCs and Tregs on each other's T cell suppressive capacities was analyzed by measurement of [^{3}H]-thymidine incorporation. At first, the effect of Tregs on the suppressive capacity of various ASCs concentrations was examined. The addition of Tregs 1:10 (ratio Tregs/effector cells) permitted the immunomodulatory function of ASCs (Figure 4A). In the reverse set-up, it was analyzed whether ASCs (1:40; ratio ASCs/

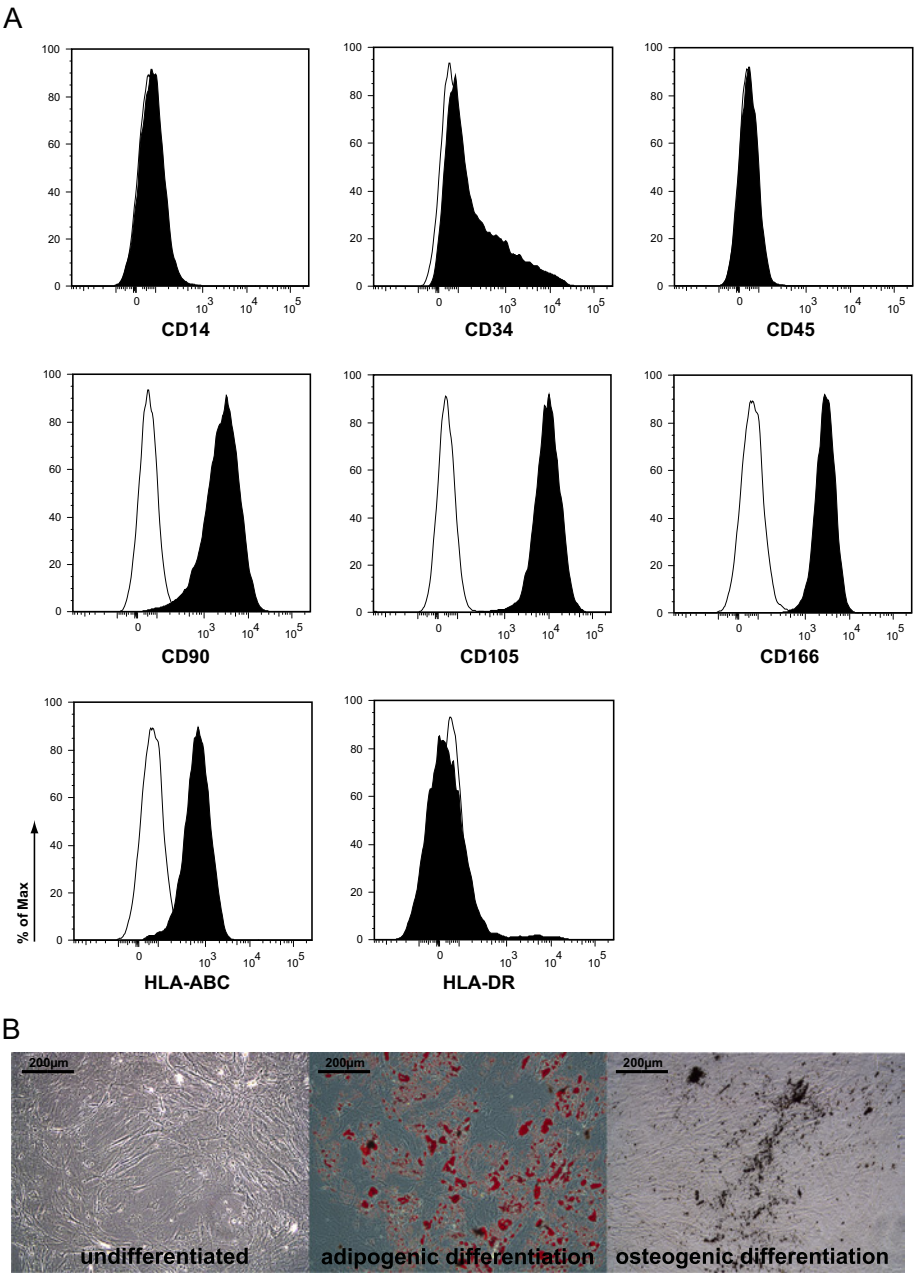

Figure 2. Characterization of ASCs. (A) Immunophenotyping of ASCs for the cell surface proteins CD14, CD34, CD45, CD90, CD105, CD166, HLA-ABC and HLA-DR. Open histograms show non-specific staining for the indicated marker, solid histograms show specific staining for indicated marker. (B) Microscopic images of undifferentiated ASCs, and ASCs differentiated into adipocytes and osteocytes. Adipogenic differentiation (14 days) induced the development of lipid-filled vesicles; these were detected by Oil Red O staining. Osteogenic differentiation (21 days) induced the deposition of calcified nodules which were blackened by von Kossa staining. Scale bars indicate 200µm.

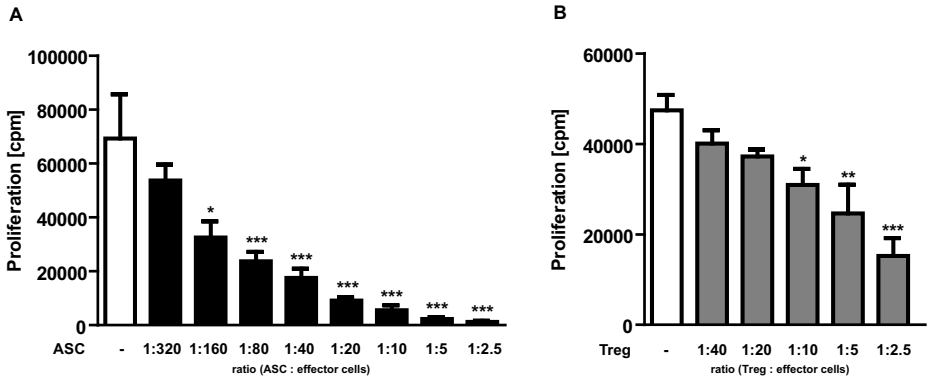

Figure 3. Effect of ASCs or Tregs on the proliferation of allo-activated CD25$^{-/dim}$ lymphocytes. MLRs (white bars) consisted of CD25$^{-/dim}$ effector cells stimulated with γ-irradiated allogeneic PBMCs (both derived from healthy individuals) with a minimum of four HLA mismatches. MLRs were performed in the presence of various concentrations of ASCs (A; n=6, mean ± SEM, 1-way ANOVA: p<0.0001) or Tregs (B; n=5, mean ± SEM, 1-way ANOVA: p<0.0001) for 7 days. Effector cell proliferation was determined by [^{3}H]-thymidine incorporation. Bonferroni multiple comparison test was used to compare conditions with MLRs. *p<0.05, **p<0.01, ***p<0.001.

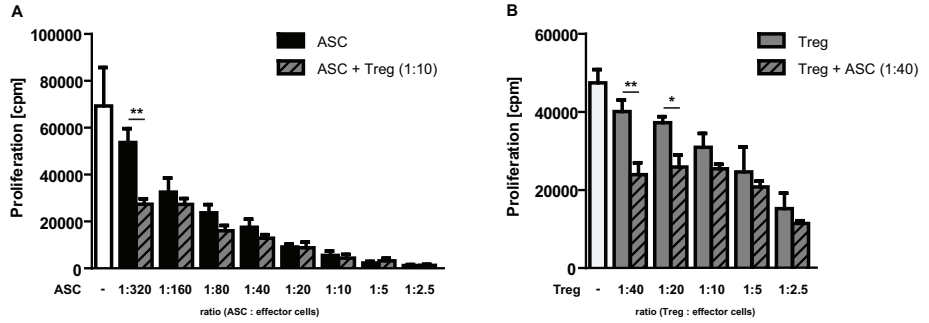

Figure 4. Effect of ASCs and Tregs on the proliferation of allo-activated CD25$^{-/dim}$ lymphocytes. MLRs (white bar) consisted of CD25$^{-/dim}$ effector cells stimulated with γ-irradiated allogeneic PBMCs (both derived from healthy individuals). Effector cell proliferation was determined by [^{3}H]-thymidine incorporation. (A) Varying ASC concentrations were added to MLRs in the absence (black bars; n=6; mean ± SEM) and in the presence of a constant Treg concentration (1:10; hatched bars; n=5; mean ± SEM). (B) Various Treg concentrations were added to MLRs in the absence (grey bars; n=5; mean ± SEM) and in the presence of a constant ASC concentration (1:40; hatched bars; n=3; mean ± SEM). Paired t-test, *p<0.05, **p<0.01.

effector cells) had an effect on the inhibition of responder cell proliferation by diverse Treg concentrations; ASCs did not hamper Treg function (Figure 4B).

ASCs and Tregs from kidney donor-recipient pairs have immunosuppressive capacities

In order to examine ASC-Treg interaction in a transplantation setting, it was investigated whether ASCs derived from kidney donors and Tregs obtained from the respective graft

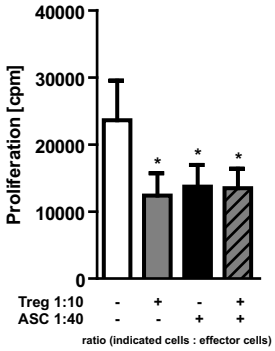

Figure 5. Effect of ASCs or Tregs on the proliferation of allo-activated CD25$^{-/dim}$ lymphocytes using cells from kidney donor-recipient pairs. MLRs (white bar) consisted of recipient-derived CD25-/dim effector cells stimulated with g-irradiated allogeneic PBMCs from the corresponding donor. ASCs were obtained from the kidney donor (n=7; mean age 54.9 years, range 43.0–63.2 years; 5 male, 2 female). Tregs and CD25-/dim effector cells were isolated from PBMCs of the respective graft recipient (n=7; mean age 51.7 years, range 18.6–75.7 years; 6 male, 1 female) at 6 months posttransplantation. Effector cell proliferation was determined by [3H]-thymidine incorporation. The condition MLR was used as reference for statistical analyses (mean ± SEM). Paired t-test, *p<0.05.

recipient 6 months post-transplantation inhibited recipient effector cells in a similar way as observed for healthy controls. The effect of both cell types was examined by means of MLRs and [^{3}H]-thymidine incorporation. ASCs (1:40; ratio ASCs/effector cells) and Tregs (1:10; ratio Tregs/effector cells) inhibited the proliferation of allo-activated CD25$^{-/dim}$ effector cells by 42% and 48%, respectively (Figure 5). The combination of ASCs and Tregs did not negatively influence the T cell suppressive capacity of each cell type. Therefore, ASC-Treg interaction in a transplantation setting is comparable to a setting using healthy controls.

ASCs and Tregs inhibit the proliferative capacities of various lymphocyte subsets

The effect of ASCs and Tregs on the proliferation of different lymphocyte subsets within the CD25$^{-/dim}$ effector cell population was investigated by PKH dilution assay. The results of a representative experiment are shown in Figure 6; a summary of a series of experiments is presented in Table 1. High proliferative activities (≥74%; Table 1) were observed for total CD4$^+$, total CD8$^+$, CD4$^+$ memory (CD45RO$^+$) and CD8$^+$ memory (CD45RO$^+$) T cells following stimulation with γ-irradiated allogeneic PBMCs for 7 days in the absence of ASCs and Tregs. Individually, ASCs (1:40; ratio ASCs/effector cells) as well as Tregs (1:10; ratio Tregs/effector cells) abrogated the proliferative capacity of all analyzed lymphocyte subsets. In addition, the presence of Tregs (1:10) to ASCs did not diminish the suppressive effects achieved by ASCs alone. Further, in comparison to the CD45RO$^+$ subpopulations, the CD45RO$^-$ subsets were found to be more susceptible to the suppressive capacities of ASCs and Tregs.

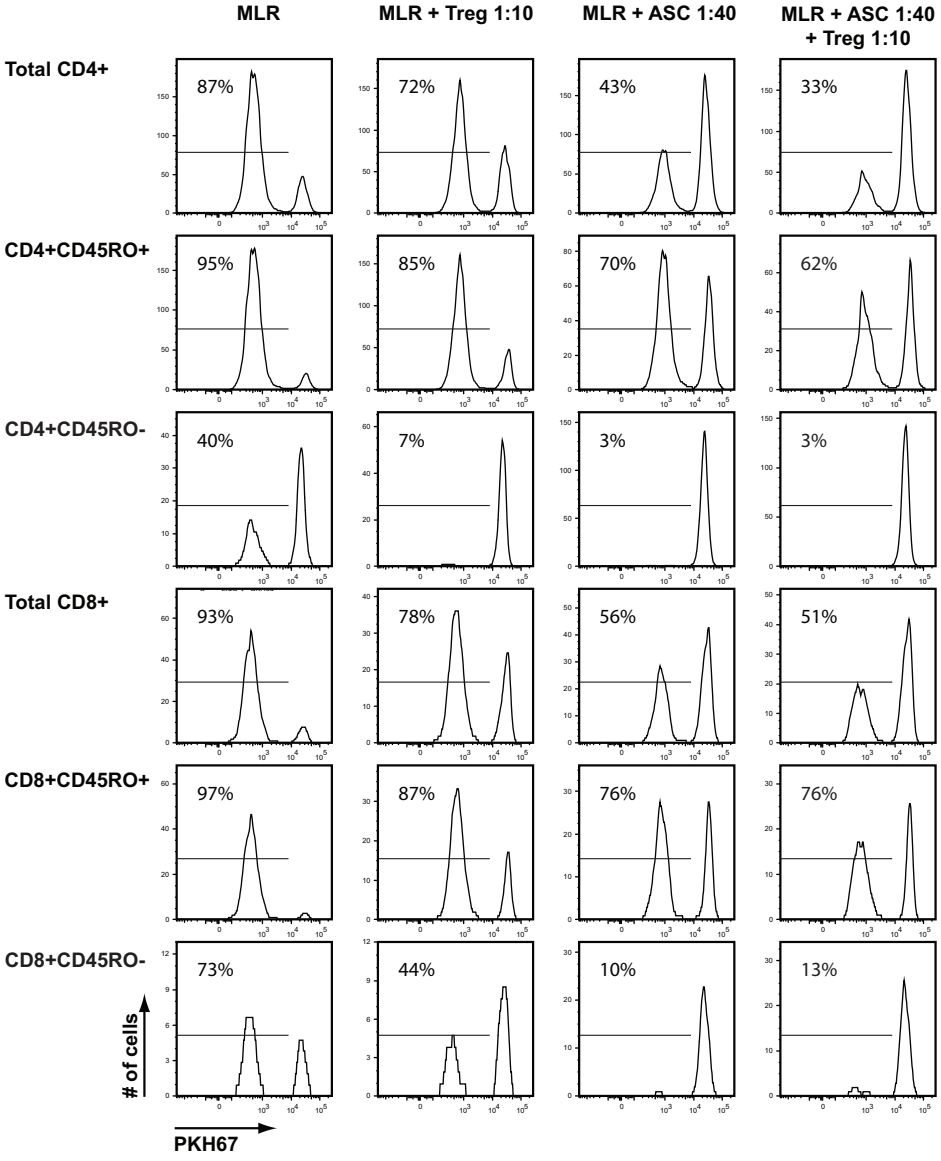

Figure 6. Effect of ASCs or Tregs on the proliferative capacities of various lymphocyte subsets. MLRs consisted of $CD25^{-/dim}$ effector cells stimulated with γ-irradiated allogeneic PBMCs (both derived from healthy individuals). $CD25^{-/dim}$ effector cells were labeled with PKH67. PKH67 dilution due to proliferation was measured by flow cytometry on day 7. For discrimination purposes Tregs (1:10; when applicable) and allogeneic stimulator PBMCs were labeled with PKH26. Cell concentrations stated refer to the ratio of indicated cells to effector cells. The percentages indicate the fraction of proliferating cells of the respective lymphocyte subset. A representative experiment is shown. Data of multiple experiments is shown in Table 1.

Table 1. Effect of ASCs and Tregs on the proliferation of allo-activated $CD25^{-/dim}$ lymphocytes.

	Proliferation [%]			
	MLR	MLR + Tregs (1:10)	MLR + ASCs (1:40)	MLR + ASCs (1:40) + Tregs (1:10)
Total CD4+[1]	74 ± 3.2	62 ± 5.4*	54 ± 7.9*	41 ± 9.3
CD4+CD45RO+[2]	90 ± 2.9	84 ± 0.4	70 ± 8.3	64 ± 9.7
CD4+CD45RO-[2]	35 ± 14.8	12 ± 6.2	7 ± 5.0	6 ± 3.7
Total CD8+[1]	81 ± 3.4	67 ± 5.5*	57 ± 9.3*	48 ± 13.1
CD8+CD45RO+[2]	89 ± 4.5	82 ± 4.1	62 ± 19.1	60 ± 20.8
CD8+CD45RO-[2]	52 ± 21.5	40 ± 18.3	17 ± 10.8	21 ± 14.3

mean ± SEM; [1]n = 6, [2]n = 3; * $p<0.05$ *versus* MLR; paired t-test

Immunosuppressive ASCs overexpress INDO

In order to examine which genes were involved in the immunosuppressive effect of ASCs, the gene expression profile of ASCs cultured in the absence or in the presence of MLRs was assessed by qPCR after 7 days. MLRs were separated from ASCs by cell culture inserts with permeable membrane supports. Changes in expression of the target genes relative to the GAPDH reference gene were quantified using the comparative C_T method. When compared to untreated ASCs, co-cultured ASCs expressed substantially more INDO (120,000 fold change; Figure 7), a gene coding for an enzyme involved in the inhibition of lymphocyte proliferation. Gene expression of the anti-inflammatory cytokine TGF-β was down-regulated by 5.7 fold, whereas the expression of the pro-inflammatory cytokine IL-6 was up-regulated by 15.5 fold. Fold changes of less than 3 were considered insignificant. Therefore, changes in gene expression as a result of ASC stimulation were not observed for the anti-inflammatory cytokine IL-10, nor for the pro-inflammatory cytokine TNF-α. IFN-γ expression by ASCs was not detectable.

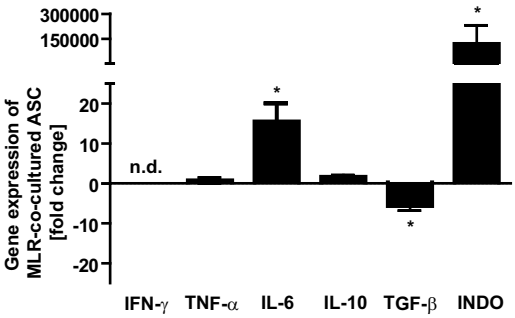

Figure 7. Gene expression of ASCs co-cultured with MLRs in comparison to ASCs cultured alone. ASCs were cultured alone or in co-culture with MLRs for 7 days. MLRs were separated from ASCs by cell culture inserts with permeable membrane supports. Quantitative real-time PCR (qPCR) was performed using 500ng cDNA. Changes in expression of the target genes relative to the GAPDH gene were quantified using the comparative C_T method (n=5; mean ± SEM). Fold changes in gene expression between ASCs and ASCs co-cultured with MLRs are shown. *significant fold changes (≥ 3); n.d., not detectable.

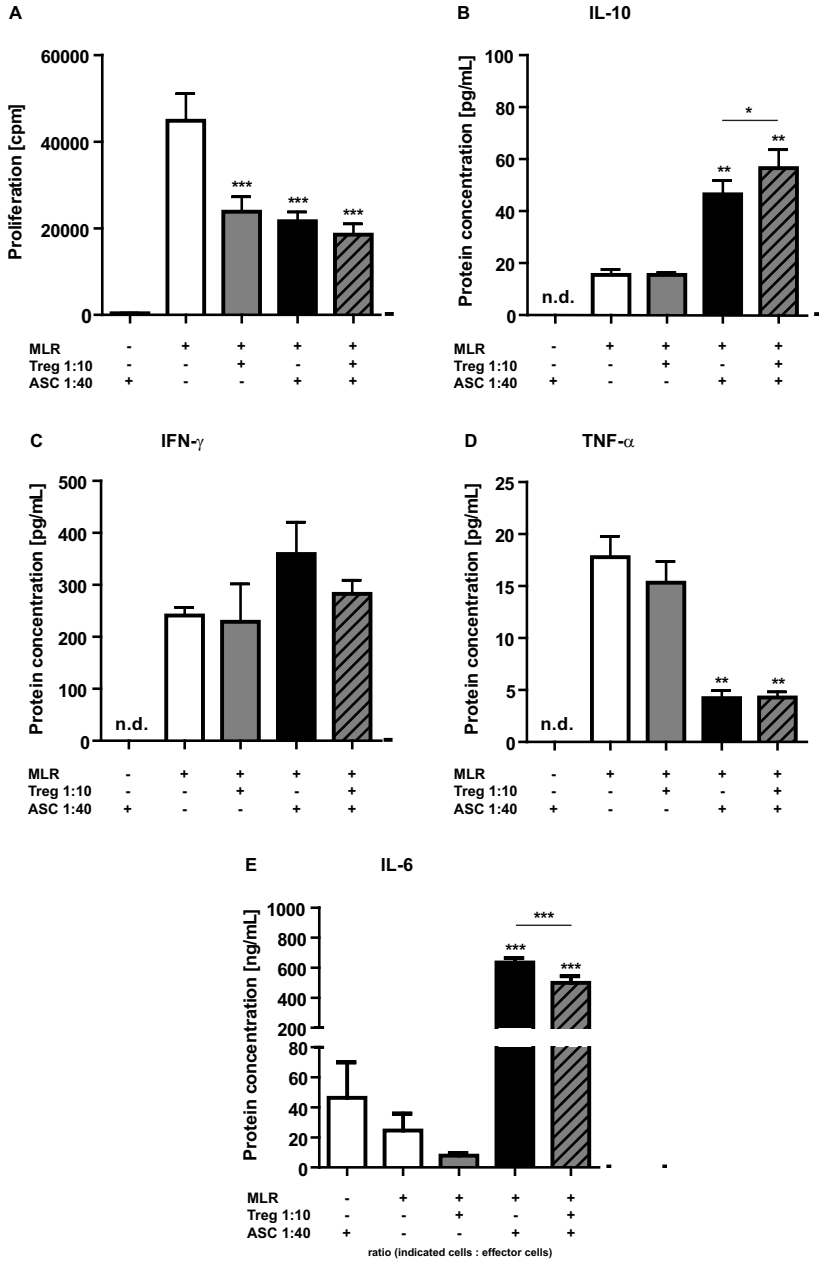

Figure 8. Determination of cytokine concentrations secreted into cell culture supernatants. (A) MLRs consisted of $CD25^{-/dim}$ effector cells stimulated with γ-irradiated allogeneic PBMCs (both derived from healthy individuals). Proliferative capacity of effector cells following the addition of various concentrations of ASCs and/or Tregs was analysed by [^{3}H]-thymidine incorporation on day 7. The secretion of IL-10 (B), IFN-γ (C) and TNF-α (D) into the cell culture supernatant was determined by CBA. Concentrations of IL-6 (E) were determined by ELISA. Unless indicated differently, the MLR condition was used as reference for statistical analyses (n=4; mean ± SEM). Paired t-test, *p<0.05, **p<0.01, ***p<0.001; n.d., not detectable.

ASCs contribute to an anti-inflammatory microenvironment

The effect of ASCs and Tregs on the generation of an immunosuppressive microenvironment was examined using cytometric bead array technology. Concentrations of secreted cytokines were analyzed in cell culture supernatants after co-culture of MLRs with ASCs (1:40; ratio ASCs/effector cells) and/or Tregs (1:10; ratio Tregs/effector cells). The proliferation data corresponding to the analyzed supernatants are shown in Figure 8A. IL-10 is not expressed by ASCs. However, ASCs mediated an elevated production of IL-10 by the effector cell population (31pg/mL; Figure 8B). Although no increase in IL-10 secretion was observed when Tregs alone were added, in the presence of both Tregs and ASCs IL-10 levels increased by 41pg/mL. The addition of Tregs, ASCs or both cell types in combination to MLRs did not lead to significant changes of IFN-γ concentrations (Figure 8C). Further, ASCs mediated a reduction of TNF-α secretion into the culture medium (Figure 8D); Tregs did not have the capacity to decrease levels of TNF-α. In addition, unstimulated ASCs secreted IL-6. In the presence of ASCs and allo-activated effector cells a strong increase in IL-6 production was observed when compared to the MLR condition (Figure 8E). Tregs had no effect on the IL-6 production by the immune cells. However, Tregs influenced the amount of secreted IL-6 when ASCs were present; the IL-6 concentration was reduced by 135ng/mL.

DISCUSSION

As cellular therapy presents itself as an emerging alternative to current drug-based immunosuppressive regimens in the field of transplantation, it becomes essential to not only investigate the potential of this new treatment to suppress effector cells, but also to evaluate its influence on existing immunomodulatory mechanisms operating in the cell therapy recipient. Focusing on the interactions between donor and acceptor immunomodulatory cells following treatment, the present study provides evidence that kidney donor perirenal adipose-tissue derived MSCs (ASCs) and Tregs, originating from healthy donors, do not impair each other's suppressive effect on allo-reactivity. Further, this study confirmed earlier reports that Tregs isolated from kidney recipients 6 months after transplantation maintained their suppressive capacities despite being exposed to high-dose medication with strong effects on T cell activation and proliferation [49]. Importantly, these Tregs were found to permit the inhibitory effect of donor ASCs. *Vice versa*, ASCs did not abolish the suppressive effect of Tregs despite their potent inhibitory influence on effector T cells. ASCs and Tregs inhibited the same lymphocyte subsets within the $CD25^{-/dim}$ effector cell population. However, in comparison to non-memory cells, $CD4^{+}CD45RO^{+}$ and $CD8^{+}CD45RO^{+}$ memory cells were less efficiently suppressed by ASCs and Tregs, possibly due to the highly activated nature of these cells. The limited

effectiveness of ASCs and Tregs in altering the proliferation of memory T cells is not unique. Similar observations were made for other clinically available immunosuppressive agents [50]. MSCs exert their immunosuppressive function on effector cells by secreting anti-inflammatory factors and inducing the reduction of pro-inflammatory factors. A possible explanation for the absence of repressive influences between ASCs and Tregs may be that both cell types exercise their immunosuppressive abilities by orchestrating non-interfering mechanisms. A mechanism employed by MSCs to create a local immunosuppressive microenvironment is tryptophan starvation. This process is mediated by INDO, a gene coding for the tryptophan-catabolizing enzyme indoleamine 2,3-dioxygenase (IDO). We and other research groups observed that activated MSCs produce increased concentrations of IDO which contribute to the inhibition of various effector cell populations [51, 52]. Yet, we found that elevated levels of IDO did not have a negative impact on Treg function. Low levels of tryptophan and the resulting presence of kynurenine have also been reported to induce and amplify the suppressive functions of Tregs in the periphery [53]. Furthermore, we found that ASCs exerted their inhibitory capacities by mediating the secretion of IL-10 by immune cells, an anti-inflammatory cytokine which MSCs do not produce themselves. IL-10 is also one of the main molecules implicated in the functional activity of Tregs [41]. As Tregs not only react upon but also produce IL-10, we were surprised not to find increased levels of the anti-inflammatory cytokine in the supernatant during the Treg-mediated suppression of allo-activated effector cells. The lack of an IL-10 increase can be explained by autocrine IL-10 signaling on Tregs and therefore consumption of IL-10. However, when ASCs and Tregs in combination inhibited allo-reactivity, we found increased concentrations of IL-10 when compared to ASC-mediated suppression. This indicates that ASCs boost the IL-10 production of Tregs. In addition, we observed that not Tregs but only ASCs were able to reduce concentrations of the pro-inflammatory cytokine TNF-α. On the other hand, both cell types were not able to decrease IFN-γ concentrations. Partly, this is in contrast to the finding by Park et al. [54] that Tregs diminish the production of IFN-γ by $CD4^+$ and $CD8^+$ T cells in an autoimmune setting. Although not expressed by MSCs, IFN-γ is required for their activation and therefore crucial for the immunosuppressive function of MSCs [16]. Thus, the availability of IFN-γ in the presence of Tregs allows optimal functionality of MSCs. It has been shown that IFN-γ-stimulated MSCs prevent GVHD mortality in patients receiving bone marrow transplants [55]. In contrast to the effect of ASCs on TNF-α and IFN-γ, we found that activated ASCs secreted levels of IL-6, demonstrating the duality of ASC immunomodulation. However, IL-6 has been reported to play a role in the induction of regulatory T cells [56], suggesting that ASCs indirectly promote immunosuppression via this pathway. In addition, MSCs also employ non-soluble factors to achieve their immunosuppressive effect. Upon activation MSCs show increased expression of the ligand of the co-stimulatory molecule programmed death-1 (PD-L1) and the intercellular adhe-

sion molecule-1 (ICAM-1) [57, 58]. ICAM-1 facilitates the binding of lymphocytes to MSCs and thus promotes MSC-mediated immunosuppression. Binding of PD-L1 to its receptor PD-1 results in the inhibition of IL-2 production and leads to a reduction of target cell proliferation [59]. In summary, MSCs and Tregs appear to utilize both distinct and partially overlapping mechanisms to alter immune responses.

The present study demonstrates that donor ASCs and recipient Tregs do not impair each other's function. This finding clearly encourages the use of MSC therapy for the prevention of graft rejection in solid organ transplantation. MSCs, in contrast to some currently applied immunosuppressive interventions, preserve the function of Tregs and therefore potentially mediate the establishment of an advanced immunosuppressive environment in transplant patients without inflicting the known side effects observed for T-cell inhibitory drugs.

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Chapter 3

Human adipose-tissue derived mesenchymal stem cells induce functional *de novo* regulatory T cells with methylated FOXP3 gene DNA

Anja U. Engela, Martin J. Hoogduijn, Karin Boer, Nicolle H.R. Litjens, Michiel G.H. Betjes, Willem Weimar, Carla C. Baan

Department of Internal Medicine, Section Nephrology and Transplantation, Erasmus University Medical Center, Rotterdam, the Netherlands

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ABSTRACT

Due to their immunomodulatory properties mesenchymal stem cells (MSC) are interesting candidates for cellular therapy for autoimmune disorders, graft-versus-host disease and allograft rejection. MSC inhibit the proliferation of effector T cells and induce T cells with a regulatory phenotype. So far it is unknown whether human MSC-induced $CD4^+CD25^+CD127^-FOXP3^+$ T cells are functional and whether they originate from effector T cells or represent expanded natural regulatory T cells (nTreg). Perirenal adipose-tissue derived MSC (ASC) obtained from kidney donors induced a 2.1-fold increase in the percentage of $CD25^+CD127^-FOXP3^+$ cells within the $CD4^+$ T-cell population from allo-stimulated $CD25^{-/dim}$ cells. IL-2 receptor blocking prevented this induction. The ASC-induced T cells (iTreg) inhibited effector cell proliferation as effectively as nTreg. The vast majority of cells within the iTreg fraction had a methylated FOXP3 gene Treg-specific demethylated region (TSDR) indicating that they were not of nTreg origin. In conclusion, ASC induce Treg from effector T cells. These iTreg have immunosuppressive capacities comparable to those of nTreg. Their induction is IL-2 pathway dependent. The dual effect of MSC of inhibiting immune cell proliferation while generating *de novo* immunosuppressive cells emphasizes their potential as cellular immunotherapeutic agent.

INTRODUCTION

Mesenchymal stem cells (MSC) can be isolated from an abundance of human tissue sites including adipose tissue and bone marrow, and their expansion is easily accomplished [1-4]. MSC possess immunosuppressive capacities and as a consequence, over the past decennium MSC have been extensively studied as a prospective cellular therapeutic agent to prevent or treat autoimmune diseases, graft-versus-host disease (GVHD) and allograft rejection [5-12]. Upon activation, MSC prevent the proliferation of various immune cells, in particular T cell proliferation [13-19]. MSC mediate their suppressive effect through cell-cell contact and the secretion of various soluble factors such as transforming growth factor-beta (TGF-β), hepatocyte growth factor (HGF), IL-10, nitric oxide (NO), human leukocyte antigen G5 (HLA-G5), indoleamine 2,3-dioxygenase (IDO) and prostaglandins [20-28]. While MSC strongly inhibit T cell proliferation via these mechanisms, they preserve the function of $CD4^+CD25^+CD127^-FOXP3^+$ regulatory T cells (Treg) [29]. Beyond this, in vitro studies and studies in animal models have indicated that MSC have the capacity to generate Treg [9, 30-34]. Recent evidence was provided that this phenomenon might also occur in renal transplant patients undergoing MSC therapy [35]. Intravenous administration of autologous MSC post-transplant led to a proportional increase of $CD4^+CD25^+CD127^-FOXP3^+$ T cells. Despite their regulatory phenotype it remains essential to investigate the characteristics and function of these cells. Further, due to the heterogeneity of the Treg population these $CD4^+CD25^+CD127^-FOXP3^+$ T cells could represent expanded natural Treg (nTreg) or newly induced Treg (iTreg).

nTreg and iTreg are distinct from each other with regard to their place of origin, the stability of their transcription factor forkhead box P3 (FOXP3) expression and in their methylation pattern of the Treg-specific demethylated region (TSDR) in the FOXP3 gene [36, 37]. nTreg develop intra-thymically, constitutively express FOXP3 and have a fully demethylated FOXP3 TSDR. In contrast, iTreg development takes place in the periphery, their FOXP3 expression is inducible and their FOXP3 TSDR is fully methylated. The MSC-mediated generation of cells with an immunosuppressive function is of particular importance if one considers the fate of MSC after infusion; Eggenhofer et al. [38] recently showed that after intravenous administration into mice, MSC survive not longer than 24 hours. This evident short lifespan of MSC in connection with their proven ability to prolong graft survival prompts further investigation to reveal how MSC accomplish long-term immunosuppression, and which mediators and mechanisms are involved in this phenomenon.

While we previously studied the interaction between human adipose-tissue derived MSC (ASC) and natural Treg [29], the aim of this study was to determine whether human ASC can generate functional *de novo* iTreg from $CD25^{-/dim}$ effector T cells and to find evidence for the mechanisms involved in MSC-mediated Treg induction.

MATERIAL AND METHODS

Origin, isolation and culture of human ASC

Perirenal adipose tissue was surgically removed from living kidney donors and collected in minimum essential medium Eagle alpha modification (MEM-α) (Sigma-Aldrich, St. Louis, MO, USA) supplemented with 2mM L-glutamine (Lonza, Verviers, Belgium), 1% penicillin/streptomycin solution (P/S; 100IU/mL penicillin, 100IU/mL streptomycin; Lonza). Samples were obtained with written informed consent as approved by the Medical Ethical Committee at Erasmus University Medical Center Rotterdam (protocol no. MEC-2006-190).

ASC were isolated, cultured and characterized as previously described [29]. In brief, perirenal adipose tissue was mechanically disrupted and enzymatically digested with collagenase type IV (Life Technologies, Paisley, UK). ASC were expanded using ASC culture medium consisting of MEM-α with 2mM L-glutamine, 1% P/S and 15% fetal bovine serum (FBS; Lonza) in a humidified atmosphere with 5% CO_2 at 37°C. Culture medium was refreshed twice weekly. At subconfluency, ASC were removed from culture flasks using 0.05% trypsin-EDTA (Life Technologies) and reseeded at 1,000cells/cm^2. ASC were characterized by means of immunophenotyping and by their ability to differentiate into adipocytes and osteoblasts. ASC cultured between 2 to 6 passages were used. ASC from these passages did not differ in their ability to differentiate or to exert their immunosuppressive functions.

Isolation of peripheral blood mononuclear cells

Peripheral blood mononuclear cells (PBMC) were isolated from buffy coats of healthy blood donors (Sanquin, Rotterdam, the Netherlands) by density gradient centrifugation using Ficoll-Paque PLUS (density 1.077g/mL; GE Healthcare, Uppsala, Sweden). Cells were frozen at -150°C until further use in Roswell Park Memorial Institute (RPMI) 1640 medium with GlutaMAX™-I (Life Technologies) supplemented with 1% P/S, 10% human serum (Sanquin) and 10% dimethyl sulfoxide (DMSO; Merck, Hohenbrunn, Germany).

Isolation of effector cells and nTreg from PBMC

CD25$^{-/dim}$ cells (effector cells) and CD25^{bright} cells (nTreg) were separated by means of CD25 MicroBeads II (Miltenyi Biotec, Bergisch Gladbach, Germany) and magnetic cell sorting as previously described [29]. Cell fraction purity was determined by flow cytometry using monoclonal antibodies (mAbs) against CD3-AmCyan (clone SK7), CD4-Pacific Blue (RPA-TA), CD25-PE-Cy7 (epitope B; M-A251), CD127-PE (HIL-7R-M21; all BD Biosciences, San Jose, CA, USA); and FOXP3-APC (PCH101; eBioscience, San Diego, CA, USA). Intracellular FOXP3 staining was carried out following the manufacturer's instructions of the Anti-Human FOXP3 Staining Set APC (eBioscience). Flow-cytometric analyses were

performed using the BD FACSCanto II flow cytometer and BD FACSDiva software (both BD Biosciences).

Mixed lymphocyte reaction and suppression assay

Mixed lymphocyte reactions (MLR) consisted of $5x10^4$ $CD25^{-/dim}$ effector cells stimulated with $5x10^4$ γ-irradiated (40 Gy) allogeneic PBMC in round-bottom 96-well plates (Nunc, Roskilde, Denmark) using PBMC culture medium (PCM) consisting of MEM-α supplemented with 2mM L-glutamine, 1% P/S and 10% heat-inactivated human serum. Effector-stimulator cell combinations were chosen on the basis of a minimum of four HLA mismatches. The immunomodulatory capacities of ASC (various concentrations), nTreg (1:10), ASC-induced $CD4^+CD25^+CD127^-$ T cells (1:10) and control $CD4^+CD25^-$ T cells (1:10) (all ratios: indicated cells/effector cells) on the MLR were determined in suppression assays. After an 8-hour incubation period on day 7, [^{3}H]-thymidine incorporation (0.25μCi/well; PerkinElmer, Groningen, the Netherlands) was measured using the Wallac 1450 MicroBeta TriLux (PerkinElmer). When MLR were performed in microtiter plates with different well sizes, the number of cells was adjusted accordingly. When applicable, 50μL cell-culture supernatant was harvested prior to the addition of [^{3}H]-thymidine and frozen at -80°C until further use.

Induction of $CD4^+CD25^+CD127^-FOXP3^+$ T cells by ASC

$CD25^{-/dim}$ effector cells were labelled using the PKH67 Green Fluorescent Cell Linker Kit (Sigma-Aldrich). For discrimination, allogeneic stimulator PBMC were labelled with PKH26 (PKH26 Red Fluorescent Cell Linker Kit, Sigma-Aldrich) according to the manufacturer's instructions. MLR were performed for 7 days in the absence or presence of ASC (1:40; ASC/effector cells). The PKH-label dilution caused by proliferation was measured by flow cytometry. After a 7-day incubation period in the absence or presence of 4μg/mL basiliximab (Novartis Pharma, Nürnberg, Germany), cells were stained with mAbs against CD3-AmCyan (clone SK7), CD4-Pacific Blue (RPA-TA), CD8-PerCP (SK1), CD25-APC-Cy7 (epitope B; M-A251) and CD127-PE-Cy7 (HIL-7R-M21; all BD Biosciences); and FOXP3-APC (PCH101; eBioscience). Allogeneic stimulator PBMC were excluded from the analysis based on their PKH26-label. To confirm that basiliximab does not interfere with the binding of the monoclonal anti-CD25 antibody on epitope B, a competition staining was performed. In the presence of basiliximab no weakening of the CD25 staining was observed.

Isolation and function test of ASC-induced $CD4^+CD25^+CD127^-$ T cells

ASC-induced $CD4^+CD25^+CD127^-$ T cells were generated in primary MLR consisting of $CD25^{-/dim}$ effector cells and allogeneic stimulator PBMC in the presence or absence of ASC (1:40; ASC/effector cells). Allogeneic stimulator PBMC were labelled with PKH67

(Sigma-Aldrich). After 7 days, cells were stained with mAbs against CD3-AmCyan (clone SK7), CD4-Pacific Blue (RPA-TA), CD25-PE-Cy7 (epitope B; M-A251), CD127-PE (HIL-7R-M21) and BD Via-Probe™ (7-AAD-PerCP) (all BD Biosciences). ASC-induced Treg were defined as PKH67$^-$7-AAD$^-$CD3$^+$CD4$^+$CD25$^+$CD127$^-$ cells. Cell sorting was performed using the BD FACSAria II cell sorter (BD Biosciences).

Sorted PKH67$^-$7-AAD$^-$CD3$^+$CD4$^+$CD25$^+$CD127$^-$ cells (1:10) were reseeded into secondary MLR. PKH67$^-$7-AAD$^-$CD3$^+$CD4$^+$CD25$^-$ cells (1:10) were used as negative control; nTreg (1:10) obtained from PBMC by magnetic cell separation served as positive control (all ratios: indicated cells/effector cells). After an 8-hour incubation period on day 7, [^{3}H]-thymidine incorporation (0.25µCi/well; PerkinElmer) was measured. Alternatively, sorted cell samples were washed twice with PBS-DEPC and frozen at -80°C until further use.

Quantitative DNA methylation analysis of the FOXP3 gene TSDR

To quantify DNA methylation of the FOXP3 gene TSDR, the EZ DNA Methylation-Direct™ Kit (Zymo Research, Irvine, CA, USA) was used according to the manufacturer's instructions. Cell pellets were digested with proteinase K prior to bisulfite conversion. During DNA bisulfite treatment unmethylated cytosines are converted into uracils while methylated cytosines remain unmodified. After bisulfite treatment, the TSDR of the FOXP3 gene was amplified by quantitative real-time PCR (qPCR) using the StepOnePlus™ Real-Time PCR System and the TaqMan® Genotyping Master Mix (all Applied Biosystems). Methylation-specific and demethylation-specific amplification primers and probes were chosen as suggested by Wieczorek et al. [39]. The percentage of cells within a cell fraction with a methylated TSDR was calculated using the ratio of amplified methylated TSDR copies and the sum of amplified methylated and unmethylated TSDR copies. To correct for the X-linked nature of the FOXP3 gene, results obtained from female PBMC donors were multiplied by 2. Intraassay and interassay variations were determined by negative controls and positive reference samples.

Flow cytometric characterization of CD4$^+$CD25$^+$CD127$^-$FOXP3$^+$ T cells and CD4$^+$CD25$^+$FOXP3$^-$ T cells

The induction of CD4$^+$CD25$^+$CD127$^-$FOXP3$^+$ T cells by ASC (1:40; ASC/effector cells) was initiated as described above. Allogeneic stimulator PBMC were labelled with either PKH67 or BD Horizon Violet Cell Proliferation Dye 450 (VPD450; BD Biosciences). After 7-days, cells were stained with mAbs against CD3-AmCyan (clone SK7), CD4-PerCP (SK3), CD25-APC-Cy7 (epitope B; M-A251), CD127-PE-Cy7 (HIL-7R-M21), cytotoxic T-lymphocyte antigen-4 (CTLA-4)-APC (BNI3; all BD Biosciences), glucocorticoid-induced TNFR-related protein (GITR)-FITC (110416; R&D Systems Europe Ltd., Abingdon, UK) and Helios-Pacific Blue (22F6; BioLegend, San Diego, CA, USA), and FOXP3-PE or FOXP3-APC (both PCH101;

eBioscience). Fluorescence minus one (FMO) controls were used to determine negative expression.

mRNA expression analysis

ASC were cultured alone or co-cultured with MLR (1:5; ASC/effector cells) for 7 days. In co-cultures, MLR were separated from ASC by cell culture inserts with permeable membrane supports (0.4µm pore size; Greiner Bio-One). ASC were harvested and washed twice with PBS-DEPC. Cells were either handled immediately or snap frozen in liquid nitrogen and stored at -80°C. Total RNA was purified using the High Pure RNA Isolation Kit (Roche Diagnostics) according to the manufacturer's instructions. Complementary DNA (cDNA) was synthesized by reverse transcription using random primers. Quantitative real-time PCR (qPCR) was performed using 500ng cDNA, the StepOnePlus™ Real-Time PCR System, TaqMan Universal PCR Master Mix and the assay-on-demand primer/probes for IL-2 (Hs00174114.m1) (Applied Biosystems, Foster City, CA, USA). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA served as endogenous control for normalization (Hs99999905.m1; Applied Biosystems). Intraassay and interassay variations were determined by negative controls and positive reference samples. Changes in target gene expression relative to the GAPDH gene were quantified using the comparative C_T method [40]. Fold changes of less than 3 were considered insignificant.

Cytometric bead array (CBA)

Supernatants were obtained from ASC cultures, MLR and ASC-MLR co-cultures (1:40; ASC/effector cells) in the absence or presence of 4µg/mL basiliximab (Novartis Pharma) after a 7-day incubation period. They were frozen until further use at -80°C. IL-2 concentrations were measured using the BD Cytometric Bead Array Human Th1/Th2/Th17 Cytokine Kit (BD Biosciences) according to the manufacturer's instructions.

Flow cytometric analysis of IL-2 expression by $CD4^+CD25^+CD127^-FOXP3^+$ T cells and $CD4^+CD25^+FOXP3^-$ T cells

$CD4^+CD25^+CD127^-FOXP3^+$ T cells were induced by ASC (1:40; ASC/effector cells) as described above. $CD25^{-/dim}$ effector cells were labelled with VPD450 (BD Biosciences). For discrimination, allogeneic stimulator PBMC were labelled with PKH67 (Sigma-Aldrich). MLR were performed in the absence or presence of ASC and 4µg/mL basiliximab (Novartis). After 7 days, cells were stimulated with 50ng/mL phorbol 12-myristate 13-acetate (PMA) and 1µg/mL calcium ionomycine (both Sigma-Aldrich) in the presence of BD GolgiStop (BD Biosciences) for 4 hours. Subsequently cells were stained with mAbs against CD3-AmCyan (clone SK7), CD4-PerCP (SK3), CD25-APC-Cy7 (epitope B; SK1), CD127-PE-Cy7 (HIL-7R-M21), IL-2-APC (5344.111; all BD Biosciences) and FOXP3-PE

(PCH101; eBioscience). Fluorescence minus one (FMO) controls were used to determine negative expression.

Statistical analysis

Statistical analyses were performed by means of one-way analysis of variance (ANOVA), Bonferroni multiple comparison tests and (un)paired t-tests using GraphPad Prism 5 software (GraphPad Software, San Diego, CA, USA). A p-value lower than 0.05 was considered statistically significant. Two-tailed p-values are stated.

RESULTS

ASC dose-dependently inhibit the proliferation of $CD25^{-/dim}$ effector cells and induce $CD4^+CD25^+CD127^-FOXP3^+$ T cells

The immunosuppressive effect of ASC on the proliferation of $CD25^{-/dim}$ effector cells was examined by means of [^{3}H]-thymidine incorporation. $CD25^{-/dim}$ effector cells were stimulated with γ-irradiated allogeneic PBMC for 7 days resulting in a strong proliferative activity of these cells (Figure 1A). Co-culture with 3rd party ASC suppressed the effector cell proliferation in a dose-dependent manner (one-way ANOVA, $p < 0.0001$), confirming our previously published data [29]. ASC significantly reduced the proliferation from a ratio of 1:80 (ASC/effector cells). ASC suppressed the proliferation of $CD4^+$ T cells and $CD8^+$ T cells (Figure 1B).

The effect of ASC on the generation of $CD4^+CD25^+CD127^-FOXP3^+$ T cells from $CD25^{-/dim}$ cells was determined by flow cytometry. At a 1:40 ratio, ASC reduced the proliferation of $CD25^{-/dim}$ effector cells by 59% (Figure 1A) and mediated a 2.1-fold increase in the percentage of $CD25^+CD127^-FOXP3^+$ cells within the $CD4^+$ T cell population (Figure 2).

ASC-induced $CD4^+CD25^+CD127^-$ T cells are immunosuppressive

The suppressive capacity of ASC-induced $CD4^+CD25^+CD127^-$ T cells was determined by means of [^{3}H]-thymidine incorporation and compared to the effect of nTreg. Sorted ASC-induced $CD4^+CD25^+CD127^-$ T cells (median purity: 98.7%; range: 95.3%-99.7%) were added at a 1:10 ratio to secondary MLR. They inhibited the proliferation of $CD25^{-/dim}$ effector cells as effectively as nTreg (1:10; 61% suppression vs. 48%, respectively; $p = 0.402$; Figure 3). Hence, ASC induce functional regulatory T cells. MLR-induced $CD4^+CD25^+CD127^-$ T cells (median purity: 99.5%; range: 98.5%-99.8%) also suppressed proliferation (1:10; 87%). Sorted $CD4^+CD25^-$ T cells from an MLR or MLR-ASC co-culture (1:10; median purities: 99.8% and 99.7%; ranges: 99.5%-99.8% and 99.3%-99.9%), as a negative controls, did not inhibit the proliferative activity of the effector cells.

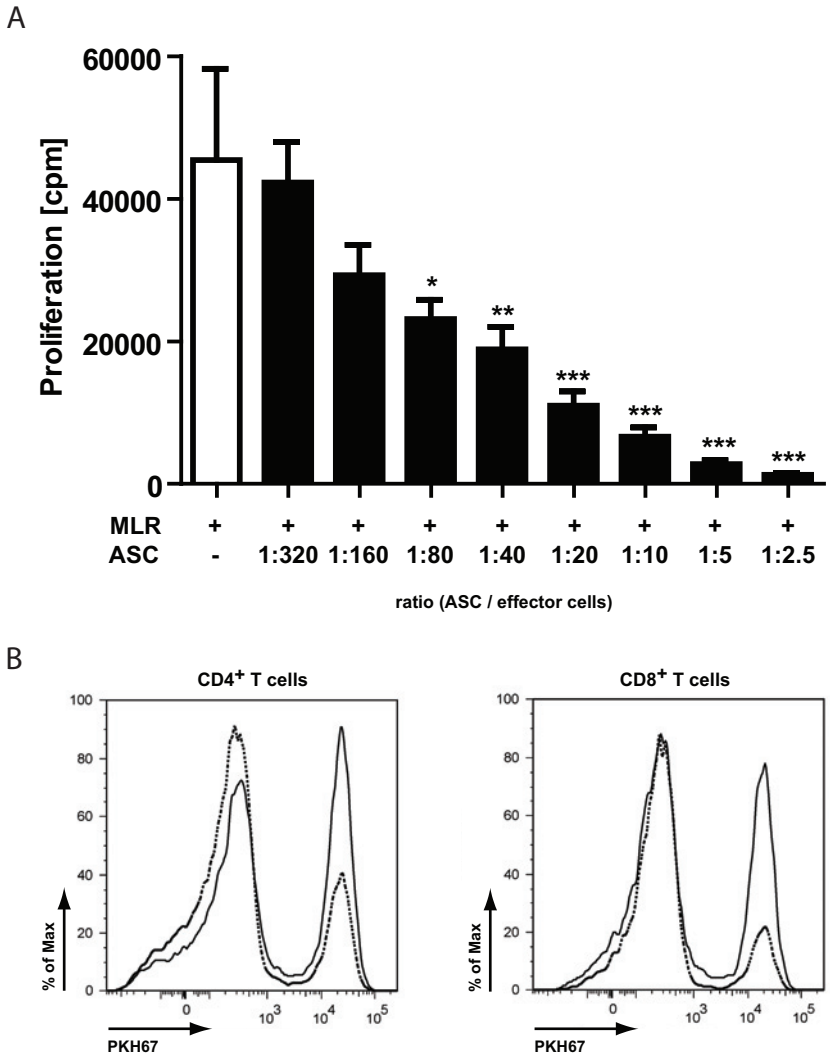

Figure 1. Effect of ASC on the proliferation of allo-activated CD25$^{-/dim}$ cells. (A) MLR (white bar) consisted of CD25$^{-/dim}$ effector cells stimulated with γ-irradiated allogeneic PBMC with a minimum of four HLA-mismatches. MLR were performed in the presence of various ASC concentrations for 7 days (n=9, mean ± SEM, one-way ANOVA: p<0.0001). The Bonferroni multiple comparison test was used to compare the different conditions with the MLR condition. *p<0.05, **p<0.01, ***p<0.001. (B) Proliferation of CD4$^+$ T cells and CD8$^+$ T cells in a 7-day MLR in the absence (dotted histograms) and presence of ASC (1:40 ratio, ASC/effector cells; solid histograms) was tracked using PKH67-labeling.

Functional ASC-induced CD4$^+$CD25$^+$CD127$^-$ Treg are *de novo* cells

To determine the origin of the ASC-induced Treg, i.e. iTreg or nTreg, the methylation status of the TSDR was investigated. The percentage of cells with a methylated FOXP3 gene TSDR present in different cell fractions was determined by means of quantitative

DNA methylation analysis. The $CD25^{-/dim}$ fraction, obtained from PBMC by magnetic cell separation, consisted almost entirely (98.1%) of cells with a methylated FOXP3 TSDR (Figure 4A). In contrast, the nTreg fraction consisted to 67.3% of cells with a demethylated FOXP3 TSDR, confirming their thymic origin. After 7-day MLR in the presence of ASC (1:40; ASC/effector cells) using allo-stimulated $CD25^{-/dim}$ cells as effector cells, sorted fractions of $CD4^+CD25^+CD127^-$ iTreg and $CD4^+CD25^-$ T cells contained 83.7% and 99.9% cells with a methylated FOXP3 TSDR, respectively. The small percentage of cells (16.3%) with a demethylated FOXP3 TSDR in the $CD4^+CD25^+CD127^-$ iTreg fraction likely represents proliferated nTreg which were present in the initial $CD25^{-/dim}$ fraction (1.9%). This demonstrates that ASC expand nTreg, but that the majority of the $CD4^+CD25^+CD127^-$ Treg found after 7-day MLR-ASC co-culture are induced from $CD25^{-/dim}$ cells.

To further characterize the ASC-induced $CD4^+CD25^+CD127^-FOXP3^+$ Treg (Figure 4B), their expression of GITR, CTLA-4 and Helios was investigated (Figure 4C-D). While the expression levels of GITR were similar for iTreg and $CD4^+CD25^+$ $FOXP3^-$ T cells, iTreg showed the tendency to have higher Helios levels and expressed 3.2-fold more CTLA-4 than $FOXP3^-$ T cells ($p = 0.003$).

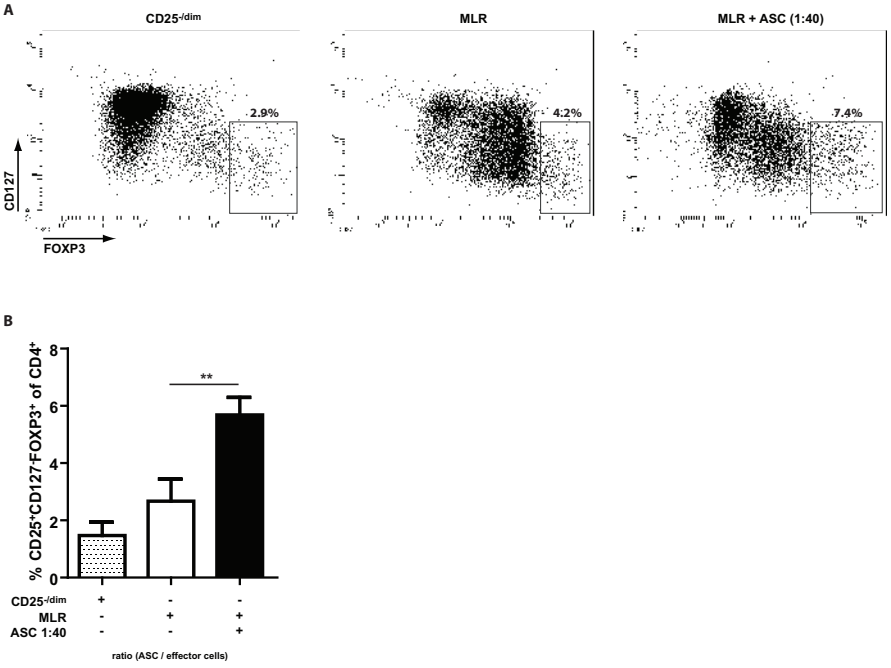

Figure 2. Effect of ASC on the induction of $CD4^+CD25^+CD127^-FOXP3^+$ T cells. MLR (white bar) consisted of $CD25^{-/dim}$ effector cells stimulated with γ-irradiated allogeneic PBMC and were performed for 7 days. In MLR-co-cultures ASC were used at a 1:40 ratio (ASC/effector cells). The percentage of $CD25^+CD127^-FOXP3^+$ cells within the $CD4^+$ T cell population was determined by flow cytometry. (A) Representative examples are shown. (B) Data of multiple experiments are shown. n=6, mean ± SEM; paired t-test; **p<0.01.

The induction of CD4⁺CD25⁺CD127⁻FOXP3⁺ Treg by ASC coincides with increased IL-2 levels and is IL-2 pathway dependent

IL-2 is known to be required for the expansion and function of nTreg. Therefore, its involvement in the ASC-mediated induction of CD4⁺CD25⁺CD127⁻FOXP3⁺ Treg was investigated (Figure 5). 7-day MLR were cultured in the absence or presence of ASC (1:40; ASC/effector cells) and concentrations of secreted IL-2 in the supernatant were analyzed. In the supernatant of an MLR of $CD25^{-/dim}$ effector cells stimulated with allogeneic PBMC 20.1pg/mL IL-2 was detected. Non-activated ASC and MLR-activated ASC do not express IL-2 (data not shown). Yet, activated ASC mediated a 15.1-fold increase in IL-2 levels by effector cells during MLR suppression and induction of CD4⁺CD25⁺CD127⁻FOXP3⁺ Treg. The monoclonal anti-IL-2 receptor antibody basiliximab (4µg/mL) effectively inhibited the IL-2 consumption in MLR and MLR-ASC co-cultures (1:40; ASC/effector cells; Figure 5A); in the presence of basiliximab IL-2 concentrations in MLR and MLR-ASC co-cultures accumulated to 217pg/mL and 525pg/mL, respectively.

To gain knowledge about which cell subset contributed most to the IL-2 concentrations detected in the supernatant, the percentage of IL-2-expressing cells within the CD4⁺CD25⁺CD127⁻FOXP3⁺ iTreg and CD4⁺CD25⁺FOXP3⁻ T cells was investigated. The ma-

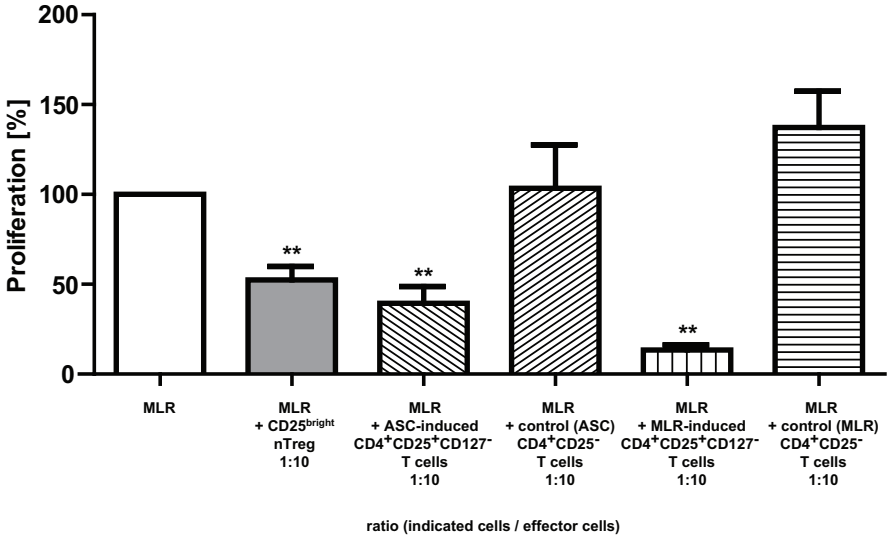

Figure 3. Effect of ASC-induced CD4⁺CD25⁺CD127⁻ T cells on the proliferation of allo-activated $CD25^{-/dim}$ effector cells. Primary MLR consisted of $CD25^{-/dim}$ effector cells stimulated with γ-irradiated allogeneic PBMC. For the induction of CD4⁺CD25⁺CD127⁻ T cells, ASC (1:40; ASC/effector cells) were added to primary MLR. After an incubation period of 7 days, CD4⁺CD25⁺CD127⁻ T cells and CD4⁺CD25⁻ T cells were separated from the total lymphocyte populations from MLR or ASC-MLR co-cultures by cell sorting and added to secondary MLR at a 1:10 ratio (indicated cells/effector cells). $CD25^{bright}$ nTreg (1:10) were incubated with the same MLR to compare the function of the sorted fractions to the suppressive function of nTreg. For statistical analyses, the MLR condition was used as reference. n=4, mean ± SEM; paired t-test; **p<0.01.

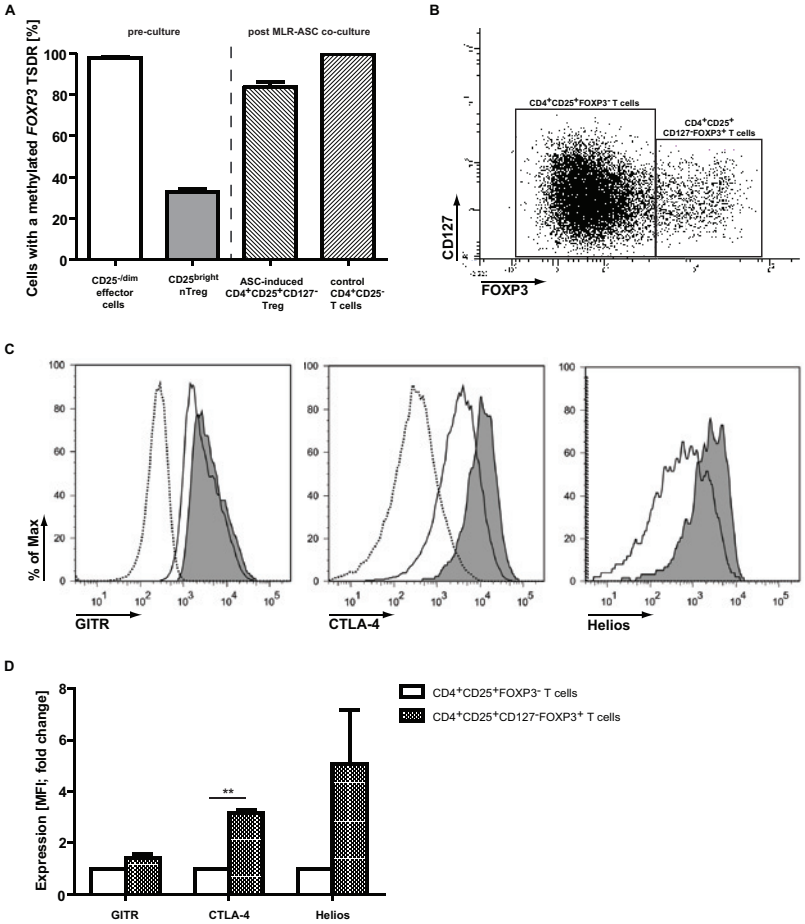

Figure 4. Characterization of ASC-induced CD4⁺CD25⁺CD127⁻ Treg. (A) Methylation analysis of the *FOXP3* gene TSDR. $CD25^{-/dim}$ effector cells and $CD25^{bright}$ nTreg were obtained from PBMC by means of cell separation. $CD4^+CD25^-$ T cells and ASC-induced $CD4^+CD25^+CD127^-$ Treg were isolated from lymphocyte populations by cell sorting after 7-day MLR-ASC co-cultures; co-cultures consisted of $CD25^{-/dim}$ effector cells, γ-irradiated allogeneic PBMC and ASC (1:40; ASC/effector cells). The methylation status of the *FOXP3* TSDR in the different cell populations was determined. The percentage of cells with a methylated TSDR was calculated using the ratio of amplified methylated TSDR copies and total TSDR copies. Results obtained from female PBMC donors were multiplied by 2. n=7; 6 male, 1 female; mean ± SEM. (B) Gating strategy to discriminate $CD4^+CD25^+CD127^-FOXP3^+$ T cells from $CD4^+CD25^+FOXP3^-$ T cells. A representative example for MLR-ASC co-culture (1:40; ASC/effector cells) is shown. (C) Expression of GITR, CTLA-4 and Helios. Flow cytometric analyses of protein expression by $CD4^+CD25^+FOXP3^-$ T cells (white histogram) and $CD4^+CD25^+CD127^-FOXP3^+$ T cells (grey histogram) are shown. FMO controls are depicted as dotted histogram. Representative examples for MLR-ASC co-culture (1:40; ASC/effector cells) are shown. Data of multiple experiments are shown in D. (D). Expression of GITR, CTLA-4 and Helios by $CD4^+CD25^+FOXP3^-$ T cells (white bars) and $CD4^+CD25^+CD127^-FOXP3^+$ T cells (checkered bars). Protein expression is shown as fold changes of median fluorescence intensity (MFI) between $CD4^+CD25^+FOXP3^-$ T cells and $CD4^+CD25^+CD127^-FOXP3^+$ T cells. Expression levels of $CD4^+CD25^+FOXP3^-$ T cells were used as reference. n=3, mean ± SEM; paired t-test; **p<0.01.

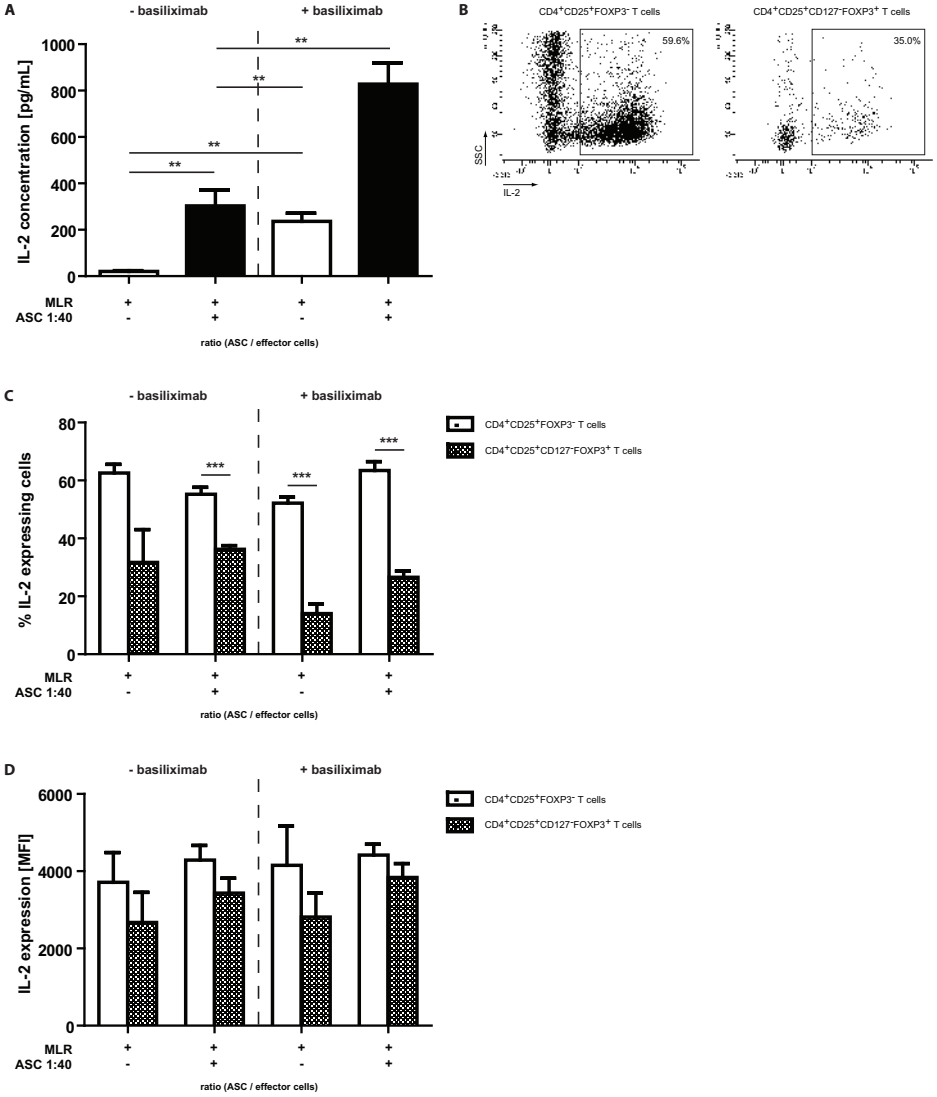

Figure 5. Effect of MLR-ASC co-culture and basiliximab on IL-2 expression. (A) IL-2 levels in cell culture supernatants. MLR (white bars) consisted of $CD25^{-/dim}$ effector cells stimulated with γ-irradiated allogeneic PBMC and were performed for 7 days in the presence and absence of basiliximab. In co-cultures with MLR, ASC were used at a 1:40 ratio (ASC/effector cells; black bars). The IL-2 concentration in the cell culture supernatants was determined by CBA. n=6, mean ± SEM; paired t-test; **p<0.01. (B) Percentage IL-2-expressing cells within the $CD4^+CD25^+FOXP3^-$ T cells and $CD4^+CD25^+CD127^-FOXP3^+$ T cells after 7-day MLR-ASC co-culture (representative examples are shown). (C) Data of multiple experiments showing the percentage IL-2-expressing cells within the $CD4^+CD25^+FOXP3^-$ T cells (white bars) and $CD4^+CD25^+CD127^-FOXP3^+$ T cells (chequered bars). n=5; mean ± SEM; paired t-test; ***p<0.001. (D) Levels of expressed IL-2. Median fluorescence intensity (MFI); n=5.

jority of the IL-2-expressing cells were $CD4^+CD25^+FOXP3^-$ T cells (Figure 5B-C). Produced IL-2 levels were similar between the IL-2 expressing $CD4^+CD25^+CD127^-FOXP3^+$ iTreg and $CD4^+CD25^+FOXP3^-$ T cells (Figure 5D).

To examine the effect of the IL-2 pathway on the ASC-mediated generation of functional $CD4^+CD25^+CD127^-FOXP3^+$ iTreg from $CD25^{-/dim}$ cells, IL-2 binding to its receptor was blocked with basiliximab. MLR were performed for 7 days in the absence and presence of ASC (1:40; ASC/effector cells). Basiliximab reduced the percentage of $CD25^+CD127^-FOXP3^+$ cells within the $CD4^+$ T cell population (Figure 6). In the presence of basiliximab, ASC did not induce a proportional increase of $CD25^+CD127^-FOXP3^+$ cells within the $CD4^+$ T cell population.

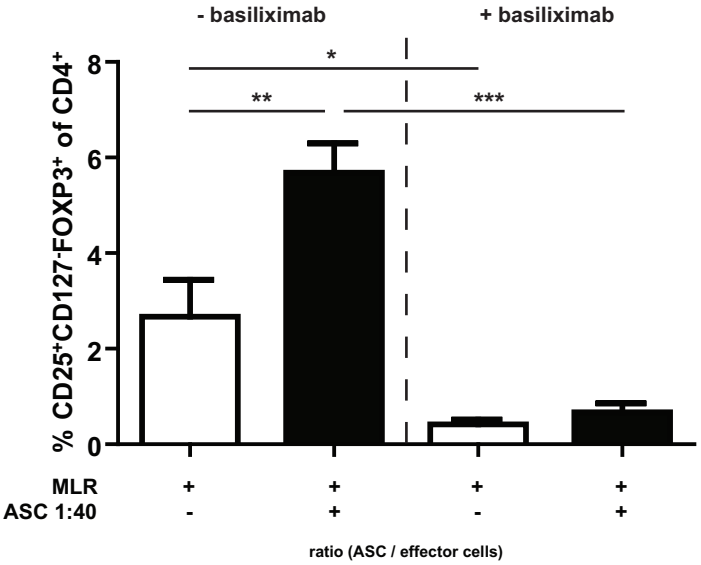

Figure 6. Effect of basiliximab on the induction of $CD4^+CD25^+CD127^-FOXP3^+$ Treg. MLR (white bars) consisted of $CD25^{-/dim}$ effector cells stimulated with γ-irradiated allogeneic PBMC and were performed for 7 days. In MLR-co-cultures ASC were used at a 1:40 ratio (ASC/effector cells; black bars). In the absence and presence of basiliximab, the percentage of $CD25^+CD127^-FOXP3^+$ cells within the $CD4^+$ T cell population was determined by flow cytometry. n=6, mean ± SEM; paired t-test; *p<0.05, **p<0.01, ***p<0.001.

DISCUSSION

Knowledge about the underlying mechanisms of how ASC contribute to a reduced responsiveness of immune effector cells is scarce. The present study provides the first evidence that human ASC mediate their immunosuppressive effect via the formation of functional *de novo* iTreg. Our results are in line with earlier in vivo studies with bone-marrow MSC in animal models and a recent case report of MSC administration to renal

transplant patients; these studies also observed proportional increases of Treg [9, 35, 41, 42]. However, as these groups did not investigate the Treg origin and Treg functionality, it is not clear whether the observed rises in Treg percentages are a consequence of the *de novo* formation of Treg or a result of the expansion of existing nTreg. We found that ASC mediate the generation of iTreg from effector T cells and that these newly formed cells have immunosuppressive capacities. iTreg also formed in MLR without ASC, but at lower numbers. While ASC-induced Treg showed similar suppressive capacities to nTreg, MLR-induced Treg had a stronger inhibitory effect. The induction of a more potent immunosuppressive phenotype in these cells might be due to their generation under highly pro-inflammatory conditions. In the presence of ASC effector cell proliferation is reduced. Because of this milder environment higher numbers of less effective iTreg are generated. Therefore, the suppressive strength of iTreg might be proportional to the stimulus under which they are induced. Further, due to the presence of a few nTreg in our initial effector T cell population we were able to infer that ASC also mediate an increase in nTreg.

Although first evidences of extrathymic conversion of conventional T cells into iTreg were found almost a decade ago, no iTreg-specific marker has been identified yet [43-45]. The only tool currently available to distinguish iTreg from nTreg is the determination of the methylation status of the TSDR. While nTreg have a demethylated TSDR, this specific region of the FOXP3 gene is methylated in iTreg [37]. The high percentage of cells with a methylated TSDR in the ASC-induced Treg fraction indicates that the vast majority of these cells originated from $CD25^{-/dim}$ effector cells. To further characterize the ASC-induced Treg we investigated their expression of GITR, CTLA-4 and Helios. GITR expression was similar in both iTreg and $CD4^+CD25^+FOXP3^-$ T cells. In contrast, iTreg showed the tendency to have a higher Helios expression and expressed significantly higher CTLA-4 levels than $FOXP3^-$ T cells. It has been described that CTLA-4 expression is up-regulated upon binding of FOXP3 to the promoter of the *CTLA-4* gene [46-50]. Therefore, the observed difference in CTLA-4 expression between iTreg and $FOXP3^-$ T cells can be attributed to their differing FOXP3 expression. Whether Helios is a potential marker for human iTreg is an ongoing debate in the field as conflicting results have been reported [51-53].

In search of possible underlying mechanisms involved in MSC-mediated iTreg generation, recently it has been reported that programmed death ligand-1 (PD-L1), a protein expressed by MSC, promotes the differentiation of T helper type 1 (Th1) cells into Treg [22, 54]. Other molecules that were suggested to play a role in the induction of Treg by MSC are IDO, prostaglandin E2 (PGE2), TGF-β and heme oxygenase-1 (HO-1) [41, 55-58]. In this study we focused on the role of IL-2 in ASC-mediated Treg induction as IL-2 drives T cell proliferation and paradoxically is also essential for tolerance induction by regulating nTreg function [59]. We observed that in the presence of ASC the IL-2 concentration in the cell culture supernatant increased. Since IL-2 is not expressed by ASC, the IL-2 surplus

originated from the allo-activated effector cells. Upon T-cell-receptor engagement and co-stimulation, activated effector T cells consume IL-2. During the inhibition of effector T cell proliferation by ASC IL-2 concentrations in the cell culture supernatant rose. This indicates that although their proliferation is suppressed, effector T cells remain activated and continue to secrete IL-2. The diminished proliferation rate of effector T cells causes a reduced IL-2 consumption and hence a surplus of IL-2. High levels of IL-2 are instrumental for Treg induction. In the presence of basiliximab, we found further accumulation of IL-2 in the supernatant. In its function as anti-rejection therapy in kidney transplant patients, basiliximab prevents the binding of IL-2 to its receptor and the subsequent uptake of IL-2. As a result, basiliximab inhibits T cell proliferation but also iTreg generation, indicating that Treg induction by ASC is IL-2 pathway dependent. Surprisingly, IL-2 was not only expressed by $CD4^+CD25^+FOXP3^-$ T cells but also by a smaller percentage of immunosuppressive ASC-induced Treg. Phenotypic adaptation of Treg in response to the cytokines present in their environment is a known phenomenon [60]. The inhibition of T cell proliferation is not an exclusive effect of MSC as other stromal cells share this characteristic [61, 62]. Hence, it is possible that these cells can also create an environment that favours iTreg generation.

The graft-supporting effects of MSC in animal models and the positive results of MSC treatment for graft-versus-host disease (GVHD) in patients who underwent hematopoietic stem cell transplantation (HSCT) are strongly convincing [8, 63]. The reported short lifespan of MSC after infusion, however, indicates that MSC are only initiators of these effects. Long-term, MSC do not actively promote immunosuppression themselves. Yet, they are able to pass on their immunosuppressive capabilities through the induction of functional *de novo* Treg, the expansion of nTreg or possibly through other immune cells which remain to be identified.

Despite continuous efforts, the translation of the experimental success of Treg-mediated immune regulation to its application in clinical settings proves difficult. In animal models, *ex vivo* or *in vivo* generated Treg prevent type 1 diabetes, reduce the severity of experimental autoimmune encephalomyelitis (EAE), control the acute and chronic rejection of allografts, and attenuate or prevent GVHD [64-70]. In humans, thus far, *ex vivo* expanded Treg have only been used for treatment of patients undergoing HSCT. In these few clinical studies, Treg were able to reduce the incidence of GVHD or to prevent GVHD [71-73]. Due to the heterogeneity of the Treg population and the lack of a specific marker for human Treg, *ex vivo* Treg expansion bares the risk of contamination with effector T cells. Therefore, *in vivo* induction of functional Treg mediated by MSC represents a beneficial effect of MSC therapy in addition to its immunosuppressive effect on effector T cells. Once the functionality of MSC-induced Treg has been confirmed in autoimmune disease patients and recipients of allografts, strategies to enhance the MSC-mediated *in vivo* generation of iTreg may be considered. Possible approaches

could be MSC treatment combined with low-dose IL-2 therapy or the use of rapamycin and rabbit anti-thymocyte globulin (rATG), anti-rejection drugs which were found to advance nTreg expansion and to allow Treg induction, respectively [70, 74-76].

In conclusion, our study demonstrates that human adipose-tissue derived MSC induce Treg from effector T cells and that these *de novo* Treg are immunosuppressive. In conjunction with the well-known MSC function of preventing immune cell proliferation, our finding encourages advancing MSC therapy into clinical development for autoimmunity, GVHD and allograft rejection.

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Chapter 4

Mesenchymal stem cells control allo-reactive $CD8^{+}CD28^{-}$ T cells

Anja U. Engela, Carla C. Baan, Nicolle H.R. Litjens, Marcella Franquesa, Michiel G.H. Betjes, Willem Weimar, Martin J. Hoogduijn

Department of Internal Medicine, Section Nephrology and Transplantation, Erasmus University Medical Center, Rotterdam, the Netherlands

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ABSTRACT

CD28/B7 co-stimulation blockade with belatacept prevents allo-reactivity in kidney transplant patients. Cells lacking CD28 are however not susceptible to belatacept treatment. As $CD8^+CD28^-$ T-cells have cytotoxic and pathogenic properties, we investigated whether mesenchymal stem cells (MSC) are effective in controlling these cells. In mixed lymphocyte reactions (MLR) MSC and belatacept inhibited PBMC proliferation in a dose-dependent manner. MSC at MSC/effector cell ratios 1:160 and 1:2.5 reduced proliferation by 38.8% and 92.2%. Belatacept concentrations 0.1µg/mL and 10µg/mL suppressed proliferation by 20.7% and 80.6%. Both treatments in combination did not inhibit each other's function. Allo-stimulated $CD8^+CD28^-$ T cells were able to proliferate and expressed the cytolytic and cytotoxic effector molecules granzyme B, IFN-γ and TNF-α. While belatacept did not affect the proliferation of $CD8^+CD28^-$ T cells, MSC reduced the percentage of $CD28^-$ T cells in the proliferating $CD8^+$ T-cell fraction by 45.9% ($p=0.009$). $CD8^+CD28^-$ T cells as effector cells in MLR in the presence of $CD4^+$ T-cell help gained CD28 expression, an effect independent of MSC. In contrast, allo-stimulated $CD28^+$ T cells did not lose CD28 expression in MLR-MSC co-culture suggesting that MSC control pre-existing $CD28^-$ T cells and not newly induced $CD28^-$ T cells.

In conclusion, allo-reactive $CD8^+CD28^-$ T cells that remain unaffected by belatacept treatment are inhibited by MSC. This study indicates the potential of an MSC-belatacept combination therapy to control allo-reactivity.

INTRODUCTION

CD28/B7 co-stimulation blockade to prevent T cell activation and proliferation has been of interest for many therapeutic areas [1]. Belatacept, the latest immunosuppressive drug approved for therapy of kidney transplant recipients, utilises this blocking mechanism. It is a fusion protein consisting of the extracellular domain of cytotoxic T-lymphocyte antigen-4 (CTLA-4) and the Fc region of a human IgG1 immunoglobulin. By binding to CD80 (B7.1) and CD86 (B7.2) with a higher affinity than CD28 belatacept blocks the co-stimulatory signal [2]. Yet, as a consequence, belatacept treatment is not effective in impairing T cells that lack CD28 expression. While at birth all T cells express CD28, the $CD8^+$ T cell compartment of an adolescent individual contains $CD28^-$ cells at a frequency of up to 20-30% [3, 4]. Persistent antigenic stimulation during ageing or, in an accelerated manner, through infection with cytomegalovirus (CMV) causes down-regulation of CD28 expression on $CD8^+$ T cells [5, 6]. The presence of these $CD8^+CD28^-$ T cells is associated with oncological diseases and autoimmune diseases such as rheumatoid arthritis, multiple sclerosis and diabetes [7-10]. In addition, their highly antigen-experienced nature and cytotoxic phenotype may pose a risk for graft rejection after organ transplantation. The insusceptibility of allo-reactive $CD8^+CD28^-$ T cell to belatacept discloses a gap in the immunosuppressive activity of this drug. Therefore, CD28/B7-blocking agents may need to be combined with a therapy that targets $CD28^-$ T cells.

A potential therapeutic approach could be the administration of mesenchymal stem cells (MSC). MSC possess immunomodulatory properties and their function has been established in vitro and in animal models [11, 12]. First MSC trials in humans for multiple disease areas such as autoimmune diseases, graft-versus-host disease (GVHD) and allograft rejection produced encouraging results [13-16]. Activated MSC inhibit cells of the innate and adaptive immune system and of central interest in MSC research is their suppression of T-cell mediated immunity as MSC inhibit the proliferation of $CD4^+$ and $CD8^+$ T cells [17]. MSC mediate their immunosuppressive effect in a CD28-independent manner through direct contact with their target cells and through various soluble factors such as human hepatocyte growth factor (HGF), indoleamine 2,3-dioxygenase (IDO), interleukin (IL)-10, prostaglandins and transforming growth factor (TGF)-β [18].

The aim of our study was to investigate whether MSC can inhibit the allo-reactivity of $CD8^+CD28^-$ T cells which escape belatacept treatment and to explore whether MSC are a potential candidate for combination therapy with belatacept.

MATERIAL AND METHODS

Origin, isolation and culture of human MSC

Perirenal adipose tissue was surgically removed from living kidney donors and collected in minimum essential medium Eagle alpha modification (MEM-α) (Sigma-Aldrich, St. Louis, MO, USA) supplemented with 2mM L-glutamine (Lonza, Verviers, Belgium), 1% penicillin/streptomycin solution (P/S; 100IU/mL penicillin, 100IU/mL streptomycin; Lonza). Samples were obtained with written informed consent as approved by the Medical Ethical Committee at Erasmus MC, University Medical Center Rotterdam (protocol no. MEC-2006-190).

MSC were isolated, cultured and characterized as previously described [19]. In brief, perirenal adipose tissue was mechanically disrupted and enzymatically digested with collagenase type IV (Life Technologies, Paisley, UK). MSC were expanded using MSC culture medium consisting of MEM-α with 2mM L-glutamine, 1% P/S and 15% fetal bovine serum (FBS; Lonza) in a humidified atmosphere with 5% CO_2 at 37°C. Culture medium was refreshed twice weekly. At subconfluency, MSC were removed from culture flasks using 0.05% trypsin-EDTA (Life Technologies) and reseeded at 1,000cells/cm^2. MSC were characterized by means of immunophenotyping and by their ability to differentiate into adipocytes and osteoblasts. MSC cultured between 2 to 6 passages were used. MSC from these passages did not differ in their ability to differentiate or to exert their immunosuppressive functions.

Isolation of peripheral blood mononuclear cells

Peripheral blood mononuclear cells (PBMC) were isolated from buffy coats of healthy blood donors (Sanquin, Rotterdam, the Netherlands) by density gradient centrifugation using Ficoll-Paque PLUS (density 1.077g/mL; GE Healthcare, Uppsala, Sweden). Cells were frozen at -150°C until further use in Roswell Park Memorial Institute (RPMI) 1640 medium with GlutaMAX™-I (Life Technologies) supplemented with 1% P/S, 10% human serum (Sanquin) and 10% dimethyl sulfoxide (DMSO; Merck, Hohenbrunn, Germany).

Mixed lymphocyte reaction and suppression assays

Mixed lymphocyte reactions (MLR) were set up with 5x10^4 effector PBMC and 5x10^4 γ-irradiated (40 Gy) allogeneic PBMC in round-bottom 96-well plates (Nunc, Roskilde, Denmark). MLR were cultured in MEM-α supplemented with 2mM L-glutamine, 1% P/S and 10% heat-inactivated human serum for 7 days in a humidified atmosphere with 5% CO_2 at 37°C. Effector-stimulator cell combinations were chosen on the basis of a minimum of four HLA mismatches.

The immunomodulatory capacities of MSC and belatacept (Bristol-Myers-Squibb, New York, NY, USA) on MLR were determined in suppression assays. For flow cytometric

analysis, effector PBMC were labelled with BD Horizon Violet Cell Proliferation Dye 450 (VPD450; BD Biosciences, San Jose, CA, USA). For distinction from effector PBMC, γ-irradiated allogeneic stimulator PBMC (40 Gy) were labelled using the PKH26 Red Fluorescent Cell Linker Kit (Sigma-Aldrich). When cell proliferation was assessed by thymidine incorporation, [^{3}H]-thymidine (0.25μCi/well; PerkinElmer, Groningen, the Netherlands) was added on day 7, incubated for 8 hours and its incorporation was measured using the Wallac 1450 MicroBeta TriLux (PerkinElmer).

MLR with sorted $CD8^+CD28^-$ T cells, $CD28^-$ T cells or $CD28^+$ T cells

PBMC were stained with mAbs against CD3 (AmCyan), CD4 (APC), CD8 (FITC), CD28 (PerCP-Cy5.5), and either $CD3^+CD8^+CD28^-$ cells and $CD3^+CD4^+$ cells or $CD3^+CD28^-$ cells and $CD3^+CD28^+$ cells were sorted on the BD FACSAria II cell sorter (BD Biosciences). Effector populations for MLR consisted either of $CD3^+CD28^-$ cells only (mean purity 97.8%, range 96.3%-98.8%), $CD3^+CD28^+$ cells only (mean purity 96.2%, range 93.0%-99.5%) or of a combination of 10% $CD3^+CD8^+CD28^-$ cells (mean purity 92.3%, range 88.4%-94.72%) and 90% $CD3^+CD4^+$ cells to provide help (mean purity 98.2%, range 97.2%-99.5%). All effector fractions were labelled with VPD450 (BD Biosciences) to track cell proliferation. To distinguish irradiated allogeneic stimulator PBMC from effector cells they were labelled with PKH26 (Sigma-Aldrich). Effector-stimulator cell combinations were chosen on the basis of a minimum of four HLA mismatches. MLR were set up in the absence or presence of MSC (1:10; MSC/effector cells) and belatacept (1μg/mL). After a 7-day incubation period, cells were re-stained with mAbs against CD3 (AmCyan), CD4 (APC), CD8 (FITC), CD28 (PerCP-Cy5.5) and analysed on the BD FACSCanto II flow cytometer using the BD FACSDiva software (BD Biosciences).

Intracellular and extracellular staining of $CD8^+CD28^-$ T cells

MLR were set up in the absence of MSC. To track cell proliferation, effector PBMC were labelled with VPD450. After 7-days, cells were re-stimulated with phorbol 12-myristate 13-acetate (PMA; 50ng/mL; Sigma-Aldrich) and ionomycin (1μg/mL; Sigma-Aldrich) in the presence of GolgiPlug (BD Biosciences). Following a 4-hour incubation period, cells were stained with monoclonal antibodies (mAbs) against CD3 (AmCyan), CD4 (APC), CD8 (FITC), CD28 (PerCP-Cy5.5), tumour necrosis factor (TNF)-α (PE), interferon (IFN)-γ (PE; all BD Biosciences) and granzyme B (PE; Sanquin). Intracellular staining for TNF-α, IFN-γ and granzyme B was performed according to protocol B for staining of intracellular antigens for flow cytometry (eBioscience, San Diego, CA, USA) using the described buffers. For the identification of extracellular CTLA-4 expression and the expression of programmed death ligand-1 (PD-L1) in proliferating $CD8^+CD28^-$ T cells, MLR were set up as described above, but cells were not re-stimulated. After 7-days, cells were harvested and stained with monoclonal antibodies (mAbs) against CD3 (AmCyan), CD4 (PE), CD8 (FITC), CD28

(PerCP-Cy5.5), CTLA-4 (APC) (all BD Biosciences) and PD-L1 (PE-Cy7; eBioscience). Fluorescence minus one (FMO) controls were used to determine negative expression. Flow cytometric analysis was performed using the BD FACSCanto II flow cytometer using the BD FACSDiva software (both BD Biosciences).

Flow cytometric analysis of apoptotic cells

MLR were set up in the absence or presence of MSC (1:10; MSC/effector cells). Effector PBMC were labelled with VPD450 (BD Biosciences) and γ-irradiated, allogeneic stimulator PBMC were labelled using the PKH67 Green Fluorescent Cell Linker Kit (Sigma-Aldrich). Cells were incubated for 4 days or 7 days. Apoptotic cells were identified using the Annexin V PE Apoptosis Detection Kit I (BD Biosciences) according to the manufacturer's instructions in combination with mAb labelling against CD3 (AmCyan), CD8 (APC), CD28 (PerCP-Cy5.5). Flow cytometric analysis was performed using the BD FACSCanto II flow cytometer and BD FACSDiva software (both BD Biosciences).

Statistical analysis

Statistical analyses were performed by means of paired t-tests using GraphPad Prism 5 software (GraphPad Software, San Diego, CA, USA). A p-value lower than 0.05 was considered statistically significant. Two-tailed p-values are stated.

RESULTS

$CD8^+CD28^-$ T cells proliferate upon allo-stimulation and have a pro-inflammatory and cytotoxic phenotype

The proliferative capacity of the $CD8^+CD28^-$ T cells and their ability to express cytotoxic effector molecules was investigated in 7-day MLR by means of VPD450 dilution and flow cytometric analysis. Allogeneically stimulated $CD8^+CD28^-$ T cells proliferated as strongly as allo-stimulated $CD8^+CD28^+$ T cells (Figure 1A). Both cell types expressed granzyme B, IFN-γ and TNF-α (Figure 1B and C). Granzyme B was expressed by equal percentages of $CD8^+CD28^-$ T cells and $CD8^+CD28^+$ T cells (85% and 90%, respectively). In contrast, more $CD8^+CD28^-$ T cells than $CD8^+CD28^+$ T cells expressed the pro-inflammatory cytokines IFN-γ and TNF-α (83% vs. 57% and 83% vs.43%, respectively). The proliferating fractions of $CD8^+CD28^-$ T cells and $CD8^+CD28^+$ T cells expressed more granzyme B and IFN-γ than the respective non-proliferating fractions; expression of granzyme B and IFN-γ in proliferating $CD8^+CD28^-$ T cells was increased by 26% ($p=0.039$) and 19% ($p=0.041$), respectively. Proliferating $CD8^+CD28^+$ T cells expressed 84% ($p=0.003$) more granzyme B and 54% more IFN-γ ($p=0.022$) than non-proliferating $CD8^+CD28^+$ T cells. TNF-α expression did not differ between the proliferating and non-proliferating fractions.

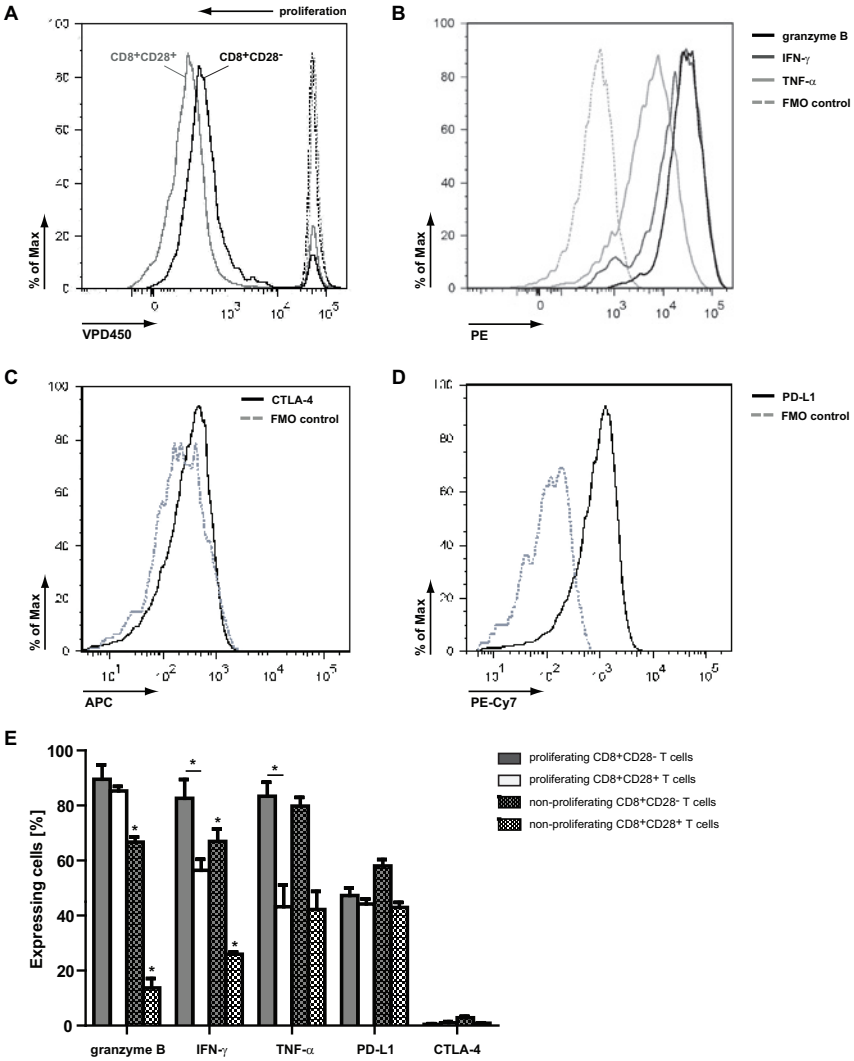

Figure 1. Characterisation of CD8+CD28- T cells. (A) Effector PBMC were labelled with the proliferation marker VPD450 and stimulated with γ-irradiated allogeneic PBMC for 7 days. Representative examples of proliferating CD8+CD28- T cells (black histogram) and CD8+CD28+ T cells (grey histogram) are shown. Dashed histograms depict unstimulated CD8+CD28- T cells (black) and CD8+CD28+ T cells (grey). (B). Expression of granzyme B (black histogram), IFN-γ (dark grey) and TNF-α (light grey) by allo-stimulated, proliferating CD8+CD28- T cells in 7-day MLR following a re-stimulation with PMA/ionomycin for 4 hours in the presence of GolgiPlug. Expression of CTLA-4 (black histogram; C) and PD-L1 (black histogram; D) by allo-stimulated, proliferating CD8+CD28- T cells in 7-day MLR. FMO control is depicted as dotted histogram. Representative examples are shown. Data of multiple experiments are depicted in C. (C) Expression of granzyme B, IFN-γ, TNF-α, PD-L1 and CTLA-4 by proliferating CD8+CD28- T cells (grey bars), proliferating CD8+CD28+ T cells (white bars), non-proliferating CD8+CD28- T cells (chequered grey bars) and non-proliferating CD8+CD28+ T cells (chequered white bars). Unless indicated otherwise, statistically significant changes between the corresponding proliferating and non-proliferating fractions are displayed. n=3, mean ± SEM; paired t-test; *p<0.05.

PD-L1 expression was similar in proliferating $CD8^+CD28^-$ T cells and $CD8^+CD28^+$ T cells (47% vs. 44%, respectively; Figure 1C and E). CTLA-4 was expressed at very low levels by both cell types (Figure 1D and E).

MSC and belatacept permit each other's immunosuppressive function

To study the combined effect of MSC and belatacept on effector cell proliferation, the appropriate concentrations and the effect of both immunosuppressive agents on each other's function had to be established. Therefore, MLR were set up in the presence of various concentrations of MSC and/or belatacept. Inhibition of proliferation was assessed by means of [^{3}H]-thymidine incorporation. MSC and belatacept inhibited PBMC proliferation in a dose-dependent manner (Figure 2). The two highest concentrations of belatacept and MSC tested (10µg/mL and 1:2.5; MSC/effector cells) reduced proliferation of effector cells to 19.4% ($p=0.0002$) and 7.8% ($p<0.0001$), respectively. When applied in combination both immunosuppressants permitted each other's anti-proliferative function. At low concentrations the combination of MSC and belatacept had an additive suppressive effect. While belatacept (0.1µg/mL) inhibited the proliferation of effector cells by 20.7% ($p=0.0086$), MSC reduced proliferation by 38.8% ($p=0.0037$). Belatacept-MSC co-treatment suppressed effector cell proliferation by an additional 15.1% compared to the inhibition achieved by MSC alone ($p=0.029$).

MSC reduce the percentage of proliferating, allo-reactive $CD8^+CD28^-$ T cells

In its function as co-stimulation blocker belatacept only constrains the interaction of CD28 expressing $CD8^+$ T cells with APC. To examine whether MSC can control $CD8^+CD28^-$ T cells which are unaffected by belatacept treatment, the effect of MSC (1:10; MSC/effector cells) and 1µg/mL belatacept on the proliferation of $CD8^+$ T cells and their

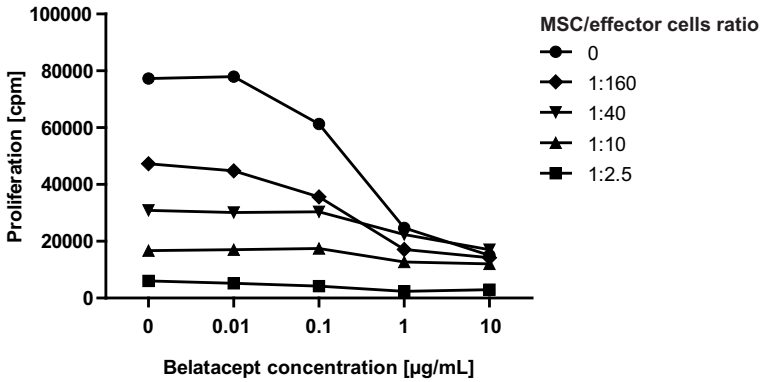

Figure 2. Immunosuppressive effects of MSC and belatacept. Using PBMC as effector cells, MLR were set up in the presence of various MSC concentrations (ratio MSC/effector cells) and belatacept concentrations. After 7 days proliferation was assessed by means of [^{3}H]-thymidine incorporation. n=5 (mean).

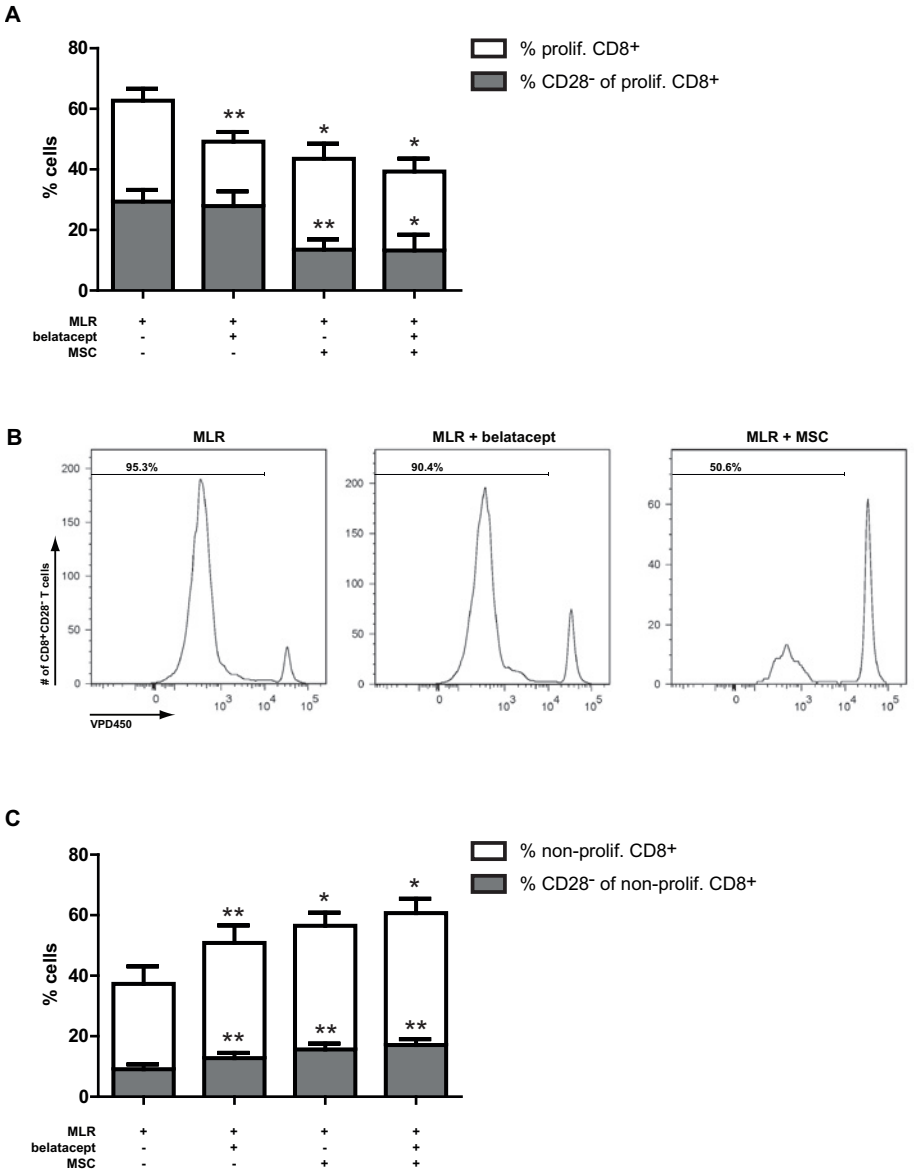

Figure 3. MSC reduce the percentage of proliferating, allo-reactive CD8+CD28- T cells. Effector PBMC were labelled with the proliferation marker VPD450 and stimulated with γ-irradiated allogeneic PBMC (PKH26 label) in the presence or absence of MSC (1:10; ratio MSC/effector cells) and/or 1μg/mL belatacept. After 7 days, flow cytometric analyses were performed. (A) The percentages of proliferating CD8+ T cells (white bar) and the percentages of CD28- cells within the proliferating CD8+ T cells (grey bar) are shown. n=8, mean ± SEM; paired t-test; *p<0.05, **p<0.01. (B) Representative examples of allo-stimulated CD8+CD28- T cells in the absence and presence of belatacept and/or MSC are shown. Percentages of proliferating CD8+CD28- T cells are stated. (C) The percentages of non-proliferating CD8+ T cells (white bar) and the percentage of CD28- cells within the non-proliferating CD8+ cells (grey bar) are shown. n=8, mean ± SEM; paired t-test; *p<0.05, **p<0.01.

CD28$^-$ subpopulation was assessed. Both agents were added alone or in combination to MLR for 7 days. Belatacept and MSC reduced the percentage proliferating CD8$^+$ T cells by 13.6% (p=0.0034) and 19.2% (p=0.012), respectively (Figure 3A); the combination of both treatments led to a reduction by 26.7%. At these concentrations a synergistic effect of MSC and belatacept was not observed. While belatacept reduced the proliferation of CD8$^+$ T cells, it did not have an effect on the proliferation of the CD28$^-$ cells within the proliferating CD8$^+$ T cells (Figure 3A and 3B). In contrast, MSC reduced the percentage of CD28$^-$ cells within the proliferating CD8$^+$ T cell population by 45.9% (p=0.009). MSC and belatacept in combination inhibited the proliferation of CD8$^+$CD28$^-$ T cells by 44.9% (p=0.036) indicating that belatacept did not impair the immunosuppressive function of MSC.

To elucidate the fate of the CD28$^-$ cells, we studied the non-proliferating T cell fraction. MSC increased the percentage of CD28$^-$ cells within the non-proliferating CD8$^+$ T cell fraction by 58% (Figure 3C). Further, as MSC are able to induce apoptosis, we also inves-

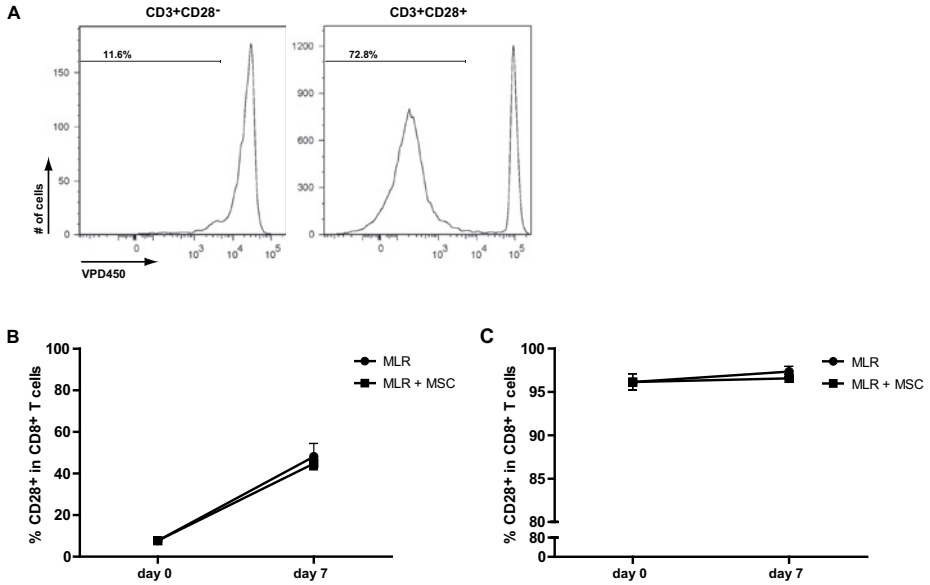

Figure 4. Effect of MSC on CD28 expression of CD8$^+$ T cells. (A) Sorted CD28$^-$ T cells and sorted CD28$^+$ T cells were allo-stimulated with γ-irradiated, allogeneic PBMC for 7 days. Proliferation of both cell populations is shown by means of VPD450 dilution. (B) Effector cell population in MLR consisted of CD8$^+$CD28$^-$ T cells (10%) and CD4$^+$ T cells (90%). Effector cells were stimulated with γ-irradiated, allogeneic PBMC in the absence and presence of MSC. The percentage CD28$^+$ cells within the CD8$^+$ T cell population was determined in the starting effector cell population (day 0) and in MLR (●) and MLR-MSC co-culture (1:10; ratio MSC/effector cells; ■) after 7 days by flow cytometry. n=6, mean ± SEM. (B) Sorted CD28$^+$ T cells were used as effector cells in 7-day MLR. MLR were set up in the absence (●) or presence of MSC (1:10; ratio MSC/effector cells; ■). The percentages CD28$^+$ cells within the CD8$^+$ T cell population was determined by flow cytometry. n=8, mean ± SEM.

tigated this option by means of annexin-V staining. At day 4 and day 7, the percentage of annexinV+CD8+CD28- T cells was similar in MLR and MLR-MSC co-culture indicating that MSC did not render CD8+CD28- T cells apoptotic (day 4 [mean]: 35.5% vs. 32.3%; day 7: 19.9% vs. 23.45%).

MSC do not affect CD28 expression of CD8+ T cells

The reduction of allo-reactive CD8+CD28- T cells in the proliferative fraction may not solely be attributed to the anti-proliferative effect MSC exert on these cells. Therefore, we investigated whether MSC influenced CD28 expression of CD8+ T cells. First the effect of MSC on a potential gain of CD28 expression was determined. When used in MLR as single effector-cell population, proliferation of CD28- T cells was limited, while allo-stimulated CD28+ T cells strongly proliferated (Figure 4A). To provide sufficient help enabling CD28- T-cell proliferation, the MLR-effector population consisted of 10% sorted CD8+CD28- T cells and 90% sorted CD4+ T cells. After 7-days, 48.2% of the originally CD8+CD28- T cells had gained CD28 expression in MLR (Figure 4B). MSC did not influence this effect on CD28 expression. In the reverse experiment to investigate whether loss of CD28 expression would be mediated by MSC, sorted CD28+ T cells were used as effector cells in 7-day MLR. Full CD28 expression was sustained in MLR and MSC did not affect this (Figure 4C).

DISCUSSION

Belatacept is the first intravenous long-term immunosuppressive therapy for kidney transplantation and is believed to challenge the position of calcineurin inhibitor (CNI) tacrolimus as most prescribed drug for the prevention of graft rejection in solid organ transplantation [20, 21]. Despite their success as immunosuppressants, next to adverse side effects such as hypertension, malignancies and diabetes, CNIs have the major drawback of causing nephrotoxicity, indicating a need for alternative agents [22]. The BENEFIT study compared the CNI cyclosporine A with belatacept in kidney transplantation [23, 24]. Three-year outcomes of this study showed that patient and graft survival rates were similar for cyclosporine A and belatacept, but belatacept-treated patients had superior renal function and less adverse events [25]. In contrast, administration of belatacept led to higher frequencies of acute rejections. An underlying cause for these acute rejections might be CD8+CD28- T cells that escape inhibition by belatacept. In the present study we investigated the effect of MSC on CD8+CD28- T cells.

We identified CD8+CD28- T cells as potentially harmful cells that express granzyme B, TNF-α and IFN-γ and are highly proliferative upon allogeneic stimulation. Expression of these cytolytic and pro-inflammatory molecules by CD8+CD28- T cells has been

observed by others [26-29]. However, data about the ability of $CD8^+CD28^-$ T cells to proliferate is ambiguous. While some reports confirm our finding [30, 31], other research groups describe that the proliferative response of $CD8^+CD28^-$ T cells is inhibited [32, 33]. Critical for $CD8^+CD28^-$ T-cell proliferation are the stimulation conditions. Plunkett et al. describe that anti-CD3 stimulation only leads to mild proliferation, while in the presence of irradiated PBMC $CD8^+CD28^-$ T cells strongly proliferate [34]. Contrary to these results, we found that $CD8^+CD28^-$ T cells stimulated with allogeneic PBMC had restrained proliferative abilities. Only when $CD4^+$ T-cell help was provided $CD8^+CD28^-$ T cells proliferated as strongly as their counterparts in total PBMC. This indicates that certain cytokines or co-stimulatory signals other than CD28 ligands are required for the activation and proliferation of $CD8^+CD28^-$ T cells. We determined that proliferating $CD8^+CD28^-$ T cells expressed PD-L1 but lacked CTLA-4. Upon binding to the CD80/86 complex, both molecules transmit inhibitory signals [2, 35-37]. Control of cell proliferation through these inhibiting pathways can therefore be jeopardised by belatacept. However, next to its inhibitory function, PD-L1 has also been described to enhance T cell activation and thereby might contribute to the proliferative capacities of $CD8^+CD28^-$ T cells [38, 39].

$CD8^+CD28^-$ T cells are predominantly found within the (terminally differentiated) effector memory $CD8^+$ T cell subset [40] and they can have cytotoxic [29, 41-43] or immunosuppressive functions [10, 44-47]. Thus, inhibition of $CD8^+CD28^-$ T cells by MSC could not only involve the suppression of the cytotoxic subset, but also affect the regulatory subset. Our study shows, however, that MSC inhibited $CD8^+CD28^-$ T cells that express the cytotoxic molecules granzyme B, TNF-α and IFN-γ. In contrast, CTLA-4, which is associated with a regulatory function, was hardly detectable on the $CD8^+CD28^-$ T cells. Earlier studies by our group demonstrated that terminally differentiated $CD8^+$ T cells contain a large proportion of $CD28^-$ cells, and these cells showed no immunosuppressive capacity in vitro [48]. This suggests that under the inflammatory conditions, as set up in the present experiments, MSC target only the effector $CD8^+CD28^-$ T cell subset. It is possible that under different conditions $CD8^+CD28^-$ T cells with regulatory properties are more prominent and under these circumstances the use of MSC should be reconsidered.

A cytokine that promotes $CD8^+CD28^-$ T cell proliferation is IL-15 [30]. Interestingly, IL-15, next to IL-7, is crucial for the homeostatic maintenance of T cells in the absence of antigenic stimuli and expedites the loss of CD28 expression [49]. During normal exposure to antigen CD28 expression is transiently reduced but quickly returns to basal expression levels. Repeated antigen exposure due to the natural aging process, viral infections or viral reactivation in immunocompromised patients causes a decline in CD28 expression, eventually leading to total loss of CD28. Surprisingly, we found that in our setting $CD28^+$ T cells did not lose CD28 during allogeneic stimulation with PBMC, confirming that extended rounds of antigen exposure are required to initiate reduction of CD28. Permanent decline of CD28 expression entails telomere shortening and reduction

of telomerase activity and is attributed to a defect in the CD28 promoter leading to transcriptional inactivation [50-54]. We, however, found that CD8+ T cells that were initially CD28- gained CD28 expression during allogeneic stimulation with PBMCs. Re-induction of CD28 expression in CD4+CD28- T cells is a known phenomenon and only possible until CD28- T cells have reached terminal differentiation. Warrington et al. described that combined stimulation of T cell receptor (TCR) and IL-12 receptor restored CD28 transcription and protein expression, while single-stimulation of either the TCR or the IL-12 receptor was not sufficient [55]. IL-12 is produced by phagocytic cells, B cells and other antigen-presenting cells [56] and therefore potentially contributes to the CD28 re-expression in originally CD8+CD28- T cells in MLR. Although CD28 expression can be influenced up to a certain stage during T cell differentiation, MSC did not affect the immunophenotypical changes of CD8+CD28- T cells nor did they cause loss of CD28 expression in CD8+CD28+ T cells. Further, we found that MSC did not induce apoptosis in CD8+CD28- T cells, despite their ability to express Fas ligand (FasL) or to initiate the programmed death (PD)-1/PD-ligand 1 (PD-L1) pathway [57, 58]. These observations indicate that MSC solely have an anti-proliferative effect on CD8+CD28- T cells.

Co-administration of MSC with other immunosuppressive drugs is not always encouraged; agents such as tacrolimus, mammalian target of rapamycin (mTor) inhibitor rapamycin and rabbit anti-thymocyte globulin (rATG) negatively affect the suppressive capacity of MSC in vitro [59-61]. At same time MSC are able to reduce the efficacy of tacrolimus and rapamycin [59, 60]. As MSC lack expression of the CTLA-4 ligands CD80 and CD86 it was not surprising that belatacept did not diminish MSC function [62]. Vice versa, MSC did not affect the immunosuppressive capability of belatacept. In the presence of belatacept and lower MSC/effector cell ratios we even observed an additive suppressive effect.

MSC exert their immunomodulatory function not only by suppressing the proliferation of various immune cells. In a previous study we have shown that MSC also induce functional *de novo* regulatory T cells (Treg) [63]. CD28/B7 co-stimulation in Treg is required for their differentiation [64]. Treg-specific deficiency of CD28 and CTLA-4 leads to an impaired immunosuppression by Treg and the development of autoimmunity and rejection in transplant models [65, 66]. The effect of CTLA-4-Ig therapy on Treg is controversial. Administration of CTLA-4-Ig to a skin transplant mouse model abolished Treg-dependent graft acceptance and expansion of Treg [67]. In contrast, CTLA-4-Ig therapy in rheumatoid arthritis patients reduced the frequency of peripheral Treg but enhanced their function [68]. Therefore, alongside the allo-reactive CD8+CD28- T cells that escape belatacept therapy, the possible diminution of Treg in patients receiving belatacept might contribute to the increased frequency of acute rejections reported for belatacept-treated kidney graft recipients [25].

In conclusion, $CD8^+CD28^-$ T cells sustain their proliferative capacity in the presence of belatacept, and secrete cytolytic and cytotoxic effector molecules. As MSC are able to control these $CD8^+CD28^-$ T cells by inhibiting their proliferation, our study suggests a potential for MSC-belatacept combination therapy to prevent allo-reactivity after solid organ transplantation.

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Chapter 5

Genetic variants of FOXP3 influence graft survival in kidney transplant patients

Anja U. Engela[1], Karin Boer[1], Joke I. Roodnat[1], Annemiek M.A. Peeters[1], Paul H. Eilers[4], Judith A. Kal-van Gestel[1], Fernando Rivadeneira[2,3], Willem Weimar[1], Carla C. Baan[1]

Department of Internal Medicine, [1]Section Nephrology and Transplantation and [2]Endocrinology Laboratory, [3]Department of Biostatistics, [4]Department of Epidemiology, Erasmus University Medical Center, Rotterdam, the Netherlands

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ABSTRACT

FOXP3$^+$ regulatory T cells (Treg) play a role in controlling alloreactivity. It has been shown that short $(GT)_n$ dinucleotide repeats ($\leq (GT)_{15}$; S) in the promoter region of the FOXP3 gene enhance the promoter activity when compared to long $(GT)_n$ repeats ($\geq (GT)_{16}$; L). The present study retrospectively investigated the influence of this $(GT)_n$ FOXP3 gene polymorphism on renal allograft survival. A total of 599 consecutive first-time kidney transplant patients (median follow-up time 7.7 years) were subdivided according to their FOXP3 genotype into the S-genotype group (SG) and the L-genotype group (LG). The SG was superior to the LG in both general graft survival censored for death (logrank test, $p = 0.013$) and graft survival following acute rejection ($p = 0.021$). Multivariate analysis defined the $(GT)_n$ FOXP3 dinucleotide repeat polymorphism as an independent factor and confirmed an advantage for the SG in renal allograft survival (HR = 0.67, 95% CI 0.48 - 0.94, $p = 0.02$). This gene association study identified a beneficial effect of FOXP3 genetic variants on graft survival in kidney transplant patients.

INTRODUCTION

Kidney transplants are susceptible to numerous unfavorable conditions including ischemic injury and anti-donor reactivity. $CD4^+CD25^+$ regulatory T cells (Treg) are involved in controlling tissue damage and alloreactivity [1-7]. Treg exert these tasks by inhibiting the functions of various cell types such as $CD4^+$ helper T cells, $CD8^+$ cytotoxic T cells and antigen-presenting cells [8, 9]. The master gene responsible for the development, maintenance and regulatory function of Treg is the transcription factor forkhead box P3 (FOXP3) [10, 11]. In humans, the FOXP3 gene is located on the p arm of the X chromosome (Xp11.23), an area of autoimmune disorder linkage; autoimmune diseases such as multiple sclerosis, rheumatoid arthritis and type 1 diabetes are linked to Xp11 [12-14]. The importance of FOXP3 in restraining autoimmunity is indicated in patients suffering from immunodysregulation polyendocrinopathy enteropathy X-linked syndrome (IPEX) were loss-of-function mutations within the FOXP3 gene lead to an aggressive lymphoproliferative immune-mediated disorder due to lack of functioning Treg. The FOXP3 gene consists of 11 exons and it has been reported that the FOXP3 promoter activity is modulated by a $(GT)_n$ dinucleotide repeat polymorphism within the FOXP3 promoter region [15]. Increased promoter activity is associated with 15 or less (GT) repeats which may lead to elevated levels of FOXP3 mRNA and possibly to an increase of regulatory T cell activity.

In this retrospective study the significance of the FOXP3 $(GT)_n$ dinucleotide repeat polymorphism was investigated with regard to graft survival in 599 first-time kidney transplant patients who received their graft between September 1995 and March 2005. It was hypothesized that patients expressing $\leq(GT)_{15}$ repeats would experience an advantage in renal graft survival over patients with $\geq(GT)_{16}$ repeats due to a proposed increase in FOXP3 activity. The different FOXP3 genotypes were studied with respect to their impact on general graft survival and on graft survival following acute rejection (AR) episodes.

MATERIAL AND METHODS

Patient population

A total of 657 end-stage renal disease patients consecutively underwent first-time kidney transplantations between September 1995 and March 2005. Material for determination of the FOXP3 $(GT)_n$ gene polymorphism was available from 632 (96%) cases. Of those, 599 (95%) patients were included in the study population; 33 patients were excluded due to the occurrence of surgery-related complications and other non-immunological complications leading to immediate graft loss. Main reasons for patient exclusion were

never-functioning grafts from non-heart-beating donors (52%) and vascular problems (36%). The analysis was performed with data obtained in May 2011. Median follow-up time of the study cohort was 7.7 years (range 0 - 15.7 years). Maintenance immunosuppressive therapy consisted of the calcineurin inhibitors cyclosporine or tacrolimus, mycophenolate mofetil and corticosteroids. Four percent of the patients received basiliximab as induction therapy. The majority (58%) of the biopsy proven acute rejections occurred within the first 3 months after transplantation. Anti-rejection therapy consisted of high-dose methylprednisolone and was administered when kidney pathology was classified as acute cellular rejection according to Banff criteria. Written consent was obtained from all patients.

Genotype analysis of the $(GT)_n$ dinucleotide repeat polymorphism in the FOXP3 gene

Genomic DNA was extracted from peripheral blood mononuclear cells (PBMC) obtained prior to kidney transplantation. DNA was isolated using the MagNA Pure LC instrument and the MagNA Pure LC DNA Isolation Kit I (Roche Diagnostics GmbH, Mannheim, Germany) according to the manufacturer's instructions. Intron zero containing the $(GT)_n$ polymorphism of the FOXP3 gene on chromosome Xp11.23 was amplified by PCR. The following primer pair was used to amplify fragments of 255-267bp containing the $(GT)_n$ repeats: 5'-GGCGGTATGAGATACTCGACCA-3' (sense) and 5'-CAACCATTGCCCT-CATAGAGG-3' (antisense). The 5'-end of the sense primer was labeled with the dye 6-carboxyfluorescein (FAM). The PCR reaction contained 40ng DNA, 10x PCR Buffer II, 2mM dNTP, 2.5mM MgCl2, 0.5µM of each primer and 1.1U AmpliTaq GOLD™ DNA polymerase (all Applied Biosystems, Foster City, CA, USA). PCR was performed by means of the GeneAmp™ PCR System 9700 (Applied Biosystems). Initial DNA denaturation was achieved at 94°C for 10min. Further, DNA samples underwent 35 cycles of denaturation at 94°C for 30s, annealing at 60°C for 30s and extension at 72°C for 30s. The last cycle was followed by additional 7min at 72°C. Amplified PCR products were denaturated and samples were processed for genotype analysis on the ABI Prism™ 3100 Genetic Analyzer (Applied Biosystems). Resulting data were analyzed using GeneScan™ Analysis Software (Applied Biosystems). GeneScan™ 500 ROX™ Size Standard (Applied Biosystems) was used as internal fragment size standard. Allele sizes were confirmed by sequencing DNA of homozygous patients.

Statistical analysis

Allele frequencies of the study cohort were tested for consistency with Hardy-Weinberg equilibrium proportions. In brief, logarithms of the probabilities were used in a generalized linear model (GLM), assuming that observed counts follow Poisson distributions, with expected values of which the logarithms are linear combinations of parameters

to be estimated. The ten observed counts for females were modeled this way. The four observed counts for males had expected values that were proportional to the allele probabilities themselves, because single alleles were involved not pairs. The observed counts for males were also fit into the GLM. To combine females and males in the model, offsets were used. Offsets were equal to the logarithms of the total values for females and males, respectively. The function glm() in R was used to fit the GLM into software and delivered parameter estimates with standard errors and the deviance, a measure of discrepancy between data and model [16]. A p-value was computed assuming chi-squared distribution.

Cumulative renal allograft survival censored for death was analyzed by means of the Kaplan-Meier method. Potential associations of variables with graft survival were analyzed using the Cox proportional hazards regression model; apart from the variable graft failure, the remaining 15 variables listed in Table 1 represented candidate predictor variables for this analysis. Because the number of available variables exceeded the number of variables allowed to be entered in the model (number of events = 146), a univariate analysis was performed on all variables individually. The twelve variables with the lowest p-values ($p < 0.489$) were included in the first run of the multivariate Cox proportional hazards regression model. Variable selection was accomplished by backward elimination using likelihood ratio tests. When no further variables met the criteria for exclusion, the three variables that were initially excluded from the model (gender, primary disease and warm ischemia period) were added to the variables of the first analysis run. The backward elimination procedure was applied to the second run of the analysis. Statistical analyses were carried out using IBM SPSS Statistics 17.0.2 and GraphPad Prism 5 (GraphPad Software Inc.). Values of $p < 0.05$ were considered to be statistically significant.

RESULTS

Clinical data

In order to review the data not biased by surgical complications and immediate graft loss, 599 (95%) out of the 632 first-time renal transplant patients with available material for genotype determination were included in the study population; characteristics of the study cohort are shown in Table 1. 146 (24%) cases of the study cohort experienced graft failure. Primary cause of graft failure was interstitial fibrosis and tubular atrophy (IF/TA; 51%).

Table 1. Characteristics of the study population and univariate Cox proportional hazards regression analysis with regard to graft survival.

	Study population (n = 599)	Univariate Cox regression analysis p-value
Recipient		
Age [year]	47.6 ± 13.7	0.036
Gender [M/F]	348 / 251	0.990
Graft failure [GF-/GF+]	453 / 146	n/a
FOXP3 gene polymorphism [SG/LG]	375 / 224	0.014
Primary disease [NIB/IB][1]	409 / 157	0.991
PRA maximum [NI/I/HI][2]	385 / 176 / 34	0.026
PRA at transplantation [NI/I/HI][3]	517 / 69 / 9	0.089
Induction therapy [-/+]	578 / 21	0.290
Donor		
Age [year][4]	46.3 ± 14.9	0.065
Gender [M/F]	294 / 305	0.139
Transplant		
Graft origin [LD/DD]	288 / 311	0.002
Cold ischemia period [minute]	719.8 ± 617.4	0.010
Warm ischemia period [minute]	35.1 ± 15.1	0.489
HLA mismatch[5]	2.7 ± 1.6	0.012
Delayed graft function [DGF-/DGF+]	492 / 107	0.224
Acute rejection [AR-/AR+]	413 / 186	0.000

[1-5] Missing information: [1]n = 33, [2]n = 4, [3]n = 4, [4]n = 10, [5]n = 3; mean ± SD
M, male; F, female; SG, S-genotype group; LG, L-genotype group; NIB, non-immunological background; IB, immunological background; PRA, panel reactive antibody; NI, non-immunized (PRA ≤10%); I, immunized (PRA 11% - 84%); HI, highly immunized (PRA ≥ 85%); LD, living (un-)related donor; DD, deceased donor; HLA, human leukocyte antigen.

FOXP3 genotypes in renal transplant patients

Within the study population, six different FOXP3 alleles were identified ranging from $(GT)_{12}$ - $(GT)_{18}$ (Figure 1). Allele frequencies were found to be consistent with Hardy-Weinberg equilibrium proportions (p = 0.23); observed and expected allele frequencies are shown in Table 2. $(GT)_{15}$ and $(GT)_{16}$ alleles were most prevalent in the study cohort, similar to the population frequencies reported by Sánchez et al. [17]. Based on the allelic distribution and the observation described by Bassuny et al. [15] that the $(GT)_{15}$ allele shows higher FOXP3 promoter activity when compared to the $(GT)_{16}$ allele, the FOXP3 alleles were divided into the short (S) class comprising all alleles ≤ $(GT)_{15}$ and the long (L) class containing all alleles ≥ $(GT)_{16}$. As proposed by Clayton [18], the hemizygous patients were included in the respective homozygous genotype group, leading to the genotype groups S/SS, SL and L/LL. By means of determining graft survival censored for death us-

Table 2. Hardy-Weinberg equilibrium (HWE) analysis of the allele frequencies.

Allele probability		0.009	0.009	0.501	0.425	0.054	0.001
Females	$(GT)_n$	12	14	15	16	17	18
	12	0 (0.0)	1 (0.0)	1 (2.4)	0 (2.0)	1 (0.3)	1 (0.0)
	14		0 (0.0)	1 (2.4)	2 (2.0)	0 (0.3)	0 (0.0)
	15			64 (63.0)	114 (106.9)	11 (13.6)	0 (0.3)
	16				45 (45.3)	9 (11.5)	0 (0.3)
	17					1 (0.7)	0 (0.0)
	18						0 (0.0)
Males		4 (3.3)	4 (3.3)	171 (174.4)	146 (147.8)	23 (18.8)	0 (0.4)

Observed values and expected values (in brackets).

ing Kaplan-Meier analysis, it was investigated whether the SL genotype was comparable to the S/SS- or the L/LL-genotype groups. Since no significant difference in graft survival between the SL genotype and the S/SS group ($p = 0.188$) was determined, and due to the observation that the SL genotype had an advantage in graft survival when compared to the L/LL group ($p = 0.007$), the SL genotype was combined with the S/SS group forming the S-genotype group (SG); accordingly, the L-genotype group (LG) comprised the L/LL group. The frequencies of SG and LG within the study population are shown in Table 1.

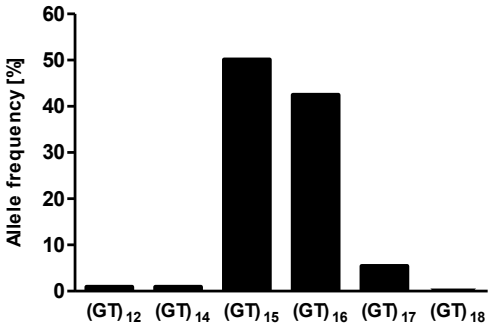

Figure 1. Allele frequencies. Within the study population (number of alleles = 850), six different FOXP3 alleles were identified ranging from $(GT)_{12}$ - $(GT)_{18}$.

Effect of the FOXP3 genetic variants on graft survival

The effect of the FOXP3 $(GT)_n$ dinucleotide repeat polymorphism on graft survival censored for death was studied in relation to the two patients genotype groups SG and LG

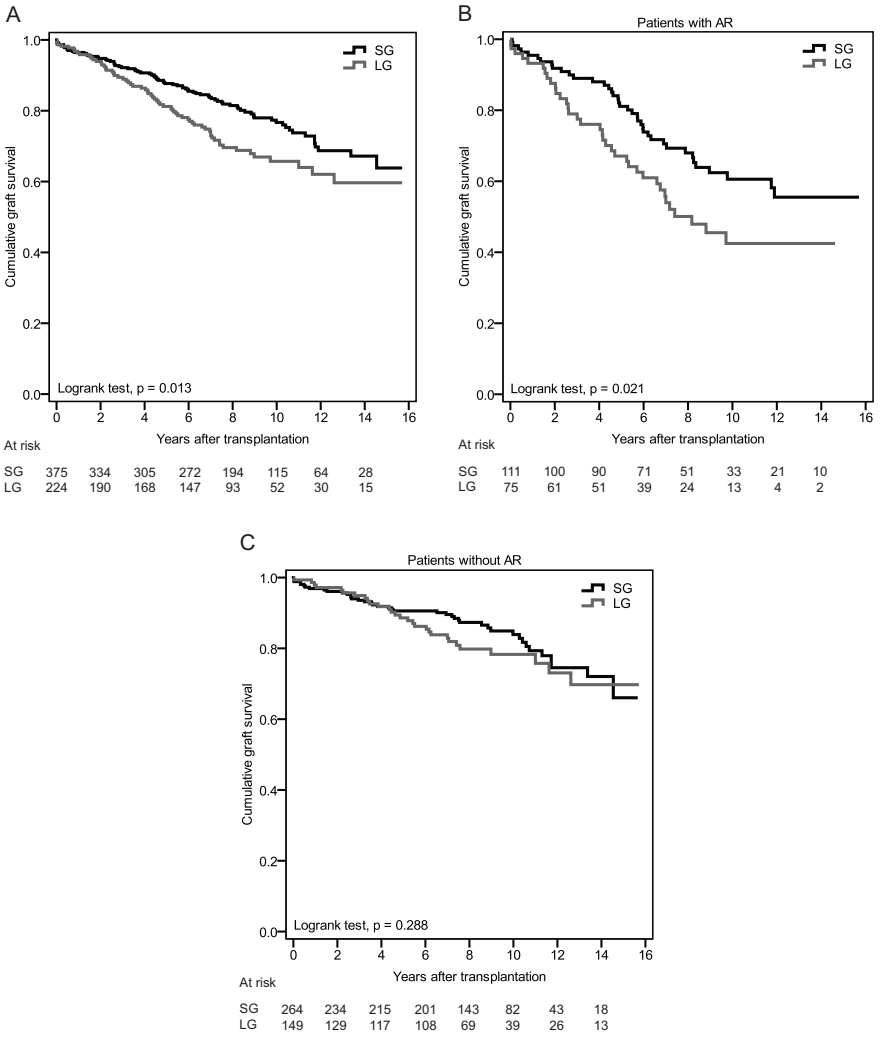

Figure 2. Impact of the FOXP3 genetic variants on renal allograft survival censored for death in kidney transplant patients. The interrelation between the two different FOXP3 genotype groups and graft survival censored for death in kidney transplant patients is shown as Kaplan-Meier curves. The black curve represents the S-genotype group (SG); the L-genotype group (LG) is depicted in grey. (A) Graft survival censored for death in the entire study population. SG patients showed an advantage in graft survival over LG patients (logrank test, p = 0.013). (B) Graft survival censored for death in patients with AR episodes. SG patients with AR episodes showed superior graft survival when compared to LG patients (logrank test, p = 0.021). (C) Graft survival censored for death in patients without AR episodes. The FOXP3 genetic variants had no impact on renal graft survival in patients who did not experience AR (logrank test, p = 0.288).

using Kaplan-Meier analysis (Figure 2A); a significant difference between the SG patients and the LG patients was observed, identifying an advantage in graft survival for the SG over the LG (logrank test, p = 0.013).

Effect of the FOXP3 genetic variants on the occurrence of acute rejection

It was investigated whether the presence of a specific FOXP3 genotype prevented the occurrence of acute rejection. At least one acute rejection episode was experienced by 30% (111/375) of the patients within the SG and by 34% (75/224) of the LG. No association between the occurrence of acute rejection and the two different FOXP3 genotype groups was found (Pearson Chi-square test, p = 0.32).

Effect of the FOXP3 genetic variants on graft survival following acute rejection

It was analyzed whether the FOXP3 $(GT)_n$ dinucleotide repeat polymorphism influenced graft survival in patients with or without acute rejection episodes. Within the study cohort 31% (186/599) patients experienced acute rejection that required anti-rejection therapy. When acute rejection occurred, it was observed that SG patients had an advantage in graft survival censored for death over LG patients (logrank test, p = 0.021; Figure 2B). The FOXP3 genetic variants had no impact on renal graft survival when patients did not experience acute rejection (logrank test, p = 0.288; Figure 2C).

Univariate and multivariate Cox proportional hazards regression analyses

The results of the univariate Cox regression analysis with regard to graft survival are shown in Table 1. Amongst other variables, the FOXP3 $(GT)_n$ dinucleotide repeat polymorphism presented as a factor associated with graft survival. In order to investigate

Table 3. Multivariate Cox proportional hazards regression analysis of factors associated with renal allograft failure in the study population.

	Hazard ratio (HR)	95% CI for HR	p-value
Recipient age (per year)	0.98	0.97 – 0.99	0.001
FOXP3 gene polymorphism (LG)	0.67	0.48 – 0.94	0.020
PRA maximum			0.019
PRA maximum (I)	1.22	0.84 – 1.77	0.309
PRA maximum (HI)	2.36	1.29 – 4.32	0.005
Donor age (per year)	1.02	1.01 – 1.03	0.005
Graft origin (LD)	1.97	1.36 – 2.86	0.000
HLA mismatch (per mismatch)	1.15	1.03 – 1.28	0.017
Acute rejection (AR-)	2.62	1.87 – 3.67	0.000

CI, confidence interval; SG, S-genotype group; PRA, panel reactive antibody; I, immunized (PRA 11% - 84%); HI, highly immunized (PRA ≥ 85%); LD, living (un-)related donor; HLA, human leukocyte antigen. For categorical variables reference categories are stated in brackets.

whether the FOXP3 $(GT)_n$ polymorphism represented an independent factor in terms of renal graft survival, a multivariate Cox regression analysis was performed (Table 3). The analysis revealed that the FOXP3 $(GT)_n$ polymorphism had an independent influence on graft survival. A protective impact on graft survival was confirmed for the genetic variants of the FOXP3 $(GT)_n$ polymorphism of the SG (HR = 0.67, 95% CI 0.48 – 0.94, p = 0.02).

Effect of the FOXP3 genetic variants on graft failure

Analyses were performed to explore whether an association existed between the FOXP3 $(GT)_n$ dinucleotide repeat polymorphism and the causes of graft loss. The cause of graft failure was reported in 112 (77%) out of the 146 cases. IF/TA (51%) was found to be the main cause leading to functional loss of the graft. No association between the two FOXP3 genotype groups and the occurrence of IF/TA was observed (Pearson Chi-square test, p = 0.88).

DISCUSSION

The present gene polymorphism association study retrospectively investigated the influence of different FOXP3 gene variants on graft survival in kidney transplant patients. In order to avoid selection bias, all 599 (95%) first-time kidney transplant recipients with available DNA samples and initially functioning grafts from the center's kidney transplant population of the years 1995-2005 were included in the study. In the group of patients who experienced acute rejection a correlation between the FOXP3 genotypes and an advantage in graft survival was observed; no association between graft survival and the FOXP3 gene variants was found when acute rejection episodes did not occur. In cases of acute rejection, SG patients showed a significant advantage in graft survival censored for death when compared to kidney recipients who belonged to the LG. The presence of one S-allele therefore seems to be sufficient to reach a threshold effect of FOXP3 promoter activity which infers protection against graft loss. Interestingly, graft survival of patients with an SL genotype was not negatively affected by the presence of an L-allele. A similar effect was observed in females heterozygous for FOXP3 mutations [19, 20]. Although these women carried a mutated FOXP3 gene on one of their X-chromosomes, which caused the development of IPEX in their sons, they were healthy. Surprisingly, as opposed to other X-linked diseases such as chronic granulomatous disease [21], no intermediate phenotypes were found in female carriers of IPEX. Studies on these women revealed that no preferential X-chromosome inactivation (XCI) occurred; both wild type FOXP3 alleles and mutated FOXP3 alleles were expressed to similar degrees in $CD4^+$ effector T cells and $CD8^+$ T cells [19, 20]. However, random XCI was not observed for natural Treg of females carrying FOXP3 mutations; only X-chromosomes carrying the wild type FOXP3 were

found to be active. In contrast, $CD4^+CD25^{high}$ and $CD4^+CD25^-$ T cells from healthy females showed a random XCI pattern [20], further substantiating the fact that in females carrying FOXP3 mutations only natural Treg with an active wild type FOXP3 allele are selected to generate the Treg subset. These results imply that Treg with a mutated FOXP3 gene do not mature due to wild type Treg stimulus, or are depleted during Treg differentiation. In the patient populations of the present study and Sánchez et al. [17], the S-allele was found to be the most prominent allele. It can be assumed that in patients with the SL genotype, the S-allele of FOXP3 is active in Treg and Treg with the L-allele are cleared via the same mechanism which depletes Treg with mutated FOXP3 genes in female carriers of IPEX. Thus, with reference to the FOXP3 promoter activity in Treg and its correlation to the FOXP3 $(GT)_n$ dinucleotide repeat polymorphism, the effect of the SL genotype on graft survival is comparable to the S/SS-genotype group; all of these patients only develop Treg with the S-allele on the active X-chromosome.

Higher FOXP3 promoter activity in the S-genotype group might be associated with increased Treg potency and possibly contributes to kidney recovery after rejection, explaining the advantage in graft survival of SG patients over LG patients. In fact, studies demonstrated a correlation between increased FOXP3 mRNA levels in urinary cells, peripheral blood and kidney tissue and a favorable renal graft outcome in the event of acute rejection [22-24]. Further, recruitment of FOXP3-expressing Treg to the allograft advances the facilitation of transplant engraftment; increased renal Treg-infiltrates in patients with subclinical rejection positively correlated with graft function [25]. T cells with a regulatory phenotype were also found in grafts of tolerant liver transplant patients [26] and cardiac grafts after acute rejection [27]. Although studies with conflicting results have been published [28, 29], there is substantial evidence that FOXP3-expressing Treg play an important role in graft amelioration after acute rejection episodes leading to better long-term graft outcomes.

Albeit Treg execute their immunosuppressive role through a variety of processes [8], the study by Thornton et al. [30] portends that an initiation stimulus is required to establish Treg function and to activate the FOXP3 up-regulation process. This assumption correlates with the finding of the present study that none of the different FOXP3 genotypes was capable of preventing the occurrence of acute rejection. However, when acute rejections arose, SG patients showed superior graft survival when compared to LG patients.

Treg contribution to graft survival can also be explained by their favorable impact on ischemia-reperfusion injury (IRI). Gandolfo et al. [1] demonstrated that Treg were present in post-ischemic kidneys. Depletion of Treg worsened tubular damage and increased renal failure while Treg administration promoted healing and kidney repair. Complementary findings indicating a beneficial role of Treg in renal repair after IRI were reported by Kinsey et al. and Baan et al. [2, 31]. Different mutations in the FOXP3 gene that affect the gene function have been described [32-35]. These mutations are associated with

autoimmune diseases. A single nucleotide polymorphism (SNP) in the FOXP3 gene at the rs3761548 locus is associated with the prevention of allergic rhinitis in the Chinese and Hungarian population [34, 35]. The FOXP3 $(GT)_n$ gene polymorphism that was the focus of this study has been associated with type 1 diabetes in the Japanese population [15]. Currently it is unknown whether the SNP at the rs3761548 locus in the FOXP3 gene is in linkage disequilibrium with the studied FOXP3 $(GT)_n$ dinucleotide repeat polymorphism.

Many factors contribute to the outcome of graft survival. Multivariate analysis by means of Cox proportional hazards regression analysis revealed that the FOXP3 $(GT)_n$ dinucleotide repeat polymorphism is an independent protective factor for allograft survival after kidney transplantation. FOXP3 gene polymorphisms have also been reported to be associated with the onset of autoimmune diseases. Iwase et al. found the FOXP3 $(GT)_n$ dinucleotide repeat polymorphism to be related to the onset of type 1 diabetes in adult patients [36]. Further, studies on other FOXP3 polymorphisms indicated a correlation between FOXP3 mutations and the susceptibility to autoimmune disorders such as systemic lupus erythematosus (SLE) and psoriasis [37, 38]. These findings suggest a relation between mutations within the FOXP3 gene and alterations in immune tolerance. Yet, contradictory results exist as other studies did not find associations of FOXP3 gene polymorphisms with the advent of autoimmune diseases such as SLE, rheumatoid arthritis (RA), inflammatory bowel disease, and type 1 diabetes [17, 39, 40].

Currently, there is a lot of debate about the value of result replication in confirmation cohorts and whether replication requirement is valid [41-45]. However, this single-center study was performed in a cohort consisting of 599 patients who consecutively underwent first-time kidney transplantation. The high number of patients included in the study allows confidence that gene associations can be unequivocally detected. Moreover, the role of regulatory $FOXP3^+$ T cells in alloreactivity and tissue injury is well established [46, 47]. Our gene polymorphism study supports the controlling role of regulatory $FOXP3^+$ T cells in tissue injury after organ transplantation.

In conclusion, this study is the first gene association analysis in renal transplant patients that identified a beneficial effect of FOXP3 gene variants on graft survival. In the event of acute rejection, patients with an S-, SS- or SL genotype with regard to the FOXP3 $(GT)_n$ dinucleotide repeat polymorphism have a better graft outcome when compared to patients expressing an L- or LL genotype.

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Chapter 6

Summary and discussion

Partly based on "On the interactions between MSC and Treg for immunomodulation in transplantation"

Anja U. Engela[1], Carla C. Baan[1], Frank M.J.F. Dor[2], Willem Weimar[1], Martin J. Hoogduijn[1]

[1]Department of Internal Medicine, Section Nephrology and Transplantation, [2]Department of Surgery, Erasmus University Medical Center, Rotterdam, the Netherlands

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SUMMARY

The research described in this thesis aimed to investigate the potential of adipose tissue-derived MSC and Treg to be used as cellular therapy for controlling anti-donor reactivity in solid organ transplantation. Long-term administration of drug-based immunosuppressive therapies has detrimental consequences such as a higher risk of tumour development, occurrence of diabetes and increased susceptibility to infection. Thus, the development of alternative treatments is required to minimise drug exposure. Cells with immunomodulatory properties are regarded as a viable treatment option and their safety and feasibility as cell therapy for different indications is currently being established in clinical trials as outlined in **chapter 1**. Despite a substantial improvement in understanding how immunosuppressive cells mediate their function over the past years, much more in-depth knowledge is required to optimally apply their capacities to the prevention of graft rejection. In particular, interactions of the administered cells with the recipient's effector cells and immunosuppressive cells should be evaluated.

In **chapter 2**, the interaction between MSC and nTreg was investigated. MSC were characterized based on their expression of cell surface molecules and their differentiation ability. MSC expressed CD90, CD105, CD166 and HLA-I molecules while lacking expression of CD14, CD34, CD45 and HLA-II. MSC were able to differentiate into adipocytes and osteoblasts. Both MSC and nTreg suppressed the proliferative activity of allo-stimulated $CD25^{-/dim}$ effector T cells in a dose-dependent manner. MSC inhibited the proliferation of allo-stimulated $CD25^{-/dim}$ effector T cells from a concentration of 1:160 (MSC/effector cell ratio). nTreg suppressed $CD25^{-/dim}$ effector cell proliferation from a concentration of 1:10 (nTreg/effector cells). In co-cultures, MSC and nTreg did not affect each other's immunosuppressive function. To examine MSC-Treg interaction in a transplant setting, MSC (1:40; MSC/effector cells) from the kidney donor were co-cultured with nTreg (1:10) obtained from the respective kidney recipient 6 months post-transplant. Similar to the healthy setting, both cell types did not negatively influence each other's suppressive capacities. The immunosuppressive functions of MSC and nTreg affected $CD4^+$ T cells and $CD8^+$ T cells; within these populations non-memory T cells were more susceptible to the inhibitory effect of MSC and Treg than memory T cells. MSC mediated their immunosuppressive function largely through the expression of IDO leading to tryptophan starvation. Further, MSC establish an anti-inflammatory environment by influencing the secretion of TNF-α and IL-10 by immune cells.

Another mechanism which MSC employ to facilitate their immunosuppressive function was investigated in the study performed in **chapter 3**. While MSC (1:40; MSC/effector cell ratio) reduced the proliferation of $CD25^{-/dim}$ effector T cells by 59%, they mediated

a 2-fold increase in the percentage of $CD25^+CD127^-FOXP3^+$ cells within the $CD4^+$ T cell population. These $CD4^+CD25^+CD127^-FOXP3^+$ iTreg were functional and as potent as nTreg in suppressing effector T cell alloreactivity. To confirm the *de novo* generation of the iTreg, the methylation status of the Treg-specific-demethylated-region (TSDR) within the FOXP3 gene was analysed. This specific gene locus is fully methylated in iTreg and demethylated in thymus-derived nTreg. The nTreg fraction comprised 32.7% of cells with a methylated TSDR. Contrarily, the iTreg fraction contained 83.7% of TSDR-methylated cells, confirming their non-thymic origin. Further characterisation of iTreg showed that they expressed CTLA-4, GITR and Helios. As IL-2 is essential for nTreg expansion and function, its involvement in the MSC-mediated generation of iTreg was assessed. Despite the reduced T cell proliferation in MSC-MLR co-culture, under these conditions the IL-2 concentration was 15.1-fold higher than in MLR alone. IL-2 is not expressed by resting or activated MSC, indicating that the IL-2 was secreted by effector cells. In fact, activated $CD4^+CD25^+FOXP3^-$ T cells were the population with the highest percentage of IL-2-expressing cells. Inhibition of the IL-2 pathway by the IL-2 receptor antagonist basiliximab reduced the percentage of iTreg in MLR and prevented MSC-mediated iTreg generation, demonstrating the involvement of IL-2 in the induction of *de novo* Treg.

Chapter 4 presents research on the effect of MSC on $CD8^+CD28^-$ T cells. $CD8^+CD28^-$ T cells had proliferative capacities after allogeneic stimulation similar to those of $CD8^+CD28^+$ T cells indicating that they are able to inflict alloreactivity in transplant patients. Proliferating $CD8^+CD28^-$ T cells had a cytotoxic and cytolytic phenotype; they expressed IFN-γ, TNF-α and granzyme B. The CTLA-4-Ig belatacept binds to CD80/CD86 on APC and thereby blocks the CD28-CD80/86 co-stimulation pathway required for T cell activation and proliferation. Consequently, $CD8^+CD28^-$ T cells escape the immunosuppressive function of belatacept. MSC were considered as co-therapeutic agent and the combined effect of belatacept and MSC on effector cells was studied. Both belatacept and MSC inhibited effector cell proliferation in a dose-dependent manner. In combination both immunosuppressants did not influence each other's function; at low concentrations belatacept and MSC had an additive inhibitory effect. Belatacept suppressed the alloreactivity of $CD8^+CD28^+$ T cells, but did not affect $CD8^+CD28^-$ T cell proliferation. In contrast, the proliferative capacity of both $CD8^+CD28^-$ T cells and $CD8^+CD28^+$ T cells was inhibited by MSC. MLR-co-culture with belatacept and MSC also prevented the proliferation of $CD8^+CD28^-$ T cells and $CD8^+CD28^+$ T cells, implying that belatacept did not restrain MSC function. Further, MSC did not render $CD8^+CD28^-$ T cells apoptotic nor did they influence CD28 expression on $CD8^+$ T cells, confirming a true anti-proliferative effect of MSC on $CD8^+CD28^-$ T cells.

In **chapter 5** a retrospective study is described evaluating the impact of a FOXP3 $(GT)_n$ dinucleotide repeat polymorphism on graft survival in 599 first-time kidney transplant

patients. Six different FOXP3 alleles were identified ranging from $(GT)_{12}$-$(GT)_{18}$. As the promoter of the $(GT)_{15}$ allele was reported to have a higher activity than the $(GT)_{16}$-allele promoter, all alleles $\leq(GT)_{15}$ were included in the short (S) class, while alleles $\geq(GT)_{16}$ were comprised in the long (L) class. Since the FOXP3 gene is located on the X chromosome, male patients only possess one FOXP3 allele. These hemizygous patients were combined with the respective homozygous genotype group resulting in the genotype groups S/SS, SL and L/LL. Graft survival censored for death was similar for the SL and the S/SS genotype group, while the SL group had an advantage over the L/LL genotype group. Therefore, the SL group and the S/SS group were combined and formed the S-genotype group (SG); the L-genotype group (LG) consisted of the L/LL genotypes. SG patients had an advantage in graft survival over LG patients in a Kaplan-Meier analysis. The protective association of the SG FOXP3 polymorphism with graft survival was confirmed by multivariate Cox regression analysis. The occurrence of acute rejection was not associated with the FOXP3 $(GT)_n$ polymorphism; in both genotype groups similar percentages of patients experienced at least one acute rejection episode. However, when acute rejection occurred, patients of the SG group had an advantage in graft survival over LG patients. This association was not found in patients without acute rejection, demonstrating the beneficial effect of the S-, SS-, and SL-genotypes on graft survival.

DISCUSSION

The goals of immunosuppression in organ transplantation are the prevention and treatment of acute rejection episodes, and the optimization of long-term patient and graft survival. Balancing the dosage of anti-rejection drugs is essential to minimise the risk of rejection and toxicity. Cells with immunosuppressive capacities are being investigated regarding their suitability to prevent graft rejection and hence present a potential mean to reduce conventional immunosuppressive medication. Administration of therapeutic cells to transplant patients potentially inflicts unfavourable effects on the function of resident immunosuppressive cells. A study described in this thesis demonstrates that MSC derived from kidney donors permitted the function of Treg from healthy blood donors as well as Treg from renal transplant patients. Similarly, Treg did not hinder the MSC-mediated suppression of alloreactivity. This important property of MSC is not shared by all currently prescribed immunosuppressive drugs. While some immunosuppressants are permissive for Treg function such as corticosteroids, anti-CD3 antibodies, ATG, anti-CD52 antibodies and mTOR inhibitors [1], cyclosporine A and tacrolimus can negatively influence the development and suppressive activity of Treg [2].

Another result of this thesis is that adipose tissue-derived MSC are able to generate de novo iTreg with anti-proliferative functions. Treg induction mediated by bone marrow-

derived MSC has already been observed in pre-clinical studies [3, 4]. In a semi-allogeneic murine heart transplant model, a single pre-transplant infusion of donor-MSC into the portal vein led to T-cell hyporesponsiveness, prolonged cardiac allograft survival and expanded donor-specific Treg [3]. Similar observations were made after administration of recipient-derived MSC. Of relevance for the translation into a clinical setting, this group noticed that double pre-transplant intraportal and intravenous infusions were more tolerogenically effective than the single MSC infusion. In contrast, peri-transplant or 24h post-transplant administration of MSC was less effective. This suggests that pre-exposure to MSC is required and that pre-activation of MSC might be necessary for MSC to successfully exert their protolerogenic function when graft alloantigens challenge the recipient's immune system. Additional evidence that the generation of Treg by MSC contributes to graft survival is provided by a kidney allograft mouse model [5]. In this mouse model, in contrast to the findings by Casiraghi et al. [3], intravenous administration of MSC 24h after renal transplantation inhibited T-cell proliferation. In tolerant recipients a Th2-dominant cytokine shift was observed; IFN-γ production was significantly decreased and IL-4 levels augmented. Further, in recipient spleens, frequencies of Treg were higher in MSC-treated mice. Ge and colleagues also found a significant increase of intragraft $FOXP3^+$ cells after MSC treatment, suggesting Treg recruitment to the renal allograft. In this model, depletion of Treg using an anti-CD25 monoclonal antibody also reversed the beneficial effect of MSC therapy. $CD25^+$ T-cell depletion caused graft rejection despite MSC treatment.

To date the results of six clinical studies with MSC in kidney transplantation have been published (Table 1). The objective of all trials was to evaluate the safety and feasibility of MSC administration to living kidney transplant recipients. All patients tolerated MSC infusions well without local complications or adverse events. Stable graft function was reported for up to 1 year after MSC treatment. However, in a case study the two enrolled patients experienced a dramatic increase of serum-creatinine levels 1-2 weeks after cell infusion which were not related to cellular or humoral rejections, indicating reduced kidney function [6]. In contrast, the 106 MSC-treated patients in the study by Tan et al. had a better recovery of kidney function during the first month after transplantation based on increased estimated glomerular filtration rate (eGFR) compared to the control group. The MSC-group further showed reduced incidences of acute rejections and glucocorticoid-resistant rejections and had a decreased risk of opportunistic infections [7]. In contrast, in a trial performed at the LUMC, three of the six MSC-treated patients developed opportunistic infections caused by cytomegalovirus (CMV) or BK virus [8]. The other 4 trials did not report on opportunistic infections. Due to the low number of subjects treated in some trials, the results of these studies are little conclusive. The Chinese investigation, however, suggests that MSC have a beneficial effect on graft function recovery while not exposing the patient to a higher risk of developing opportunistic infections.

Table 1. Overview of published clinical trials with mesenchymal stem cells in solid organ transplantation.

Study population (MSC-treated patients)	MSC source	MSC dose* [cells/kg BW]	Experimental group		Control group	Main outcomes	Reference
			Induction therapy	Maintenance therapy	Complete i.s. therapy		
Living kidney transplant patients; n=2	Autologous, bone marrow-derived	$1.7x10^6$ or $2x10^6$ (day 7)	Basiliximab (day 0+4), Low-dose ATG (day 0-6)	CsA, MMF, steroids (day ≤7)	as experimental group; n=3	MSC infusion is feasible; increase in serum creatinine (day 14-21); no cellular or humoral rejection; increased peripheral Treg; decreased memory CD8+ T cells; stable graft function up to 1 year	Perico et al., 2011
Living kidney transplant patients; n=2	Autologous, bone marrow-derived	$2x10^6$ (day -1)	Low-dose ATG (day 0-6)	CsA, MMF, steroids (day ≤7)	as experimental group; induction therapy: (I) Low-dose ATG (day 0-6); n=6 (II) Basiliximab (day 0+4), low-dose ATG (day 0-6); n=6	Uneventful MSC infusion; one acute rejection; increased peripheral Treg; decreased memory CD8+ T cells and CD8+ T cell cytolytic function; stable graft function up to 1 year	Perico et al., 2013
Living kidney transplant patients; n=106; (I) 53, (II) 53	Autologous, bone marrow-derived	$1-2x10^6$ (day 0+14)	-	(I) Standard-dose CsA or Tacro, MMF, steroids (II) Low-dose CsA or Tacro, MMF, steroids	Anti-IL-2R (day 0+4), standard-dose CsA or Tacro, MMF, steroids; n=53	MSC infusion is safe; reduced incidence of acute rejections and glucocorticoid-resistant rejections; increased eGFR; improved renal function; reduced risk of opportunistic infections; stable graft function at 1 year	Tan et al., 2012

Table 1. (continued)

Study population (MSC-treated patients)	MSC source	MSC dose* [cells/kg BW]	Experimental group		Control group	Main outcomes	Reference
			Induction therapy	Maintenance therapy	Complete i.s. therapy		
Living kidney transplant patients; n=6	Kidney donor, bone marrow-derived	$5x10^6$ (day 0); renal allograft artery) + $2x10^6$ (day 30)	Cyclophospha-mide, methyl-prednisolone (day 0-3)	Low-dose Tacro (day ≥4), MMF, steroids	Cyclophosphamide + methylprednisolone (day 0-3), standard -dose Tacro (day ≥4), MMF, steroids; n=6	MSC infusion is safe; less (no) occurrence of acute rejection; increased B cell frequency at 3 months; no peripheral chimerism; stable graft function at 1 year with reduced Tacro concentrations	Peng et al., 2013
Living kidney transplant patients with SCR and IF/TA; n=6	Autologous, bone marrow-derived	1-$2x10^6$ (SCR and/or IF/TA at 24-week biopsy post-transplant; infusion 7 days apart)	Basiliximab (day 0+4)	CsA or Tacro, MMF, steroids	Basiliximab (day 0+4), CsA or Tacro, MMF, steroids; n=9	MSC infusion is feasible and was well-tolerated; occurrence of opportunistic viral infection in 3 patients; resolution of tubulitis without IF/TA after rejection; decreased donor-specific lymphocyte proliferation; stable graft function up to 24 weeks after MSC infusion	Reinders et al., 2013
Living kidney transplant patients; n=7	Kidney donor, bone marrow-derived	$1x10^6$ (day 0; bone marrow of iliac bone) + $2x10^6$ (day 30)	ATG (day ≤8-10)	CsA or Tacro, MMF, steroids	ATG (day 0-10), CsA or Tacro, MMF, steroids; n=4	MSC infusion is feasible and was well-tolerated; no increase in rejection rates; no peripheral chimerism; stable graft function at 1 year	Lee et al., 2013

* intravenous administration unless stated otherwise.

BW, body weight; i.s., immunosuppressive; ATG, rabbit anti-thymocyte globulin; CsA, cyclosporine A; MMF, mycophenolate mofetil; Tacro, tacrolimus; eGFR, estimated glomerular filtration rate; SCR, subclinical rejection; IF/TA, interstitial fibrosis/tubular atrophy.

Because the published trials were of exploratory nature with their main focus on MSC safety, only a few trial protocols allowed first insights into MSC pharmacology in a human solid organ transplantation setting. Four of the six trials, conducted in three different centres, looked into the aspect of Treg expansion by MSC. The Italian case studies implied the enlargement of the Treg population one month after MSC infusion [6, 9]. Since low-dose ATG was used as induction therapy in these patients, a treatment known to expand functional Treg in vitro [10-12], it cannot be ruled out that the effects on Treg expansion are due to ATG therapy and not evoked by MSC. Lee and colleagues reported for two patients an increased FOXP3 mRNA expression after the first MSC infusion, which further enhanced after the second MSC dose [13]. In the other five patients FOXP3 mRNA levels were unaffected by MSC treatment. The pilot study by Peng et al. did not observe differences in Treg proportions between the experimental group and the control group during the first year after transplantation [14]. Although the observations result from a small set of patients, they indicate that MSC potentially have the ability to enlarge the Treg pool in kidney recipients. If this finding still holds true in clinical trials with larger patient populations, it would in part confirm the results described in this thesis, that MSC induce de novo Treg and expand existing Treg. However, available techniques such as TSDR determination will then have to be applied to identify Treg origin and to fully confirm the data obtained in the scope of this thesis. MSC-mediated Treg expansion and possibly Treg induction in humans has also been indicated in a non-transplantation setting. Ten patients suffering from fistulising Crohn's disease, a chronic inflammatory bowel disease with dysregulation of immune tolerance towards intestinal bacteria were enrolled in this trial [15]. They received a median of four intrafistular injections (range 2-5) of autologous MSC, scheduled four weeks apart. Closure of fistula tracks in seven patients and disease improvement in the other three patients coincided with a suggested increase in mucosal and peripheral Treg. Once more, the number of patients who underwent MSC treatment in this trial limits the conclusions that can be drawn.

Although the clinical trials listed in Table 1 are very different with regard to their study set-up, all protocols succeeded to prove that MSC administration is safe and feasible. Moreover, despite the use of autologous or donor-derived MSC, up to 5-fold differences in MSC dose and large variations in the time of MSC infusion (pre-, peri- or post-transplant), all patients enjoyed stable graft function. However, when MSC studies will advance to the next development stage, MSC origin, dose and the time-point of cell administration will become crucial for optimising MSC therapy and demonstrating its efficacy. To better understand MSC-mediated immunomodulation in vivo, the distribution of MSC in the body after administration was explored by multiple studies [16-21]. After intravenous infusion MSC accumulate in the lung. The size of cultured MSC is significantly larger than the size of other immune cells in circulation, presumably causing MSC to be trapped in the

capillaries of the lung. When MSC are administered via alternative routes, they are also found in other organs such as liver and spleen [22]. Despite the consistency of the data, the drawback of these studies is that researchers cannot be certain that label detection confirms the presence of living MSC. Retrieved label can originate from viable MSC, deceased MSC or possibly phagocytosed debris of MSC. To gain more clarity on this issue, a different approach has been examined [23]. After intravenous administration of labelled MSC to mice, organs were harvested, and MSC were isolated and re-cultured. Interestingly, labelled MSC were found in the lung up to 24h after infusion, but in none of the other observed organs at any time-point after administration. The apparent shortevity of MSC after infusion fortifies the importance of rapid Treg induction by MSC. While MSC are retained in different tissues shortly after administration and subsequently cleared, they pass on their immunomodulatory effect to other immunosuppressive mediators. Next to Treg expansion or peripheral de novo induction from effector T cells, MSC are able to educate macrophages [24], a capacity also described for Treg [25]. In close proximity with MSC, macrophages adapt an anti-inflammatory and immunosuppressive phenotype. They produce elevated levels of anti-inflammatory IL-10 and IL-6, while the secretion of the pro-inflammatory cytokines TNF-α and IL-12 is reduced [24]. Administration of regulatory macrophages (Mreg) to two recipients of living-donor kidney grafts allowed drug weaning to low-dose tacrolimus monotherapy within 24 weeks and resulted in stable graft function [26]. Evidence from one patient suggests that Mreg transfer elevates FOXP3 mRNA levels which are related to a small increase in circulating Treg. In addition, MSC have the ability to induce tolerance through dendritic cells (DC) [27]. Combined in vivo treatment of MSC and rapamycin was associated with the appearance of non-matured, tolerogenic DC with lowered allogeneic stimulatory capacities and increased Treg frequencies. Hence, despite their short survival after infusion, MSC seem to enforce graft acceptance by manipulating the development or differentiation of immune cells, leading to the generation of cells with newly acquired immunosuppressive and protolerogenic properties.

In addition to the infusion timing and dose of cellular therapy, the accompanying immunosuppressive regimens should be carefully selected. To ensure optimal efficacy of both therapeutic MSC or Treg and immunosuppressive medication in a combined regimen, interplays between cells and drugs have to be studied. While some cell-drug combinations may enhance each other's suppressive function, other combinations may cause impaired efficacy. Research presented in this thesis evaluated the effect of basiliximab and belatacept on MSC and/or Treg function. Both belatacept and basiliximab did not negatively influence the suppressive capacities of MSC in vitro. At low concentrations the combination of MSC and belatacept resulted in an additive immunosuppressive effect. This effect was caused by MSC-mediated suppression of $CD8^{+}CD28^{-}$ T cells that

are not susceptible to belatacept treatment. In vivo and clinical studies on belatacept/MSC co-therapy still have to be performed to verify whether the in vitro findings translate to these settings. While basiliximab did not hamper the direct immunosuppression by MSC, IL-2 receptor blocking averted the MSC-mediated induction of Treg. Basiliximab induction therapy combined with MSC infusion was used in the clinical studies by Reinders et al. and Perico et al. [6, 8]. Reinders and colleagues administered MSC a minimum of 24 weeks after transplantation. Due to its short half-life of approximately 7 days [28], basiliximab was already eliminated from the patient's body at the time of MSC infusion. Therefore, this study does not allow conclusions about the interaction of MSC and basiliximab in humans. In contrast, in the Italian study MSC infusion at day 7 post-transplant coincided with basiliximab treatment (day 0 and day 4) [6]. In spite of the relatively high basiliximab concentrations in the patient's periphery when MSC were infused, a tendency to increased Treg populations was noticed. The discrepancy between the clinical data and the in vitro observation described in this thesis may arise due to the high basiliximab concentration used in vitro or due to the in vivo presence of Treg-promoting cytokines such as IL-7 and IL-15 [29, 30]. The clinical observation suggests that low-doses of basiliximab may be permissive for the expansion or induction of Treg, but confirmation in larger study cohorts is required.

Attenuation of immunosuppressive medication by MSC (and vice versa), has been observed in vitro. MSC reduce the efficacy of immunosuppressants including rapamycin and tacrolimus and, conversely, these immunomodulatory agents negatively affect MSC function [31, 32]. On the other hand, cumulative inhibition of effector cell proliferation has been reported for combination therapy of MSC with mycophenolic acid (MPA), while dexamethasone did not influence MSC functionality. A synergistic effect of MSC and mycophenolate mofetil (MMF), the prodrug of MPA, on prolonged graft survival was observed in a fully allogeneic heart transplant mouse model [33]; treatment with MSC and cyclosporine A failed to prolong allograft survival in this model. In contrast to in vitro results, combination therapy of MSC and rapamycin achieved long-term heart allograft tolerance in mice and increased the frequency of splenic Treg [27]. This finding is important as rapamycin is currently used for the ex vivo expansion of Treg [34]. In renal transplant patients, rapamycin led to an increase of Treg numbers [35]. A recent in vitro study by Ma et al. further indicated that rapamycin had better synergistic effects on Treg function than cyclosporine A and tacrolimus [36]. Subsequent adoptive infusion of donor-alloantigen-specific Treg in combination with low-dose of rapamycin delayed the acute rejection of kidney allografts in *Cynomolgus* monkeys. In addition, selective expansion of donor-specific Treg after in vivo administration of rapamycin in combination with IL-2 suppressed acute GVHD in mice [37]. These interactions between both immunomodulatory cell types and immunosuppressive drugs demonstrate that the

choice of immunosuppressive regimen will affect the outcome of combined cellular and drug-based therapies.

Another aspect of this thesis was to retrospectively investigate a potential association of the FOXP3 $(GT)_n$ dinucleotide gene polymorphism with graft survival and acute rejection in 599 consecutive first-time kidney transplant recipients. The $(GT)_n$ polymorphism occurs in the promoter region of the FOXP3 gene modulating its activity. Bassuny et al. described that 15 or less (GT) repeats caused increased FOXP3 promoter activity, while promoter function was reduced with 16 or more (GT) repeats [38]. Based on this study, kidney transplant recipients were grouped according to their FOXP3 $(GT)_n$ genotype. Superior overall graft survival and an advantage in graft survival after acute rejection were determined for patients with 15 or less (GT) repeats. The prominent role of the transcription factor FOXP3 in regulating the development and function of Treg is well established as well as its involvement in Treg-mediated tolerance [39, 40]. Various FOXP3 gene polymorphisms have been studied and their presence is commonly related to an imbalance of the immune system leading to autoimmune diseases of different severities [41]. Hence, the gene association study reported in this thesis is one of the few FOXP3 gene association studies that describes a beneficial effect of FOXP3 gene variants. Higher FOXP3 promoter activity as observed in the patients with 15 or less (GT) repeats might be associated with increased Treg potency and possibly contributes to organ recovery after rejection, explaining the advantage in graft survival this kidney recipient group. Other studies support this notion as increased FOXP3 mRNA levels in urinary cells, peripheral blood and kidney tissue are related to favourable renal graft outcome in the event of acute rejection [42-44]. Further, recruitment of FOXP3-expressing Treg to the allograft facilitates transplant engraftment; increased renal Treg-infiltrates in patients with subclinical rejection positively correlated with graft function [45]. T cells with a regulatory phenotype were also found in grafts of tolerant liver transplant patients [46] and cardiac grafts after acute rejection [47]. Although studies with conflicting results have been published [48, 49], there is substantial evidence that FOXP3-expressing Treg play an important role in graft amelioration after acute rejection episodes leading to better long-term graft outcomes. Treg and MSC therapy may benefit from effector T cells and Treg with increased FOXP3 promoter activity. Ex vivo expanded Treg with a $(GT)_{\leq 15}$ genotype may be more competent in the prevention of donor-specific alloreactivity due their potentially enhanced immunosuppressive function. Moreover, the induction of Treg by MSC from effector T cells with elevated FOXP3 levels could ameliorate the efficacy of MSC therapy and allow the reduction of drug-based immunosuppression.

Based on the research presented in this thesis the following main conclusions can be drawn: Treg and MSC share certain immunological targets and pathways, but also

employ cell type-distinct suppressive mechanisms. They are able to convey their immunomodulatory capacities to other cell types including the MSC-mediated induction of Treg. Direct interaction between MSC and Treg did not influence their respective immunosuppressive functions. Further, MSC are able to control the proliferation of $CD8^+CD28^-$ T cells, cells that are not susceptible to belatacept-mediated immunosuppression. The main incentive to use cellular therapy for the maintenance of stable graft function after transplantation is to reduce detrimental drug-related side effects. Therefore, data of this thesis suggest that belatacept/MSC combination therapy may not only provide this benefit, but may also have increased efficacy. Both Treg and MSC have proven to be efficacious as immunomodulatory therapies in animal models. In humans, the safety of both cell therapies has been confirmed in first trials of haematopoietic stem cell transplantation. To date only MSC, not Treg, have been administered to recipients of solid organ grafts and based on these few experiences are deemed safe. The suitability of Treg and MSC for the prevention of anti-donor immune responses has been suggested for haematopoietic stem cell transplantation and/or solid organ transplantation. Yet, our knowledge about the exact mechanisms employed by both cell types to achieve graft acceptance and possibly also about their potential to induce tolerance is still limited. The work described in this thesis contributed to a better understanding of the mechanisms of action of MSC and Treg by elucidating capacities that may play a role in the MSC- and Treg-mediated modulation of immune responses in vivo and in clinical research.

FORWARD-LOOKING REMARKS

Due to lack of an MSC-specific marker, MSC identification is based on a set of functional criteria in combination with phenotypical features. As MSC are a heterogenic population, these criteria should be further refined to identify the most potent MSC subset. To facilitate MSC donation the field should endeavour to select MSC whose source is abundantly available and minimal-invasively accessible such as MSC derived from adipose tissue, and MSC expansion procedures should be internationally standardised. Logistically, allogeneic or third-party MSC are the most suitable option as these cells can be prepared in advance and be readily available for therapy. Despite initial believes, MSC are not immune privileged and a target for $CD8^+$ T cells and NK cell lysis [50-52]. The ability of allogeneic MSC to prevent allo-reactivity, induce tolerance and work in synergy with immunosuppressive drugs is controversial [3, 27, 33, 53, 54]. The fact that autologous MSC are also cleared after infusion [23] implies that in vitro expansion induces alterations in MSC such as the expression of novel adhesion molecules which are regarded as foreign by the MSC donor's immune systems after reinfusion. To minimize the potential impact of in vitro manipulation on MSC immunogenicity, culture conditions should be optimized.

MSC expansion protocols possibly will have to be adapted to reduce MSC size. Cell enlargement during culture causes infused MSC to be trapped in the small capillaries of the lung and may hinder MSC to display their full immunosuppressive potential. Further, short-passaging of MSC should be considered, because MSC of early passages have been shown to be more potent [55] and it will avoid the occurrence of senescent cells which may develop pro-tumourigenic properties. Moreover, it should be investigated whether MSC viability is required for the suppression of anti-donor responses or whether certain molecules on the cell surface of MSC are sufficient; for example, MSC-derived microvesicles are able to protect against kidney injury [56]. In addition, the mediation of immunosuppression through MSC-conditioned medium, cell membrane debris of dead or fixed MSC should be explored. Currently, investigators continue to combine MSC infusion in humans with traditional drug-based regimens in order not to jeopardise patient and graft survival. This approach makes it difficult to define MSC-mediated immunosuppression. Therefore, present and future animal studies should be carefully evaluated and designed; accompanying immunosuppressive drugs should be reduced to gain maximal information about in vivo MSC function and to possibly optimise MSC infusion timing and location.

Next to these more general suggestions to improve the outcome of MSC therapy, more specific considerations based on this thesis can be made:

- Basiliximab should not be used in combination with MSC therapy. If induction therapy is required the anti-CD52 monoclonal antibody alemtuzumab or ATG should be administered. ATG creates lymphopenia and the subsequent leukocyte repopulation can favour immune regulation resulting from the outnumbering of effector cells by Treg. Next to its depleting effect ATG promotes Treg generation [10].
- Combining belatacept treatment with MSC infusions allows CNI-free therapies and reduces nephrotoxicity in transplant patients.
- Due to the in vitro induction and expansion of Treg by MSC, patients receiving MSC therapy should be carefully monitored to assess whether MSC therapy induces a local or global immunosuppressive effect.
- An MSC-mediated increase of the Treg population can be used as surrogate endpoint of MSC efficacy in clinical trials.
- Not only the most potent MSC subset should be identified, also the MSC population that is most efficient in inducing and expanding Treg.
- Vice versa, the most stable and effective Treg population should be determined, possibly through more in-depth knowledge about methylation patterns, and its induction by MSC should be optimised.

- MSC therapy in transplant recipients will be combined with drug regimens proven to lead to good graft outcomes. Hence, identifying a beneficial effect of MSC on clinical outcomes will be challenging; the use of current clinical parameters to assess the efficacy of MSC treatment will have to be complemented by in vitro assays which determine the function of donor-specific effector T cells or in vivo Treg expansion, e.g. the evaluation of Treg phenotype, numbers and function.
- It should be investigated whether biomarkers identified in patients with operational tolerance can contribute to determine beneficial effects of MSC infusions on graft survival [57-59].

In summary, the momentum and current excitement about immunomodulatory cells as potential alternative for drug-based immunosuppression should be used to further advance this treatment to the clinical arena. Although immunosuppressive drugs are effective in preventing immune responses to alloantigen, some achieve their effects in a non-specific manner. Cell-based therapies offer the opportunity to modulate or activate inherent regulatory pathways. During three decades of intense research to promote cell therapies, much progress has been made in the translation of in vitro data to the pre-clinical stage and from animals into humans. Yet, many questions remain to be answered. Filling the current gaps in our knowledge about MSC function will allow us to better comprehend and exploit the ability of MSC to suppress alloreactivity and to induce tolerance in organ transplant patients.

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Chapter 7

Dutch summary (Samenvatting)

German summary (Zusammenfassung)

SAMENVATTING

Het onderzoek beschreven in dit proefschrift is gericht op het bestuderen van het potentieel van mesenchymale stamcellen (MSC) geïsoleerd uit vetweefsel en regulatoire T cellen (Treg) om als celtherapie de reactiviteit van geactiveerde donor T cellen na orgaan transplantatie te controleren. Behandelingen met de huidige immunosuppressieve medicijnen hebben op de lange termijn nadelige bijwerkingen, bijvoorbeeld een toenemend risico op het ontwikkelen van tumoren, een hogere incidentie van suikerziekte en een grotere ontvankelijkheid voor infecties. Er is dus behoefte aan de ontwikkeling van alternatieve behandelingen om de blootstelling van patïenten aan de medicijnen te verminderen. Cellen met immunomodulatoire eigenschapen worden beschouwd als potentiele behandelingsopties en hun veiligheid en haalbaarheid als celtherapieën voor verschillende indicaties wordt momenteel onderzocht in klinische studies zoals beschreven in **hoofdstuk 1**. Ondanks het toegenomen begrip van immunomodulatie door immunosuppressieve cellen in de afgelopen jaren, is er veel meer basale kennis nodig om de capaciteiten van deze cellen optimaal voor de preventie van transplantaat rejecties te kunnen toepassen. Met name interacties van de toegediende cellen met de geactiveerde cellen en de immunosuppressive cellen van de ontvanger behoeven verder onderzoek.

In **hoofdstuk 2** wordt de interactie tussen MSC en natural Treg (nTreg) geëxploreerd. MSC worden gekarakteriseerd aan de hand van hun expressie van oppervlakte markers en hun differentiatie vermogen. MSC brachten de moleculen CD90, CD105, CD166 en HLA-I tot expressie terwijl de expressie van CD14, CD34, CD45 and HLA-II ontbrak. MSC waren in staat om in vetcellen en botcellen te differentiëren. Zowel MSC als nTreg onderdrukten op een dosisafhankelijke manier de proliferatie van allo-gestimuleerde $CD25^{-/dim}$ T effectorcellen. MSC remden de proliferatie van allo-gestimuleerde $CD25^{-/dim}$ T cellen vanaf een concentratie van 1:160 (ratio MSC/effectorcellen). nTreg remden $CD25^{-/dim}$ effectorcel proliferatie vanaf een concentratie van 1:10 (ratio nTreg/effectorcellen). In gecombineerde kweken hadden MSC en nTreg geen ïnvloed op elkaars immunosuppressieve functies. Om de interactie tussen MSC en nTreg in een in vitro transplantatie situatie te onderzoeken, werden MSC (1:40; ratio MSC/effectorcellen) afkomstig van nierdonoren gekweekt met nTreg (1:10) verkregen van de bijbehorende ontvanger 6 maanden na niertransplantatie. Vergelijkbaar met de situatie waar nTreg van gezonde donoren werden gebruikt, verhinderden beide celtypen elkaars suppressieve capaciteiten niet. De immunosuppressieve effecten van MSC en nTreg hadden ïnvloed op $CD4^+$ T cellen en $CD8^+$ T cellen; binnen deze populaties waren naïve T cellen meer ontvankelijk voor de remmende werking van MSC en nTreg dan geheugen T cellen. MSC bewerkstelligden hun immunosuppressieve functies onder andere door de expressie van IDO dat

zorgt voor de afbraak van tryptofaan. Verder, MSC zorgden voor een anti-inflammatoir milieu middels hun invloed op de secretie van TNF-α and IL-10 door immuuncellen.

Andere mechanismen die MSC gebruiken voor hun immunosuppressieve functies werden bestudeerd in de studie uitgevoerd in **hoofdstuk 3**. Terwijl MSC (1:40; ratio MSC/effectorcellen) de proliferatie van $CD25^{-/dim}$ T effectorcellen met 59% verminderden, medieerden zij een 2-voudige toename van het percentage $CD25^{+}CD127^{-}FOXP3^{+}$ cellen binnen de $CD4^{+}$ T cel populatie. Deze $CD4^{+}CD25^{+}CD127^{-}FOXP3^{+}$ iTreg waren functioneel en even potent als nTreg in het onderdrukken van de allogene reactiviteit van effector T cellen. Om te bevestigen dat deze iTreg nieuw werden gevormd werd de methylatie status van de Treg-specifieke niet-gemethyleerde regio (TSDR) binnen het FOXP3 gen geanalyseerd. Deze specifieke positie in het FOXP3 gen is volledig gemethyleerd in iTreg en gedemethyleerd in nTreg afkomstig vanuit de thymus. De nTreg fractie bevatte 32,7% cellen met een gemethyleerde TSDR. In tegenstelling, de iTreg fractie bevatte 83,7% TSDR-gemethyleerde cellen en dit resultaat bekrachtigt dat zij niet in de thymus worden gevormd. De verdere karakterisatie van iTreg demonstreerde dat zij CTLA-4, GITR en Helios tot expressie brengen. Aangezien IL-2 essentieel is voor de expansie en functie van nTreg, werd de rol van IL-2 in de generatie van iTreg door MSC bepaald. Ondanks verminderde T cel proliferatie in een gecombineerde MSC-MLR kweek, waren IL-2 concentraties onder deze condities 15,1 keer hoger dan in MLR alleen. IL-2 wordt niet tot expressie gebracht door rustende of geactiveerde MSC. De IL-2 was dus afkomstig van effectorcellen. Inderdaad, geactiveerde $CD4^{+}CD25^{+}FOXP3^{-}$ T cellen waren de populatie met het hoogste percentage IL-2-producerende cellen. Inhibitie van de IL-2 route met de IL-2 receptor antagonist basiliximab reduceerde het percentage iTreg in MLR en voorkwam de MSC-gemedieerde generatie van iTreg. Deze bevinding toont de betrokkenheid van IL-2 in de inductie van *de novo* Treg door MSC aan.

Hoofdstuk 4 presenteert onderzoek naar het effect van MSC op $CD8^{+}CD28^{-}$ T cellen. Na allogene stimulatie toonden $CD8^{+}CD28^{-}$ T cellen proliferatieve capaciteiten vergelijkbaar met die van $CD8^{+}CD28^{+}$ T cellen. Dit resultaat geeft aan dat $CD8^{+}CD28^{-}$ T cellen het vermogen hebben om alloreactiviteit in transplantaat patiënten te veroorzaken. Prolifererende $CD8^{+}CD28^{-}$ T cellen hadden een immuunactiverend en cytotoxisch phenotype; ze brachten IFN-γ, TNF-α en granzyme B tot expressie. Het CTLA-4 immunoglobuline (Ig) belatacept bindt aan CD80/CD86 op de oppervlakte van antigen-presenterende cellen (APC). Daardoor blokkeert belatacept de CD28-CD80/86 co-stimulatie route die nodig is om T cellen te activeren en te laten prolifereren. Dientengevolge ontkomen $CD8^{+}CD28^{-}$ T cellen aan de immunosuppressieve functie van belatacept. Om te bekijken of MSC de cellen die ontkomen aan belatacept remmen, werd het gecombineerde effect van belatacept en MSC op effectorcellen gebestudeerd. Zowel belatacept als MSC rem-

den effectorcel proliferatie op een dosisafhankelijke manier. De combinatie van beide immunosuppressieve middelen had geen negatieve ïnvloed op elkaars functie. Lage concentraties van belatacept en MSC hadden een additief remmend effect. Belatacept onderdrukte de alloreactiviteit van $CD8^+CD28^+$ T cellen, maar beïnvloedde $CD8^+CD28^-$ T cel proliferatie niet. In tegenstelling, MSC remden de proliferatieve capaciteit van zowel $CD8^+CD28^-$ als $CD8^+CD28^+$ T cellen. Het gelijktijdig kweken van MLR met belatacept en MSC zorgde voor inhibitie van de proliferatie van $CD8^+CD28^-$ en $CD8^+CD28^+$ T cellen, wat betekent dat belatacept de functie van MSC niet belemmert. MSC induceerden geen apoptose in $CD8^+CD28^-$ T cellen en evenmin beïnvloedden MSC de CD28 expressie van $CD8^+$ T cellen. Deze bevinding bekrachtigt dat MSC een anti-proliferatief effect op $CD8^+CD28^-$ T cellen hebben.

In **hoofdstuk 5** wordt een retrospectieve studie beschreven die de uitwerking van een FOXP3 $(GT)_n$ dinucleotide repeat polymorphisme op de transplantaat overleving van 599 primaire nier transplantatie patiënten evalueert. Zes verschillende FOXP3 allelen werden geïdentificeerd die varieerden van $(GT)_{12}$ tot $(GT)_{18}$. Omdat beschreven is dat de promotor van het $(GT)_{15}$-allel een hogere activiteit heeft dan de promotor van het $(GT)_{16}$-allel, werden alle allelen $\leq(GT)_{15}$ geïncludeerd in de korte (S) klasse, terwijl alle allelen $\geq(GT)_{16}$ werden geïncludeerd in de lange (L) klasse. Aangezien het FOXP3 gen is gesitueerd op het X chromosoom, bezitten mannelijke patiënten slechts één FOXP3 allel. Deze hemizygote patiënten werden gecombineerd met de respectievelijke homozygote genotype groep. Patienten werden dus gegroepeerd over drie groepen; S/SS, SL en L/LL. Transplantaat overleving gecensureerd voor overlijden was vergelijkbaar tussen de SL en de S/SS genotype groepen, terwijl de SL groep een voordeel had over de L/LL genotype groep. De SL groep en de S/SS groep werden daarom samengevoegd en vormden de S-genotype groep (SG).; de L-genotype groep (LG) bestond uit de L/LL genotypen. In een Kaplan-Meier analyse hadden SG patiënten een voordeel in transplantaat overleving vergeleken met de LG patiënten. Multivariant Cox regressie analyse bevestigde deze analyse. Acute rejectie was niet geassocieerd met FOXP3 polymorphismes; in beide genotype groepen maakten evenveel patiënten tenminste een acute rejectie episode door. Daarentegen, na een acute rejectie hadden patiënten van de SG group een voordeel in orgaan overleving over de LG patiënten. Deze associatie werd niet gevonden in patiënten zonder acute rejectie en dit resultaat demonstreert een voordelig effect op transplantaat overleving van de S-, SS-, and SL-genotypen.

ZUSAMMENFASSUNG

Die Arbeiten, die in dieser Dissertation beschrieben werden, untersuchen das Potenzial von mesenchymalen Stammzellen (MSC; isoliert aus perirenalem Fettgewebe) und regulatorischen T-Zellen (Treg), um als Zelltherapie nach einer Organtransplantation die Reaktivität von aktivierten Spender-T-Zellen zu kontrollieren. Nach einer Transplantation muss verhindert werden, dass das Immunsystem des Empfängers das nicht körpereigene Organ abstößt. Hierzu werden Medikamente eingesetzt, die das Immunsystem des Patienten künstlich schwächen. Der langfristige Gebrauch dieser Immunsuppressiva kann Nebenwirkungen, z.B. ein erhöhtes Risiko für das Auftreten von Tumorerkrankungen, Diabetes mellitus und Infektionen, zur Folge haben. Aus diesem Grund besteht ein Bedarf an alternativen Behandlungsmöglichkeiten, um die Dosis der Medikamente zu verringern. Als mögliche Alternative zu den gegenwärtig angewandten Immunsuppressiva werden Zellen mit immunmodulatorischen Eigenschaften angesehen. Die Sicherheit und Realisierbarkeit dieser Zelltherapien wird derzeit, wie in **Kapitel 1** beschrieben, für verschiedene Indikationen in klinischen Studien untersucht. In den vergangenen Jahren wurden viele wertvolle Erkenntisse über Immunmodulation durch immunsuppressive Zellen gewonnen. Dennoch ist weitere Grundlagenforschung notwendig, um die Funktionen dieser Zellen optimal für die Prävention von Organabstoßungen anzuwenden. Insbesondere die Interaktionen zwischen den verabreichten, therapeutischen Zellen und den aktivierten und immunsuppressiven Zellen des Transplantatempfängers müssen näher erforscht werden.

In **Kapitel 2** wurde die Interaktion zwischen MSC und natürlichen Treg (nTreg) untersucht. MSC wurden anhand ihrer Zelloberflächenmarker und ihrem Differenzierungsvermögen charakterisiert. MSC exprimierten die Moleküle CD90, CD105, CD166 und HLA-I, jedoch nicht CD14, CD34, CD45 und HLA-II. MSC differenzierten sich in Adipozyten und Osteoblasten. Sowohl MSC als auch $CD25^{+}$ nTreg (isoliert von gesunden Blutspendern) unterdrückten dosisabhängig die Proliferation von allo-stimulierten $CD25^{-/dim}$ Effektor-T-Zellen in gemischten Lymphozytenreaktionen (MLR). MSC verhinderten die Vermehrung von allo-stimulierten $CD25^{-/dim}$ T-Zellen ab einer Konzentration von 1:160 (Verhältnis MSC/Effektorzellen). nTreg unterdrückten die Proliferation der $CD25^{-/dim}$ Zellen ab einer Konzentration von 1:10 (Verhältnis nTreg/Effektorzellen). In Co-Kulturen beeinflußten beide Zelltypen ihre gegenseitigen immunsuppressiven Funktionen nicht. Um die Wechselwirkung von MSC und nTreg in einer In-vitro-Transplantationssituation zu untersuchen, wurden MLR mit MSC von Nierenspendern (1:40; Verhältnis MSC/Effektorzellen) und nTreg von den entsprechenden Nierenempfängern (1:10; entnommen 6 Monate nach der Operation) kultiviert. Vergleichbar mit der Situation, in der nTreg von gesunden Blutspendern benutzt wurden, behinderten MSC und nTreg ihre gegenseiti-

gen suppressiven Fähigkeiten nicht. Die immununterdrückenden Effekte beider Zelltypen beeinflußten $CD4^+$ T-Zellen und $CD8^+$ T-Zellen. Innerhalb dieser Zellpopulationen waren naive T-Zellen empfänglicher für die antiproliferative Wirkung von MSC und nTreg als T-Gedächtniszellen. MSC erzielten ihre immunsuppressive Funktion unter anderem durch die Expression von IDO, einem Enzym, das den Abbau von Tryptophan katalysiert. Zudem sorgten MSC für den Aufbau eines antiinflammatorischen Milieus, indem sie Immunzellen zur Sekretion der Zytokine TNF-α und IL-10 anregten.

Weitere Mechanismen, die MSC anwenden, um ihre immunsuppressive Funktionen auszuüben, wurden in der in **Kapitel 3** beschriebenen Studie untersucht. Während MSC (1:40; Verhältnis MSC/Effektorzellen) die Proliferation von $CD25^{-/dim}$ Effektor-T-Zellen um 59% verringerten, verdoppelte sich der prozentuale Anteil an $CD25^+CD127^-FOXP3^+$ Zellen innerhalb der $CD4^+$ T-Zellpopulation. Diese $CD4^+CD25^+CD127^-FOXP3^+$ induzierten Treg (iTreg) hatten immunsuppressive Eigenschaften und unterdrückten die Alloreaktivität von Effektor-T-Zellen ebenso wirksam wie nTreg. Um zu bestätigen, dass die iTreg aus den $CD25^{-/dim}$ Effektor-T-Zellen enstanden sind, wurde der Methylierungsstatus der Treg-spezifischen, unmethylierten Region (TSDR) des FOXP3-Gens bestimmt. Die TSDR ist in iTreg vollständig methyliert und unmethyliert in nTreg, die im Thymus entstehen. Die nTreg-Fraktion enthielt 32,7% Zellen mit einer methylierten TSDR. Im Gegensatz dazu bestand die iTreg-Fraktion zu 83,7% aus Zellen mit unmethylierter TSDR. Dieses Ergebnis verdeutlicht, dass die iTreg in vitro und nicht im Thymus gebildet wurden. Eine weitere Charakterisierung der iTreg zeigte, dass sie CTLA-4, GITR und Helios exprimieren. Da IL-2 essenziell für das Wachstum und die Funktion von nTreg ist, wurde die Rolle von IL-2 bei der Entstehung von iTreg durch MSC untersucht. Trotz verringerter T-Zellproliferation bei der Co-Kultur von MSC und MLR waren die IL-2-Konzentrationen unter diesen Bedingungen 15,1-fach höher als in der MLR ohne MSC. IL-2 wurde nicht von ruhenden oder aktivierten MSC exprimiert. Deshalb stammte das IL-2 von den Effektorzellen. Die Population mit dem höchsten prozentualen Anteil an IL-2-exprimierenden Zellen waren aktivierte $CD4^+CD25^+FOXP3^-$ T-Zellen. Die Inhibierung des IL-2-Signalweges durch den IL-2-Rezeptorantagonisten Basiliximab reduzierte den prozentualen Anteil an iTreg in der MLR und verhinderte somit die MSC-vermittelte Entstehung von iTreg. Dieses Resultat beweist die Beteiligung von IL-2 bei der Entstehung von *de novo* iTreg durch MSC.

Kapitel 4 beschreibt die Untersuchung des Effektes von MSC auf $CD8^+CD28^-$ T-Zellen. Nach allogener Stimulation zeigten $CD8^+CD28^-$ T-Zellen ein Proliferationsvermögen, das vergleichbar war mit dem von $CD8^+CD28^+$ T-Zellen. Dies deutet an, dass $CD8^+CD28^-$ T-Zellen das Potenzial haben Alloreaktivität in Transplantationspatienten zu verursachen. Diese proliferierenden $CD8^+CD28^-$ T-Zellen zeigten einen immunaktivierten und zytotoxischen Phänotyp; sie exprimierten IFN-γ, TNF-α und Granzyme B. Das CTLA-4 Immunglo-

bulin (Ig) Belatacept bindet an CD80/86 auf der Oberfläche von antigenpräsentierenden Zellen (APC). Dadurch blockiert Belatacept den CD28-CD80/86 Co-Stimulations-Signalweg, der für die Aktivierung und Proliferation von T-Zellen notwendig ist. Aufgrund der Abwesenheit des CD28-Moleküls hat Belatacept keine immunsuppressive Wirkung auf $CD8^+CD28^-$ T-Zellen. Um zu ermitteln, ob MSC die Proliferation von $CD8^+CD28^-$ T-Zellen inhibieren können, wurde zunächst der Effekt einer MSC-Belatacept-Kombinationstherapie auf Effektor-T-Zellen untersucht. Sowohl Belatacept als auch MSC unterdrückten dosisabhängig die Effektorzellproliferation. Die Kombination beider Immunsuppressiva beeinflusste ihre jeweiligen inhibierenden Funktionen nicht. Geringe Konzentrationen von Belatacept und MSC hatten einen additiven immununterdrückenden Effekt. Belatacept hemmte die Alloreaktivität von $CD8^+CD28^+$ T-Zellen, beeinflusste jedoch nicht das Proliferationsvermögen der $CD8^+CD28^-$ T-Zellen. MSC hingegen inhibierten die proliferativen Fähigkeiten von $CD8^+CD28^-$ und $CD8^+CD28^+$ T-Zellen. Auch in MLR-Co-Kulturen mit MSC und Belatacept wurden sowohl $CD8^+CD28^-$ T-Zellen als auch $CD8^+CD28^+$ T-Zellen in ihrer Proliferation supprimiert. Belatacept beeinträchtigte demnach die Funktion der MSC nicht. Darüber hinaus induzierten MSC keine Apoptose in $CD8^+CD28^-$ T-Zellen und beeinflussten auch nicht die CD28-Expression von $CD8^+$ T-Zellen. Diese Ergebnisse bestätigen, dass MSC einen antiproliferativen Effekt auf $CD8^+CD28^-$ T-Zellen haben.

In **Kapitel 5** wird eine retrospektive Studie beschrieben, die die Auswirkung eines FOXP3 $(GT)_n$ Dinukleotid-Wiederholungspolymorphismus auf das Transplantatüberleben von 599 Erst-Nierentransplantationspatienten beurteilt. Sechs verschiedene FOXP3-Allele wurden identifiziert und variierten von $(GT)_{12}$ bis $(GT)_{18}$. Da festgestellt wurde, dass der Promotor des $(GT)_{15}$-Allels eine höhere Aktivität besitzt als der Promotor des $(GT)_{16}$-Allels, wurden alle Allele $\leq(GT)_{15}$ in der kurzen (S) Klasse zusammengefasst. Alle Allele $\geq(GT)_{16}$ bildeten die lange (L) Klasse. Da sich das FOPX3-Gen auf dem X-Chromosom befindet, besitzen männliche Patienten nur ein FOXP3-Allel. Diese hemizygotischen Patienten wurden mit der entsprechenden homozygotischen Genotypgruppe kombiniert. Demzufolge konnten alle Patienten in eine der drei Gruppen S/SS, SL und L/LL eingeteilt werden. Das Transplantatüberleben der S/SS- und der SL-Genotypgruppe war vergleichbar. Deshalb wurden diese zwei Genotypgruppen zur S-Genotypgruppe (SG) zusammengefasst; die L-Genotypgruppe (LG) bestand aus den L/LL-Genotypen. In einer Überlebenszeitanalyse (Kaplan-Meier-Methode) hatten die SG-Patienten, im Vergleich zu den LG-Patienten, einen Vorteil im Überleben ihrer Transplantate. Dieser Vorteil wurde von einer multivariaten Cox-Regressionsanalyse bestätigt. Das Auftreten von akuten Abstoßungsreaktionen stand nicht im Zusammenhang mit den unterschiedlichen FOXP3-Polymorphismen; in beiden Gruppen erlebten vergleichbare Anzahlen von Patienten mindestens eine akute Abstoßungsreaktion. Im Gegensatz dazu zeigten SG-Patienten, verglichen mit den LG-Patienten, nach dem Auftreten einer akuten Ab-

stoßungsreaktion ein besseres Transplantatüberleben. Diese Assoziation wurde nicht für Patienten ohne akute Abstoßungsreaktionen gefunden. Demnach haben Patienten mit S-, SS- oder SL-Genotypen nur einen Vorteil im Überleben von Nierentransplantaten gegenüber Transplantationspatienten mit L- oder LL-Genotypen, wenn akute Abstoßungsreaktionen auftreten.

Appendix

ACKNOWLEDGEMENTS (DANKWOORD, DANKWORT)

The very first time I visited Rotterdam, I went to the Museum Boijmans Van Beuningen, smiled at the oversized bunnies in the Museumpark, walked through Het Park, ate a 'lekker soepje' at the top of the Euromast and glanced at the not-to-be-missed tall, white building with the big light-blue letters which overlooks the Maas – not knowing or even imagining back then, that I would spend more than four years behind the porthole-like windows working on my PhD. And eventful years they were: full of learning, experiences, challenges and a lot of fun. My time at the Erasmus would not have been so eventful and successful without the support of many people. I am a bit fearful of singling people out, in case I miss anyone, but I feel there are a few names in particular I have to mention:

Prof.dr. Willem Weimar, my promoter. Dear Prof. Weimar, thank you for the opportunity to conduct my research in your department. Your experience and your critical evaluation of my work improved my abstracts, manuscripts and presentations, and contributed to the success of the abstracts at conferences and the relatively swift manuscript submissions. I would also like to thank you for enabling me to attend various national and international congresses – a real privilege that many PhD students from other departments or universities do not share. The presentations I was fortunate enough to give at these events were nerve-racking (especially in the beginning), but they are experiences that will live on and so will my memories of the 'gezelligheid' during the annual labweekends.

Dr. Carla C. Baan and Dr. Martin J. Hoogduijn, my co-promoters. In general, I would like to thank you both for giving me the freedom to work independently, but at the same time always giving me your full support when I needed it (even after my time in the lab). **Dear Carla**, the enthusiasm you have for your research is contagious. Your general knowledge about the field is admirable. I greatly appreciate that despite your many roles and functions you always made time for discussions or the review of manuscripts. **Dear Martin**, thank you for simply being your calm and patient self, even when I gave you the umpteenth version of a manuscript or when I asked you (again) to double-check my doings on the flow cytometer. Although my sentences are still on the long side, I have learned from your concise way of writing and your ability to present data in such a way that readers get the message 'at first glance'. Thank you for allowing work discussions that started with me asking 'Do you have 10 minutes, please?' to last for more than an hour, for always being positive and encouraging, and for last but not least being a fantastic emergency-'fietsenmaker'.

Marieke and Marcel·la, my paranymphs. You two not only shared my interest in MSC, you became friends. I am honoured and very happy that you agreed to support me on this special day in December. **Dear Marieke**, despite having a very busy private and professional life, you always cared about not only my scientific but also my personal well-being. By doing so, you helped me sail through small gusts and heavier storms. I know, I have mentioned this a few times to you, but I am truly inspired by your attitude to make the most of the here & now. **Dear Marcel·la**, with you joining the MSC group, I had someone in the lab who understood what it is like to live and work as a foreigner in the Netherlands. Your fresh and lively Catalan spirit lifted the atmosphere in our 'AIO hok'. 'Crisis management' with you always meant putting things into perspective and getting a clear view of what really matters. And when I thought it would never happen, your approach to setting compensations on the Canto finally put me a bit more at ease when I did it myself. For this in particular and many other things a heartfelt 'Applause, applause!'

Anne, you have been so many persons in one for me over the past few years: loyal 'kamergenoot' during congresses, concert & travel companion, patient 'lerares Nederlands', asylum provider in Rotterdam on occasions when it did not make sense to travel home to Breda, decorator of my work desk (on the few spots I left for you) and co-enthusiast for certain TV series. Thank you for all of this and more.

Martijn, when you became my neighbour in the 'AIO hok', the bad habit of having lunch in front of the computer was over (at least for a while), because you were quite adamant in getting me away from my desk. I find your optimistic attitude very refreshing and I appreciated your general IT support and help with FlowJo.

Ruud, we both worked with blood and we both shared a soft spot for a professional living in a warmer climate, analysing its spatters. It has been a real pleasure working with you and our little banters lightened up my days on many occasions. Remember that you have joined the 'concert club'; we will keep you to it.

Gretchen, I like the fact that although you are determined in reaching your professional goals, you do not take yourself too seriously. Thank you for all the good laughs.

All 'AIO hok' alumni, although we did complain at times that the room was too small for the number of people working in it, and despite the constant (seemingly impossible) mission to find a 'one-fits-all' room temperature, having moved to the office spaces in the new building showed me, that 'the hok' was a special place. During our time as PhD students (and postdoc), we all went through similar phases and had comparable ex-

periences. Being able to celebrate progress together (small and big), share frustrations (small and big), receive and give support or just take one's mind off work for a moment by bantering was what made this room and its inhabitants so memorable. In one way or another you all contributed to this thesis.

Nicolle, you were always very interested in the progress of my work and had an 'open ear' for the problems I stumbled upon along my way. Your ideas for the design of certain experiments surely shortened the time it took to get the answers I was looking for. You very often provided 'first-aid' when the flow cytometer and I had a little quarrel (again) and not only for that I am very grateful.

Karin, your help during my 'fight with the FOX' was very valuable and the TSDR assay you established in the lab was a great tool for one of my projects. For a short (and fun) time we both looked into the option of replacing the pipette with a racket, but I think we soon came to the conclusion to stick to our scientific careers. I have enjoyed exploring the Charles River and its surroundings with you.

All colleagues from the Tx lab, you have trained me in many techniques and the use of different machines in the lab, shared your experience in various subjects with me, indirectly taught me Dutch (that's why 'het Brabantse zachte G' never made it into my diction), helped me with my labwork, ficolled blood samples for me or just said nice things about my cakes. By simply doing your day-to-day work, you all have been supportive and contributed to my research in innumerable ways and I am very appreciative of this.

I would also like to thank **Saida** for taking care of the administrative tasks involved in a PhD, all the **transplant surgeons** for providing the MSC group with adipose tissue to isolate MSC, the **'nierpoli' and 'prikkamer' staff**, and all my **co-authors** for their contributions to this thesis.

Family and friends, Danke für euer Interesse an meiner Arbeit, das Mitfreuen, die ermutigenden Worte, wenn sie nötig waren, die IT-Betreuung und die Ablenkung von der wissenschaftlichen Welt, wenn ich Zeit mit euch verbracht habe. **Mami und Papi**, durch euer Vertrauen und eure Weitsicht in Zeiten als noch nicht so recht absehbar war, was die Zukunft bringen wird, habt ihr die Grundlagen gelegt für die Möglichkeiten, die ich bisher ergreifen konnte, und für alles, was ich bisher erreicht habe. Dafür bin ich euch sehr, sehr dankbar.

Jean, a PhD student can come in many flavours and goes through various phases. Who knows this better than you – you had one at home. Without your love, patience, unwavering support and confidence in me the past years would not have been the same. With these words I almost literally close, not only this last chapter of my thesis, but also the chapter of being a student. I am curious and excited about all the new chapters lying ahead, waiting to be opened and experienced, and I am proud and very thankful that I will share them with you.

Thank you. Dank je wel. Danke.

Anja

Breda, October 2013

PhD PORTFOLIO

Name PhD student:	Anja U. Engela
Erasmus MC department:	Internal Medicine, Section Nephrology and Transplantation
PhD period:	April 2009 – June 2013
Research school:	Postgraduate School Molecular Medicine
Promoter:	Prof.dr. Willem Weimar
Co-promoters:	Dr. Carla C. Baan and Dr. Martin J. Hoogduijn

Courses and workshops

2009	Infection prevention for paramedics, Erasmus MC, Rotterdam
2009	Irradiation hygiene expertise level 5B, Erasmus MC, Rotterdam
2009	Classical methods for data analysis, NIHES, Erasmus MC, Rotterdam
2010	Anatomy/Physiology (self-study course), DWM Utrecht
2010	Research management for PhD students and Postdocs, MolMed, Erasmus MC, Rotterdam
2011	Laboratory animal competence (Article 9), Erasmus MC, Rotterdam
2011	Molecular Immunology for PhD students, Erasmus MC, Rotterdam
2011	Workshop on Photoshop and Illustrator CS5, MolMed, Erasmus MC, Rotterdam
2012	Biomedical English Writing Course for MSc and PhD students, MolMed, Erasmus MC, Rotterdam
2012	Workshop on InDesign CS5, MolMed, Erasmus MC, Rotterdam

Participation and presentations at (inter)national conferences

2009	Nederlandse Transplantatie Vereniging (NTV) Symposium, Utrecht	participation
2010	Science days of the Department of Internal Medicine, Antwerp, Belgium	poster
2010	2nd MiSOT, Rotterdam	oral
2010	22nd Annual Meeting NTV (Bootcongres), Rotterdam	oral
2010	3rd Dutch Stem Cell meeting, Utrecht	participation
2010	10th American Transplantation Congress (ATC), San Diego, CA, USA	oral
2010	23rd TTS, Vancouver, Canada	mini-oral, poster
2010	2nd Regional Meeting ISCT-Europe, Belgirate, Italy	poster
2011	Science days of the Department of Internal Medicine, Antwerp, Belgium	participation
2011	15th MolMed Day, Rotterdam	poster
2011	23rd Annual Meeting NTV (Bootcongres), Amsterdam	oral
2011	3rd MiSOT, Leuven, Belgium	poster
2011	11th American Transplantation Congress (ATC), Philadelphia, PA, USA	poster
2011	17th Annual ISCT Meeting, Rotterdam	poster
2011	12th TTS Basic Science Symposium and 2nd ESOT Basic Science Meeting,	oral
	Boston (Cape Cod), MA, USA	poster
2011	15th ESOT Congress, Glasgow, UK	oral (2)

2012	24th Annual Meeting NTV (Bootcongres), Maastricht	oral
2012	12th American Transplantation Congress (ATC), Boston, MA, USA	oral
2012	24th TTS, Berlin, Germany	poster
2012	4th MiSOT, Barcelona, Spain	oral
2013	25th Annual Meeting NTV (Bootcongres), Duiven	oral

Awards

2010 Young Investigator Award, 10th ATC, San Diego, CA, USA

2011 Poster Award, 15th MolMed Day, Rotterdam

2011 Best Poster Abstract Award, 17th Annual ISCT Meeting, Rotterdam

2011 Poster with distinction, 11th ATC, Philadelphia, PA, USA

2011 TTS-ESOT Mentee-Mentor Award, 12th TTS Basic Science Symposium and European Society for Organ Transplantation (ESOT) 2nd ESOT Basic Science Meeting, Boston (Cape Cod), MA, USA

2012 Young Investigator Award, 12th ATC, Boston, MA, USA

Grants

2010 Travel grant Novartis B.V. (10th ATC)

2011 Travel grant NTV (Annual Meeting NTV)

2011 Travel grant Novartis B.V. (TTS/ESOT Basic Science)

Teaching activities

2011/12 Supervision of Esther Jongste (Bachelor student), November 2011 – June 2012

Memberships

2009 – 2013 Dutch Transplantation Society (Nederlandse Transplantatie Vereniging, NTV)

2010 – 2013 Mesenchymal Stem Cells in Solid Organ Transplantation (MiSOT) study group

2010 – 2012 American Society of Transplantation (AST)

2010 – 2012 International Society for Cellular Therapy (ISCT)

2010 – 2012 European Society for Organ Transplantation (ESOT)

CURRICULUM VITAE AUCTORIS

Anja Ulrike Engela (née Grohnert) was born on June 2nd, 1978 in Luckenwalde, Germany. In 1998 she completed secondary school at the Marie-Curie-Gymnasium in Ludwigsfelde. The following four years Anja studied at the University of Applied Sciences in Köthen (Anhalt) and in 2002 - after completing a 3-month research project on the expression and purification of a ribonuclease inhibitor at the department of Microbiology of the Academy of Sciences of Czech Republic in Prague - she became 'Diplom-Ingenieur' in Biotechnology. Following her interests in cell biology and drug development, Anja worked at SiRENADE Pharmaceuticals AG in Martinsried as a Research Associate. At the end of 2005 she decided to gain international experience and started working at GlaxoSmithKline R&D at the Biopharmaceutical Centre of Excellence for Drug Discovery (BioPharm CEDD) in Beckenham, UK. As a Scientist in Cell Biology she was responsible for developing potency and functional assays for therapeutic monoclonocal antibodies. During her time at GSK, Anja was given the opportunity to study part-time for a Master of Science in Medical Molecular Biology at the University of Westminster in London. She was awarded this degree with distinction in 2008, after finishing her research project "Elucidation of mechanism of action and development of a biological assay for a monoclonal antibody". Then Anja crossed the English Channel again and in spring 2009 she began her doctoral studies at the Section Nephrology and Transplantation of the Department of Internal Medicine at the Erasmus MC in Rotterdam, the Netherlands. Under the supervision of her promoter Prof.dr. Willem Weimar and co-promoters Dr. Carla C. Baan and Dr. Martin J. Hoogduijn, she studied the two immunosuppressive cell types mesenchymal stem cells and regulatory T cells in the context of their potential application for the prevention of alloreactivity after organ transplantation. Anja is married to Jean and their current home is Breda.

PUBLICATIONS

Mesenchymal stem cells control allo-reactive CD8+CD28- T cells.
Engela AU, Baan CC, Litjens NH, Franquesa M, Betjes MG, Weimar W, Hoogduijn MJ.
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Stem Cells Dev. 2013.

Mesenchymal Stem Cells in Solid Organ Transplantation (MiSOT) Fourth Meeting: Lessons Learned from First Clinical Trials.
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The effect of rabbit antithymocyte globulin on human mesenchymal stem cells.
Franquesa M, Baan CC, Korevaar SS, Engela AU, Roemeling-van Rhijn M, Weimar W, Betjes MG, Grinyo JM, Hoogduijn MJ.
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On the interactions between mesenchymal stem cells and regulatory T cells for immunomodulation in transplantation.
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Interaction between adipose tissue-derived mesenchymal stem cells and regulatory T-cells.
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Immunological aspects of allogeneic and autologous mesenchymal stem cell therapies.
Hoogduijn MJ, Roemeling-van Rhijn M, Korevaar SS, Engela AU, Weimar W, Baan CC.
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ABBREVIATIONS

7-AAD	7-aminoactinomycin D
ACR	acute cellular rejection
ANOVA	analysis of variance
APC	antigen-presenting cell or allophycocyanin
AR	acute rejection
ASC	adipose-tissue derived MSC
AZA	azathioprine
CBA	cytometric bead array
CD	cluster of differentiation
(c)DNA	(complementary) deoxyribonucleic acid
CMV	cytomegalovirus
CNI	calcineurin inhibitor
CsA	cyclosporine A
CTL	cytotoxic T lymphocyte
CTLA-4	cytotoxic T-lymphocyte antigen-4
DC	dendritic cell
DD	deceased donor
DEPC	diethylpyrocarbonate
DMSO	dimethyl sulfoxide
eGFR	estimated glomerular filtration rate
ESRD	end-stage renal disease
F	female
FAM	6-carboxyfluorescein
FasL	Fas ligand
FBS	fetal bovine serum
FITC	fluorescein isothiocyanate
FMO	fluorescence minus one
FOXP3	forkhead box P3
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GF	graft failure
GFR	glomerular filtration rate
GITR	glucocorticoid-induced TNF-receptor related protein
GLM	generalized linear model
GVHD	graft-versus-host disease
HGF	hepatocyte growth factor
HI	highly immunized
HLA	human leukocyte antigen

HO-1	heme oxygenase-1
HSCT	haematopoietic stem cell transplantation
HWE	Hardy-Weinberg equilibrium
I	immunized
IB	immunological background
IDO	indoleamine 2,3-dioxygenase
IFN-γ	interferon-gamma
IF/TA	interstitial fibrosis and tubular atrophy
Ig	immunoglobulin
IL	interleukin
IPEX	immunodysregulation polyendocrinopathy enteropathy X-linked syndrome
IRI	ischemia-reperfusion injury
iTreg	induced Treg
KTx	kidney transplantation
L	long $(GT)_n$ dinucleotide repeat
LD	living (un-)related donor
LG	L-genotype group
M	male
mAbs	monoclonal antibodies
MACS	magnetic cell sorting
MEM-α	minimum essential medium Eagle alpha modification
MFI	median fluorescence intensity
MLR	mixed lymphocyte reaction
MMF	mycophenolate mofetil
Mreg	regulatory macrophage
(m)RNA	(messenger) ribonucleic acid
MSC	mesenchymal stem cell
mTOR	mammalian target of rapamycin
NFAT	nuclear factor of activated T cells
NI	non-immunized
NIB	non-immunological background
NK	natural killer
NO	nitric oxide
nTreg	natural Treg
PBMC	peripheral blood mononuclear cells
PCM	PBMC culture medium
PD-1	programmed death-1
PD-L1	programmed death ligand-1
PE	phycoerythrin

PerCP	peridinin chlorophyll protein
PGE2	prostaglandin E2
PHA	phytohaemagglutinin
PMA	phorbol 12-myristate 13-acetate
P/S	penicillin/streptomycin solution
PRA	panel reactive antibody
qPCR	quantitative real-time PCR
RA	rheumatoid arthritis
rATG	rabbit anti-thymocyte globulin
RPMI	Roswell Park Memorial Institute
S	short $(GT)_n$ dinucleotide repeat
SEM	standard error of the mean
SG	S-genotype group
SLE	systemic lupus erythematosus
SNP	single nucleotide polymorphism
TCR	T cell receptor
TGF-β	transforming growth factor-beta
Th1	T helper type 1
TNF-α	tumour necrosis factor-alpha
Treg	regulatory T cell
TSDR	Treg-specific demethylated region
VPD450	violet proliferation dye 450
XCI	X-chromosome inactivation